COMPARATIVE PHYTOCHEMICAL ANALYSIS AND GENE EXPRESSION PROFILING OF LEAF GALLS ON SELECTED MEDICINAL PLANTS

Thesis

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BY

RESHMA K RAMESH

Under the guidance of

Dr. SURESH V

DEPARTMENT OF BOTANY

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GOVERNMENT VICTORIA COLLEGE, PALAKKAD
Kerala, India, PIN 678001
Affiliated to the University of Calicut NAAC
Accreditation - A Grade (3.2, Fourth Cycle)

Phone: 0491 2576773 (Office) | Mobile: +91 8281716773 | Email: victoriapkd@gmail.com | Web: www.gvc.ac.in

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Dr. Suresh V

Associate Professor, P. G. & Research Department of Botany

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This is to certify that the thesis entitled **"COMPARATIVE PHYTOCHEMICAL ANALYSIS AND GENE EXPRESSION PROFILING OF LEAF GALLS ON SELECTED MEDICINAL PLANTS"** submitted to the University of Calicut by **Mrs. Reshma K Ramesh** in partial fulfilment for the award of the Degree of Doctor of Philosophy in Botany is a bonafide record of the research work carried out by her under my guidance and supervision at Research Department of Botany, Govt. Victoria College, Palakkad.

Place: Palakkad

Date: 27-11-25

Dr. Suresh V

(Research Guide)

Dr. Suresh V
Associate Professor of Botany
Govt. Victoria College, Palakkad - 1
PEN: 656830

DECLARATION

I hereby declare that the work presented in the thesis entitled ***"COMPARATIVE PHYTOCHEMICAL ANALYSIS AND GENE EXPRESSION PROFILING OF LEAF GALLS ON SELECTED MEDICINAL PLANTS"*** *is a genuine record of research work carried out by me at P. G. & Research Department of Botany, Govt. Victoria College, Palakkad under the guidance and supervision of* ***Dr. Suresh V, Associate Professor,*** *P. G. & Research Department of Botany, Govt. Victoria College, Palakkad and has not been included in any other thesis submitted previously for the award of any degree. The contents of the thesis are undergone plagiarism check using iThenticate software at C.H.M.K. Library, University of Calicut, and the similarity index found within the permissible limit. I also declare that the thesis is free from AI generated contents.*

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Name: **Dr. Suresh V**

Place: Palakkad

Date: 27-11-25

Dr. Suresh V
Associate Professor of Botany
Govt. Victoria College, Palakkad - 1
PEN: 656830

LIST OF PLATES

Plate 1.1: Habit of *Alstonia scholaris* (L.) R. Br.

Plate 1.2: Habit of *Ficus racemosa* L.

Plate 3.1: Figure A: *A. scholaris* plant with branches, leaves and leaf galls, Figure B: *A. scholaris* twig with healthy leaves and Figure C: *A. scholaris* twig possessing leaves with leaf galls.

Plate 3.2: Figure A: *A. scholaris* single leaf with galls (Adaxial surface), Figure B: *A. scholaris* abaxial surface of leaf with galls, Figure C: Unilocular gall, bilocular gall and multilocular galls of *A. scholaris* and Figure D: Each gall is cut into two pieces to view the larval chamber.

Plate 3.3: Figure A: The photograph of inducer insect *Pauropsylla tuberculata* developing inside the leaf galls of *A. scholaris*, Figure B: Photograph showing the insect *P. tuberculata*, Figure C: Mature stage of *P. tuberculata*, Figure D: The leaf gall inducer insect *Pauropsylla depressa* developing inside the leaf galls of *F. racemosa*, Figure E: Photograph of insect *P. depressa* and Figure F: Matured stage of *P. depressa* with wings.

Plate 3.4: Figure A: A twig of *A. scholaris* with clumped galls and Figure B: *F. racemosa* twig turned completely into galls.

Plate 3.5: Figure A: *F. racemosa* plant with branches, leaves and leaf galls, Figure B: *F. racemosa* twig with healthy leaves and Figure C: *F. racemosa* twig possessing leaves with leaf galls.

Plate 3.6: Figure A: *F. racemosa* single leaf with galls (Adaxial surface), Figure B: Abaxial surface of leaf with galls in *F. racemosa*, Figure C: *F. racemosa* galls having single to multiple chambers and Figure D: Each gall is cut into two pieces to view the larval chamber.

Plate 3.7: Anatomy of normal leaves. Figure A. Transverse section (T. S.) of normal (healthy) leaves of *A. scholaris*, Figure B: T. S. of mid rib portion of *A. scholaris* leaf, Figure C: T. S. of normal leaves of *F. racemosa* and Figure D: T. S. of mid rib portion of *F. racemosa* leaf.

Plate 3.8: Anatomy of leaf galls. Figure A: T. S. of leaf galls of *A. scholaris,* Figure B: *A. scholaris* leaf galls; a portion enlarged, Figure C: T. S. of leaf galls of *F. racemosa* and Figure D: *F. racemosa* leaf galls; a portion enlarged.

Plate 3.9: Preliminary phytochemical tests of normal leaf (A), gall leaf (B) and gall (C) aqueous extract of *A. scholaris.*

Plate 3.10: Preliminary phytochemical tests of normal leaf (A), gall leaf (B) and gall (C) hexane extract of *A. scholaris.*

Plate 3.11: Preliminary phytochemical tests of normal leaf (A), gall leaf (B) and gall (C) ethanolic extract of *A. scholaris.*

Plate 3.12: Preliminary phytochemical tests of normal leaf (A), gall leaf (B) and gall (C) aqueous extract of *F. racemosa.*

Plate 3.13: Preliminary phytochemical tests of normal leaf (A), gall leaf (B) and gall (C) hexane extract of *F. racemosa.*

Plate 3.14: Preliminary phytochemical tests of normal leaf (A), gall leaf (B) and gall (C) ethanol extract of *F. racemosa*.

Plate 3.15: GC-MS chromatogram obtained through GC-MS analysis of the methanolic extract of *A. scholaris* normal leaves (AS-NL). Each peak represents a compound eluted at a specific retention time, indicating the presence of different phytoconstituents in the extract of *A. scholaris* normal leaf extracts, showing different peaks.

Plate 3.16: GC-MS profile of methanolic extract of gall leaf of *A. scholaris* (AS-GL). Each peak in this chromatogram corresponds to a bioactive compound separated based on its retention time, reflecting the metabolic diversity present in the sample.

Plate 3.17: GC-MS chromatogram of *A. scholaris* gall (AS-GA) illustrates the separation of volatile constituents present in the methanolic extract of *A. scholaris.* Each peak represents a distinct compound identified based on its retention time, highlighting the phytochemical complexity of the gall extract.

Plate 3.18: GC-MS chromatogram obtained through GC-MS analysis of the methanolic extract of normal leaf of *F. racemosa* (FR-NL). Each peak represents a

compound eluted at a specific retention time, indicating the presence of different phytoconstituents in the extract.

Plate 3.19: GC-MS profile of *F. racemosa* gall leaf (FR-GL) extract. Each peak in this chromatogram corresponds to a bioactive compound separated based on its retention time, reflecting the metabolic diversity present in the sample.

Plate 3. 20: GC-MS chromatogram *F. racemosa* gall (FR-GA) extract illustrates the separation of volatile constituents present in the methanolic extract of *F. racemosa.* Each peak represents a distinct compound identified based on its retention time, highlighting the phytochemical complexity of the gall extract.

Plate 3.21: Bar diagram representing the number of different phytochemical compound classes identified in normal leaves, gall leaves and galls of *A. scholaris and F. racemosa.*

Plate 3.22: Figure A: OHR-LC-MS-Chromatogram of ethanolic extracts of AS-NL presents the metabolic profile of the AS-NL, revealing the presence of several bioactive compounds and Figure B: OHR-LC-MS Chromatogram of ethanolic extracts of AS-GL shows the chemical profile of the gall leaf, indicating the presence of a variety of secondary metabolites based on distinct retention time peaks.

Plate 3.23: The diagram illustrates the diverse pharmacological properties of phenolic constituents present in AS-NL. These activities are represented with lines linking specific compounds to their known bioactivities, highlighting the potential therapeutic value of the identified phytochemicals.

Plate 3.24: Schematic diagram illustrating the bioactivities of phenolic compounds identified in AS-GL. Each line links a specific phenolic compound to its corresponding biological activity.

Plate 3.25: Figure A: Shows the chromatographic profile of AS-GA, characterized by several sharp and well-resolved peaks at different retention times (RT), indicating the presence of distinct secondary metabolites and Figure B: displays the OHR-LC-MS chromatogram of ethanolic extracts of FR-NL with distinct peaks corresponding to individual compounds.

Plate 3.26: The diagram illustrates the pharmacological potential of phenolic constituents specifically present in the AS-GA. Compounds and their respective activities are connected by lines and many of the compounds have multiple bioactivity.

Plate 3.27: The diagram illustrates the diverse pharmacological properties of phenolic constituents present in FR-NL. These bioactivities are represented with lines linking specific compounds, highlighting the potential therapeutic value of the identified phytochemicals.

Plate 3.28: Figure A: OHR-LC-MS chromatogram of ethanolic extracts of FR-GL represents the metabolic profile of the FR-GL, revealing the presence of several bioactive compounds and Figure B: OHR-LC-MS chromatogram of ethanolic extracts of FR-GA shows the chemical profile of the gall, indicating the presence of a variety of secondary metabolites based on distinct retention time peaks.

Plate 3.29: The schematic illustration represents the bioactivity of phenolic compounds found in the FR-GL. The lines in the diagram connect individual phenolic compounds to their respective bioactivities.

Plate 3.30: This diagram (part-1) illustrates the pharmacological relevance of phenolic constituents found in FR-GA. Each compound is connected to its corresponding biological activities through lines. This is part 1 of the complete diagram; the remaining phenolic compounds and their bioactivities are presented in Part 2.

Plate: 3.31: The diagram (Part-2) is the continuation of bioactivities of phenolic compounds identified in FR-GA. This second part completes the illustration of bioactive phenolic compounds found in FR-GA. Together; both parts emphasize the complex and diverse bioactive profile of gall-derived phenolic compounds.

Plate 3.32: Figure A: Results of quantitative estimation of chlorophyll a in normal leaves, gall leaves and galls of *A. scholaris* and *F. racemosa* and Figure B: Results of quantitative estimation of chlorophyll b in normal leaves, gall leaves and leaf galls of *A. scholaris* and *F. racemosa*.

Plate 3.33: Figure A: Results of quantitative estimation of total chlorophyll in normal leaves, gall leaves and leaf galls of *A. scholaris* and *F. racemosa* and Figure B: Results of quantitative estimation of carotenoids in normal leaves, gall leaves and leaf galls of *A. scholaris* and *F. racemosa*.

Plate 3.34: The bar diagram represents quantitative estimation of PAL enzyme activity in three tissue types - normal leaves, gall leaves and leaf galls of *A. scholaris* and *F. racemosa*. Error bars represent the standard deviation of three replicates (n = 3).

Plate 4.1: Results of reducing power analysis of *A. scholaris* extracts (100 µg/ml). Figure A-C: shows the reducing power analysis result of aqueous extracts of AS-NL, AS-GL and AS-GA; Figure D-F: represents the result of hexane extracts of AS-NL, AS-GL and AS-GA; Figure G-I: shows the result of ethanol extracts of AS-NL, AS-GL and AS-GA.

Plate 4.2: Results of reducing power analysis of *F. racemosa* extracts (100 µg/ml). Figure A-C: shows the reducing power of aqueous extracts of FR-NL, FR-GL and FR-GA; Figure D-F: shows reducing power activity of hexane extracts of FR-NL, FR-GL and FR-GA and Figure G-I: shows the result of ethanol extracts of FR-NL, FR-GL and FR-GA.

Plate 4.3: Figure A: Graphical representation of antioxidant activity analysis results of aqueous extracts of AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA using reducing power method. Ascorbic acid taken as positive control. Each bar represents reducing power of the sample with SD and Figure B: Graphical representation of results of antioxidant activity analysis of hexane extracts of AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA using RP method. Ascorbic acid taken as positive control. Each bar represents reducing power of the sample with SD.

Plate 4.4 : Figure A: Graphical representation of results of antioxidant activity analysis of ethanolic extracts of AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA using RP method. Ascorbic acid taken as the positive control. Each bar represents reducing power of the sample with standard deviation and Figure

B: IC_{50} values obtained by RP assay using aqueous, hexane and ethanolic extracts of different samples of *A. scholaris* and *F. racemosa*.

Plate 4.5: Photographs showing the results of DPPH radical scavenging activity of ethanol, aqueous and hexane extracts of *A. scholaris* - normal leaf, gall leaf and gall in different dilutions of extracts (A: 2.5 µL, B: 5 µL, C: 7.5 µL and D: 10 µL).

Plate 4.6: Photographs showing the results of DPPH radical scavenging activity of ethanol, aqueous and hexane extracts of *F. racemosa* - normal leaf, gall leaf and gall in different dilutions of extracts (A: 2.5 µL, B: 5 µL, C: 7.5 µL and D: 10 µL).

Plate 4.7: Figure A: Graphical representation of results of DPPH antioxidant activity analysis of aqueous extracts of AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA. Each bar represents the mean %RSA of a sample with standard deviation and Figure B: Graphical representation of results of antioxidant activity analysis of hexane extracts by DPPH method. Each bar represents the mean %RSA of a sample with standard deviation.

Plate 4.8: Figure A: Graphical representation of antioxidant activity of ethanolic extracts of AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA. Each bar represents the mean %RSA of a sample with standard deviation and Figure B: IC_{50} values of different samples of *A. scholaris* and *F. racemosa* determined using DPPH radical scavenging assay.

Plate 4.9: Antibacterial analysis of controls. The activity of ethanol (negative control) and streptomycin (positive control) was tested against four tested bacterial strains. Figure A: *Bacillus subtilis*, Figure B: *Escherichia coli*, Fig. C: *Pseudomonas aeruginosa* and Fig. D: *Staphylococcus aureus*. The figures demonstrate streptomycin exhibiting standard antibacterial activity, while ethanol shows no inhibition of bacterial growth.

Plate 4.10: Antibacterial activity of ethanolic extracts of *A. scholaris* and *F. racemosa*. Figure A-C: Zones of inhibition produced by AS-NL, AS-GL and AS-GA against *B. subtilis*, *E. coli* and *S. aureus*, respectively and Figure D-F: Zones

of inhibition of FR-NL, FR-GL and FR-GA against *B. subtilis*, *E. coli* and *S. aureus*, respectively.

Plate 4.11: Heat maps showing results of antibacterial analysis: The results of antibacterial analysis of AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA ethanolic extracts in 4 different dilutions with the zone of inhibition (mm) produced. Light colours indicate no activity and the increase of darkness of colours indicates increase in inhibition zone against *Staphylococcus aureus* (SA), *Bacillus subtilis* (BS) and *Escherichia coli* (EC).

Plate 4.12: Anticandidal activity of *A. scholaris* and *F. racemosa*. Figure A: positive control (Itraconazole), Figure B: negative control, Figure C: activity of AS-NL, AS-GL and AS-GA ethanolic extracts, Figure D: no activity for AS-NL, AS-GL and AS-GA hexane extracts, Figure E: AS-NL, AS-GL and AS-GA aqueous extracts (no activity), Figure F: zone of inhibition of FR-NL, FR-GL and FR-GA ethanolic extracts, Figure G: FR-NL, FR-GL and FR-GA hexane extracts (no activity) and Figure H: no activity for FR-NL, FR-GL and FR-GA aqueous extracts.

Plate 4.13: Graphical representation of antifungal activity. Figure A: Antifungal activity of ethanolic extracts of *A. scholaris* and *F. racemosa* against *C. albicans*. The X-axis represents different plant samples and positive control, while the Y-axis shows inhibition zones (mm) and Figure B: Percentage growth inhibition of ethanolic extracts of *A. scholaris* and *F. racemosa* against *A. niger*, with Itraconazole as the positive control. The X-axis represents different samples and the Y-axis shows growth inhibition (%).

Plate 4.14: Antifungal activity of ethanolic extracts of *A. scholaris* and *F. racemosa* against *A. niger*. Figure A: Growth of hyphae in medium with Itraconazole, Figure B: Growth of hyphae in medium with ethanol, Figure C: Growth of hyphae AS-NL extract, Figure D: Growth in medium with AS-GL extract, Figure E: Growth of hyphae in AS-GA extract, Figure F: Growth of hyphae with FR-NL extract, Figure G: Growth in medium with FR-GL extract and Figure H: Growth of hyphae in medium with FR-GA extract.

Plate 4.15: Figure A: Cell viability percentage of *A. scholaris* extracts - AS-NL, AS-GL and AS-GA showing mean values with standard deviation, indicating their effect on cell viability and Figure B: Cytotoxic activity of *F. racemosa* extracts- FR-NL, FR-GL and FR-GA represented as cell viability percentage with standard deviation.

Plate 4.16: Microscopic representation of 3T3 cells. Figure A: Image of untreated 3T3 cells used for cytotoxicity assay, Figure B: Image of 3T3 cells after treatment, showing the formation of formazan crystals indicating metabolically active cells and Fig C: Photograph of MTT treated 3T3 cells after addition of solubilisation reagent showing the developed color intensity used for viability measurement.

Plate 4.17: Figure A: shows the representation of a culture plate having purple formazan crystals and Figure B: The graph showing the results of cell viability percentage of ethanolic extracts of *A. scholaris* gall (AS-GA) and *F. racemosa* gall leaf (FR-GL) using MTT assay.

Plate 4.18: Anti-inflammatory activity analysis by protein denaturation inhibition assay. Figure A: Control assay showing untreated protein sample, Figure B: Activity of standard drug (Diclofenac sodium) showing inhibition of protein denaturation and Figure C-G: Anti-inflammatory activity of *F. racemosa* gall leaf (FR-GL) ethanolic extract at concentrations of 1, 2, 3, 4, and 5 µg/µL, respectively, showing progressive inhibition of protein denaturation with increasing extract concentration.

Plate 5.1: TLC analysis: Figure A: TLC analysis of FR-GL using pre-coated silica gel plate, shown under visible light, Figure B: Pre-coated silica gel plate for separation of FR-GL under UV 254 nm, Figure C: TLC on pre-coated silica gel plate visible under UV 365 nm, Figure D: The TLC plate prepared using silica gel G and water is drying at room temperature, Figure E: FR-GL extracts spotted in the plate and developing in a chamber saturated with solvent and Figure F: Separation of the extract into distinct bands inside the chromatographic chamber.

Plate 5.2: Figure A: TLC separated plate images under visible light, Figure B: TLC plate showing separated bands under UV light of 254 nm and Figure C: TLC separated plate images under UV light (365nm).

Plate 5.3: Figure A: Scraping off and separation of compounds from the silica gel plate and Figure B: Seven distinct compounds isolated from FR-GL extract, labeled as R1-R7.

Plate 5.4: Antibacterial activity of TLC separated products: Figure A: Antibacterial activity of C1 (20 µL) against *S. aureus*, Figure B: Zone of inhibition produced by C1 (80 µL) sample (S) against *S. aureus*, Figure C: Antibacterial activity shown by C5 (20 µL) and C7 (20 µL) against *E.coli*, Figure D: Zone of inhibition produced by C5 (80 µL) sample (S) against *E. coli* along with positive and negative controls, Figure E: Antibacterial activity shown by C7 (80 µL) sample (S) against *E. coli* and Figure F: No activity detected by any TLC separated compounds against *B. subtilis.*

Plate 5.5: Figure A: Heat map representing the antibacterial activity of seven fractions separated from FR-GL ethanolic extract using preparative TLC technique (BS- *B. subtilis*, SA- *S. aureus* and EC- *E. coli*) and Figure B: The 1H NMR spectrum obtained from analysis of the compound C5 in methanol.

Plate 6.1: Diagrammatic representation of shikimic acid pathway with important genes and pathway products. Genes selected for the present expression analysis are shown in yellow boxes (Liu et al., 2014).

Plate 6.2: Diagrammatic representation of phenylpropanoid pathway. Genes selected for the present expression analysis are shown in yellow boxes. Dotted line arrow represents multiple steps (Ali et al., 2011; Choi et al., 2021).

Plate 6.3: Extraction of RNA from different samples. Figure A: RNA from AS-NL, AS-GL and AS-GA, Figure B: RNA isolated from FR-NL, Figure C: RNA isolated from FR-GL and Figure D: RNA isolated from FR-GA.

Plate 6.4: Primer efficiency analysis. Figure A: Standard curve for primer efficiency analysis of DAHPS gene primer, Figure B: Standard curve of EPSPS gene primer and Figure C: Standard curve of CS gene primer.

Plate 6.5: Primer efficiency analysis. Figure A: Standard curve for primer efficiency analysis of PAL gene primer, Figure B; Primer efficiency curve of CHI gene and Figure C: Primer efficiency analysis of DFR gene primer.

Plate 6.6: Figure A: Representation of qRT-PCR analysis outcome file of *A. scholaris*, showing fluorescent profile of gene expression and Figure B: Representation of qRT-PCR analysis outcome file of *F. racemosa*, showing fluorescent profile of gene expression.

Plate 6.7: qRT-PCR expression analysis- Figure A-F: Relative fold gene expression (FGE) of DAHPS, EPSPS, CS, PAL, CHI and DFR genes in gall leaves and galls compared to control (normal leaves) in *A. scholaris*.

Plate 6.8: Comparative analysis of gene expression: Figure A: Fold gene expression of 6 selected genes in *A. scholaris* normal leaves (control), gall leaves and leaf galls and Figure B: Fold gene expression in *F. racemosa* normal leaf (control), gall leaves and leaf galls.

Plate 6.9: qRT-PCR analysis. Figure A-F: Relative expression levels of DAHPS, EPSPS, CS, PAL, CHI and DFR genes in gall leaves and galls of *F. racemosa* expressed as fold change compared to normal leaves (control).

Plate 6.10: Gene expression in gall leaves: Fold gene expression (FGE) of selected genes in gall leaves of *A. scholaris* denoted with the letter 'A' and FGE of selected genes in gall leaves of *F. racemosa* represented with letter 'F'. Different colored boxes indicated with the range of FGE. Dotted lines indicate multiple steps.

Plate 6.11: Gene expression in galls: FGE of selected genes in galls of *A. scholaris* denoted with the letter 'A' and FGE of selected genes in galls of *F. racemosa* represented with 'F'. Different colored boxes indicated with the range of FGE. Dotted lines indicate multiple steps.

LIST OF TABLES

Table 3.1: Results of preliminary phytochemical analysis of aqueous, hexane and ethanolic extracts of normal leaves, gall leaves and leaf galls of *A. scholaris.*

Table 3.2: Result of preliminary phytochemical analysis of aqueous, hexane and ethanolic extracts of normal leaves, gall leaves and leaf galls of *Ficus racemosa.*

Table 3.3: Results of quantitative phytochemical analysis on methanolic extracts of normal leaves, gall leaves and leaf galls of *A. scholaris* by GC-MS analysis.

Table 3.4: Results of quantitative phytochemical analysis on methanolic extracts of normal leaves, gall leaves and leaf galls of *F. racemosa.*

Table 3.5: List of compounds identified from *A. scholaris* and *F. racemosa* with their reported biological activities.

Table 3.6: List of compounds of AS-NL identified by OHR-LC-MS analysis with molecular formula, chemical class and reported bioactivities.

Table 3.7: List of compounds found in AS-GL through OHR-LC-MS analysis with molecular formula, chemical class and reported bioactivities.

Table 3.8: List of compounds found in AS-GA through OHR-LC-MS analysis with molecular formula, chemical class and reported bioactivities.

Table 3.9: List of compounds found in FR-NL identified by OHR-LC-MS analysis with molecular formula, chemical class and reported bioactivities.

Table 3.10: List of compounds found in FR-GL identified by OHR-LC-MS analysis with molecular formula, chemical class and reported bioactivities.

Table 3.11: List of compounds found in FR-GA identified by OHR-LC-MS analysis with molecular formula, chemical class and reported bioactivities.

Table 3.12: Result of quantitative estimation of chlorophyll content in normal leaves, gall leaves and galls of *A. scholaris* and *F. racemosa.*

Table 3.13: Result of quantitative estimation of PAL enzyme activity in normal leaves, gall leaves and galls of *A. scholaris* and *F. racemosa.*

Table 4.1: The percentage radicle scavenging activity of aqueous extracts of different samples of *A. scholaris* and *F. racemosa* determined using RP method.

Table 4.2: The percentage radicle scavenging activity of hexane extracts of different samples of *A. scholaris* and *F. racemosa* determined using RP method.

Table 4.3: The percentage radicle scavenging activity of ethanolic extracts of different samples of *A. scholaris* and *F. racemosa* evaluated through RP method.

Table 4.4: IC_{50} values of *A. scholaris* and *F. racemosa* extracts of normal leaves, gall leaves and leaf galls analysed through RP method.

Table 4.5: Radical scavenging activity percentage (%RSA) of aqueous extracts of different samples of *A. scholaris* and *F. racemosa* compared with Ascorbic acid (standard) at different concentrations through the DPPH method.

Table 4.6: The percentage radicle scavenging activity of hexane extracts of different samples of *A. scholaris* and *F. racemosa* analysed through DPPH method.

Table 4.7: The percentage radicle scavenging activity of ethanolic extracts of different samples of *A. scholaris* and *F. racemosa* analysed through DPPH method.

Table 4.8: IC_{50} values of normal leaves, gall leaves and leaf galls of *A. scholaris* and *F. racemosa* extracts determined using DPPH method.

Table 4.9: Antibacterial activity of AS-NL, AS-GL and AS-GA with the average zone of inhibition (mm) produced with SD calculated in 4 different dilutions (10µl, 20µl, 30µl and 40µl).

Table 4.10: Antibacterial activity of FR-NL, FR-GL and FR-GA with the average zone of inhibition (mm) produced with SD calculated in 4 different dilutions (10µl, 20µl, 30µl and 40µl).

Table 4.11: The average zone of inhibition produced by streptomycin (+ve control) against all tested organisms with SD.

Table 4.12: Results of antifungal activity of ethanolic extracts against *C. albicans* using AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA along with antifungal drug Itraconazole. Zone of inhibition represented in mm with standard deviation. IC_{50} values of all the samples presented in mm.

Table 4.13: Percentage growth inhibition against *A. niger* shown by ethanolic extracts of different samples of *A. scholaris* and *F. racemosa* along with SD.

Table 4.14: Percentage cell viability of aqueous, ethanolic and hexane extracts of *A. scholaris* and *F. racemosa.*

Table 4.15: Concentration dependent cell viability assay results of AL-GA and FR-GL showing the cell viability percentage of 50-500 mg/mL of sample concentrations.

Table 4.16: Anti-inflammatory activity of five different dilutions of FR-GL extracts, using protein denaturation inhibition assay.

Table 5.1: The retention factor values of the bands produced through preparative TLC analysis of FR-GL extract.

Table 5.2: Antibacterial activity analysis of TLC separated products of FR-GL, showing zone of inhibition in mm produced by 2 different dilutions of compound.

Table 6.1: List of selected genes with their reaction substrates and products.

Table 6.2: Gene accessions used for primer design, with their accession numbers and plant species.

Table 6.3: PCR programme outlining the step-wise temperature conditions, duration, and cycle number used for amplification of the selected genes.

Table 6.4: Optimized thermal cycling conditions used for the quantitative real-time PCR (qRT-PCR) analysis of the selected genes.

Table 6.5: List of forward and reverse primer sequences of selected genes used in this study with their respective product sizes (bp).

Table 6.6: Relative fold gene expression levels of selected genes in *A. scholaris* normal leaves (control), gall leaves and galls, with values expressed as mean ± SD.

Table 6.7: Relative fold gene expression levels of six selected genes in *F. racemosa* normal leaves, gall leaves and galls, with values expressed as mean ± SD.

ABBREVIATIONS

AA	- Ascorbic acid
ABTS	- 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid
AIRF	- Advanced Instrumentation Research Facility
ANOVA	- Analysis of variance
ATP	- Adenosine triphosphate
AS-GA	-*A. scholaris* - leaf galls
AS-GL	-*A. scholaris* - gall leaves
AS-NL	- *A. scholaris* - normal leaves
BLAST	- Basic local alignment search tool
C4H	- Cinnamic acid 4-hydroxylase
cDNA	- Complementary DNA
CHI	- Chalcone-flavanone isomerase
CS	- Chorismate synthase
Ct	- Cycle threshold
DAHP	- 3-Deoxy-D-arabino-heptulosonate-7-phosphate
DAHPS	- 3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase
DFR	- Dihydroflavonol 4-reductase
DHQ	- 3-Dehydroquinic acid
DHS	- 3-Dehydroshikimic acid
DMEM	- Dulbecco's modified Eagle's medium
DMSO	- Dimethyl sulfoxide
DPPH	- 2, 2-Diphenyl-1-picrylhydrazyl
E4P	- Erythrose-4-phosphate
EPSP	- 5-Enolpyruvyl shikimic acid 3-phosphate
EPSPS	- 5-enolpyruvylshikimate-3-phosphate synthase
FBS	- Fetal bovine serum
FGE	- Fold gene expression
FHT	- Flavanone 3-hydroxylase
FRAP	- Ferric reducing antioxidant power
FR-GA	- *F. racemosa*- leaf galls
FR-GL	- *F. racemosa*- gall leaves
FR-NL	- *F. racemosa*- normal leaves
GC-MS	- Gas chromatography-mass spectrometry
GTC	- Guanidine thiocyanate
HCN	- Hydrogen cyanide
HPTLC	- High-performance thin layer chromatography
IAA	- Indole-3-acetic acid

ISR	- Induced systemic resistance
LC-MS	- Liquid chromatography-mass spectrometry
LC-MS/MS	- Liquid chromatography-tandem mass spectrometry
LPA	- Lysophosphatidic acid
MCF-7	- Michigan cancer foundation-7
MEP	- Methylerythritol phosphate
MTT	-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	- Nicotinamide adenine dinucleotide phosphate
NCBI	- National Center for Biotechnology Information
NGS	- Next generation sequencing
NH3	- Ammonia
NMR	- Nuclear magnetic resonance
OD	- Optical density
OHR-LC-MS	- Orbitrap high-resolution liquid chromatography-mass spectrometry
PAL	- Phenylalanine ammonia-lyase
PBS	- Phosphate buffered saline
PCR	- Polymerase chain reaction
PDA	- Potato dextrose agar
PEP	- Phosphoenolpyruvate
PR proteins	- Pathogenesis-related proteins
PTLC	- Preparative thin-layer chromatography
PVP	- Polyvinylpyrrolidone
qRT-PCR	- Quantitative real-time PCR
ROS	- Reactive oxygen species
RSA	- Radical scavenging activity
SAGE	- Serial analysis of gene expression
SAIF	- Sophisticated Analytical Instrument Facility
SAR	- Systemic acquired resistance
SD	- Standard deviation
SK	- Shikimate kinase
TAE	- Tris-acetate-EDTA
TCA	- Tricarboxylic acid
TLC	- Thin layer chromatography
WHO	- World Health Organization
XTT	- Methoxynitrosulfophenyl-tetrazolium carboxanilide

CONTENTS

ACKNOWLEDGEMENTS 5

PREFACE 7

ABSTRACT 8

ABSTRACT IN MALAYALAM - ᗰᐯᐅᐲᓇᐱᐅ 10

CHAPTER 1 - GENERAL INTRODUCTION 11

1.1. Plant galls 11

1.1.1. Gall induction 12

1.1.2. Structure of gall 13

1.1.3. Biochemical alterations due to gall development 14

1.1.4. Molecular alterations due to gall development 14

1.2. Significance of phytochemical and molecular study of plant galls 15

1.3. Aim of the study 16

1.4. Objectives of the study 16

1.5. Plants selected for the study 17

CHAPTER 2 - LITERATURE REVIEW 19

2.1. Medicinal plants and importance of secondary metabolites 19

2.2. Introduction to plant galls 20

2.2.1. Gall induction and development 21

2.2.2. Effects of gall inducers on host plants 22

2.2.3. Use of plant galls 23

2.3. *Alstonia scholaris* (L.) R. Br. 23

2.4. *Ficus racemosa* L. 25

2.5. Phytochemical composition of the selected plants 26

2.5.1. Phytochemical composition of normal leaves 26

2.5.2. The effect of insect attack on phytochemical composition of plants 26

2.5.3. Phytochemical profile of plant galls 27
2.5.4. Analytical techniques used to find metabolomic profiles 28
2.6. Biological properties of different parts of selected plants 29
2.6.1 Antioxidant activity 29
2.6.2. Antibacterial activity 29
2.6.3. Antifungal activity 30
2.6.4. Cytotoxic activity 31
2.6.5. Anti-inflammatory activity 31
2.7. Genes involved in regulation of secondary metabolic pathways 32
2.7.1. Gall induction and gene expression 33
2.7.2. Genes selected for the study 33
CHAPTER 3 - STRUCTURE AND PHYTOCHEMISTRY OF LEAF GALLS ON SELECTED MEDICINAL PLANTS 35
3.1. INTRODUCTION 35
3.1.1. Plant galls 35
3.1.2. Overview of plant secondary metabolites and their functions 36
3.2. METHODOLOGY 39
3.2.1. Structure of normal leaves and leaf galls 39
3.2.2. Phytochemical analysis of plant extracts 40
3.2.3. Other preliminary quantitative analysis 45
3.3. RESULTS AND DISCUSSION 47
3.3.1. Structure of normal leaves and leaf galls 47
3.3.2. Results of phytochemical analysis of plant extracts 53
3.3.3. Other preliminary quantitative analysis 97
3.4. CONCLUSIONS 101
CHAPTER 4 - BIOACTIVITY ANALYSIS OF NORMAL LEAVES, GALL LEAVES AND LEAF GALL EXTRACTS 103
4.1. INTRODUCTION 103
4.1.1. Antioxidant activity 105
4.1.2. Antimicrobial activity 105

4.1.3. Cytotoxic activity 106
4.1.4. Anti-inflammatory activity 106
4.2. METHODOLOGY 107
4.2.1. Antioxidant activity analysis 107
4.2.2. Antibacterial activity analysis 108
4.2.3. Antifungal activity analysis 109
4.2.4. Cytotoxic activity analysis 111
4.2.5. Anti-inflammatory activity analysis 113
4.3. RESULTS AND DISCUSSION 114
4.3.1. Results of antioxidant activity analysis 114
4.3.2. Results of antibacterial activity analysis 132
4.3.3. Results of antifungal activity analysis 138
4.3.4. Results of cytotoxic activity analysis 143
4.3.5. Results of anti-inflammatory activity analysis 149
4.4. CONCLUSIONS 151
CHAPTER 5 - ISOLATION OF BIOACTIVE FRACTIONS RESPONSIBLE FOR POTENT BIOLOGICAL ACTIVITY 152
5.1. INTRODUCTION 152
5.2. METHODOLOGY 154
5.2.1. Selection of most bioactive plant sample 154
5.2.2. Optimization of chromatographic conditions of the targeted plant extracts 155
5.2.3. Preparative TLC analysis for separation of compounds 155
5.2.4. Antibacterial assay of separated fractions using disc diffusion method 156
5.2.5. Characterization of most active fractions using NMR technique 157
5.3. RESULTS AND DISCUSSION 157
5.3.1. Selection of most bioactive sample 157
5.3.2. Optimization of chromatographic conditions of FR-GL plant extracts 158
5.3.3. Preparative TLC analysis 159
5.3.4. Results of antibacterial activity assay of separated and purified fractions 160
5.3.5. Results of Nuclear magnetic resonance (NMR) spectrometry analysis 162
5.4. CONCLUSIONS 163

CHAPTER 6 - EXPRESSION PROFILING OF GENES INVOLVED IN THE SECONDARY METABOLIC PATHWAY FROM DIFFERENT SAMPLES 164
6.1. INTRODUCTION 164
6.1.1. Secondary metabolites and plant defence 165
6.1.2. Secondary metabolic pathways and important genes 166
6.1.3. Gene expression profiling 167
6.2. METHODOLOGY 170
6.2.1. Plant material collection and storage 170
6.2.2. Extraction of total RNA 170
6.2.3. Complementary DNA (cDNA) synthesis 172
6.2.4. Primer design and synthesis 173
6.2.5. Quantitative real-time PCR for expression analysis of selected genes 177
6.3. RESULTS AND DISCUSSION 179
6.3.1. RNA quality evaluation for qRT-PCR analysis 179
6.3.2. Primer design and synthesis 180
6.3.3. Primer efficiency curve 180
6.3.4. Gene expression profile of *A. scholaris* 182
6.3.5. Gene expression profile of *F. racemosa* 188
6.3.6. Comparison of gene expression 194
6.4. CONCLUSIONS 197
CHAPTER 7 – SUMMARY 198
CHAPTER 8 – RECOMMENDATIONS 200
CHAPTER 9 – REFERENCES 202
INDEX

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PREFACE

This study focuses on the structural, phytochemical and molecular characteristics of leaf galls on selected medicinal plants and the thesis is organized into 9 chapters. The research used available AI tools to improve language and grammar. Chapter 1 provides a general introduction to medicinal plants and plant galls. In addition to outlining the aim and objectives of the study, the chapter provides details about the plants selected for the present study. Chapter 2, literature review, this chapter reviews the literature on gall formation and its biochemical and molecular aspects.

Chapter 3 investigates the morphological and anatomical modifications in gall-affected leaves compared to normal leaves. Additionally, it explores the phytochemical profile of galls, in respect to healthy leaves and gall forming leaves. GC-MS and OHR-LC-MS analysis reports were used for producing the phytochemical profile of the selected plants. In forth chapter the details on the bioactivity analysis of normal leaves, gall leaves and leaf gall extracts are given for understanding the biological significance of the phytochemical modifications produced through gall induction. This chapter evaluates the antimicrobial, antioxidant and cytotoxic properties of different extrats of selected plants. By comparing their bioactivity, this study aims to determine whether gall formation enhances or diminishes the medicinal potential of the host plant.

Chapter 5 explains the separation of fractions from the sample having the greatest bioactivity using TLC technique. This chapter also provides the details of the antibacterial activity analysis of the separated fractions for identifying the most bioactive component. In chapter 6, we describe the expression profiling of selected genes from secondary metabolic pathway in different samples. Through this molecular basis of phytochemical alterations in gall tissues is explored. Quantitative real-time PCR analyses were performed to examine the expression of key genes and are profiled to determine their differential regulation in normal and gall tissues.

Chapter 7 summarizes key findings and suggests directions for future research. Chapter 8 contains the recommendations; this chapter provides the list of recommendations suggested for further study. Finally all the references cited in the thesis are listed in chapter 9.

CONTENTS

ACKNOWLEDGEMENTS 5

PREFACE 7

ABSTRACT 8

ABSTRACT IN MALAYALAM - ᗰᐯᐝᔑᓇᑐᐝ 10

CHAPTER 1 - GENERAL INTRODUCTION 11

1.1. Plant galls 11

1.1.1. Gall induction 12

1.1.2. Structure of gall 13

1.1.3. Biochemical alterations due to gall development 14

1.1.4. Molecular alterations due to gall development 14

1.2. Significance of phytochemical and molecular study of plant galls 15

1.3. Aim of the study 16

1.4. Objectives of the study 16

1.5. Plants selected for the study 17

CHAPTER 2 - LITERATURE REVIEW 19

2.1. Medicinal plants and importance of secondary metabolites 19

2.2. Introduction to plant galls 20

2.2.1. Gall induction and development 21

2.2.2. Effects of gall inducers on host plants 22

2.2.3. Use of plant galls 23

2.3. *Alstonia scholaris* (L.) R. Br. 23

2.4. *Ficus racemosa* L. 25

2.5. Phytochemical composition of the selected plants 26

2.5.1. Phytochemical composition of normal leaves 26

2.5.2. The effect of insect attack on phytochemical composition of plants 26

2.5.3. Phytochemical profile of plant galls 27
2.5.4. Analytical techniques used to find metabolomic profiles 28
2.6. Biological properties of different parts of selected plants 29
2.6.1 Antioxidant activity 29
2.6.2. Antibacterial activity 29
2.6.3. Antifungal activity 30
2.6.4. Cytotoxic activity 31
2.6.5. Anti-inflammatory activity 31
2.7. Genes involved in regulation of secondary metabolic pathways 32
2.7.1. Gall induction and gene expression 33
2.7.2. Genes selected for the study 33
CHAPTER 3 - STRUCTURE AND PHYTOCHEMISTRY OF LEAF GALLS ON SELECTED MEDICINAL PLANTS 35
3.1. INTRODUCTION 35
3.1.1. Plant galls 35
3.1.2. Overview of plant secondary metabolites and their functions 36
3.2. METHODOLOGY 39
3.2.1. Structure of normal leaves and leaf galls 39
3.2.2. Phytochemical analysis of plant extracts 40
3.2.3. Other preliminary quantitative analysis 45
3.3. RESULTS AND DISCUSSION 47
3.3.1. Structure of normal leaves and leaf galls 47
3.3.2. Results of phytochemical analysis of plant extracts 53
3.3.3. Other preliminary quantitative analysis 97
3.4. CONCLUSIONS 101
CHAPTER 4 - BIOACTIVITY ANALYSIS OF NORMAL LEAVES, GALL LEAVES AND LEAF GALL EXTRACTS 103
4.1. INTRODUCTION 103
4.1.1. Antioxidant activity 105
4.1.2. Antimicrobial activity 105

4.1.3. Cytotoxic activity 106
4.1.4. Anti-inflammatory activity 106
4.2. METHODOLOGY 107
4.2.1. Antioxidant activity analysis 107
4.2.2. Antibacterial activity analysis 108
4.2.3. Antifungal activity analysis 109
4.2.4. Cytotoxic activity analysis 111
4.2.5. Anti-inflammatory activity analysis 113
4.3. RESULTS AND DISCUSSION 114
4.3.1. Results of antioxidant activity analysis 114
4.3.2. Results of antibacterial activity analysis 132
4.3.3. Results of antifungal activity analysis 138
4.3.4. Results of cytotoxic activity analysis 143
4.3.5. Results of anti-inflammatory activity analysis 149
4.4. CONCLUSIONS 151
CHAPTER 5 - ISOLATION OF BIOACTIVE FRACTIONS RESPONSIBLE FOR POTENT BIOLOGICAL ACTIVITY 152
5.1. INTRODUCTION 152
5.2. METHODOLOGY 154
5.2.1. Selection of most bioactive plant sample 154
5.2.2. Optimization of chromatographic conditions of the targeted plant extracts 155
5.2.3. Preparative TLC analysis for separation of compounds 155
5.2.4. Antibacterial assay of separated fractions using disc diffusion method 156
5.2.5. Characterization of most active fractions using NMR technique 157
5.3. RESULTS AND DISCUSSION 157
5.3.1. Selection of most bioactive sample 157
5.3.2. Optimization of chromatographic conditions of FR-GL plant extracts 158
5.3.3. Preparative TLC analysis 159
5.3.4. Results of antibacterial activity assay of separated and purified fractions 160
5.3.5. Results of Nuclear magnetic resonance (NMR) spectrometry analysis 162
5.4. CONCLUSIONS 163

CHAPTER 6 - EXPRESSION PROFILING OF GENES INVOLVED IN THE SECONDARY METABOLIC PATHWAY FROM DIFFERENT SAMPLES 164
6.1. INTRODUCTION 164
6.1.1. Secondary metabolites and plant defence 165
6.1.2. Secondary metabolic pathways and important genes 166
6.1.3. Gene expression profiling 167
6.2. METHODOLOGY 170
6.2.1. Plant material collection and storage 170
6.2.2. Extraction of total RNA 170
6.2.3. Complementary DNA (cDNA) synthesis 172
6.2.4. Primer design and synthesis 173
6.2.5. Quantitative real-time PCR for expression analysis of selected genes 177
6.3. RESULTS AND DISCUSSION 179
6.3.1. RNA quality evaluation for qRT-PCR analysis 179
6.3.2. Primer design and synthesis 180
6.3.3. Primer efficiency curve 180
6.3.4. Gene expression profile of *A. scholaris* 182
6.3.5. Gene expression profile of *F. racemosa* 188
6.3.6. Comparison of gene expression 194
6.4. CONCLUSIONS 197
CHAPTER 7 – SUMMARY 198
CHAPTER 8 – RECOMMENDATIONS 200
CHAPTER 9 – REFERENCES 202
INDEX

ABSTRACT

Leaf galls are abnormal plant structures that are often caused by gall-inducing organisms like insects, mites, fungi or bacteria. These structures are the result of complex physiological and biochemical interactions between the gall inducer and the host plant. Traditionally viewed as detrimental, galls are increasingly recognized as sites of complex metabolic reprogramming, with the potential to modify the production of bioactive secondary metabolites in host plants. The purpose of this study is to investigate the molecular, phytochemical and structural characteristics of leaf galls in selected medicinal plants: *Alstonia scholaris* (L.) R. Br. and *Ficus racemosa* L., in which galls are induced by insects, *Pauropsylla tuberculata* and *Pauropsylla depressa*, respectively. The study aims to elucidate how gall formation influences secondary metabolism and address a gap in understanding the biochemical and molecular responses of medicinal plants to biotic stress.

The methodology combines morphological, anatomical, phytochemical and molecular techniques. Microscopy was used to undertake morphological and anatomical investigations of gall-affected tissues. Phytochemical profiling entails both qualitative and quantitative investigations utilizing techniques such as Gas Chromatography-Mass Spectrometry (GC-MS) and Orbitrap High-Resolution Liquid Chromatography-Mass Spectrometry (OHR-LC-MS). Extracts from normal leaves, galled leaves and galls were evaluated for bioactivity analysis. The molecular approach used Quantitative Real-Time PCR (qRT-PCR) to characterize the expression of six essential genes involved in the shikimic acid and phenylpropanoid pathways that regulate phenolic compound biosynthesis.

Morphological analysis revealed that galls are distinct, with specialized structures like larval chambers and nutritive tissues. The key findings show that gall formation profoundly changes plant physiology. Phytochemical analysis leads to the finding that galls and galled leaves contain more secondary metabolites, particularly phenolics, flavonoids and tannins, than normal leaves, indicating stronger defense responses against inducers. GC-MS and OHR-LC-MS revealed a diverse range of secondary metabolites with numerous compounds elevated or expressed only in gall tissues. These results suggest that gall enhances the phytochemical diversity and potency of the plant. When compared to normal leaves,

bioactivity analysis revealed that ethanolic extracts from galls and galled leaves exhibited superior antioxidant, antibacterial, antifungal and cytotoxic properties. The highest bioactivity was detected in *F. racemosa* gall leaf extracts. According to qRT-PCR analysis, all the selected genes were significantly upregulated in galls. *F. racemosa* had higher fold gene expression than *A. scholaris*, suggesting a more robust metabolic response to *P. depressa*. Nonetheless, there was little gene upregulation in gall leaves, suggesting metabolites were being translocated to gall leaves from galls.

The findings show that gall induction enhances secondary metabolism, boosting the production of bioactive compounds with therapeutic potential. These gall induced changes offer valuable applications in phytopharmacology, from improving herbal formulations to supporting drug discovery. Overall, the study provides a useful model for understanding inducible secondary metabolism and its relevance to agriculture and medicine. Future studies should compare other gall-forming species, evaluate ecological functions and investigate post-transcriptional regulations.

Keywords: Leaf galls, Insects, Secondary metabolism, Antioxidant activity, Antibacterial activity.

ABSTRACT IN MALAYALAM - സംഗ്രഹം

പ്രാണികൾ, ഫംഗസുകൾ, ബാക്ടീരിയകൾ തുടങ്ങിയ ജീവികളും അവയുടെ ആതിഥേയ സസ്യങ്ങളും തമ്മിലുള്ള സങ്കീർണ്ണമായ ജൈവ-രാസ പ്രവർത്തനങ്ങളുടെ ഫലമായി സസ്യങ്ങളിൽ ഉണ്ടാകുന്ന അസാധാരണ വളർച്ചയാണ് ഗാളുകൾ. സസ്യങ്ങളിലെ ഉപാപചയ പ്രവർത്തനങ്ങളിൽ മാറ്റം വരുത്തി ഔഷധഗുണമുള്ള ദ്വിതീയ ഉപാപചയ ഉൽപ്പന്നങ്ങളുടെ നിർമ്മാണം വർദ്ധിപ്പിക്കാൻ കഴിയുന്ന സവിശേഷ ഇടങ്ങളായാണ് ഗാളുകൾ അറിയപ്പെടുന്നത്. *Pauropsylla tuberculata, Pauropsylla depressa* എന്നീ കീടങ്ങൾ യഥാക്രമം *Alstonia scholaris, Ficus racemosa* എന്നീ ഔഷധസസ്യങ്ങളിൽ ഉണ്ടാക്കുന്ന ഗാളുകളെക്കുറിച്ചുള്ള തന്മാത്രാപരവും ഘടനാപരവുമായ സവിശേഷതകളാണ് ഈ പഠനം അന്വേഷിക്കുന്നത്. സാധാരണ ഇലകൾ, ഗാൾ ബാധിച്ച ഇലകൾ, ഗാളുകൾ എന്നിവയിലെ സസ്യജന്യ രാസഘടകങ്ങൾ, ജൈവിക പ്രവർത്തനങ്ങൾ, ജീനുകളുടെ പ്രവർത്തനം എന്നിവ താരതമ്യം ചെയ്ത്, ഗാൾ രൂപീകരണം വഴി ഈ സസ്യങ്ങളുടെ ചികിത്സാപരമായ സാധ്യതകൾ വർദ്ധിക്കുന്നുണ്ടോ എന്ന് പരിശോധിക്കുകയാണ് ഈ ഗവേഷണത്തിന്റെ ലക്ഷ്യം.

ഗാളുകൾക്കുള്ളിൽ ലാർവ ചേമ്പറുകളും പ്രത്യേക പോഷക കലകളും ഉള്ളതായി മൈക്രോസ്കോപ്പിക് പരിശോധനയിൽ കണ്ടെത്തി. GC-MS, OHR-LC-MS തുടങ്ങിയ പരിശോധനകളിൽ, സാധാരണ ഇലകളെ അപേക്ഷിച്ച് ഗാളുകളിലും ഗാൾ ഇലകളിലും ഫിനോളുകൾ, ഫ്ലേവനോയ്ഡുകൾ തുടങ്ങിയ രാസഘടകങ്ങൾ കൂടുതലായി അടങ്ങിയിട്ടുണ്ടെന്ന് തെളിഞ്ഞു. ചില പ്രത്യേക രാസസംയുക്തങ്ങൾ ഗാളുകളിൽ മാത്രമാണ് കണ്ടെത്തിയത് എന്നത്, ഗാൾ രൂപീകരണം സസ്യങ്ങളിലെ രാസഘടനയിൽ വൈവിധ്യം കൊണ്ടുവരുന്നു എന്നതിനെ സാധൂകരിക്കുന്നു.

സാധാരണ ഇലകളെക്കാൾ ഗാളുകളിൽ നിന്നും ഗാൾ ഇലകളിൽ നിന്നും വേർതിരിച്ചെടുത്ത സത്തിന് ആന്റിഓക്സിഡന്റ്, ആന്റിബാക്ടീരിയൽ, ആന്റിഫംഗൽ ഗുണങ്ങളും കോശങ്ങളെ നശിപ്പിക്കാനുള്ള ശേഷിയും കൂടുതലാണെന്ന് പരീക്ഷണങ്ങളിൽ കണ്ടെത്തി. ഇതിൽ *F. racemosa* -യുടെ ഗാൾ ഇലകളാണ് ഏറ്റവും മികച്ച ഫലം നൽകിയത്. Shikimic acid, Phenylpropanoid എന്നീ പാതകളിലെ പ്രധാന ജീനുകളെക്കുറിച്ചുള്ള പഠനത്തിൽ (qRT-PCR), ഔഷധമൂല്യമുള്ള രാസവസ്തുക്കളുടെ നിർമ്മാണത്തിന് സഹായിക്കുന്ന ജീനുകൾ ഗാളുകളിൽ, പ്രത്യേകിച്ച് *F. racemosa* -യിൽ, വളരെ ശക്തമായി പ്രവർത്തിക്കുന്നതായി കണ്ടെത്തി. എന്നാൽ ഗാൾ ഇലകളിൽ ഈ ജീനുകളുടെ പ്രവർത്തനം കുറവായിരുന്നു. ചുരുക്കത്തിൽ, ഗാൾ രൂപീകരണം സസ്യങ്ങളിലെ ദ്വിതീയ ഉപാപചയ പ്രവർത്തനങ്ങളെ ഉത്തേജിപ്പിക്കുകയും അതുവഴി ഔഷധമൂല്യമുള്ള രാസവസ്തുക്കളുടെ ഉത്പാദനം വർദ്ധിപ്പിക്കുകയും ചെയ്യുന്നു എന്ന് ഈ പഠനം വ്യക്തമാക്കുന്നു. ഗാളുകൾ സജീവമായ ജൈവ-രാസ നിയന്ത്രണ കേന്ദ്രങ്ങളാണെന്ന് ഈ കണ്ടെത്തലുകൾ തെളിയിക്കുന്നു. സസ്യങ്ങളും കീടങ്ങളും തമ്മിലുള്ള ഇത്തരം പ്രതിപ്രവർത്തനങ്ങളുടെ ഔഷധപരമായ പ്രാധാന്യം ഈ പഠനം എടുത്തു കാണിക്കുന്നു.

സൂചക പദങ്ങൾ: ഗാളുകൾ, പ്രാണികൾ, ദ്വിതീയ ഉപാപചയ പ്രവർത്തനങ്ങൾ, ആന്റിഓക്സിഡന്റ് ഗുണങ്ങൾ, ആന്റിബാക്ടീരിയൽ ഗുണങ്ങൾ.

CHAPTER 1 - GENERAL INTRODUCTION

India is one among the most biodiverse countries globally and is home to approximately 8,000 plant species used in both traditional and modern medicine (Kala et al., 2006; Sharma et al., 2014). India has varied climatic zones including Himalayan heights, tropical rainforests and arid desert, which supports the growth of wide range of medicinal plants. Plants have been an essential part of Indian traditional medicinal system for centuries. Plants have been used in systems like Ayurveda, Unani and Siddha, as well as being recognized in modern pharmacology. The Ayurvedia documented over 2,000 plants have therapeutic value and used as medicine (Chandra, 2016; Mehta et al., 2021).

Plants produce different secondary metabolites including alkaloids, flavonoids, terpenoids, tannins and polyphenols, which are essential for defense, adaptation and ecological interactions. These phytochemical compounds exhibit diverse pharmacological properties such as antibacterial, anti-inflammatory, antioxidant and anticancer activities. Phytochemistry is the study of plant-derived compounds especially their structure, biosynthesis and beneficial functions. It played a significant role in the development of many modern drugs from traditional herbal medicines. These modern drugs derived from bioactive phytochemical constituents, highlights the importance of scientific validation for the development of new herbal medications (Kala et al., 2006; Marrelli, 2021).

1.1. Plant galls

Plants continually interact with biotic and abiotic environmental stresses throughout their growth and development. These stresses trigger the modification of physiological, biochemical and genetic processes of plants. Plant galls, known as cecidia, are abnormal growths induced by insects, mites, fungi, bacteria or viruses, representing a plant-parasite relationship. Due to the activities of these foreign organisms, the normal plant developmental patterns will change and these modifications will result in the development of galls. The significant changes due to plant-parasite interaction will appear in the morphology, biochemistry and molecular composition of affected plant tissues. In this relationship, the host plant will provide nutrition and shelter to the inducing organisms. Cecidology is the branch of science that deals with the scientific study of plant galls, explores these complex interactions

Reshma K Ramesh. Comparative phytochemical analysis and gene expression profiling of leaf galls on selected medicinal plants. Thesis 2025. Govt. Victoria college, Palakkad, University of Calicut.

between plant and inducer organism (Albert et al., 2011; Mani, 2000; Raman, 2011; Shorthouse and Rohfritsch, 1992).

Galls show a varied pattern from normal plant development as the inducing organisms use the gall for meet their own needs. Therefore plant galls are often considered an extension of the phenotype of an inducer organism (Bailey et al., 2015; Lu et al., 2021). Galls are formed entirely of plant tissue; but they exhibit reprogrammed transcriptional pathways. So, galls are unique structures that example for how biotic stimuli like insects or other organisms can redirect plant developmental processes. After gall induction, the affected plant tissues produce much higher amounts and often different types of secondary metabolites. The research indicating higher concentrations of phytochemicals in gall tissues than unaffected plant tissue may potentially enhancing plant defense systems against herbivores (Hartley, 1998; Lu et al., 2021).

Approximately 13,000 species from various insect groups produce galls. The insect induced galls are the most complex and diverse among plant galls (Stone and Schönrogge, 2003). These galls may be develop on different plant parts like leaves, flowers, stems and roots, comprising highly organized structures with specialized tissue types (Raman, 2011; Stone and Schönrogge, 2003). Gall-inducing insects significantly modify the host plants for producing specialized tissues that are capable of provide nourishment, shelter and protection from natural enemies. Plants undergo morphological, anatomical, physiological, biochemical and molecular alterations during gall formation due to their dynamic environmental interactions (Rawat et al., 2012; Shorthouse and Rohfritsch, 1992).

1.1.1. Gall induction

Although elicitors or effectors responsible for gall formation have been reported, their precise mechanisms remain largely unknown and vary across insect classes. Plant growth regulators, such as auxins, cytokinins, indole-3-acetic acid (IAA), certain amino acids and proteins may act as potential inducing stimuli generated by the gall inducer (Gätjens-Boniche, 2019; Raman, 2011; Uma and Verghese, 2008).

Gall induction may begin when insects feed on immature leaves, then their salivary secretions acting as stimuli. Alternatively, oviposition can initiate gall formation, with female venom gland secretions triggering the process. In mites, stylets puncture epidermal cells, injecting saliva, while in Diptera and Lepidoptera, wounds formed by larval feeding release induction signals. Post-induction, insect excrement or other signals may contribute to gall development (Albert et al., 2011; Desnitskiy et al., 2023; Gätjens-Boniche, 2019; Raman, 2011; Tooker and Helms, 2014). Two fundamental events occurring during gall formation are cellular hypertrophy (cell enlargement) and hyperplasia (cell proliferation), leading to the development of galls. Reactive oxygen species (ROS) produce some metabolic reactions leading to the enhanced production of phenolics and hormones (auxins and cytokinins), that will leads to the development of gall (Dos et al., 2014).

1.1.2. Structure of gall

Galls are formed on any parts of plants and leaf galls are the most common in occurrence. Leaf galls produced on different flowering plants differ in their appearance, shape and color. Based on their complexity, they can be classified from simple thickenings to complex pouch galls enclosing the insect within differentiated organs. Complex insect-induced galls differ markedly from the host organ, forming unique tissues like nutritive tissue that nourishes the developing insect (Schultz et al., 2019).

Galls mainly consist of three regions; they are (1) lignified zone; protecting the nymphs from environmental damage or enemies, (2) larval chamber; where nymphs develop and (3) nutritive area; surrounding the chamber. The cells of nutritive region are rich in cytoplasm, organelles, lipids, carbohydrates, nitrogen, proteins and other nutrients, with fragmented vacuoles and hypertrophied nuclei (Gätjens-Boniche, 2019; Raman, 2011; Shorthouse and Rohfritsch, 1992). The outer tissues of the gall are rich in defensive chemicals such as phenolics and tannins, which protect the insect from pathogens and predators. In contrast, the inner tissues function as nutrient sinks, providing the food necessary for the growth and development of the inducer insect (Hall et al., 2017).

1.1.3. Biochemical alterations due to gall development

Plants produce diverse bioactive secondary metabolites that have antibacterial and antioxidant properties. The phenolic compounds are critical for plant defense are often more abundant in gall tissues than in normal plant tissues. The flavonoid profiles in galled leaves are modified by stress responses produced by the gall-inducer organisms. Tannins are frequently occurring in higher amounts in galled tissues, which have astringency and therapeutic properties. Alkaloids exhibit difference in their composition during gall formation. Many of these exhibited neuroprotective and antibacterial activities. A similar observation was made for terpenoids, which showed different composition in galled tissues and are widely used in medicine for their diverse therapeutic effects (Biswas et al., 2014; Eshwarappa et al., 2015; Khurshid et al., 2021; Nyman and Julkunen-Tiitto, 2000; Shorthouse and Rohfritsch, 1992; Ushir et al., 2015). These modifications in phytochemical composition are the result from altered gene expression in biosynthetic pathways of secondary metabolites. In galls, stress-induced metabolic reprogramming increasing phytochemical production to strengthen plant defenses against the inducer (Chen et al., 2018; Oates et al., 2021; Wang et al., 2017a).

These modifications in secondary metabolite composition due to gall induction may serve as defensive mechanism of plants and potentially enhance therapeutic efficacy by altering biosynthesis of phytochemical compounds (Rawat et al., 2012; Shorthouse and Rohfritsch, 1992). Gall induction also affects phytohormone metabolism, stress-response pathways, photosynthesis, organ development and nutrient mobilization. Gall inducers alter source-sink interactions by making galls as nutrient sinks vital for nymph growth in insects like *Pauropsylla* (Hirano et al., 2020; Raman, 2011; Rawat et al., 2012). Phytohormones such as auxins, cytokinins and jasmonic acid regulate plant growth and defense, with elevated levels in galled tissues causing abnormal development and altered secondary metabolite synthesis (Roy and Das, 2023; Tooker and Helms, 2014; Yamaguchi et al., 2012).

1.1.4. Molecular alterations due to gall development

Gall development results from molecular reprogramming within the host plant, with inducers altering gene expression to favor their survival. Differential expression of genes linked to secondary metabolic pathways drives these changes, enabling inducers to reorganize

host metabolic pathways (Chen et al., 2018; Oates et al., 2021; Shorthouse and Rohfritsch, 1992; Wang et al., 2017a). Transcriptomic studies of gall tissues reveal the enhanced expression of genes for alkaloid, phenylpropanoid, flavonoid and shikimic acid biosynthesis. This will leads to increased production of bioactive compounds during gall induction and thus that may enhance medicinal value of plants (Mizuki et al., 2024; Shorthouse and Rohfritsch, 1992).

The shikimic acid pathway is takes place in plastids and it links primary and secondary metabolism. It is a seven step pathway producing chorismate, a precursor to aromatic amino acids (L-phenylalanine, L-tyrosine, L-tryptophan) and secondary metabolites like phenolics and flavonoids (Herrmann and Weaver, 1999; Maeda and Dudareva, 2012; Santos-Sánchez et al., 2019). The phenylpropanoid pathway in plants produces flavonoids, tannins and lignins. During gall development key genes of this pathway get upregulated and leads to increased phenolic synthesis, potentially enhancing medicinal properties of plants (Chung et al., 2019; Hartley, 1998; Yang et al., 2022).

1.2. Significance of phytochemical and molecular study of plant galls

In plants, gall development results from complex interactions between biotic stresses produced by inducer and plant metabolism. This interaction will drive significant biochemical and molecular changes in metabolism. So, the study of these types of interactions in medicinal plants is crucial for understanding plant responses to biotic stress and their potential to enhance therapeutic compounds. Galled plant tissues show enhanced synthesis of flavonoids, tannins, alkaloids and terpenoids suggests that gall induction modifies secondary metabolism. Increased phenolics compunds protects inducers from pathogens and herbivores and also helps to improve therapeutic properties of plants (Boava et al., 2011; Duan et al., 2014; Ferreira et al., 2017; Hall et al., 2017).

The accumulation of particular compounds in gall tissue highlights the co-evolutionary interaction between gall inducers and host plants. Increased synthesis of bioactive compounds may enhance the effectiveness of herbal formulations; while reduction in significant medicinal metabolites reflects the need of strict quality assurance of traditional medicine formulations (Nacoulma et al., 2013). Gall-induced biochemical and molecular alterations could enhance therapeutic substance concentrations, supporting improved herbal

formulations. By studying the differentially expressed genes in gall tissues may reveal targets for metabolic engineering to produce phytochemicals under controlled conditions (Albert et al., 2011; Dsouza and Ravishankar, 2014; Ferreira et al., 2017; Kariñho-Betancourt et al., 2019; Raman, 2011). Understanding the gall induced changes is essential for finding how the therapeutic value of affected plants gets modified. It also helps in development of new plant-based medication approaches.

Morphological, phytochemical and molecular investigations are necessary to find the impacts of gall formation on medicinal plants. Morphological studies reveal structural alterations of normal plant tissues, phytochemical analyses identify difference in phytochemical composition and molecular investigations provide insights into regulatory mechanisms of biosynthetic pathways (Nyman and Julkunen-Tiitto, 2000; Schultz et al., 2019).

This study directly compares the gall-induced changes in two medicinally significant plants. This comparative approach is unique because it examines how distinct biochemical and molecular responses to gall induction occurs in these plants. By identifying the bioactive compounds and gene expression changes due to gall induction, the present study aims to elucidate whether gall formation enhances or diminishes medicinal properties, offering insights for phytopharmacology and plant defense strategies.

1.3. Aim of the study

The study focuses on gene expression profiling and phytochemical analysis of leaf galls of *Alstonia scholaris* (L.) R. Br. and *Ficus racemosa* L.

1.4. Objectives of the study

1. Extraction and identification of the phytochemicals in galls and regular (normal) leaves of selected plants both qualitatively and quantitatively.
2. Comparison of the gall and regular leaves for phytochemical profile.
3. Bioactivity analysis of the phytochemicals for its anti-microbial, cytotoxic and anti-inflammatory activity.
4. Bioactivity guided fractionation of the active phytochemicals.

5. Genetic profiling of the regular leaves and galls for the expression of selected genes in different phytochemical pathways involved in secondary metabolites production through quantitative RT-PCR.

1.5. Plants selected for the study

Through a thorough literature search, lists of plants that produce galls on their leaves were prepared. In order to guarantee the scientific rigor, relevance and potential for novel discoveries of the research, a number of important variables were thoroughly evaluated before the plants were chosen. The criteria adopted for selection of plants were, the therapeutic value of the plants, having medicinally important plant parts, taxonomic diversity and ecological considerations, availability and prevalence of gall formation, taxonomic position and life cycle of the inducer, phytochemical potential of their galls, practical considerations including accessibility and ensuring sufficient material for phytochemical and molecular analyses. Also based on the gall size and structures, those are helpful for precise sampling of galled and non-galled tissues, enabling accurate comparisons. The details of the selected plants are given below;

1. *Alstonia scholaris* (L.) R. Br.

Systematic position

Kingdom	: Plantae
Clade	: Tracheophytes
Clade	: Angiosperms
Clade	: Eudicots
Clade	: Asterids
Subclade	: Lamiids
Order	: Gentianales
Family	: Apocynaceae
Genus	: *Alstonia*
Species	: *Alstonia scholaris* (L.) R. Br.

A. scholaris is a medium to large sized tree, grows up to 30 m height. It is commonly known as ‘Devil Tree’, ‘Milk wood pine’ or ‘Saptaparna’. In Kerala, the plant is known as *Ezhilamppala* or *Yekshippala*. It has corky, grey to grey-white, rough bark, which is

lenticellate. Produce latex when injured, which is milky white. Branches whorled; branchlets terete, glabrous. Leaves are in a whorl of 4-7, coriaceous, glabrous, elliptic to oblanceolate, entire margin, acute base and rounded apex. The inflorescence is an umbellate cyme. Flowers bisexual, light green, pubescent, strongly perfumed. Fruits are follicles, linear with more than 20 cm long; pendulous, cylindrical and green. The Seeds are many, cylindrical, glabrous, and smooth with a tuft of hairs on both ends. Plate 1.1 represents the habit of the plant *A. scholaris.*

2. *Ficus racemosa* L.

Systematic position

Kingdom : Plantae
Clade : Tracheophytes
Clade : Angiosperms
Clade : Eudicots
Clade : Rosids
Order : Rosales
Family : Moraceae
Genus : *Ficus*
Species : *Ficus racemosa* L.

The *F. racemosa* is a large deciduous evergreen tree, which grows up to a height of 15-25 m (Plate 1.2). It is commonly known as 'Cluster Fig' or 'Indian fig'. In *Malayalam*, the plant is known as *'Atthi*'. It is one of the four species of *Ficus* that make up the well-known Ayurvedic preparation known as *'Nalpamara'*. Young shoots are glabrous, pubescent. Bark rusty brown with a fairly smooth and soft surface. Leaves are simple, dark green colored and rough when mature. Flowers are unisexual. Male and female flowers within the same fig. Male flowers found near apical pore, sessile, calyx lobes 3 or 4, stamens 2. Female flowers are pedicellate, calyx lobes linear, style lateral, stigma clavate. Fruits, 1-2 cm in diameter, subglobose or pyriform, found in large clusters on the main trunk or large branches (cauliflorous). When ripe, they are orange, reddish or dark crimson.



Plate 1.1: Habit of *Alstonia scholaris* (L.) R. Br.



Plate 1.2: Habit of *Ficus racemosa* L.

CHAPTER 2 - LITERATURE REVIEW

Plant galls are abnormal growths induced by organisms like insects, mites, fungi, bacteria and viruses and are a striking example of plant-parasite interactions. The leaf galls are not only morphologically unique but also exhibit distinct biochemical and molecular profiles, serving as nutrient-rich shelters for inducers (Gätjens-Boniche, 2019; Mani, 2000; Raman, 2011). In medicinal plants, such as *Alstonia scholaris* and *Ficus racemosa*, galls may alter the production of bioactive compounds, impacting their therapeutic potential (Biswas et al., 2014; Chauhan and Chauhan, 2020; Eshwarappa et al., 2015; Muhammad et al., 2023; Ushir et al., 2015). There are inconsistencies in the findings about gall physiology and phytochemistry particularly in medicinal species, so this chapter critically evaluates the literature on plant galls, focusing on their formation and biochemical changes, focusing on *A. scholaris* and *F. racemosa*, to identify gaps that the current study addresses.

2.1. Medicinal plants and importance of secondary metabolites

In India, for more than thousands of years, medicinal plants are the essential part of traditional medicine, where about 7,500 species are used in traditional health care systems like Ayurveda, Siddha and Unani (Kala et al., 2006). The World Health Organization (WHO) estimates that 80% of people in developing countries rely on plant-based drugs, with over 21,000 plant species being utilized for medicinal purposes globally (Mehta et al., 2021). The secondary metabolites such as alkaloids, flavonoids, phenolics and terpenoids, which are produced from plants, possess different pharmacological properties, including antibacterial, antioxidant and anti-inflammatory effects (Jain et al., 2019; Pagare et al., 2015).

The oldest form of medicine is the direct use of medicinal plants, which have been employed as traditional medicine for thousands of years in different countries worldwide (Marrelli, 2021). The pharmaceutical research has improved its reliability by identifying the function of the active phytochemical constituents found in medicinal plants and identifying their action in both human and animal systems (Jain et al., 2019). The phytochemical constitution and possible health advantages of numerous plants have not yet been thoroughly examined and require further research. Future herbal medications will thus be made possible

Reshma K Ramesh. Comparative phytochemical analysis and gene expression profiling of leaf galls on selected medicinal plants. Thesis 2025. Govt. Victoria college, Palakkad, University of Calicut.

by the essential scientific validation of these plant-based medical benefits (Kala et al., 2006; Marrelli, 2021).

Chemical defense is one of the most important mechanisms in plant resistance to diseases and herbivores. The secondary metabolites present in plants can shield them from a wide range of herbivores (such as arthropods and vertebrates) and microorganisms (such as bacteria, fungus and viruses). Additionally, these compounds play a significant role in the adaptation of plants to their environment. Thus these phytochemicals are important in defending plants against both biotic and abiotic stresses (Pagare et al., 2015; Zaynab et al., 2018). They also serve a variety of purposes, such as providing vitality, structure, signaling, stimulating and inhibiting enzymes and enhancing their own catalytic activity. Their range within the plant kingdom is relatively constrained and unique. For a given plant species growing in different environmental conditions, the phytochemicals may vary in both the quantity and quality. They are usually generated by particular cell types at different developmental stages in lower quantities (Jain et al., 2019; Pagare et al., 2015).

2.2. Introduction to plant galls

Plant galls are induced by a diverse array of organisms, including bacteria, nematodes, insects, mites and fungi, with insects and mites being the most common inducers (Bailey et al., 2015; Raman, 2011; Uma and Verghese, 2008; Ushir et al., 2015). Galls can be developed on various plant organs, among them leaf galls are predominant (Schultz et al., 2019; Ushir et al., 2015). Globally, approximately 13,000 insect species induce galls, with 2,000 reported in India and 87 gall inducing insects found from Northern Kerala (Saleem and Nasser, 2015; Stone and Schönrogge, 2003). Galls vary in shape, size and color, influenced by the host plant, inducer species and plant developmental stage (Ananthakrishnan, 1998; Mani, 2000). Uma and Abraham reported that gall making habit was found in 6 different Orders such as Diptera (flies), Hemiptera (aphids), Coleoptera (beetles), Hymenoptera (sawflies and wasps), Lepidoptera (moths and butterflies) and Thysanoptera (thrips), 90% of these insects are host specific (Uma and Verghese, 2008). The development of galls proceeds through distinct stages, including initiation, growth and differentiation, maturation and finally dehiscence (Gätjens-Boniche, 2019; Ushir et al., 2015). According to Takeda and colleagues, gall

development can be divided into 4 steps: secretion of signaling molecules from insects, perception of the signals by plants, plant cell regeneration and differentiation and organization of gall tissue (Takeda et al., 2019).

Martinson and colleagues (2021) demonstrated that gall is transcriptionally as well as anatomically complex structures (Martinson et al., 2022). Galls arise as an extension of the host-plant phenotype (Datta and Tomar, 2022) and some researchers report galls as an extension of inducers phenotype (Bailey et al., 2015; Lu et al., 2021). Galls provides food and protection for the larvae of the inducer and also protecting them from abiotic stresses and other predators (Davidovich-Rikanati et al., 2022; Gätjens-Boniche, 2019; Ushir et al., 2015). A rich cytoplasm with numerous cell organelles, broken vacuoles, a hypertrophied nucleus and nucleolus and dedifferentiated plastids grouped around the nucleus are the characteristics of a typical nutritive tissue (Schultz et al., 2019; Ushir et al., 2015). According to the 'nutrition hypothesis' the gall interiors are rich in nutrients like sugars, lipids and proteins, while exteriors are enriched with defensive compounds like phenolics and tannins to deter herbivores (Hall et al., 2017; Price et al., 1987). Nyman and Julkunen-Tiitto in 2000 support this, by reporting higher phenolic concentrations in gall exteriors to deter herbivores (Nyman and Julkunen-Tiitto, 2000). However, there exist some conflicting findings, that uniform distribution of secondary metabolites, such as terpenoids, throughout galls (Davidovich-Rikanati et al., 2022).

2.2.1. Gall induction and development

The exact mechanism of gall induction is still unknown, so that there exist different hypotheses on the induction process of galls. A physiological fluid injected during oviposition by insects would mediate gall induction. Another hypothesis proposes that gall formation is the result of mechanical irritation produced by an external organism. The third hypothesis proposes that the saliva of the inducing insect contains some active proteins along with lytic enzymes that acts as a chemical shock and leads to induction of galls. Some research suggested that rather than adult feeding, larval feeding damages the host epidermal cells, which act as a stimulus for gall induction. Finally, it has also been hypothesized that the

excretion of metabolic products from the insect may act as inductive stimuli for gall initiation (Desnitskiy et al., 2023; Gätjens-Boniche, 2019; Raman, 2011).

Researchers found that hormones like auxin and cytokinins are found to accumulate during gall induction. The interaction between these plant hormones and phenolic compounds will helps in the growth of the galls (Gätjens-Boniche, 2019; Hall et al., 2017; Hartley, 1998; Isaias et al., 2014). In combination with auxin, the cytokinins promote cell division and thus help in gall development. There are reports that, the cytokinins have been found to upregulate genes controlling the phenylpropanoid pathway, which is responsible for the synthesis of various phenolic compounds. So, they helps in the production of significantly higher amounts of phenolics and tannins in insect induced galls (Hall et al., 2017). There are no details available in literature about the working of these chemicals and the general processes by which the insect could regulate and alter the physiology and development of plants (Gätjens-Boniche, 2019; Raman, 2011).

2.2.2. Effects of gall inducers on host plants

The relationship between gall development and photosynthesis continues to be a controversial and actively researched topic. There exist several studies suggesting the reduced photosynthetic activity of galls. In this case, galls will functioning as nutrient sinks that take nutrients from surrounding tissues (Desnitskiy et al., 2023; Martinson et al., 2022). Martinson and colleagues found that the photosynthetic genes in gall tissues are downregulated, supporting the ‘sink hypothesis’ (Martinson et al., 2022). Similarly, Florentine and colleagues reported decreased transpiration and stomatal conductance in galled tissues (Florentine et al., 2005). These results suggest that the galls function as sinks, taking nutrients from surrounding non-gall tissues (Hall et al., 2017). In contrast, Fay and colleagues found enhanced photosynthesis in *Silphium integrifolium* galls and they suggest it as a compensatory response to overcome stress induced by the inducer. The reaerschers found that, this discrepancy may arise from differences in plant species, gall inducers or experimental conditions (Fay et al., 1993). Retuerto and colleagues in 2004 demonstrated that gall tissue itself increases photosynthesis relative to uninjured tissue (Retuerto et al., 2004). For instance, Takeda and colleagues in 2019 found consistent downregulation of photosynthetic genes that is in

contradictory to the compensatory hypothesis (Takeda et al., 2019). These conflicting findings highlight the important gap in understanding the effect of gall induction on photosynthetic efficiency in medicinal plants, where energy allocation could influence secondary metabolite production. Pathways for amino acid synthesis and degradation were shown to be altered in aphid galls in a way that is advantageous for the inducer. The production of glucose was found increase after gall induction (Lu et al., 2021). There are many research studies showing that gall-inducing insects influence their host plants in various ways, including biochemically, physiologically and genetically. These alterations will be performed with the aim to promote growth and development of inducers (Biswas et al., 2014; Saini and Sarin, 2012).

2.2.3. Use of plant galls

In medicinal plants, gall induction can significantly impact the concentration of bioactive compounds, such as phenolics, flavonoids, tannins, alkaloids and terpenoids. These compounds contribute to the pharmacological activities of plants, including antioxidant, antimicrobial, anti-inflammatory and anticancer properties (Ahmed and Urooj, 2010; Yamaguchi et al., 2012). Various plant galls have been reported to possess medicinal uses. Uddin in 2011 reported that galls of *Pistacia chinensis* were used in treatment of asthma, respiratory tract infection, dysentery, bronchitis, cough, vomiting skin diseases, fever and also to increase hunger (Uddin et al., 2011). In 2008 a work by Ganesh and colleagues proved the use of leaf galls of *Pongamia glabra* in the treatment of bronchitis and cough (Ganesh et al., 2008). *F. racemosa* plant is used in different systems of medicines including Ayurveda. Bark, fruits, leaf, roots, latex and seeds were found to be used along with other herbs. But no use of leaf galls of *F. racemosa* reported (Ushir et al., 2015).

2.3. *Alstonia scholaris* (L.) R. Br.

The plant belongs to the Family Apocynaceae. It is a large evergreen tree growing up to 200 m height. Almost all parts of the plant are used for treatment of different ailments in various systems of medicines like Ayurvedic, Unani and Siddha. It possesses different pharmacological activities and is potentially used as an antimalarial drug. Various extracts of different plant parts have possessed anticancer, antibacterial, antifertility, antitussive, antitumour, antidiabetic, anthelmintic and antiepileptic properties (Bonvicini et al., 2014;

Kanase and Mane, 2018; Khan et al., 2003; Saini and Sarin, 2012). The parts were used as febrifuge, also used as tonic for digestion and stimulation of appetite. Used in treatment of liver and intestinal troubles, malaria, diarrhea, dysentery, gonorrhea, hypertension, fever, leprosy, skin diseases, bronchitis, asthma and lung cancer. The bark is anthelmintic, astringent and antiperiodic. It is used against chronic diarrhoea, dysentery and bowel movements. Alkaloids present in it can cause a rapid fall in the blood pressures and are hypotensives. Leaves are used against liver congestion, dropsy and ulcers. The latex is useful as an application for ulcers, sores, tumours and in rheumatoid pain (Bonvicini et al., 2014; Khan et al., 2003). The stem-bark has been used in the antimalarial drug formulation 'Ayush64', developed by the Central Council for Research in Ayurvedic Sciences, Calcutta (Das et al., 2020).

Different classes of secondary metabolic compounds have been isolated from the plant, including alkaloids, flavonoids and terpenoids. Alkaloids such as alstonidine, alstonine, chlorogenic acid, chlorogenine, echitine and echitamine. Triterpenoids include linoleate and lupeol (Bonvicini et al., 2014; Das et al., 2020). Echitamine, Echitamine chloride, Scholarine, Scholaricine, monoterpenoid indole alkaloids, iridoids, coumarins, phenolics, steroids, saponins and tannins were also reported to be present in the leaves and bark of the plant (Saini and Sarin, 2012).

Leaf galls of *A. scholaris*

Leaf galls of *A. scholaris* are pouch-like and globular formed on the upper surface of the leaf as isolated or as agglomerate structures (Tripathy et al., 2019). Young galls are green or light green in colour and when they mature change to brownish or yellowish with a hard woody texture (Bhatnagar et al., 2022). The galls are produced due to infection by the insect, *Pauropsylla tuberculata*. The life cycle of this insect passes through the egg stage, nymphal instars and then the adult stage. *Pauropsylla* is a hemimetabolous insect, which has no larval stage (Albert et al., 2011; Pasayat and Tripathy, 2020). Biochemical analysis shows that phenolic compounds were found to be higher in galls compared to leaves (Biswas et al., 2014; Muhammad et al., 2023). High proline and phenol contents and low chlorophyll level was observed in gall by Rasool and colleagues in 2019 (Rasool et al., 2019).

2.4. *Ficus racemosa* L.

F. racemosa is an important medicinal tree in India. The plant is used for treatment of diabetes, liver disorders, respiratory, urinary diseases and inflammatory conditions. Different parts of the plant such as root, bark, latex, leaves, fruit, seeds and galls were medicinally important (Eshwarappa et al., 2015; Kumari et al., 2020; Singh et al., 2023). The various extracts of plant parts have diverse biological activities such as anti-inflammatory, hepatoprotective, chemopreventive, antidiabetic, antipyretic, cardio-protective, mosquito larvicidal, gastroprotective, antidiuretic and hypolipidemic activity. The roots of the plants are used for the treatment of diarrhea and hydrophobia. The milky juice produced from the bark is used to treat piles and diarrhea. In combination with sesamum oil, there is report of use of it to treat cancer. The leaves are also useful for bronchitis and are astringent to the bowels. The bark and fruit are astringent and used in the treatment of haematuria, menorrhagia and hemoptysis (Bagyalakshmi et al., 2019; Singh et al., 2023; Ushir et al., 2015) The flavonoids and polyphenols present in the plants help in prevention and cure of many diseases (Bagyalakshmi et al., 2019; Chaware et al., 2020).

Leaf galls of *F. racemosa*

Leaf galls found in *F. racemosa* are induced by the insect, *Pauropsylla depressa*. The pouch galls developed on the leaves are simple, fleshy, globose, sessile, unilocular, sometimes agglomerate or multilocular. Colour varies from yellow to reddish brown. The galls measure about 15- 30 mm in diameter (Savitha et al., 2013; Singh et al., 2023). Galls were initially green during development, turned brown as they matured and then turned reddish brown during the senescence stage (Ushir et al., 2015).

The galls contained different active phytochemicals such as flavonoids, triterpenoids, alkaloids and phenolics. They also reported that the leaf galls are pharmacologically significant as the TLC analysis of ethanolic extract also proved the presence of active phytoconstituents (Ushir et al., 2015). The use of leaf galls of *F. racemosa*, reported by Kumari and colleagues in the preparation of ‘Chvyanprash avaleha’ and ‘Kumari Asava’ (Eshwarappa et al., 2015; Kumari et al., 2020).

2.5. Phytochemical composition of the selected plants

Notable differences between the phytochemical composition of healthy leaves and galls were reported in previous studies on selected plants, *A. scholaris* and *F. racemosa*. During the insects attack, the plants produce more defensive chemicals and thus the galls have higher levels of certain compounds compared to normal leaves. Advanced techniques like Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS) help identify these compounds, revealing the phytochemical profiles in galls.

2.5.1. Phytochemical composition of normal leaves

There are reports on the chemical constituents of *A. scholaris* and *F. racemosa* leaves. For *A. scholaris*, Labaran and colleagues (2024) found that methanolic extracts contain phenolics, flavonoids and alkaloids, but lack proteins, anthraquinone or reducing sugar. In contrast, aqueous extracts include reducing sugar, amino acids, phenolic compounds, anthraquinone and saponins (Labaran et al., 2024). For *F. racemosa*, both aqueous and methanolic leaf extracts revealed saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids (Mpala et al., 2019). Additional studies identified lanosterol, tetracyclic triterpene-glauanol acetate alkaloids, tannins and significant amounts of total phenols and flavonoids in *F. racemosa* leaves (Pahari et al., 2022; Yadav et al., 2019).

2.5.2. The effect of insect attack on phytochemical composition of plants

Plants produce defensive secondary metabolites, including nitrogenous alkaloids, terpenes, phenols and flavonoids, in response to insect attacks. This is supported by multiple studies done in 2019-2022 (Davidovich-Rikanati et al., 2022; Qi et al., 2020; Takeda et al., 2019). For instance, tobacco plants infected with *Rhodococcus fascians* exhibit significant metabolic changes, yielding new compounds with therapeutic potential (Nacoulma et al., 2013). Qi and colleagues found that, the insect attacks can trigger the production of over 200 distinct volatile compounds, which helps in enhancing plant defense (Qi et al., 2020).

2.5.3. Phytochemical profile of plant galls

Galls exhibit unique phytochemical profiles compared to normal leaf tissues. High-performance thin layer chromatography (HPTLC) fingerprinting revealed clear variations in phytoconstituents between leaves with and without galls (Das et al., 2020). Mizuki and co-workers identified that, galls typically contain higher concentrations of total phenolics, terpenes and tannins across all growth stages (Mizuki et al., 2024). However, some studies report no significant difference in volatile compounds between galled and normal tissues (Hall et al., 2017). The 'nutrition hypothesis' suggests that galls accumulate higher nutrient concentrations internally with reduced defensive compounds, while their exterior has elevated phenolics and tannins to deter herbivory (Hall et al., 2017; Price et al., 1987). This is supported by the findings of (Nyman and Julkunen-Tiitto in 2000 that bioactive phytochemicals are rich in gall exteriors to reduce predation risk (Nyman and Julkunen-Tiitto, 2000).

Each type of gall exhibits a distinct metabolomic profile. In some cases there exist similarities in metabolomic alterations following gall induction across different plant species; it may indicate the presence of common induction mechanisms. But the differences in metabolomics profile could reflect species-specific gall structures or causal agents (Markel et al., 2024).

Specific findings of phytochemical constituents in galls of *A. scholaris*

In galled leaves of *A. scholaris* reported the presence of an alkaloid Alstorisine A by Chen and colleagues in 2016 (Chen et al., 2016). Other identified compounds include ursolic acid, lupeol acetate, 17-nor-excelsinidine, 19, 20 E-vallesamine and an unidentified monoterpene indole alkaloid, 2-hydroxy deacetyl akuammiline (Pandey et al., 2023). Galls are considered a promising source of bioactive phytochemicals (Pandey and Kate, 2024). Proline content in galled tissue is significantly elevated; indicating stress, with mature galls containing approximately twice as much as young galls (Albert et al., 2011). A negative correlation exists between phenolic compound production and chlorophyll content, with perforated galls showing higher phenolic content than mature or young galls (Biswas et al., 2014; Muhammad et al., 2023).

Specific findings of phytochemical constituents in galls of *F. racemosa*

F. racemosa galls are rich in pharmacologically important phytochemicals, including alkaloids, phenolic compounds and terpenoids, with high levels of phenolics, lignins and tannins (Bagyalakshmi et al., 2019; Ushir et al., 2015). Phenolic content in galls exceeds that in healthy or galled leaves, with elevated orthodihydric and total phenols. Polyphenols and dihydroxyphenols act as IAA-oxidase inhibitors, contributing to gall development (Dsouza and Ravishankar, 2014). Methanolic gall extracts exhibit higher phenolic and flavonoid content, resulting in greater antioxidant activity (Eshwarappa et al., 2015).

2.5.4. Analytical techniques used to find metabolomic profiles

Advanced analytical methods, such as Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS), are effective for identifying bioactive phytochemicals in plants (Goyal and Shinde, 2020). For *A. scholaris,* GC-MS analysis of stem bark identified compounds like hexadecanoic acid methyl ester, 1,4-benzenedicarboxylic acid dimethyl ester and dodecanoic acid methyl ester (Bagheri et al., 2020). Carranza and colleagues found 18 volatile compounds from dichloromethane bark extracts (Carranza et al., 2020). Analysis of leaf extract confirmed alkaloids such as Akuammidine, Voacristine, Strictamine and Nicotine (Hamdiani et al., 2018), with metabolic extracts identifying seven phytoconstituents (Chakraborthy et al., 2016). In case of *F. racemosa*, GC-MS revealed bioactive secondary metabolites in leaves, with isoamyl nitrite showing the highest peak area (Sharma and Kumar, 2021). Phytochemical profiling identified 48 phytoconstituents in fruit extract and 80 in leaf extract (Rani et al., 2024), with other studies reporting six to ten compounds in fruits (Kannan et al., 2024; Swargiary et al., 2020).

LC-MS/MS analysis of *A. scholaris* latex detected quercetin, kaempferol and ursolic acid (Banik et al., 2023), while bark analysis yielded different active phytochemicals (Carranza et al., 2020). For *F. racemosa*, LC-MS of aqueous extract confirmed 11 phytochemical compounds, with phloretin (64.165%) and choline (11.933%) showing the highest area percentages (Dhas et al., 2021). Chloroform extract analysis identified 10 active compounds in *F. racemosa* leaves (Shaikh et al., 2020).

2.6. Biological properties of different parts of selected plants

2.6.1 Antioxidant activity

Research indicates that *A. scholaris* exhibit significant antioxidant activity. Methanol extracts of stem bark and leaves demonstrate strong DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical scavenging, with ethyl acetate extracts following closely. The phenolic compounds are key contributors of antioxidant activity (Itam et al., 2018). The bark shows mild to moderate DPPH free radical scavenging activity (Bellah et al., 2017), while ethanolic leaf extracts are highly effective across various assays (Arulmozhi et al., 2010). In leaf galls, antioxidant enzymes (catalase and peroxidase) correlate positively with phenolic content, enhancing the antioxidant capacity of plants (Biswas et al., 2014).

For *F. racemosa*, DPPH assays reveal that fruit extracts have greater antioxidant activity than leaf extracts (Bagyalakshmi et al., 2019). Seeds outperform leaves in antioxidant potential and leaf extracts exhibit dose-dependent reducing power (Hosain et al., 2011). Yadav conducted different methods of antioxidant assays in leaves of the plant and found that it has dose dependent reducing power activity (Yadav et al., 2019). *F. racemosa* stem bark and leaves found to exhibit significant free radical scavenging activity (Sultana et al., 2014). DPPH free radical scavenging activity, reducing power assay and nitric oxide scavenging activity analysis were done by Londhe and colleagues 2012 to prove the antioxidant activity of ethanolic extracts of leaves of *F. racemosa* and found that the leaves were potential source of plant antioxidants (Londhe et al., 2012). Sumi and colleagues also investigated reducing capacity of the leaves of the plant by using different approaches and proved the same (Sumi et al., 2016). A work done by Eshwarappa and colleagues proves the significance of *F. racemosa* leaf gall extracts as a natural antioxidant and justify its ethnobotanical use (Eshwarappa et al., 2015). With an 80% free radical scavenging potential, young galls seem to be a strong source of antioxidants (Savitha et al., 2013).

2.6.2. Antibacterial activity

Different parts of *A. scholaris* and *F. racemosa* were checked for its antibacterial property. The stem bark extracts of *A. scholaris* were determined active against reference strains (Bonvicini et al., 2014; Lalchhandama et al., 2024) and clinical isolates (Bonvicini et

al., 2014). Methanolic extracts of leaf, stem, root and bark extracts of *A. scholaris* have been reported to have potent antimicrobial activity (Khan et al., 2003). Flower extracts (benzene, methanol, water) are more effective against Gram-positive bacteria, while hexane and water extracts lack activity against *Pseudomonas aeruginosa* and *Salmonella typhi* (Thankamani, 2011). Hexane, water, benzene, isopropanol and methanol fractions of *A. scholaris* bark and leaf were tested against different bacteria and except isopropanol and methanol extract none of the other extracts showed no activity (Bagheri et al., 2020). Researchers found that metabolic extracts of the leaves of *A. scholaris* have higher activity than other extracts (Labaran et al., 2024).

The disc diffusion assay showed no antibacterial activity from methanolic and aqueous extracts of *F. racemosa* leaves against a variety of human pathogenic bacteria, such as *Staphylococcus aureus* and *Escherichia coli* (Mpala et al., 2019). But there is a report that leaves of *F. racemosa* were found active against the growth of *Actinomyces viscosus* (Shaikh et al., 2010). Antibacterial activity of leaf and fruit of *F. racemosa* proved that the extracts possess good inhibition activity against wound isolates (Bagyalakshmi et al., 2019). The seeds and leaves of *F. racemosa* were tested against Gram-positive and Gram-negative bacteria and found that both showed moderate activity (Hosain et al., 2011). Kumari and colleagues checked the antibacterial activity of leaf gall extracts of *F. racemosa* and reported that gall extracts are more inhibitory to Gram-negative bacteria in comparison to Gram-positive bacteria. The gall extracts have more inhibitory effects than leaf extracts against the fifteen bacterial isolates tested (Kumari et al., 2020). It was found that silver nanoparticles made from *F. racemosa* gall extracts had strong antibacterial action against *S. aureus* and *E. coli*. Significant anticancer activities have also been demonstrated (Sabira et al., 2024).

2.6.3. Antifungal activity

Previous studies on different parts of both plants proved its antifungal activity. Bonvicini and colleagues proved the antifungal activity of stem bark extracts of *A. scholaris* against *Candida albicans* (Bonvicini et al., 2014). Isopropanol, methanol and water extract of leaf found to inhibit the growth of *Candida* spp and *Cryptococcus* spp (Bagheri et al., 2020). The bark carbon tetrachloride and chloroform extracts showed growth inhibition to

Aspergillus niger and *C. albicans* (Bellah et al., 2017). Crude ethanolic extract of leaves showed antifungal activity against *C. albicans* (Ara et al., 2020).

Methanolic extracts of leaves of *F. racemosa* showed better antifungal action against both *C. albicans* and *A. niger* (Mishra et al., 2023). The ethanolic and ethyl acetate extract of fruits also showed antimicrobial activity against *C. albicans* and *A. niger*, these properties support the traditional use of *F. racemosa* for treating fungal infections (Pingale et al., 2019).

2.6.4. Cytotoxic activity

A. scholaris found to have strong cytotoxic effects against cancer cells. Studies show that the n-hexane fraction of *A. scholaris* is highly toxic to breast cancer (MCF-7) cells, while the ethanol fraction is less harmful to vero cells (Zuraida and Mariya, 2020). Bark extracts (methanol) also showed significant cytotoxicity in brine shrimp lethality tests (Bellah et al., 2017). Bagheri and colleagues identified that, hexane extracts of stem bark and leaves has promising anticancer activity against various cancer cell lines using different assays such as trypan blue exclusion, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and apoptotic assays (Bagheri et al., 2016). Leaf extracts (ethyl acetate fraction), show high cytotoxicity against brine shrimp (*Artemia salina*) and leaves are noted for significant bioactivity (Ara et al., 2020; Sarkar et al., 2021).

Evidence indicates that *F. racemosa* has notable cytotoxic properties. Methanol extracts of seeds show good cytotoxicity in brine shrimp lethality tests (Hosain et al., 2011). Hexane extracts exhibit strong anticancer activity against human lung cancer cell lines in XTT cell proliferation assays (Gorla and Shankar, 2016). *In-vivo* studies of methanolic extracts from bark, fruits and leaves demonstrate anticancer effects in Swiss albino mice with Dalton lymphoma ascites tumors, using trypan blue and MTT assays (Poongothai and Annapoorani, 2021).

2.6.5. Anti-inflammatory activity

Inflammation is the natural way of the body for protecting itself and is important for survival. But if it continues for too long, due to constant release of certain chemicals and ongoing harmful signals, it can turn into a long-lasting problem. So, finding safe and effective

ways to stop inflammation is important. Medicinal plants are an important source for developing new and effective medicines (Nunes et al., 2020). There are so many *in vitro* assays for finding anti-inflammatory activity; they are inhibition of protein denaturation method, human red blood cell membrane stabilization test, proteinase inhibitory assay etc. The water extracts of *F. racemosa* bark demonstrated a concentration-dependent increase in inhibition of egg albumin denaturation. Among the extracts, hot water preparations exhibited significantly higher inhibition compared to cold water extracts. The IC_{50} values were 3691.97 μg/mL for the cold water extract and 4207.1 μg/mL for the hot water extract (Dharmadeva et al., 2018). Arulmozhi and colleagues in 2007 evaluated the anti-inflammatory activity of ethanolic extracts of *A. scholaris* leaves using carrageenan-induced rat paw oedema in male albino Wistar rats and reported significant activity (Arulmozhi et al., 2007). Banik and Das demonstrated that the latex of *A. scholaris* exhibited significant anti-inflammatory activity when assessed using the egg albumin denaturation method (Banik and Das, 2023). Mandal and colleagues reported that *F. racemosa* leaf extract exhibited significant anti-inflammatory activity in carrageenan, serotonin, histamine and dextran-induced rat hind paw oedema models at different doses (Mandal et al., 2000a). Aqueous extracts of *F. racemosa* bark demonstrated significant anti-inflammatory activity in rat models, with intraperitoneal administration at 20 mg/100 g inhibiting carrageenan-induced acute inflammation and at 30 mg/100 g suppressing formalin-induced subacute inflammation (Mehta et al., 2012).

2.7. Genes involved in regulation of secondary metabolic pathways

Biotic and abiotic stresses, such as insect attacks, mechanical wounding, drought and fungal infections, induce significant metabolic shifts in host plants by altering gene expression in secondary metabolite pathways. These changes enhance the synthesis and accumulation of phytochemicals, including phenolics, terpenoids and flavonoids, which serve as defense compounds or structural components (Boava et al., 2011; Løvdal et al., 2010; Ralph et al., 2006b). The shikimic acid and phenylpropanoid pathways are critical for producing these compounds and genes regulating key steps in these pathways were selected for the current study.

2.7.1. Gall induction and gene expression

Gall-inducing insects, such as aphids manipulate host plants to enhance their ecological fitness by upregulating genes in secondary metabolic pathways. For example, aphids increase the expression of terpenoid pathway genes, leading to high monoterpene concentrations in galls (Davidovich-Rikanati et al., 2022). Gene expression in galls shows similarities with adjacent host tissues, but galls experience the greatest stress from the inducer, resulting in more pronounced gene expression changes (Lu et al., 2021). Gall inducers control host plant metabolism, upregulating genes involved in DNA synthesis, secondary metabolism, cell division, tissue development and formation of structural proteins (Lu et al., 2021). Research by Wang and colleagues (2017) and Martinson and co-workers (2022) identified numerous genes associated with secondary metabolism that are more active in galls than in normal leaf tissues (Martinson et al., 2022; Wang et al., 2017a). *De novo* transcriptome analysis of *Quercus rubra* galls, using RNA extracted from inner and outer gall tissues and adjacent leaves, revealed increased expression of genes related to morphogenesis and secondary metabolite production (Martinson et al., 2022). A study by Lu and colleagues (2021) found significant differences in gene expression between galls and normal leaves, with 18 genes involved in phenylpropanoid metabolism (e.g., flavonols, isoflavonoids, anthocyanins) upregulated and only 9 downregulated (Lu et al., 2021). Genes related to carotenoid and terpenoid backbone biosynthesis also showed altered expression in galls. Phenylpropanoids contribute to defense against herbivores, regulate hormone transport and support new cell wall formation in growing galls (Martinson et al., 2022).

2.7.2. Genes selected for the study

3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS) is an important gene in shikimic acid pathway which catalyzes the condensation of phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P). cDNA microarray and RNA-seq analyses show elevated transcript levels in response to mechanical wounding or insect feeding (Ralph et al., 2006a; Ralph et al., 2006b). 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene produces carboxyvinyl transferase enzyme, which involved in the shikimic acid pathway. The gene expression found to vary in tissue, time and abiotic stresses like drought, salt and cold (Garg et al., 2014). According to RNA-seq and transcriptome profiling, the chorismate

synthase (CS) gene, which is involved in the production of lignin, showed elevated expression during wounding. There are evidences for the accumulation of phenolic compounds linked to this gene in broccoli (Torres-Contreras et al., 2018). Boava and colleagues reported that mechanical wounding enhanced the expression of phenylalanine ammonia-lyase (PAL) gene (Boava et al., 2011). Investigations on upregulation of PAL due to fungal infections (Chen et al., 2009; Rukmini et al., 2017; Sayari et al., 2014), herbivores (Duan et al., 2014) and abiotic stresses (Løvdal et al., 2010) are reported. RNA-seq has confirmed its role in phenylpropanoid biosynthesis.

Chalcone-flavanone isomerase (CHI) gene involved in flavonoid production found to be modified by insect attacks, microbes or mechanical wounding. Dao and co-workers done RNA-seq and qPCR studies to highlight its expression in stressed condition (Dao et al., 2011). Finally RNA-seq investigations of the Dihydroflavonol 4-reductase (DFR) gene demonstrate upregulation in response to fungal infections (Yang et al., 2022), drought stress (André et al., 2009; Ma et al., 2014) and biotic stresses (Kumar et al., 2013), leading to flavonoid accumulation.

The review of literature found several unresolved aspects related to plant galls and their development, such as photosynthetic activity, secondary metabolite distribution, and differential gene expression. There are conflicting reports on photosynthetic activity whether galls reduce, maintain or increase photosynthesis (Fay et al., 1993; Martinson et al., 2022). It requires clarification, especially in medicinal plants where energy allocation affects metabolite production. Secondary metabolite distribution in normal leaves and galls need more detailed analysis to resolve the discrepancies of whether metabolites are concentrated in gall exteriors or interiors and to study the impact in their therapeutic potential. Also need to reveal the molecular basis of structural, biochemical and physiological alteration due to gall formation (Davidovich-Rikanati et al., 2022; Hall et al., 2017).

CHAPTER 3 - STRUCTURE AND PHYTOCHEMISTRY OF LEAF GALLS ON SELECTED MEDICINAL PLANTS

3.1. INTRODUCTION

The present chapter deals with the qualitative and quantitative phytochemical analysis of healthy leaves (normal leaves), galled leaves and galls of selected medicinal plants. Two plants were selected for the study: *Alstonia scholaris* (L.) R. Br. and *Ficus racemosa* L. This chapter provides insights into the differential phytochemical profiles resulting from gall formation in comparison with healthy leaves. The chapter also sheds light on the anatomical differentiations produced in healthy leaves due to gall induction. This provides a complete comprehension of anatomical and biochemical differentiation in plants due to gall formation.

3.1.1. Plant galls

Plant galls are abnormal growths on formed on different plant parts resulting from hypertrophy and hyperplasia. The galls are produced by the activity of foreign organism, which may range from microorganisms to macroorganisms such as insects, bacteria, fungi, nematodes or mites. These structures represent complex interaction between plants and gall-inducing organisms (Mani, 2000; Uma and Verghese, 2008). The galls are classified into leaf, bud, stem, flower or fruit galls according to the part of the plant in which they originate. They exhibit diverse shapes and textures (round, oblong, globular, discoid, egg-shaped, spindle-shaped or bottle-shaped). Some galls are hairy or spiny, while others have rough or glabrous surfaces. Based on the number of cavities, galls are categorized as unilocular or multilocular. Gall showed three distinct phases of growth: the developmental stage, the maturation stage and finally senescence stage (Raman, 2011; Ushir et al., 2015).

Among gall-inducing organisms, insects and midges are the most common. In India, over 2,000 species of gall-inducing insects have been identified, with 87 morphotypes of insect-induced galls reported in Northern Kerala (Saleem and Nasser, 2015; Uma and Verghese, 2008). Most gall-inducing insects are specific to particular host plants. Insect-mediated gall induction often starts with the activity of salivary secretions, which act as a chemical stimulus. Inducer insects create a small wound, allowing salivary amino acids and

Reshma K Ramesh. Comparative phytochemical analysis and gene expression profiling of leaf galls on selected medicinal plants. Thesis 2025. Govt. Victoria college, Palakkad, University of Calicut.

proteins to enter the host plant, change the osmotic balance and create stressed condition. Then stress driven excessive growth and cell division occur. This may be the result of altered enzymatic reactions and modified chemical constituents in the affected tissues. Gall inducers modify host plant biochemistry and physiology through altered metabolic pathways to support their development, resulting in a chemical composition of gall distinct from surrounding normal plant tissue. These differences arise from altered metabolic pathways of host plants that arise through differential molecular processes in response to stress (Hartley, 1998; Raman, 2011).

Gall induction causes tissue redifferentiation, transcriptional changes, increased protein content, decreased chlorophyll content, transition of leaves from source to sink, accumulation of phytochemicals such as phenolic compounds and elevated proline levels in the affected tissues. These changes are produced due to altered gene expression patterns induced by the gall inducer. The secondary metabolites like phenolics, determines therapeutic potential of plants as they have antioxidant, anti-inflammatory, anticancer and antimicrobial properties. The gall induction enhances the medicinal value of plants for applications such as antioxidant and anti-inflammatory treatments (Albert et al., 2011; Dsouza and Ravishankar, 2014; Ferreira et al., 2017). The therapeutic potential of plant galls is attributed to their unique phytochemical composition, which is distinct from that of normal plant tissues. The accumulation of secondary metabolites like phenolics, flavonoids and tannins in galls provides them with enhanced biological activities, making them valuable resources for developing natural antioxidants and other medicinal agents (Saleem and Nasser, 2015).

3.1.2. Overview of plant secondary metabolites and their functions

Secondary metabolites are diverse group of compounds produced by plants and are not directly involved in growth or development of plant, but play critical roles in plant interactions. These compounds have significant role in defense against pathogens and herbivores, facilitation of symbiosis, signaling and stress response etc. (Croteau et al., 2000; Taiz and Zeiger, 2010). Recent advancements in biochemical and sequencing technologies have significantly enhanced our understanding of these compounds, revealing their

biosynthetic pathways, genetic regulation and ecological significance (Dixon and Dickinson, 2024; Wang et al., 2024).

Plant secondary metabolites can be classified into three distict chemical catogaries; they are terpenoids, phenolics and nitrogen containing compounds. Terpenoids are the largest class of secondary metabolites (30,000 compounds). They are derived from isoprene units having five carbons. They are synthesized by two separate mechanisms; methylerythritol phosphate (MEP) pathway (plastids) and mevalonate pathway (cytosol). Terpenoids perform a variety of functions in plants, including functioning as hormones (e.g., gibberellins, abscisic acid), pigments (e.g., carotenoids) and mediators of plant-plant or plant-insect interactions. They also participate in the defense mechanisms against herbivores and diseases. Some volatile terpenoid molecules repel herbivores or attract herbivore predators, while others are feeding deterrents (Taiz and Zeiger, 2010).

Phenolic compounds have a structure consisting of one or more hydroxyl groups attached to aromatic rings. These chemicals are mostly synthesized through the shikimate pathway which results in the formation of phenylalanine, the precursor of the majority of phenolic compounds. There are different types of phenolic compounds; they are flavonoids, tannins, lignins etc. Flavonoids play important roles in UV protection, flower colouration and disease defense. Tannins deter herbivores by making plant tissues less palatable. Lignins give plants structural support by reinforcing cell walls, especially in vascular tissues. Phenolics have antioxidant properties, which helps plants to survive oxidative stress produced by environmental conditions such as high light intensity, drought and disease infections. Some of the compounds are phytoalexins, useful against micro-organisms (Johnson et al., 2021; Mamari, 2021; Taiz and Zeiger, 2010).

Nitrogen-containing secondary metabolites are a broad collection that includes alkaloids, cyanogenic glycosides and glucosinolates. These chemicals frequently play important roles in plant defense. Alkaloids are nitrogen-containing molecules produced from amino acids like tryptophan, tyrosine and ornithine. They are known for their significant biological activities like antiviral, antibacterial, anti-inflammatory and anticancer properties. Because of their bitter taste and toxicity, alkaloids are frequently used to deter herbivores,

thus helping in plant defense. Cyanogenic glycosides are produced from amino acids and can generate hydrogen cyanide (HCN) when plant tissue is injured. They are found in a number of plant species and serve as a defense mechanism against herbivores. When an animal bites on the plant, the glycosides are hydrolysed, producing cyanide, which inhibits the cellular respiration of herbivores and thus inhibits feeding (Adamski et al., 2020; Taiz and Zeiger, 2010).

Glucosinolates are sulfur containing chemicals generated from amino acids. When plant tissue is damaged, it produces isothiocyanates that are harmful to many insects and microorganisms. Saponins are glycosides from both triterpenes and steroids. This reduces serum cholesterol by preventing its reabsorption. Research suggests that saponins may be able to cure hypercholesterolaemia by preventing the uptake of cholesterol. It possesses cardiac depressant and hypotensive actions. Tannins are complex phenolic polymers. It has the ability to bind to proteins and carbohydrates, decreasing their digestibility and so inhibiting microbial development. Tannins possess astringent properties (Soetan and Aiyelaagbe, 2009).

Plant galls can be viewed as extensions of the host plant phenotype as they have the same genetic makeup as normal tissues, but exhibit distinct phytochemistry and this may due to differential gene expression (Raman, 2011). The accumulation of secondary metabolites like phenolics and flavonoids in galls not only aids in plant defense but also provides valuable bioactive compounds for medicinal applications, particularly as natural antioxidants and anti-inflammatory agents (Dsouza and Ravishankar, 2014; Ferreira et al., 2017).

In India, plant galls have been used for their therapeutic potential. Galls from genera such as *Rhus, Pistacia, Quercus* and *Terminalia* are utilized in Indian folk remedies, including Ayurveda, Unani and Siddha, for treating various ailments (Patel et al., 2018). This chapter compares the structure and phytochemistry of normal leaf tissues and gall tissues in selected plants to elucidate the biochemical differences, particularly the enhanced phenolic content in galls, which may increase their therapeutic potential.

3.2. METHODOLOGY

3.2.1. Structure of normal leaves and leaf galls

Collection of plants

Mature plants of *Alstonia scholaris* (L.) R. Br. and *Ficus racemosa* L. grown in different regions of Palakkad district were chosen to collect normal leaves and leaves with galls. Normal leaves are those that were fully grown, healthy and showed no symptoms of infection or abnormal growth. Leaf galls are abnormal outgrowths present on leaves, varying in size, shape and position. In order to prevent damage, some normal and galled leaves were carefully collected by hand using sterile scissors. The collection was done during the active growing season, when both kinds of leaves were abundant.

Every sample collected, identified and authenticated using Flora of the Presidency of Madras (Gamble and Fischer, 1936) and Flowering Plants of Kerala (Sasidharan, 2013). During collection, the sample was tagged with the date, plant part collected and species name. For reference, field photography was also done. To preserve tissue integrity, fresh samples were brought to the lab in insulated containers and wrapped in moist paper or towels. Voucher specimens of all collections prepared as herbarium following standard procedures and were deposited in Herbarium of Government Victoria College (GVCH), Palakkad, Kerala, with accession numbers GVCHP-SV 768 and GVCHP-SV 769 for *A. scholaris* and *F. racemosa* respectively. The inducer organism (insect) responsible for gall formation in *A. scholaris* and *F. racemosa* leaves were isolated from mature galls. Species identification and authentication was conducted by Dr. K. Abdul Rasheed, Assistant Professor, Department of Zoology, Government Victoria College, Palakkad, using standard taxonomic procedures. The authenticated voucher specimens were subsequently deposited in the Museum of Government Victoria College, Palakkad for future reference.

Morphological study

The healthy leaves (normal leaves) and leaf galls were collected from the field and maintained their freshness for morphological and anatomical studies. Galls were separated from galled leaves and analysed under a dissection microscope in the laboratory. For

morphological documentation, the Leica Sapo Stereo Microscope was used. Photographs of different developmental stages of leaf galls were taken from the field as well.

Anatomical study

Anatomical study was conducted by taking free hand sections. Thin sections were selected, stained with safranin, mounted in glycerin, observed through microscope and images were transferred to the computer. Leica DM 1000 Stereo Microscope with Leica DFC 295 digital camera connected to the computer and Leica Application Software LAS Version 3.6.1 was used for anatomical study. The transverse sections taken were observed and compared to find out the difference induced by the gall inducing insects in the normal leaves (Johansen, 1940).

3.2.2. Phytochemical analysis of plant extracts

Collection of plants

Healthy plants of *Alstonia scholaris* (L.) R. Br. and *Ficus racemosa* L. were selected and their leaves were collected. *A. scholaris* and *F. racemosa* plants with galls were located in different regions of Palakkad district and gall containing leaves were collected from areas of its abundance. The gall tissues were separated from the leaves immediately after collection. The normal leaves, gall leaves and the galls were separately dried in shade for pulverization and for further extraction.

Extraction of plant samples with selected solvents

The collected tissues, including normal leaves, galled leaves and galls of *A. scholaris* and *F. racemosa,* were thoroughly cleaned and air-dried. The dried samples were pulverized into a fine powder using a mechanical blender. The powdered samples were transferred into airtight containers, properly labeled and stored at room temperature for subsequent use. For extraction, 25 g of each pulverized sample was weighed and extracted using a Soxhlet apparatus with solvents of varying polarity to capture a broad spectrum of secondary metabolites.

The choice of solvents was guided by their polarity, which determines their ability to extract specific secondary metabolites, such as phenolics, flavonoids, tannins, alkaloids, terpenoids and saponins, known to be present in *A. scholaris* and *F. racemosa*. The solvents cover a polarity gradient, ensuring comprehensive extraction of both polar and nonpolar compounds, which is critical for comparing phytochemical profiles across normal leaves, gall leaves and galls. The solvents selected for our study are listed below;

1. **Water** is a highly polar solvent, it is ideal for extracting highly polar compounds such as phenolic glycosides, hydrolyzable tannins, saponins and cardiac glycosides.
2. **Ethanol** is a moderately polar solvent having amphiphilic nature, with a polar hydroxyl group and a non-polar ethyl group, allowing it to extract both polar and moderately non-polar compounds. Ethanol is highly effective for extracting flavonoids, phenolics, tannins and some alkaloids.
3. **Hexane** is a highly non-polar solvent, suitable for extracting lipophilic compounds. Targeting terpenoids, phytosteroids and fatty acids.

The extracts were concentrated by evaporating the solvents using a rotary evaporator or hot air oven, yielding dry extracts free of solvents. These extracts were stored in glass vials for further phytochemical analysis.

Qualitative phytochemical analysis

The various extracts of *A. scholaris* and *F. racemosa* were subjected to qualitative phytochemical analysis to detect the presence of various phytoconstituents. The tests were conducted using standard procedure on normal leaves, gall leaves and gall of both plant extracts and the results were compared to get the phytochemical profile of the plants (Edeoga et al., 2005; Harborne, 1998).

1. **Test for alkaloids:**
    a) Dragendorff's test: To 1 mL of the crude extract, a few drops of Dragendorff's reagent were added. The presence of alkaloids was indicated by the formation of an orange-red precipitate.

b) Hager's test: To 1 mL of each sample solution taken in a test tube, a few drops of Hager's reagent (saturated aqueous picric acid solution) were added. The formation of a yellow precipitate confirmed the presence of alkaloids.
c) Wagner's test: To a few drops of each sample solution taken in a test tube, a few drops of Wagner's reagent (iodine in potassium iodide) were added. The appearance of a brown flocculent precipitate indicated the presence of alkaloids.

2. Test for phenolics:

Ferric chloride test: To 2 mL of extract 0.5 mLof 5% aqueous ferric chloride solution was added and observed for the colour change. Formation of deep blue or black colour was taken as positive for the presence of phenols.

3. Test for flavonoids:

a) Lead acetate test: A few drops of each sample solution were taken in a test tube, and aqueous basic lead acetate was added. The formation of a white turbid precipitate indicated the presence of flavonoids.
b) Alkaline reagent test: To about 3 mL of the extract, 2 mL of 20% sodium hydroxide solution was added and the mixture was observed for the formation of an intense yellow colour, which became colourless upon the further addition of dilute hydrochloric acid, confirming the presence of flavonoids.

4. Test for saponins:

Froth test: To 1 mL of each sample solution taken in a test tube 2 mL of distilled water was added and shaken well. Appearance of froth on shaking of the mixture indicated presence of saponins.

5. Test for quinones:

HCl Test: A small amount of extract was treated with 2 mL of concentrated hydrochloric acid and observed for the formation of yellow precipitate which indicated the presence of quinones.

6. Test for coumarins:

Ferric chloride test: To 1 mL of each sample solution taken in a test tube, one drop of ferric chloride solution was added, resulting in the formation of a dark green colour. The appearance of a dark green colour that turned yellow upon the addition of five drops of concentrated nitric acid indicated the presence of coumarins.

7. Test for cardiac glycosides:

Keller killani test: To 5 mL of each sample taken in a test tube added 2 mL of acetic acid along with 1 drop of ferric chloride solution and 1 mL of concentrated sulphuric acid. The appearance of the brown ring indicates the presence of cardiac glycosides.

8. Test for triterpenoids:

a) Libberman's test: To 1 mL of each sample taken in a test tube added 3 drops of acetic anhydride and boiled. Then the sample was cooled and added concentrated sulphuric acid along the side of the test tube. A brown ring formed at the junction and deep red colour in the lower region indicated the presence of triterpenoids.
b) Salkowski test: To 1 mL of each of the samples taken in a test tube added a few drops of chloroform on the side of the test tube. Then carefully added a few drops of concentrated sulphuric acid. Reddish brown colour at the interphase indicated the presence of triterpenoids and steroids.

9. Test for tannin:

Potassium dichromate test: To 1 mL of each sample added a few drops of strong potassium dichromate solution. Yellow colour precipitate indicated the presence of tannin.

Quantitative phytochemical analysis

1) Quantitative phytochemical analysis by GC-MS

The normal leaf, gall leaf and gall extract of *A. scholaris* and *F. racemosa* were subjected to Gas Chromatography analysis along with Mass Spectrum analysis using the

equipment by Shimadzu QP-2010 Plus with Thermal Desorption System TD 20. About 1µL of ethanolic extracts of *A. scholaris* and *F. racemosa* were injected into the machine using the micro syringe and the scanning was done for 45 min. The injector and detector temperatures were maintained at 260 ºC, and the operation temperature at 50-280 ºC. The column temperature was programmed at 50-120 ºC, with a 4 ºC increase per min which was maintained for 1 min. Then it was programmed at 120-300 ºC, with 6 ºC increases per min and held on for 3 min, with retention time (Rt) 60 min. Column oven temperature was 50.0 ºC, injection temperature was 260.00 ºC and injection in split mode. Pressure was kept at 69.0 k Pa. Total flow was 16.3 mL/min and column flow was maintained at 1.21 mL/min. The identification of the compounds was done using the Software named GC-MS solution. The determination of structure of compounds was done based on the comparison of mass spectra and their fragmentation profiles using published data, Wiley, NIST library search. The analysis was done at Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University, New Delhi.

2) Identification of phytochemicals by OHR-LC-MS analysis

To obtain the detailed phytochemical profile of the normal leaves and galls, Orbitrap High Resolution Liquid Chromatography Mass Spectroscopy (OHR-LC-MS) analysis was performed in all the 3 samples of both plants. It is done using the equipment Q Exactive Plus Biopharma, Thermo Scientific Xcalibur, version 4.2.28.14. The analytic instrument is composed of two outer electrodes and a central electrode, which act as an analyzer and detector. It performs both negative and positive ionisation modes with mass (m/z) ranges of 50 to 8000 amu. The mobile phase contains 0.1% formic acid in Milli-Q water, methanol and acetonitrile. The running time is kept at 35 min. Metabolite identification was performed using Thermo Compound Discoverer 3.2 and BioPharma Finder Software 2.0. Resulted data were analysed with Compound Discoverer 3.2. The experiment was conducted at Sophisticated Analytical Instrument Facility (SAIF), IIT, Bombay. The biological properties of identified chemical compounds in normal leaves, gall leaves and galls were identified using Pub-Chem and Chem-spider.

3.2.3. Other preliminary quantitative analysis

Estimation of chlorophyll content

Photosynthetic pigments are essential biomolecules that enable plants to absorb and utilize light energy for photosynthesis. Carotenoids and chlorophyll are important among them. Chlorophylls are green pigments that absorb light energy and transform it into chemical energy. They are mostly composed of chlorophyll a and chlorophyll b. The proportion and amount of these pigments can change depending on the environment and frequently indicate the physiological state of the plant. In plant physiological investigations, the estimation of chlorophylls and carotenoids is crucial because they work together to provide the photosynthetic system with the maximum possible light absorption and protection. Each of the pigments has a characteristic absorption spectrum which can distinguish one pigment from another.

Protocol:

Fresh leaf samples (50 mg each) from normal leaves, gall-infected leaves and isolated galls were weighed and thoroughly crushed using a mortar and pestle in 10 mL of acetone. The homogenized mixture was then centrifuged at 5000 rpm for 10 min, and the resulting supernatant was carefully collected. The remaining residue was re-extracted using 80% acetone and centrifuged under the same conditions. This extraction process was repeated several times until the pellet turned colourless, indicating complete extraction of pigments. All supernatants from successive extractions were pooled together and the final volume was noted. The absorbance of the pooled extract was measured at 663 nm, 646 nm, 470 nm and 750 nm using 80% acetone as the solvent blank.

The quantities of different pigments were calculated using the following formula; (Arnon, 1949; Prabhakaran and Neravathu, 2019).

$$\text{Chlorophyll a} = 12.69\,(A_{663} - A_{750}) - 2.69\,(A_{646} - A_{750}) \times V \div W$$

$$\text{Chlorophyll b} = 22.9\,(A_{646} - A_{750}) - 4.68\,(A_{663} - A_{750}) \times V \div W$$

$$\text{Total chlorophyll} = 20.12\,(A_{646} - A_{750}) + 8.02\,(A_{663} - A_{750}) \times V \div W$$

Carotenoid = 1000 (A_{470}) + 3.72 (Chl a - Chl b) × V ÷ W× 229

Where A is the absorbance, V is volume and W is fresh weight of the sample

Estimation of phenylalanine ammonia-lyase (PAL) enzyme activity

Phenylalanine ammonia-lyase (PAL) is an important enzyme in the phenylpropanoid pathway, which converts phenylalanine into trans cinnamic acid. The amount of PAL that catalyzes the conversion of 1 µmol of phenylalanine into NH3 and trans-cinnamic acid at pH 8.5 and 30 ºC per min was regarded as the unit of activity. PAL enzyme activity was calculated based on the following protocol (Babich et al., 2020; Safari et al., 2021; Yasmin et al., 2023).

Protocol

About 10 mg each of the fresh plant samples of both *A. scholaris* and *F. racemosa* - gall, gall leaf and normal leaf was taken. Each sample was ground with 0.5 mL of 0.1 M trisodium borate buffer (pH 8.8) and mercaptoethanol was added to make up the final volume to 3 mL. The homogenates were centrifuged at 8000 × g for 20 min at 4 °C using a refrigerated centrifuge. The resulting supernatant was collected and used as the enzyme source for determining PAL activity. For the assay, 1 mL of the enzyme extract was mixed with 1 mL of borate buffer and 1 mL of 0.05 M, L-phenylalanine. The reaction mixture was incubated at 37 °C for 1 hour, after which 30% trichloroacetic acid (TCA) was added to terminate the reaction. The absorbance of the resulting solution was measured at 270 nm using a UV-Visible spectrophotometer. PAL activity was calculated based on the rate of conversion of L-phenylalanine to trans-cinnamic acid at 270 nm.

The amount of trans-cinnamic acid synthesized was calculated, by using its extinction coefficient and enzyme activity of sample was calculated by using the following equation;

$$Units/mL\ enzyme\ = \frac{(\Delta A270nm/min\,Test - \Delta A270nm/min\,Blank)\ (3)(df)}{(19.73)(0.1)}$$

Where ΔA is the change in absorbance per min, 3 is the total volume (in mm) of assay, df is the dilution factor (1), 19.73 is the mM extinction coefficient of trans-cinnamate at 270 nm and 0.1 is the enzyme volume.

3.3. RESULTS AND DISCUSSION

Gall formation is a complex phenomenon resulting from interactions between plants and various organisms, including insects, which induce pathological growths through feeding or oviposition. These galls exhibit a wide range of morphological complexities, from simple to highly elaborate structures. Key cellular processes involved include hypertrophy (cell enlargement) and hyperplasia (increased cell division), leading to the development of specialized tissues that support the growth of the inducing organism (Mani, 2000; Oliveira and Isaias, 2009). In India, over 2000 different gall forms have been documented, highlighting the richness of gall-inducing flora (Raman, 2007). This study focuses on two medicinal plants, *A. scholaris* and *F. racemosa,* which develop leaf galls induced by psyllids from the Order Hemiptera and Class Psyllidae. With approximately 350 species of psyllids known to induce galls on dicotyledonous leaves, these insects play a significant role in shaping plant morphology and phytochemistry (Raman, 2011).

3.3.1. Structure of normal leaves and leaf galls

Morphology of leaves of *A. scholaris*

The leaves are smooth and glossy, with a dark green upper surface and a paler, grayish underside. They are elliptic to broadly obovate in shape and exhibit a subcoriaceous to coriaceous (leathery) texture. The leaves measure approximately 20-25 cm in length and 5.5-6.5 cm in width. The apex is either rounded or emarginate, while the base is cuneate to decurrent. The petiole is short, typically less than 1 cm in length. Leaves are arranged in whorls of 6-7 leaflets and each leaflet displays numerous pairs of closely spaced lateral veins that are oriented nearly perpendicular to the midrib (Plate 3.1: Figure A and Figure B). *A. scholaris* leaves with galls (Plate 3.1: Figure C) and single leaf with galls, adaxial surface (Plate 3.2: Figure A) and abaxial surface (Plate 3.2: Figure B) of leaf with galls were shown.

Morphology of leaf galls of *A. scholaris*

Both young and mature leaves of *A. scholaris* are susceptible to infection by leaf gall-inducing insects. The galls predominantly develop along the lateral veins and are fleshy, yellowish-green and spherical in shape, ranging from 5-10 mm in diameter. A single leaf may bear 1-190 galls, with an average of 20-30 galls per leaf. The galls are typically unilocular, containing a single chamber; however, in cases where galls are fused, they may become multilocular with 3-4 chambers. Severe infestations result in significant morphological deformities, with the leaf lamina often becoming entirely covered by dense clusters of galls. Mature galls possess an opening called ostiole, on the abaxial surface, which facilitates the emergence of the insect after development. After the adult insect exits, the gall becomes brownish-black, hard and woody in texture. Plate 3.2: Figure C represents the images of unilocular gall, bilocular gall and multilocular galls of *A. scholaris* and Figure D photograph showing the cross sections of galls.

The leaf galls of *A. scholaris* are induced by the insect *Pauropsylla tuberculata* Crawford (Psyllidae: Homoptera). The Plate 3.3: Figure A showing the photograph of inducer insect *P. tuberculata* developing inside the leaf galls of *A. scholaris*, Figure B showing the photograph of the *P. tuberculata* and Figure C is the mature stage of *P. tuberculata*. The galls are epiphyllous, which can be unilocular or multilocular, with 3-4 chambers. The gall possesses a diameter between 0.1 and 1 cm. The galls can be single, dispersed or occasionally form agglomerated clusters. As the number of galls increases, the leaves get crumpled and distorted (Albert et al., 2011; Pasayat and Tripathy, 2020). Similar findings were made by the current study, which observed the occurrence of enormous galls that affected the natural shape and structure of *A. scholaris* leaves and led to change into totally distorted structures. The morphology of agglomerate galls of *A. scholaris* is shown in Plate 3.4: Figure A.

Morphology of the leaves of *F. racemosa*

The leaves (Plate 3.5: Figure A and Figure B) are simple and arranged alternately along the stem, with a length ranging from 8-23 cm and a width of 3-7 cm. They are ovate, elliptical, lanceolate or sub-ovate in shape, with entire margins and a distinctly petiolated base. The leaf apex is acute and the margins are slightly wavy. The adaxial (upper) surface is



Plate 3.1: Figure A: *A. scholaris* plant with branches, leaves and leaf galls, Figure B: *A. scholaris* twig with healthy leaves and Figure C: *A. scholaris* twig possessing leaves with leaf galls.

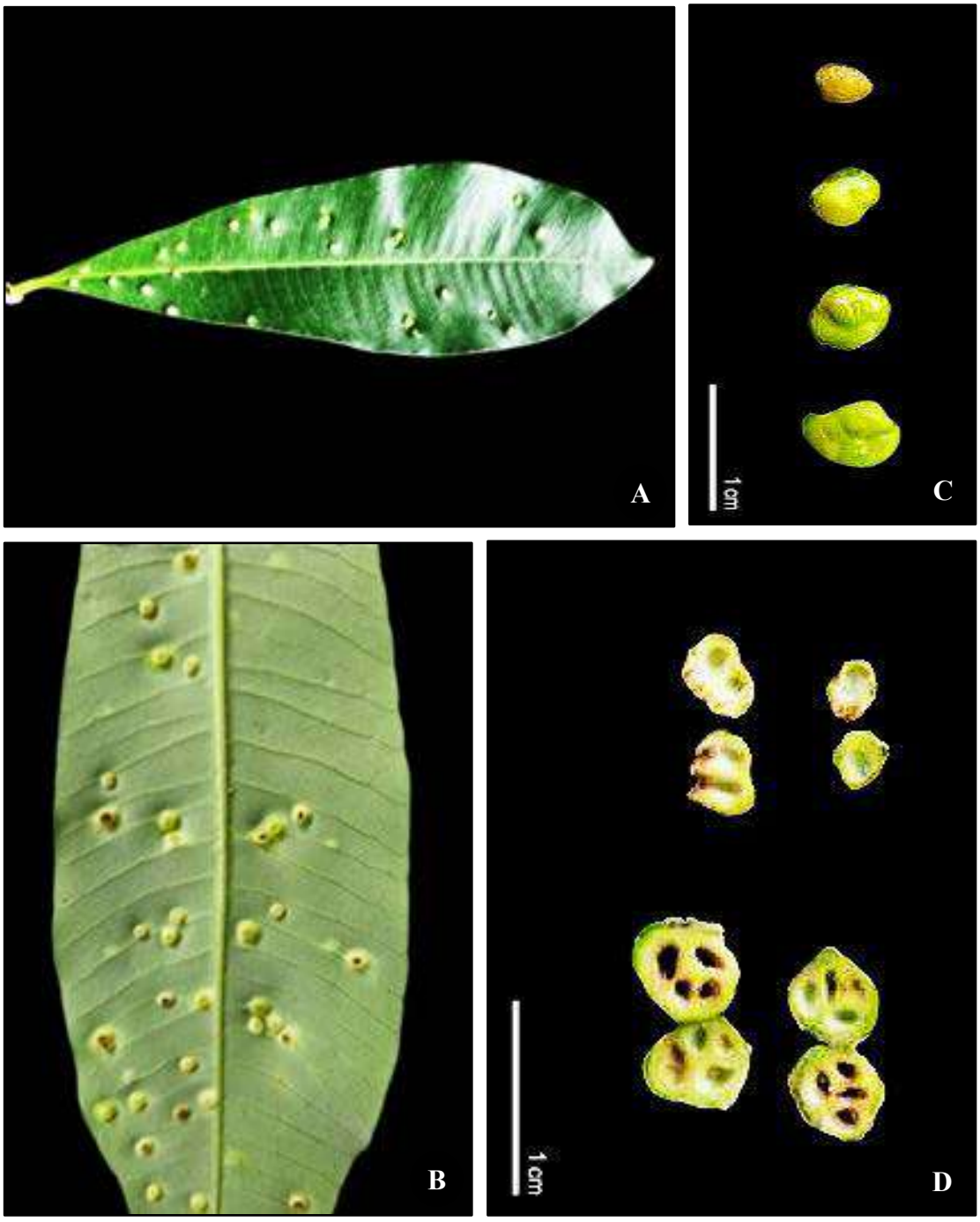


Plate 3.2: Figure A: *A. scholaris* single leaf with galls (Adaxial surface), Figure B: *A. scholaris* abaxial surface of leaf with galls, Figure C: Unilocular gall, bilocular gall and multilocular galls of *A. scholaris* and Figure D: Each gall is cut into two pieces to view the larval chamber.



Plate 3.3: Figure A: The photograph of inducer insect *Pauropsylla tuberculata* developing inside the leaf galls of *A. scholaris*, Figure B: Photograph showing the insect *P. tuberculata*, Figure C: Mature stage of *P. tuberculata*, Figure D: The leaf gall inducer insect *Pauropsylla depressa* developing inside the leaf galls of *F. racemosa*, Figure E: Photograph of insect *P. depressa* and Figure F: Matured stage of *P. depressa* with wings.

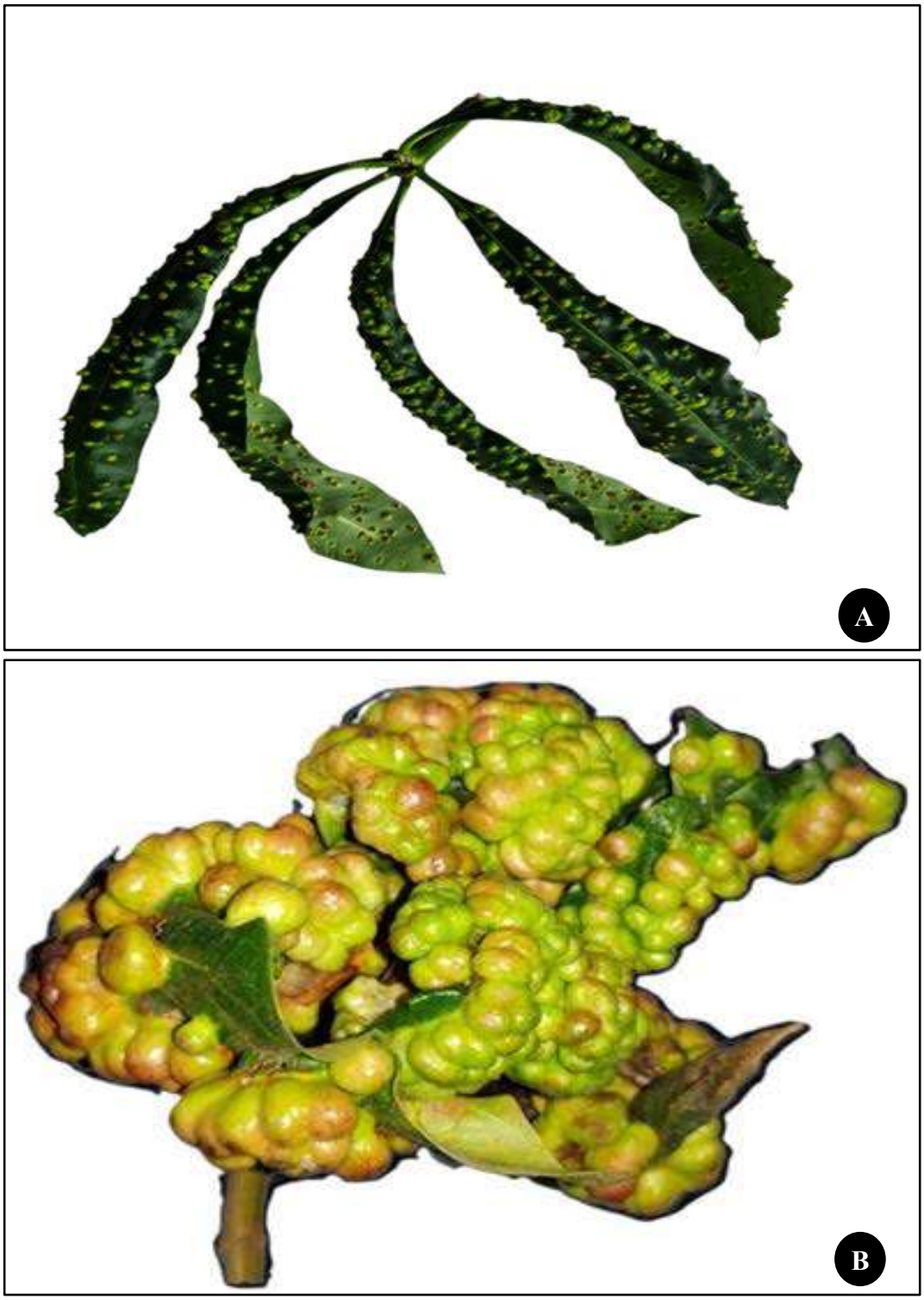


Plate 3.4: Figure A: A twig of *A. scholaris* with clumped galls and Figure B: *F. racemosa* twig turned completely into galls.



Plate 3.5: Figure A: *F. racemosa* plant with branches, leaves and leaf galls, Figure B: *F. racemosa* twig with healthy leaves and Figure C: *F. racemosa* twig possessing leaves with leaf galls.

dark green, while the abaxial (lower) surface is lighter green in colour. The petiole is angular, reddish brown in appearance and measures approximately 4.0-5.5 cm in length.

Morphology of leaf galls of *F. racemosa*

Galls are epiphyllous structures that develop on the adaxial surface of leaves, occurring singly or in multiple numbers. They may appear scattered across the leaf blade or grouped together, forming conspicuous spherical masses or convex bulges. The individual galls are simple, regular, globose, sessile, perfoliate and unilocular in structure, measuring approximately 5-10 mm in diameter. The number of galls per leaf ranges from 1 to 65, with an average of 15-20.

In cases of severe infestation, galls may coalesce, forming a single, large mass that can obscure the leaf lamina entirely. Such compound galls, resulting from the partial fusion of multiple galls, are typically irregular in shape, multilocular and measure between 15-30 mm in diameter (Plate 3.5: Figure C). Young galls are initially light green, gradually developing brownish or reddish-brown discoloration as they mature. In mature galls a small ostiole is found on the abaxial surface of the leaf, which is the way to release the adult insect out of gall after their complete development. Plate 3.6: Figure A and B represents the dorsal and ventral view of *F. racemosa* single leaf with leaf galls respectively, Figure C showing the leaf galls having single chamber to multiple chambers and Figure D represents the gall chambers of leaf galls of *F. racemosa*.

Galls induced on the leaves of *F. racemosa* by *Pauropsylla depressa* Crawf (Psyliidae, Hemiptera). Plate 3.3: Figure D shows the gall inducer *P. depressa* developing inside the leaf galls of *F. racemosa,* Figure E is the photograph of insect *P. depressa* and Figure F is the picture of the mature stage of *P. depressa* with wings. The leaf galls are epiphyllous, simple, fleshy, globose, sessile, unilocular pouch galls or multilocular aggregates, clumps or masses. Our observations on *F. racemosa* galls found that the aggregate galls developed from individual galls are combined to generate complicated masses or clumps (Plate 3.4: Figure B). The gall typically lacks chlorophyll and is reddish or reddish-brown in color when mature. Its diameter ranges from 5 to 10 mm (Dsouza and Ravishankar, 2014; Ushir et al., 2015).

Anatomy of leaves of *A. scholaris*

Normal healthy leaves are dorsiventral in structure (Plate 3.7: Figure A and Figure B). The upper epidermis is covered by a thick cuticle, whereas the lower epidermis has a relatively thinner cuticle. The upper epidermal cells are tubular in shape and compactly arranged. Stomata are confined to the lower epidermis and are sunken in nature. The mesophyll is differentiated into palisade and spongy tissues. Palisade cells are cylindrical and tightly packed, while the spongy tissue is aerenchymatous, facilitating gaseous exchange. Resin canals are present within the mesophyll region. The vascular bundles are bicollateral and are surrounded by thick-walled supportive cells, which are sclerenchymatous. The vascular bundles are traversing the mesophyll, with the xylem strand flanked by phloem on both sides.

Anatomy of leaf galls of *A. scholaris*

The transverse section of leaf galls of *A. scholaris* is shown in Plate 3.8: Figure A and Figure B. The epidermis of galled leaves consists of rectangular cells covered by a thin cuticle. Beneath the epidermis lies the mesophyll, which appears homogeneous due to the absence of tissue differentiation. The typical palisade layer is replaced by thin-walled, compactly arranged parenchyma cells. These cells are large, round and closely packed. At the center of the gall is a prominent cavity known as the larval chamber, which serves as the site for insect development. This chamber is surrounded by nutritive parenchyma cells that contain conspicuously large starch grains, providing nourishment to the developing insect. Vascular bundles are seen around the larval chamber. They are scattered and embedded within the gall parenchymatous tissue irregularly.

The normal leaf anatomy of *A. scholaris* was investigated by Albert and colleagues and found a moderately thick layer of cuticle covers the tubular, densely packed upper epidermal cells. The lower epidermal cells are papillate. The thick, echinulate cuticle covering the lower epidermis is located near the papilla. Stomata are limited to the lower epidermis and are sunken. By analysing the transverse section of a normal leaf, there is no such difference observed from normal anatomy of a dicot leaf. Leaves were dorsiventral having a mesophyll tissue differentiated into palisade and spongy tissue. Albert and colleagues also analysed foliar

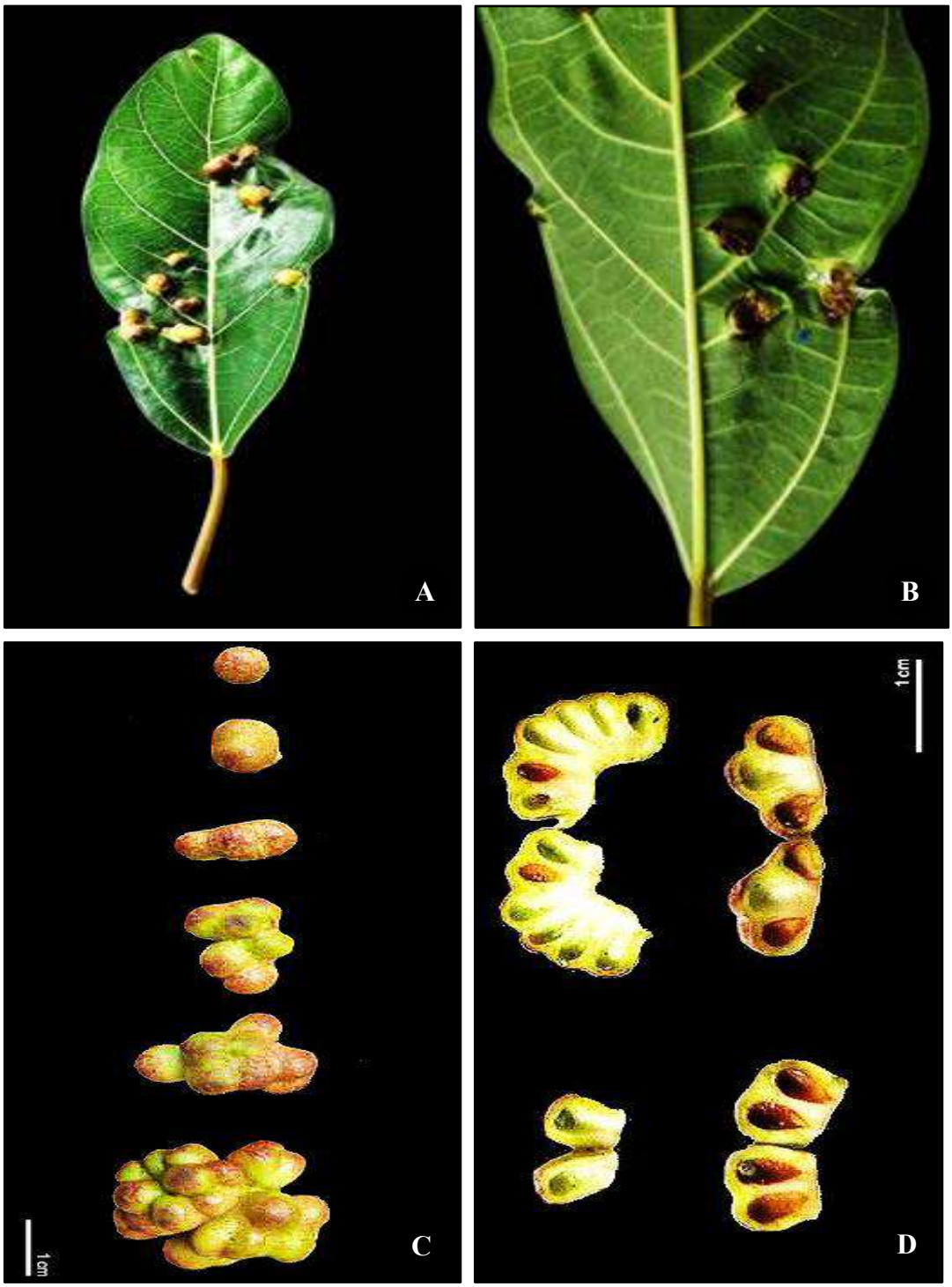


Plate 3.6: Figure A: *F. racemosa* single leaf with galls (Adaxial surface), Figure B: Abaxial surface of leaf with galls in *F. racemosa*, Figure C: *F. racemosa* galls having single to multiple chambers and Figure D: Each gall is cut into two pieces to view the larval chamber.

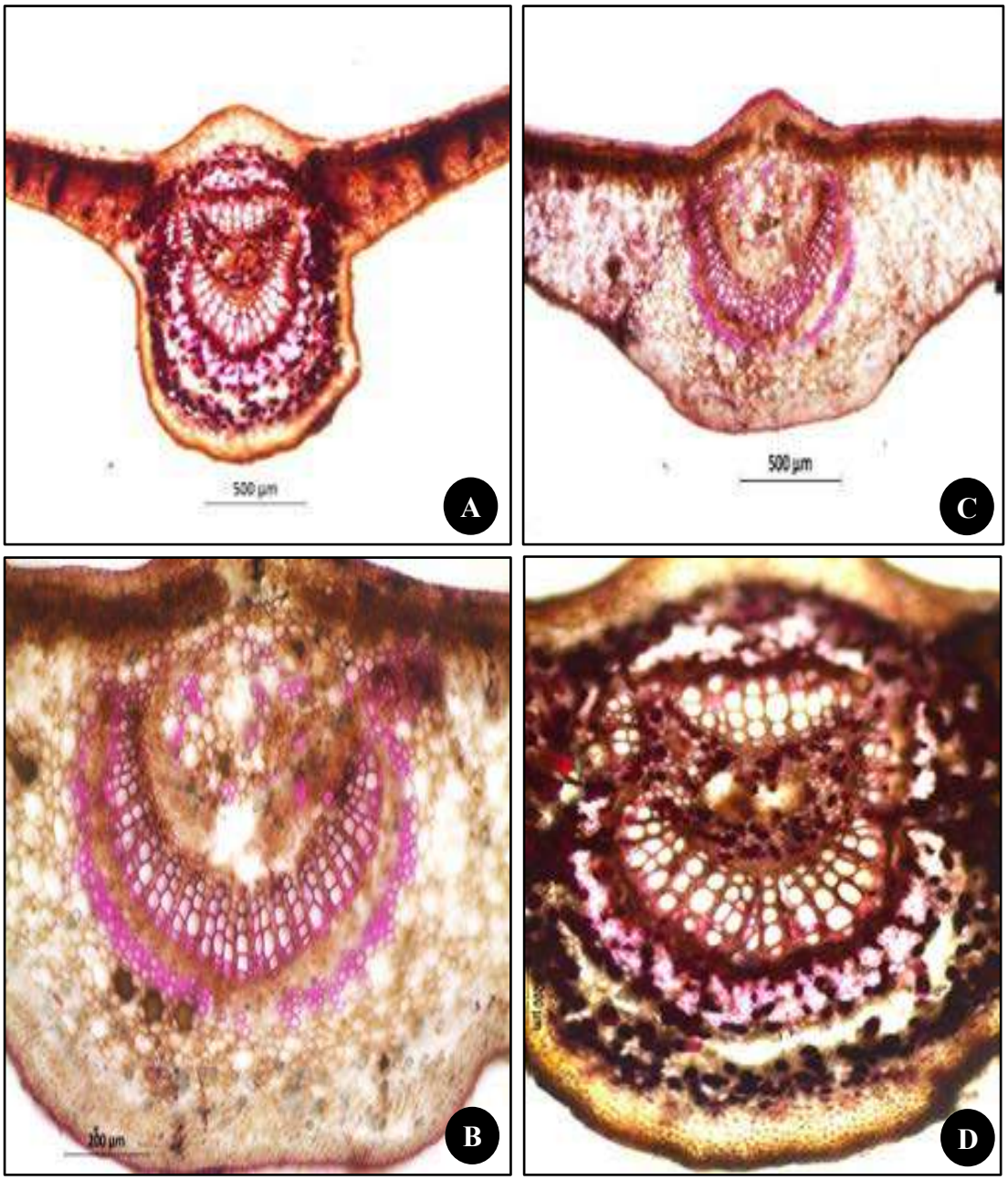


Plate 3.7: Anatomy of normal leaves. Figure A. Transverse section (T. S.) of normal (healthy) leaves of *A. scholaris,* Figure B: T. S. of mid rib portion of *A. scholaris* leaf, Figure C: T. S. of normal leaves of *F. racemosa* and Figure D: T. S. of mid rib portion of *F. racemosa* leaf.

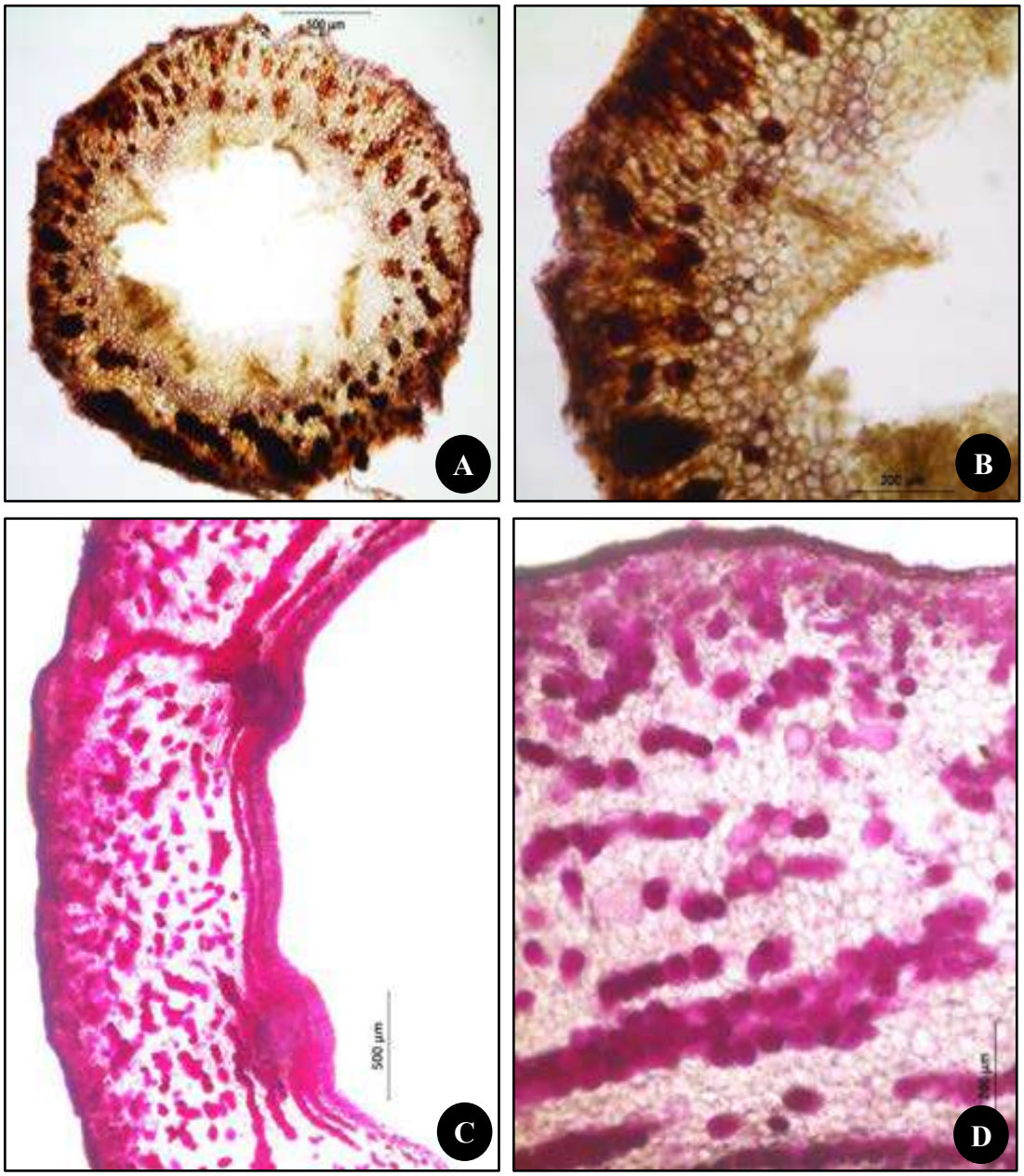


Plate 3.8: Anatomy of leaf galls. Figure A: T. S. of leaf galls of *A. scholaris*, Figure B: *A. scholaris* leaf galls; a portion enlarged, Figure C: T. S. of leaf galls of *F. racemosa* and Figure D: *F. racemosa* leaf galls; a portion enlarged.

galls of *A. scholaris* anatomically and discovered that there is a cavity at the center of the gall, surrounded by a closely packed mass of chlorophyll lacking cells, which are rich with starch grains. In this cavity, the insect development occurs and the surrounding tissue will supply nutrition for their development (Albert et al., 2011). Only a few works were reported on the anatomy of leaf galls of *A. scholaris.* A cavity in the middle of the leaf, encircled by starch-rich cells was visible in the transverse section. The former is commonly referred to as the larval chamber, while the latter is described as the nutritive tissue.

Anatomy of leaves of *F. racemosa*

The dorsiventral leaf consists of a central midrib and two lateral wings (Plate 3.7: Figure C and Figure D). The entire epidermis is covered by a thin cuticle and is composed of rectangular and cylindrical cells. Stomata are confined to the abaxial epidermis, which also bears simple trichomes. The adaxial epidermis is two-layered, while the abaxial epidermis is single-layered. Beneath the epidermis lies the mesophyll, which is clearly differentiated into palisade and spongy tissues. The adaxial side contains 2-3 layers of cylindrical, compactly arranged palisade cells. The spongy tissue comprises multiple layers of loosely arranged aerenchymatous cells with conspicuous intercellular spaces, facilitating gas exchange.

The midrib displays a convex outline on the ventral (upper) side and an arched profile on the dorsal (lower) side. Below the epidermis, the hypodermis consists of two layers. A central vascular bundle is present in the midrib region and is enclosed by a collenchymatous bundle sheath. The vascular bundle contains annular xylem vessels and 6-10 distinct phloem strands. Lithocysts are located in the adaxial region of the leaf, while crystals are observed within the bundle sheath and in the midrib parenchyma. Tanniferous cells are distributed in both adaxial and abaxial regions.

Anatomy of leaf galls of *F. racemosa*

The T.S. of leaf galls shown in plate 3.8: Figure C and Figure D. The epidermis of the gall is a direct continuation of the normal leaf epidermis and consists of narrow, small-sized cells. It is externally covered by a thin cuticle. Beneath the epidermis lie 2-3 layers of lignified sclerenchyma cells, providing mechanical support to the gall structure. The cortex is

differentiated into two distinct regions: an outer cortex and an inner cortex. The outer cortex is composed of spongy parenchyma interspersed with sclereids, while the inner cortical region contains parenchyma cells rich in starch grains. This inner cortical tissue serves as nutritive tissue essential for the development of the insect larvae.

The gall parenchyma contains a large number of superficial and deep vascular bundles that are irregularly scattered. The bundles have distinct xylem and phloem. The xylem is located on the adaxial side and the phloem is located on the inner side of the gall. At the center of the gall lies a prominent cavity known as the larval chamber, which serves as the site for the development of insects from larva to adult.

Ushir and colleagues reported that the mesophyll cells of the *F. racemosa* normal leaves are structured into layers. Adjacent to the adaxial epidermis are two layers of palisade cells. The cells of the palisade are closely spaced and cylindrical. The mesophyll tissues contain the vascular bundles buried within them. In the palisade region, cystoliths are often observed. In the case of leaf galls of *F. racemosa*, the gall tissue is made up of undifferentiated parenchyma that is several times thicker than the mesophyll of a normal leaf. These cells are large, thin-walled and are closely packed together. There are no stomata seen in galls. The parenchyma cells of mature galls are devoid of chlorophyll (Ushir et al., 2015).

Our study also detected absence of chlorophyll in the gall tissue. Previous findings reported that the cells around the gall cavity show the accumulation of starch. This region is known as the nutritive region of the gall. Numerous vascular bundles are scattered across the gall parenchyma. The xylem is on the adaxial side of the gall, as seen in normal leaves and the phloem is on the inner side (Singh et al., 2023; Ushir et al., 2015). Transverse sections of galls have been examined which show the presence of a larval chamber and the presence of vascular bundles within the parenchyma underneath the chamber. The nutritive tissue around the chamber consisted of large, thin-walled cells with dense cytoplasm.

3.3.2. Results of phytochemical analysis of plant extracts

Preliminary phytochemical analysis of *A. scholaris*

Aqueous, hexane and ethanolic extracts of normal leaves, gall leaves and galls of *A. scholaris* plant were analysed for the presence of different phytochemicals. Analysis was done in triplicates to assess the consistency and significance of the observations. The frequency of positive results (presence of a phytoconstituent) was calculated for each phytoconstituent across the three replicates for each solvent extract (water, ethanol and hexane) and tissue type (normal leaves, gall leaves and galls) of both *A. scholaris* and *F. racemosa*. This provided a qualitative overview of the distribution of phytoconstituents.

Alkaloids were tested using Dragendorff's test, Hager's test and Wagner's test; found that Hanger's test gave positive result in aqueous extract of all samples and Wagner's test showed positive for ethanolic extracts. Phenolic compounds were detected in all samples of aqueous and ethanol extracts but only hexane extract of gall showed positive result. Lead acetate test of flavonoid were positive for all samples and extracts except ethanolic extracts of normal leaves and gall leaves. Saponins and coumarins gave positive results only in aqueous extracts and quinones were absent in all extracts and samples.

Cardiac glycosides indicated their presence in all extracts except ethanolic extracts of normal leaf and gall leaf. Terpenoids were tested using Libberman's test and Salkowski test, found to be present in all extracts except hexane extract of gall leaves. Potassium dichromate test of tannins gave positive results only in ethanolic extracts. The results of qualitative phytochemical analysis of *A. scholaris* are summarized in Table 3.1. The photographs of the results of preliminary phytochemical analysis of *A. scholaris* normal leaf, gall leaf and gall aqueous extracts (Plate 3.9), hexane extracts (Plate 3.10) and ethanolic extracts (Plate 3.11) are shown.

Table 3.1: Results of preliminary phytochemical analysis of aqueous, hexane and ethanolic extracts of normal leaves, gall leaves and leaf galls of *A. scholaris.*

Compound	Name of test	*A. scholaris*								
		Water			Hexane			Ethanol		
		Normal leaf	Gall leaf	Gall	Normal leaf	Gall leaf	Gall	Normal leaf	Gall leaf	Gall
Alkaloids	Dragendorff's test	–	–	–	–	–	–	–	–	–
	Hager's test	+	+	+	–	–	+	–	–	–
	Wagner's test	–	–	–	–	–	–	+	+	+
Phenolics	Ferric chloride test	+	+	+	–	–	+	+	+	+
Flavonoid	Lead acetate test	+	+	+	+	+	+	–	–	+
	Alkaline reagent test	–	–	–	+	+	+	–	–	–
Saponin	Froth test	+	+	+	–	–	–	–	–	–
Quinones	HCl test	–	–	–	–	–	–	–	–	–
Coumarins	Ferric chloride test	+	+	+	–	–	–	–	–	–
Cardiac glycosides	Kellerkillani test	+	+	+	+	+	+	–	–	+
Terpenoids	Libberman's test	+	+	+	–	–	+	+	+	+
	Salkowski test	+	+	+	+	–	+	+	+	+
Tannin	Potassium dichromate	–	–	–	–	–	–	+	+	–

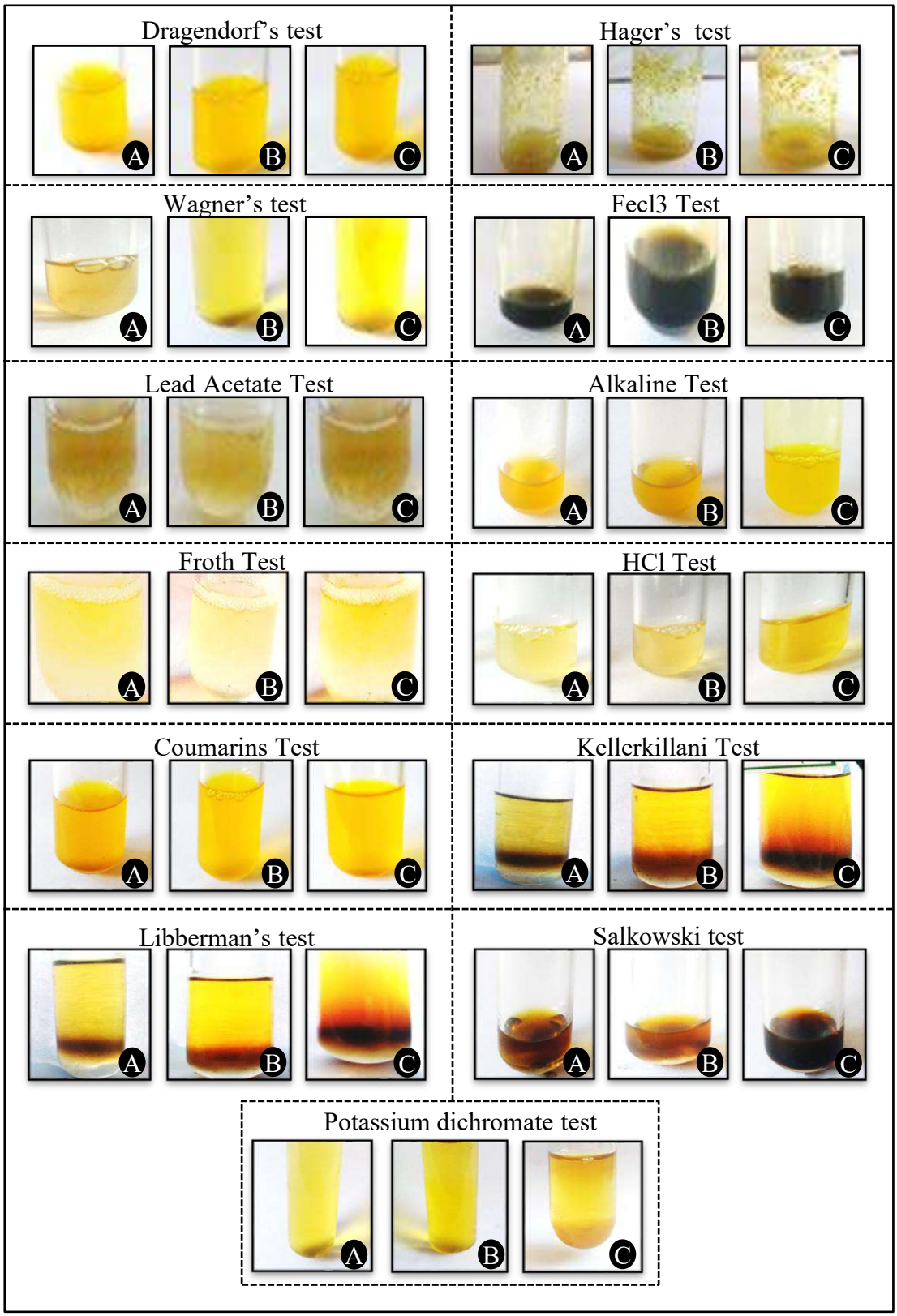


Plate 3.9: Preliminary phytochemical tests of normal leaf (A), gall leaf (B) and gall (C) aqueous extract of *A. scholaris.*

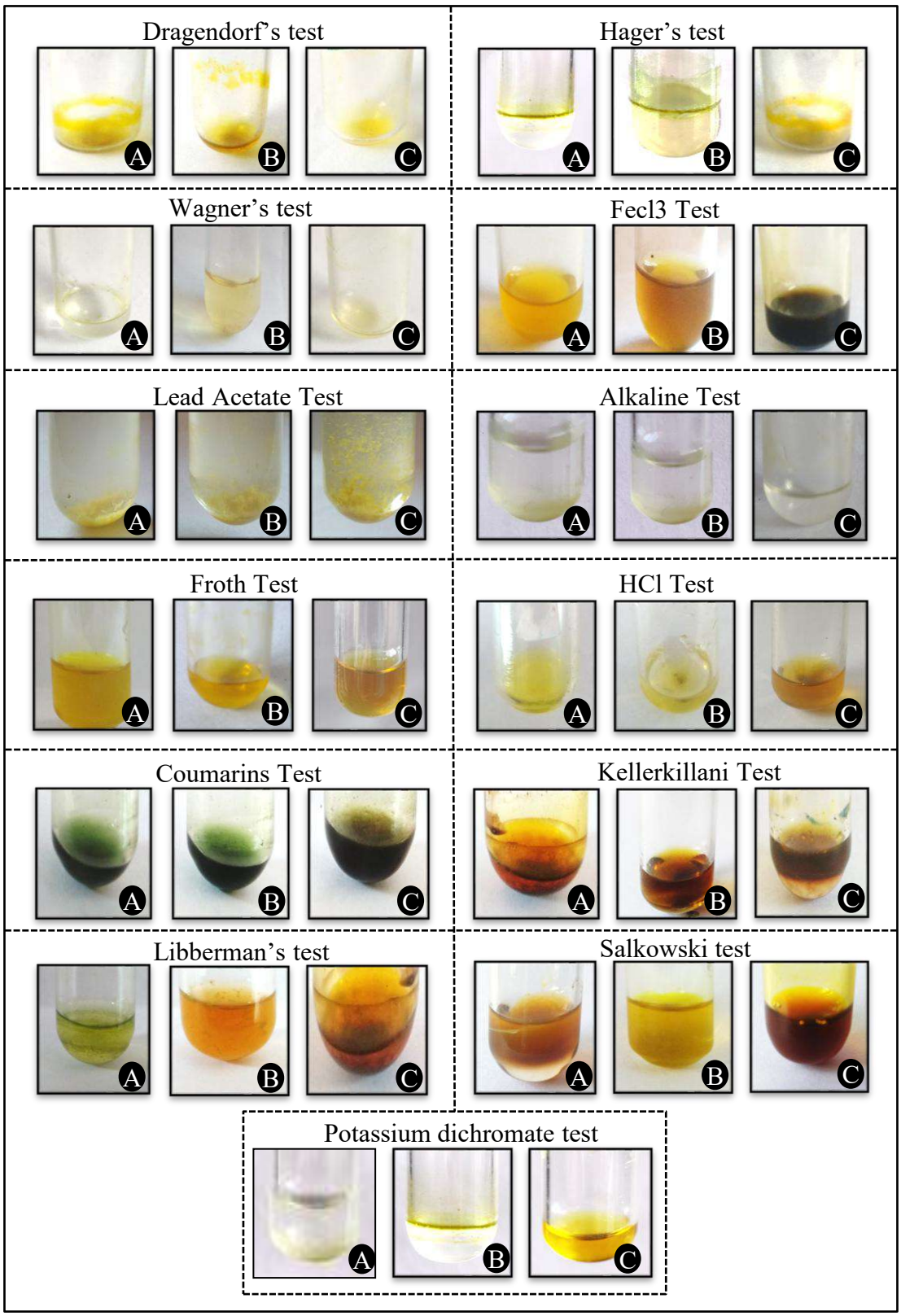


Plate 3.10: Preliminary phytochemical tests of normal leaf (A), gall leaf (B) and gall (C) hexane extract of *A. scholaris.*

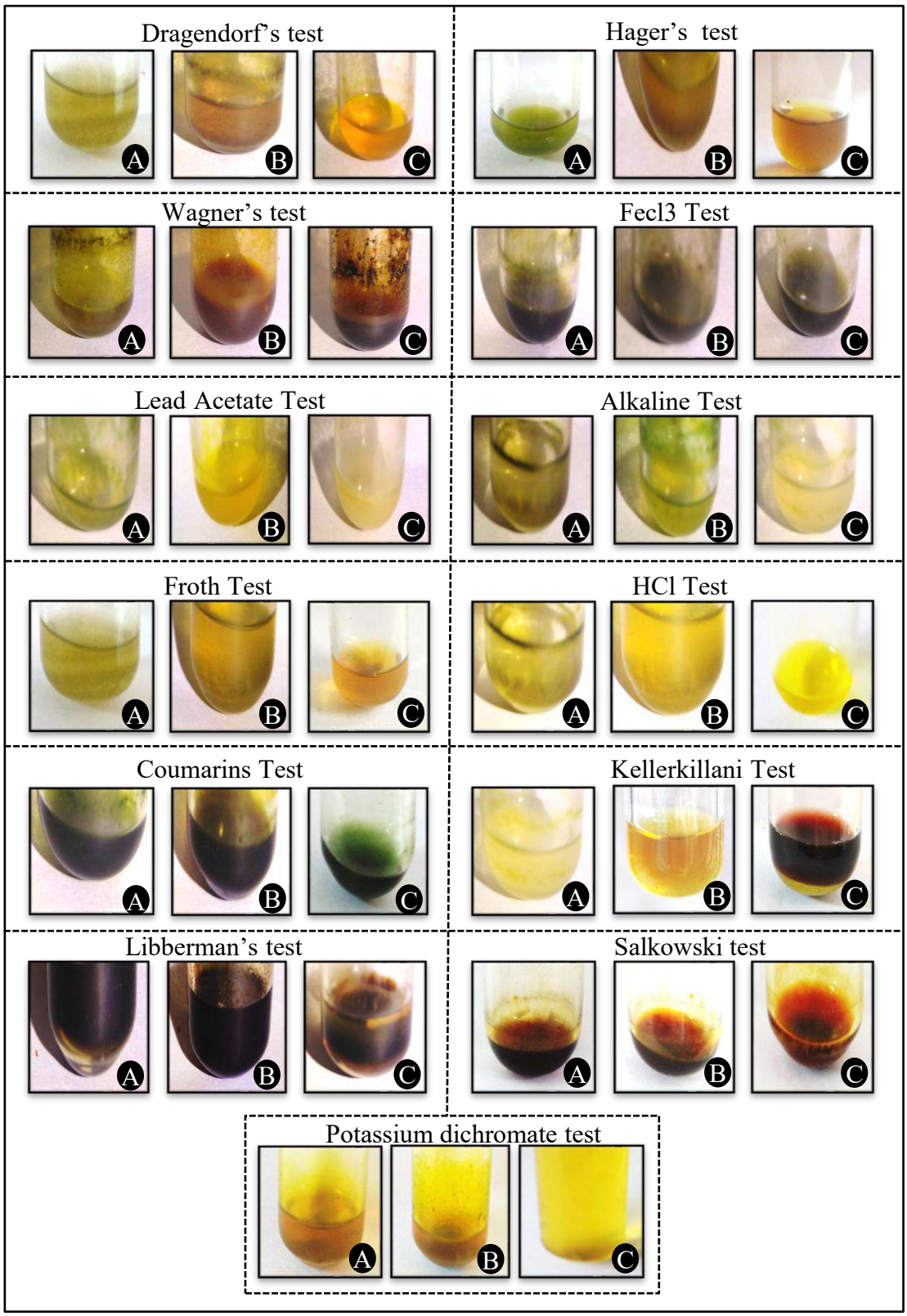


Plate 3.11: Preliminary phytochemical tests of normal leaf (A), gall leaf (B) and gall (C) ethanolic extract of *A. scholaris.*

There were previously published reports on phytochemical compounds of *A. scholaris* leaves identified in various extracts. Alkaloids and steroids were the main constituents of the leaf hexane extracts, whereas alkaloids, terpenoids and saponins were found in the water extract of healthy leaves. Phenols, alkaloids and saponins were detected in benzene extract (Misra et al., 2011). Methanol extracts showed the presence of alkaloids, coumarins, flavonoids, leucoanthocyanins, reducing sugars, simple phenolics, steroids, saponins and tannins (Khyade et al., 2009). Here in this study we analysed gall and gall leaf along with normal leaf of *A. scholaris.* Water extracts of all samples contain alkaloids, flavonoids, tannins, phenolics, coumarins, cardiac glycosides and steroids but no sample detected the presence of quinones. Gall hexane extracts indicated the presence of more compounds than other two samples. Alkaloids and phenols are only detected in gall hexane extracts. Ethanolic extracts were analyzed and detected the presence of flavonoids and cardiac glycosides in gall only.

Preliminary phytochemical analysis of *F. racemosa*

The photographs showing the results of preliminary phytochemical analysis of *F. racemosa* aqueous extracts, hexane extracts and ethanol extracts of normal leaf, gall leaf and gall are shown in Plate 3.12, Plate 3.13 and Plate 3.14 respectively. Hagers's test of alkaloids gave positive results for all samples in all three extracts but Wagner's test didn't show positive for all hexane extracts. Phenols and cardiac glycosides indicated their presence only in galls of *F. racemosa*. Flavonoids showed positive results for all samples of aqueous extracts and also in ethanolic extracts of galls. Aqueous extracts of galls indicated positive results for saponins and quinones. Terpenoids were present in all samples of aqueous extracts by Libberman's test and in the case of Salkowski test; results were positive for hexane extracts and only gall ethanolic extracts. Except for steroids and coumarins all the other phytochemical constituents showed positive results in gall aqueous and ethanolic extracts. The results of phytochemical analysis of *F. racemosa* are summarized in Table 3.2.

Table 3.2: Result of preliminary phytochemical analysis of aqueous, hexane and ethanolic extracts of normal leaves, gall leaves and leaf galls of *Ficus racemosa.*

Compound	Name of test	*F. racemosa*								
		Water			Hexane			Ethanol		
		Normal leaf	Gall leaf	Gall	Normal leaf	Gall leaf	Gall	Normal leaf	Gall leaf	Gall
Alkaloids	Dragendorff's test	–	–	–	–	–	–	–	–	–
	Hager's test	+	+	+	+	+	+	+	+	+
	Wagner's test	+	+	+	–	–	–	+	+	+
Phenolics	Ferric chloride test	–	–	+	–	–	+	–	+	–
Flavonoids	Lead acetate test	+	+	+	–	–	–	–	+	–
	Alkaline reagent test	+	+	+	–	–	–	–	+	–
Saponins	Froth test	–	–	+	–	–	–	–	–	–
Quinones	HCl test	–	–	+	–	–	–	–	–	+
Coumarins	Ferric chloride test	–	–	–	+	+	+	–	–	–
Cardiac glycosides	Kellerkillani test	–	–	+	–	–	+	–	–	+
Terpenoids	Libberman's test	+	+	+	+	–	+	–	–	–
	Salkowski test	–	–	–	+	+	+	–	–	+
Tannin	Potassium dichromate	–	–	–	–	–	–	+	–	+

The previous report on the preliminary phytochemical analysis conducted in the leaves of *F. racemosa* found the presence of triterpenoids, tannins, kaempferol, rutin, arabinose, bergapten, psoralenes, flavonoids, ficusin, coumarin, phenolic glycosides and saponins (Ahmed and Urooj, 2010). Savitha and colleagues reported that saponins, flavonoids, terpenoids, reducing sugars and tannins showed presence in both normal leaves and gall leaves. The presence of coumarins and quinones were found only in the galled leaves (Bagyalakshmi et al., 2019; Savitha et al., 2013). Here in the present study, phenolics and

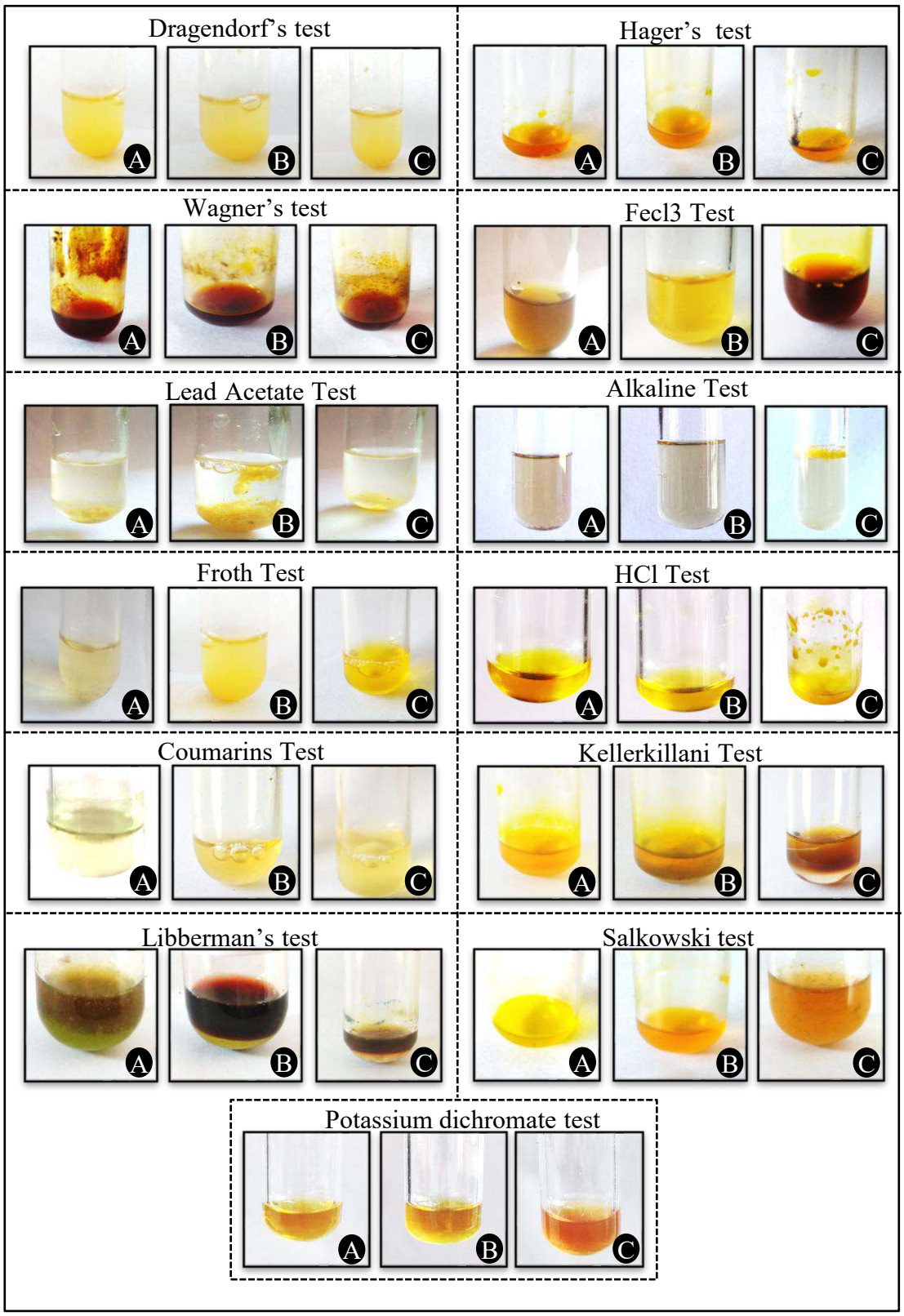


Plate 3.12: Preliminary phytochemical tests of normal leaf (A), gall leaf (B) and gall (C) aqueous extract of *F. racemosa.*

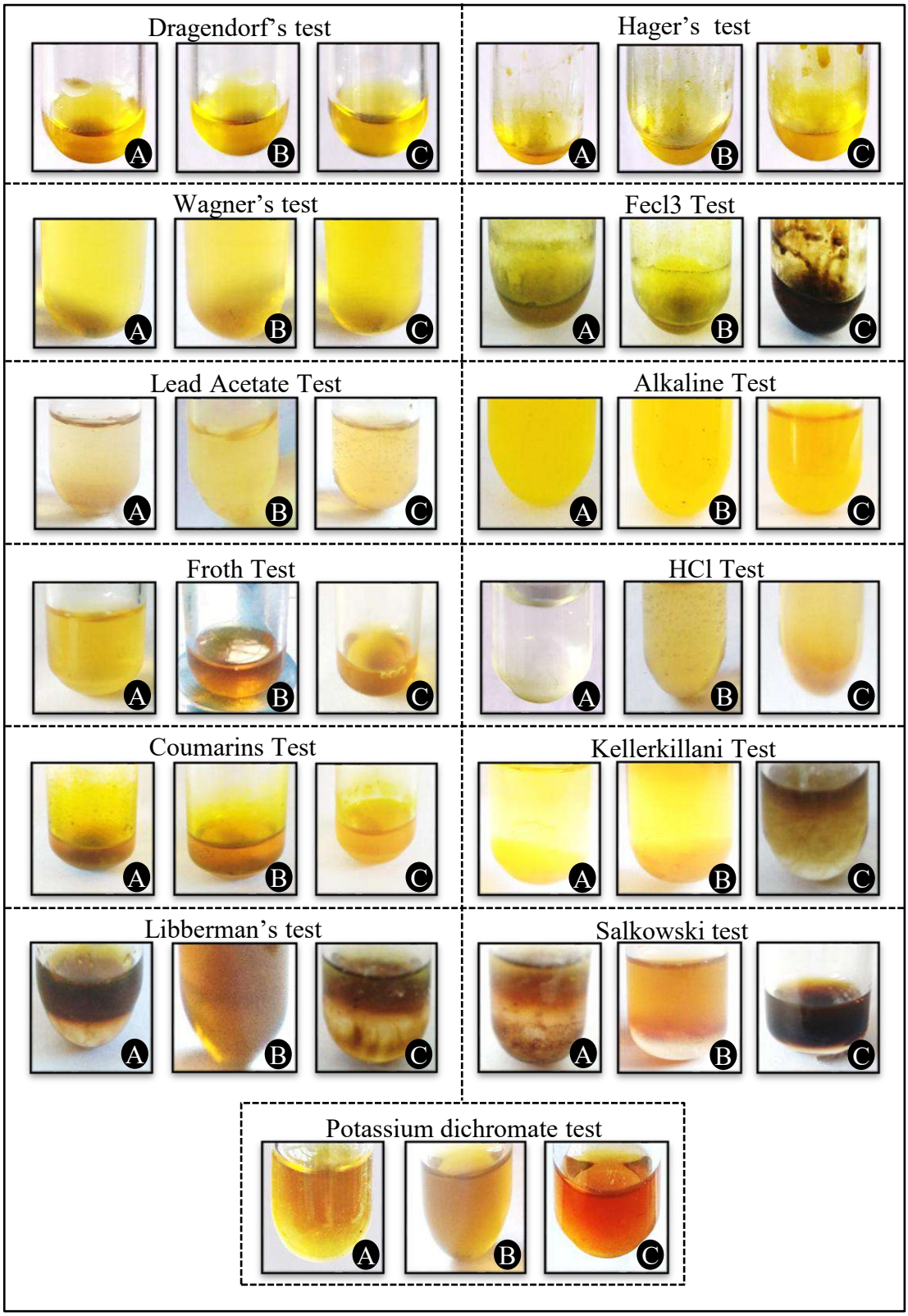


Plate 3.13: Preliminary phytochemical tests of normal leaf (A), gall leaf (B) and gall (C) hexane extract of *F. racemosa.*

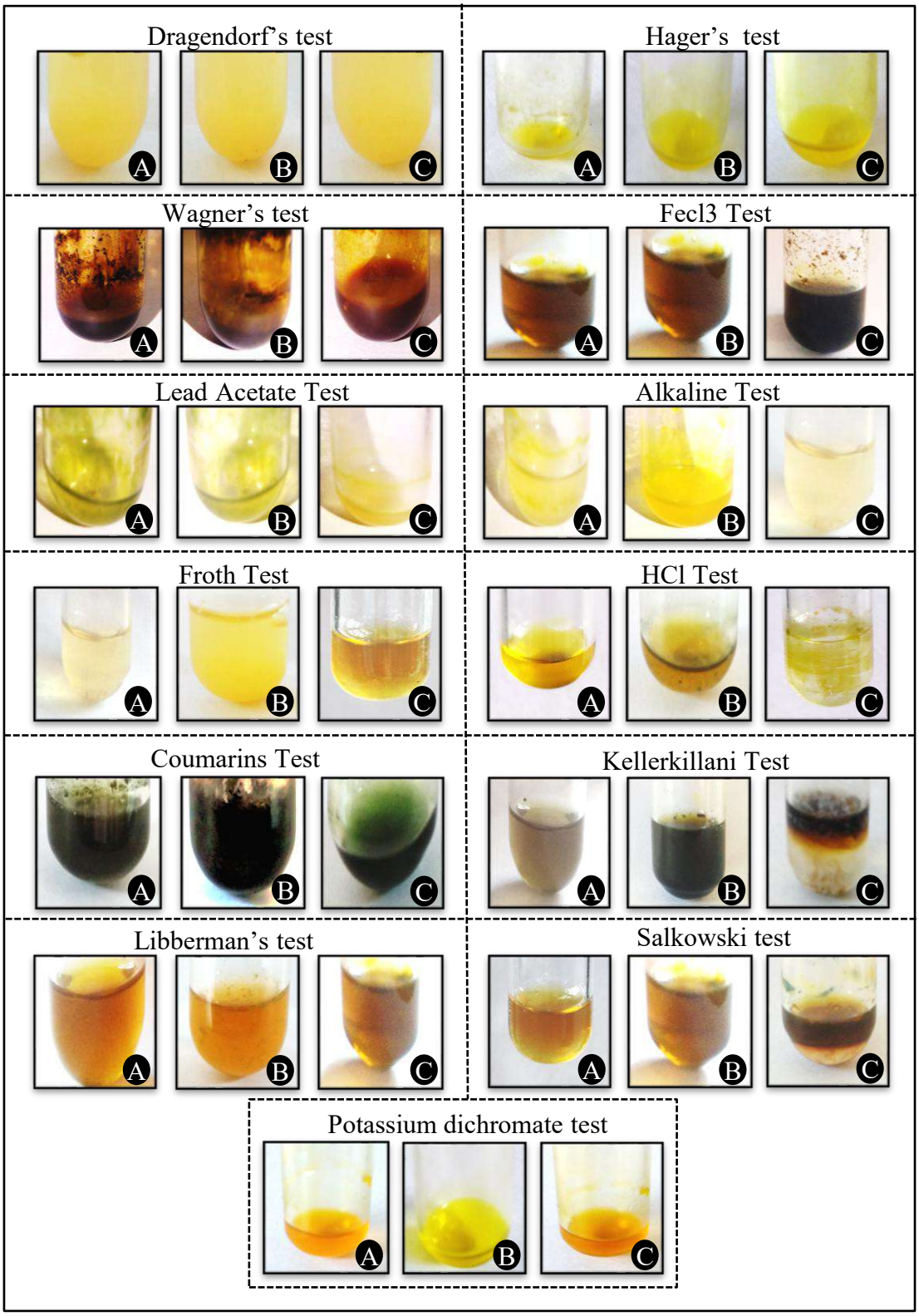


Plate 3.14: Preliminary phytochemical tests of normal leaf (A), gall leaf (B) and gall (C) ethanol extract of *F. racemosa.*

cardiac glycosides indicated their presence only in gall extracts. Quinones and saponis were found in water extracts of gall only. All aqueous extract samples contained steroids and terpenoids.

Quantitative phytochemical analysis

GC-MS analysis of *A. scholaris*

The GC-MS analysis report of methanolic extracts of normal leaves of *A. scholaris* found 54 peaks, gall leaves with 33 peaks and the gall having 63 peaks. Plate 3.15 displays the resulted chromatogram of GC-MS analysis conducted in normal leaf; Plate 3.16 represents the chromatogram of gall leaf whereas Plate 3.17 displays the chromatogram of gall. Compounds in *A. scholaris* with an area percentage of higher than 1 are listed in Table 3.3, along with their chemical formula and area percentage in different samples.

From the phytochemical compounds discovered in *A. scholaris*, 4 were unique to gall, 7 compounds were found unique to gall leaves and 6 were unique to normal leaves. 6 compounds were identified from all the analysed samples. There are 9 compounds detected as common to normal leaf and gall, but they were represented with different area percentages in different samples. Betulin is found in gall leaves and gall and 9, 19-cyclolanostan-3-ol, 24-methylene, (3.beta.) is the only compound common to normal leaf and gall leaf. By analyzing the data it has been found that from normal leaf to gall, some of the chemicals showed an increase in the area percentage.

Khyade and colleagues discovered more than 70 different types of alkaloids from different parts of *A. scholaris* such as root, stem bark, leaves, fruit and flower. The majority of alkaloids are found to occur in the leaves (Khyade et al., 2014). GC-MS analysis of *A. scholaris* was done by Goyal and Shinde in the year 2020 discovered that the methanol and hexane extracts possess 35 and 29 compounds respectively. The compounds like flavonoids, tannins, alkaloid, phenols, coumarins, ketones and terpenoids were extracted only in methanolic leaf extract whereas phenylethyl icosanoate, linoleic acid and unsaturated hydrocarbons from hexane extract (Goyal and Shinde, 2020).

Table 3.3: Results of quantitative phytochemical analysis on methanolic extracts of normal leaves, gall leaves and leaf galls of *A. scholaris* by GC-MS analysis.

Sl. No.	Name of compound	Molecular formula	Area percentage		
			Normal leaf	Gall leaf	Gall
1.	2-Furanmethanol,tetrahydro-.alpha.,.alpha., 5-trimethyl-5-(4-methyl-3-cyclohexen-1-yl)	$C_{15}H_{26}O$	3.99	-	-
2.	2H-pyran-3-ol, tetrahydro-2,2,6-trimethyl-6-(4-methyl-3-cyclohexen-1-yl)-, [3S-[3alpha,6alpha(R*)]]	$C_{15}H_{26}O$	1.19	-	-
3.	Mome inositol	$C_7H_{14}O_6$	1.5	-	4.84
4.	Neophytadiene	$C_{20}H_{38}$	5.69	-	0.48
5.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	3.97	-	4.43
6.	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	2.49	-	-
7.	(Z,Z)-6,9-cis-3,4-epoxy-nonadecadiene	$C_{19}H_{34}O$	3.81	-	-
8.	Hexadecanoic Acid, 2-hydroxy- 1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	2.79	-	0.71
9.	9-Octadecenoicacid (Z)-, 2,3-dihydroxypropyl ester	$C_{21}H_{40}O_4$	2.45	-	0.47
10.	Squalene	$C_{30}H_{50}$	3.24	1.49	4.61
11.	Gamma-tocopherol	$C_{28}H_{48}O_2$	1.97	1.35	0.7
12.	Vitamin E	$C_{29}H_{50}O_2$	3.27	1.39	1.11
13.	Ergost-5-en-3-ol, (3.beta.,24r)	$C_{28}H_{48}O$	2.51	1.8	1.46
14.	Stigmasterol	$C_{29}H_{48}O$	2.79	1.48	2.43
15.	Gamma-sitosterol	$C_{29}H_{50}O$	2.73	-	3.16
16.	Cycloeucalenyl acetate	$C_{32}H_{52}O_2$	3.46	-	-
17.	9,19-Cyclolanost-24-en-3-ol, (3.beta.)	$C_{30}H_{50}O$	4.53	-	2.22
18.	Lupeol	$C_{30}H_{50}O$	2.66	-	6.27
19.	Olean-12-en-3-ol, acetate, (3.beta.)	$C_{32}H_{52}O_2$	4.51	1.76	2.95
20.	9,19-Cyclolanostan-3-ol,24-methylene-, (3.beta.)	$C_{31}H_{52}O$	1.12	2.54	-
21.	Lup-20(29)-en-3-ol, acetate,	$C_{32}H_{52}O_2$	23.13	-	23.51

	(3.beta.)				
22.	Phytyl palmitate	$C_{36}H_{70}O_2$	1.57	-	-
23.	Catechol	$C_6H_6O_2$	-	-	1.13
24.	1,3,4,5-Tetrahydroxy-cyclohexanecarboxylic acid	$C_{15}H_{24}O$	-	-	11.12
25.	9-Octadecenoic acid (Z)	$C_{18}H_{34}O_2$	-	-	1.05
26.	Betulin	$C_{30}H_{50}O_2$	-	10.03	3
27.	Lup-20(29)-en-3-one	$C_{30}H_{48}O$	-	-	2.91
28.	Oleoyl chloride	$C_{18}H_{33}ClO$	-	1.68	-
29.	Stigmast-5-en-3-ol,(3. beta.)	$C_{29}H_{50}O$	-	3.57	-
30.	Alpha.-amyrin	$C_{30}H_{50}O$	-	2.57	-
31.	24-Norursa-3,12-diene	$C_{29}H_{46}$	-	4.14	-
32.	Ursolic aldehyde	$C_{30}H_{48}O_2$	-	1.28	-
33.	Uvaol	$C_{30}H_{50}O_2$	-	1.02	-
34.	Acetamide, 2-(diethylamino)-n-(2,6-dimethyl)	$C_{12}H_{18}N_2O$	-	1.55	-

GC-MS analysis of *F. racemosa*

When *F. racemosa* was analysed using GC-MS and found 53, 24 and 57 peaks in normal leaves, gall leaves and galls respectively. Plate 3.18 displays the resulting chromatogram for normal leaf of *F. racemosa;* Plate 3.19 represents the chromatogram of gall leaf, while Plate 3.20 displays the chromatogram for leaf gall. A list of the phytochemicals in *F. racemosa* with an area percentage of above 1 listed with molecular formula and area percentage in different samples represented in Table 3.4.

The quantitative examination of phytochemicals from *F. racemosa* normal leaves, gall leaves and galls revealed variation in their composition across the tissues examined. There were 4 compounds unique to normal leaves, 8 compounds shared with galls and no compounds shared with gall leaves. Galls have 4 unique compounds and gall leaves have 10 unique compounds. No compounds found to be shared between gall leaf and gall. Despite being reported from all three samples, N-hexadecanoic acid, olean-12-en-3-ol, acetate, (3.beta.) and lup-20(29)-en-3-ol, acetate, (3.beta.) were found to vary in their area percentage.

Ushir and colleagues reported the qualitative phytochemical analysis of *F. racemosa* leaf galls and revealed the presence of secondary metabolites such as alkaloids, flavonoids, steroids, tannins and terpenoids (Ushir et al., 2015). The gall portions extracted in methanol detected the presence of terpenoids. The chloroform extract of the galled portion indicated the presence of glycosides (Savitha et al., 2013).

Table 3.4: Results of quantitative phytochemical analysis on methanolic extracts of normal leaves, gall leaves and leaf galls of *F. racemosa.*

Sl. No.	Name of compound	Molecular formula	Area percentage		
			Normal leaf	Gall leaf	Gall
1.	1,3,4,5-tetrahydroxy-cyclohexanecarboxylic acid	$C_7H_{12}O_6$	10.45	-	15.5
2.	Neophytadiene	$C_{20}H_{38}$	4.02	-	0.61
3.	N-hexadecanoic acid	$C_{16}H_{32}O_2$	6.79	6.87	5.98
4.	2-hexadecen-1-Ol, 3,7,11,15-tetramethyl-, (R-(R*,R*-(E)))	$C_{20}H_{40}O$	3.27	-	-
5.	9,12-octadecadienoic acid (Z,Z)	$C_{18}H_{32}O_2$	1.77	-	-
6.	1-Hexacosanol	$C_{26}H_{54}O$	1.01	-	-
7.	1-Heptacosanol	$C_{27}H_{56}O$	1.97	-	-
8.	Ergost-5-en-3-ol, (3.beta.,24r)	$C_{28}H_{48}O$	1.64	-	0.76
9.	Stigmasterol	$C_{29}H_{48}O$	3.06	-	1.36
10.	Gamma.-sitosterol	$C_{29}H_{50}O$	14.77	-	5.18
11.	Methyl commate D	$C_{31}H_{50}O_4$	2.37	-	1.51
12.	Olean-12-en-3-ol, acetate, (3.beta.)	$C_{32}H_{52}O_2$	4.79	1.87	7.02
13.	Methyl commate B	$C_{31}H_{50}O_3$	21.62	-	0.89
14.	Lup-20(29)-en-3-ol, acetate, (3.beta.)	$C_{32}H_{52}O_2$	4.99	8.49	8.33
15.	Betulin	$C_{30}H_{50}O_2$	4.04	-	1.8
16.	1,2,3-propanetriol	$C_3H_8O_3$	-	-	1.09
17.	4H-pyran-4-one, 2,3- dihydro - 3,5- dihydroxy-6-methyl	$C_6H_8O_4$	-	-	2.05
18.	(Z,Z)-6,9-cis-3,4-epoxy-nonadecadiene	$C_{19}H_{34}O$	-	-	4.23
19.	Ethyl (9z,12z)-9,12-octadecadienoate	$C_{20}H_{36}O_2$	-	-	1.92

20.	Methyl beta-D-glucopyranoside	$C_7H_{14}O_6$	-	34.21	-
21.	Puchidiol	$C_{13}H_{20}O_2$	-	1.65	-
22.	Phytol	$C_{20}H_{40}O$	-	17.06	-
23.	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	-	1.41	-
24.	cis-9-hexadecenal	$C_{16}H_{30}O$	-	6.02	-
25.	9,12,15-octadecatrienoic acid, ethyl ester, (Z,Z,Z)	$C_{20}H_{34}O_2$	-	1.68	-
26.	Hexadecanoic acid, 2-hydroxy -1-(hydroxy methyl) ethyl ester (2-palmitoylglycerol)	$C_{19}H_{38}O_4$	-	3.54	-
27.	Oleoyl chloride	$C_{18}H_{33}ClO$	-	4.78	-
28.	Stigmast-5-en-3-ol,(3. beta.)	$C_{29}H_{50}O$	-	4.54	-
29.	Faradiol	$C_{30}H_{50}O_2$	-	1.25	-

Both normal leaf and gall of *A. scholaris* showed a higher area percentage for the triterpenoid lup-20(29)-en-3-ol, acetate, (3. beta.), which has an unidentified biological action. Betulin showed the highest area percentage in gall leaf of *A. scholaris*, which has reported antiviral, analgesic, anti-flu, cytotoxic, anti-inflammatory and antineoplastic activities (Bębenek et al., 2019; Kim et al., 2023). The substance with the largest area percentage in *F. racemosa* normal leaf is methyl commate B, which is known to have antibacterial and anti-inflammatory properties (Arora et al., 2017; Motlhanka et al., 2010) and in gall- 1,3,4,5-tetrahydroxy- cyclohexanecarboxylic acid, which has antimicrobial, anti-inflammatory, antioxidant and analgesic activities (Mujeeb et al., 2014). This compound has the second-highest area percentage in the gall of *A. scholaris.* Both plant galls and leaves contain n-hexadecanoic acid, which possesses cytotoxic, insecticidal and nematicide qualities (Abubakar and Majinda, 2016; Tyagi and Agarwal, 2017).

Olean-12-en-3-ol, acetate, (3.beta.) was reported from all the 3 samples of both plants, which have antidyslipidemic activity (Maurya et al., 2012). It was discovered that stigmasterol (found in all samples except the gall leaves of *F. racemosa*), has cytotoxic activity (Padmashree et al., 2018; Singh and Chaturvedi, 2019; Sudha et al., 2013; Tyagi and Agarwal, 2017). The 9-octadecenoic acid (Z) found in *A. scholaris* gall has been shown

to have insectifuge irritant effect (Kim et al., 2023; Selvan and Velavan, 2015). 1-heptacosanol, which exhibits nematicidal activity, is found in the normal leaves of *F. racemosa* (Begum et al., 2016; Raman et al., 2012). Most of the compounds were discovered to possess anti-inflammatory, antibacterial and antioxidative qualities. Table 3.5 shows the biological activity of compounds having an area greater than 1% in normal leaves, gall leaves and galls of both plants.

Table 3.5: List of compounds identified from *A. scholaris* and *F. racemosa* with their reported biological activities.

Sl. No.	Name of compounds	Reported bioactivities
1.	2-Furanmethanol, tetrahydro-.alpha., .alpha., 5-trimethyl -5-(4- methyl -3-cyclohexen -1-yl)	Anti-hyperalgesic and anti-edematous activities (Tomić et al., 2014).
2.	Mome inositol	Anti-cirrhotic, anti-neuropathic, cholesterolytic and lipotropic activities (Arora and Saini, 2017).
3.	2H-Pyran-3-ol, tetrahydro -2,2,6-trimethyl-6-(4- methyl-3-cyclohexen-1-yl) [3S-[3alpha, 6alpha (R*)]]	Antifungal activities (Tolouee et al., 2010).
4.	Neophytadiene	Analgesic, antipyretic, anti-inflammatory, antimicrobial and antioxidant activities (Kim et al., 2023; Singh and Chaturvedi, 2019).
5.	n-Hexadecanoic acid	Antioxidant, hypocholesterolemic, pesticide, nematicide, antiandrogenic, hemolytic, 5-alpha reductase inhibitor, antipsychotic, antibacterial, antifungal and cytotoxic activities (Abubakar and Majinda, 2016; Tyagi and Agarwal, 2017).
6.	9,12-Octadecadienoic acid, methyl ester	Antifungal and antioxidant activity (Pinto et al., 2017).
7.	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	Hemolytic and antioxidant activity (Arora et al., 2017; Tyagi and Agarwal, 2017).
8.	9-Octadecenoic acid (Z)-, 2,3- dihydroxypropyl ester	Antioxidant and anti- inflammatory activities (Shaheed et al., 2019).

9.	Squalene	Antibacterial, antioxidant, anti-tumour, immunostimulant, lipoxygenase-inhibitor and chemo-preventive activity (Padmashree et al., 2018; Singh and Chaturvedi, 2019; Sudha et al., 2013).
10.	Gamma.-tocopherol	Antioxidant, cardioprotective, anticancer, anti-inflammatory and hypocholesterolemic activities (Padmashree et al., 2018; Rahim et al., 2017).
11.	Vitamin E	Antioxidant, cytoprotective, anti-aging, analgesic, antidiabetic, anti-dermatitis, antileukemia, anticancer, vasodilator, hepatoprotective, hypocholesterolemic, anti-bronchitis, anti-alzheimer's, immunostimulant, anti-inflammatory, antiulcerogenic and antispasmodic activities (Arora et al., 2017; Padmashree et al., 2018; Singh and Chaturvedi, 2019).
12.	Ergost-5-en-3-ol, (3. beta.,24r)	Antimicrobial, anticarcinogenic, antiangiogenic, hypocholesterolemic and antioxidant activities (Arora et al., 2017).
13.	Stigmasterol	Anti-angiogenic, anticancer, anti-inflammatory, antioxidant, antidiabetic, cytotoxic and antimicrobial activities (Padmashree et al., 2018; Singh and Chaturvedi, 2019; Sudha et al., 2013; Tyagi and Agarwal, 2017).
14.	Gamma.-sitosterol	Antioxidant, antimicrobial, antidiabetic, antiasthma, diuretic, hypolipidemic and prophylactic activities (Padmashree et al., 2018; Singh and Chaturvedi, 2019; Sudha et al., 2013).
15.	Cycloeucalenyl acetate	Antibacterial and antifungal activities (Zubair et al., 2017).
16.	9,19-Cyclolanost-24-en-3-ol, (3.beta.)	Antibacterial, anticancer, antidiabetics and pesticidal activity (Naidoo et al., 2023).
17.	Lupeol	Cytotoxic and anti-inflammatory activities (Padmashree et al., 2018; Singh and Chaturvedi, 2019; Sudha et al., 2013).
18.	Olean-12-en-3-ol, acetate, (3. beta.)	Antidyslipidemic activity (Maurya et al., 2012).
19.	9,19-cyclolanostan-3-ol, 24-methylene-, (3. Beta.)-	Antibacterial activity and anti-HIV compound used to prevent the HIV virus (Arora et al., 2017).
20.	Lup-20(29)-en-3-ol, acetate, (3.beta.)	Data deficient.
21.	Phytyl palmitate	Antimicrobial and cytotoxic activity (Damasceno et al., 2019).
22.	Catechol	Antimicrobial, antioxidant, anti-inflammatory and

		analgesic activities (Juch and Rüedi, 1997; Kocaçalışkan et al., 2006).
23.	1,3,4,5-Tetrahydroxy-cyclohexanecarboxylic acid	Antimicrobial, anti-inflammatory, antioxidant and analgesic activities (Mujeeb et al., 2014).
24.	9-Octadecenoic acid (Z)	Antibacterial, antioxidant, allergenic, anemia genetic, antiandrogenic, anti-inflammatory, cancer preventive, choleretic, hypocholesterolemic and insectifuge irritant activities (Kim et al., 2023; Selvan and Velavan, 2015).
25.	Cis-9-hexadecenal	Antimicrobial activity (Mujeeb et al., 2014).
26.	Betulin	Antiviral, analgesic, anti-flu, cytotoxic, anti-inflammatory and antineoplastic activities (Bębenek et al., 2019; Kim et al., 2023).
27.	Lup-20(29)-en-3-one	Trypanocidal activity (Polanco-Hernández et al., 2012).
28.	2-Hexadecen-1-ol,3,7,11,15-tetramethyl-,(R-(R*, R*-(E)))	Antioxidant, anti-inflammatory, anticancer neuroprotective, antinociceptive, antioxidant, antimicrobial and diuretic activities (Rahim et al., 2017).
29.	9,12-Octadecadienoic acid (Z, Z)	Antioxidant, antiproliferative, anti-inflammatory and antiarthritic activities (Padmashree et al., 2018; Singhand Chaturvedi, 2019).
30.	1-Hexacosanol	Antileishmanial activity (Padmashree et al., 2018).
31.	1-Heptacosanol	Nematicidal, anticancer, antioxidant and antimicrobial activities (Begum et al., 2016; Raman et al., 2012).
32.	Methyl commate D	Antimicrobial and anti-inflammatory activities (Motlhanka et al., 2010).
33.	Methyl commate B	Antimicrobial and anti-inflammatory activities (Arora et al., 2017; Motlhanka et al., 2010).
34.	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	Antimicrobial, anti-inflammatory, antiproliferative, anticancer and antioxidant activities (Mujeeb et al., 2014; Yu et al., 2013).
35.	(Z,Z)-6,9-cis-3,4-epoxy- nonadecadiene	Antibacterial activity (Kumar and Sati, 2021).
36.	Ethyl (9z,12z)- 9,12-octadecadienoate	Anti-inflammatory activity (Kim et al., 2023).
37.	Methyl beta-D-glucopyranoside	Unknown activity.
38.	Puchidiol	Antioxidants, antiviral and anti-inflammatory activity (Kavitha, 2021; Lina and Eman, 2022; Zacharia et al., 2024).
39.	Phytol	Antimicrobial, anticancer, antiinflammatory antioxidant, anti-diabetic, anti-diuretic,

		neuroprotective, antidepressant, anticonvulsant and antiviral activity (Kavitha, 2021; Zacharia et al., 2024).
40.	Hexadecanoic acid ethyl ester	Antibacterial, antioxidant, hemolytic, hypocholesterolemic, nematicide and anti-androgenic activities (Shaaban et al., 2021; Tyagi and Agarwal, 2017).
41.	cis-9-Hexadecenal	Antifungal, cytotoxic and antibacterial activity (Hoda et al., 2019; Lykholat et al., 2021).
42.	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)	Anti-melanogenesis, anti-inflammatory, antioxidant and antimicrobial properties (Huh et al., 2010).
43.	Hexadecanoicacid, 2-hydroxy -1-(hydroxymethyl)ethyl ester	Sugar-phosphatase inhibitor, lipid metabolism regulator, antiinfective, anti-inflammatory, histamine release inhibitor and antiprotozoal activity (Adnan et al., 2019).
44.	Oleoyl chloride	Unknown activity.
45.	Stigmast- 5-en-3-ol,(3. beta.)	Methyltransferase inhibitor, an anti cholesteremic drug and an antioxidant (Kim et al., 2023).
46.	Faradiol	Antiinflammatory activity (Reznicek and Zitterl-Eglseer, 2003).
47.	Alpha.-amyrin	Anti-inflammatory and antioxidant agent (Melo et al., 2010).
48.	24-Norursa-3,12-diene	Antibacterial and antifungal activity (Ullah et al., 2022).
49.	Ursolic aldehyde	Unknown activity.
50.	Uvaol	Anti-proliferative and pro-apoptotic effect (Bonel-Pérez et al., 2020).
51.	Acetamide, 2-(diethylamino) -N-(2,6-dimethyl)	Unknown activity.

Quantitative phytochemical analysis using OHR-LC-MS

OHR-LC-MS analysis was performed on normal leaves, gall leaves and gall ethanolic extracts of plants, yielding different phytochemical compounds, fatty acids and essential amino acids from various tissues. The data from each sample was evaluated and a phytochemical profile was developed for each sample. The phytochemical makeup of normal leaf samples was compared to gall leaf and gall information in order to detect modifications in plant tissue associated with infection by gall causer. The composition of

phytochemical compounds in different samples of both plants, they are: *A. scholaris*-normal leaf (AS-NL), *A. scholaris*-gall leaf (AS-GL), *A. scholaris*-gall (AS-GA), *F. racemosa*-normal leaves (FR-NL), *F. racemosa*-gall leaves (FR-GL) and *F. racemosa*-gall (FR-GA) were represented graphically in Plate 3.21.

Quantitative phytochemical analysis of *A. scholaris*-normal leaf (AS-NL)

The AS-NL ethanolic extracts were analysed using OHR-LC-MS analysis to find out the quantitative phytochemical profile. The analysis expressed the presence of 22 different chemical compounds. Among the compounds 8 were phenolic compounds of different categories, they are; 1- cardiac glycoside, 2 were terpenes, 10 were fatty acids and 1-amino acid. 9 (10)-epoxy-12Z-octadecenoic acids present in AS-NL has a role as chemical mediators of insects. Another compound, chlorogenic acid is an intermediate metabolite in the biosynthesis of lignin. Lignin helps in strengthening tissues. Another fatty acid, 16-hydroxyhexadecanoic acid is a component of the plant cuticle. Cuticles are important plant cell structures that act as barriers limiting water and nutrient loss and protecting plants from radiation and pathogens. The chromatogram of AS-NL is displayed in Plate 3.22: Figure A. Table 3.6 represents the list of compounds detected in AS-NL extracted through OHR-LC-MS with molecular formula, chemical class and reported bioactivities. Plate 3.23 represents a sunburst diagram showing the phenolic compounds present in the AS-NL with their reported bioactivity.

Quantitative phytochemical analysis of *A. scholaris*-gall leaf (AS-GL)

36 distinct chemical compounds have been identified in AS-GL through OHR-LC-MS analysis. Of these, 16 were phenolic compounds (including lignin-1, phenolics-7 and flavonoids-6), 5 were terpenes, 6 were nitrogen-containing compounds, 7 were fatty acids and 1-aminoacid. There were twice as many phenolic chemicals in AS-GL compared to AS-NL. Chlorogenic acid, an intermediate in the production of lignin, was found in gall leaves. Similar to normal leaves, 16-hydroxyhexadecanoic acid, a cuticle component, is also found. Another fatty acid that was found in AS-GL was 12-oxo phytodienoic acid, which is an intermediary in the production of jasmonic acid. The chromatogram of AS-GL is displayed in Plate 3.22: Figure B. Table 3.7 represents the list of compounds detected in the AS-GL

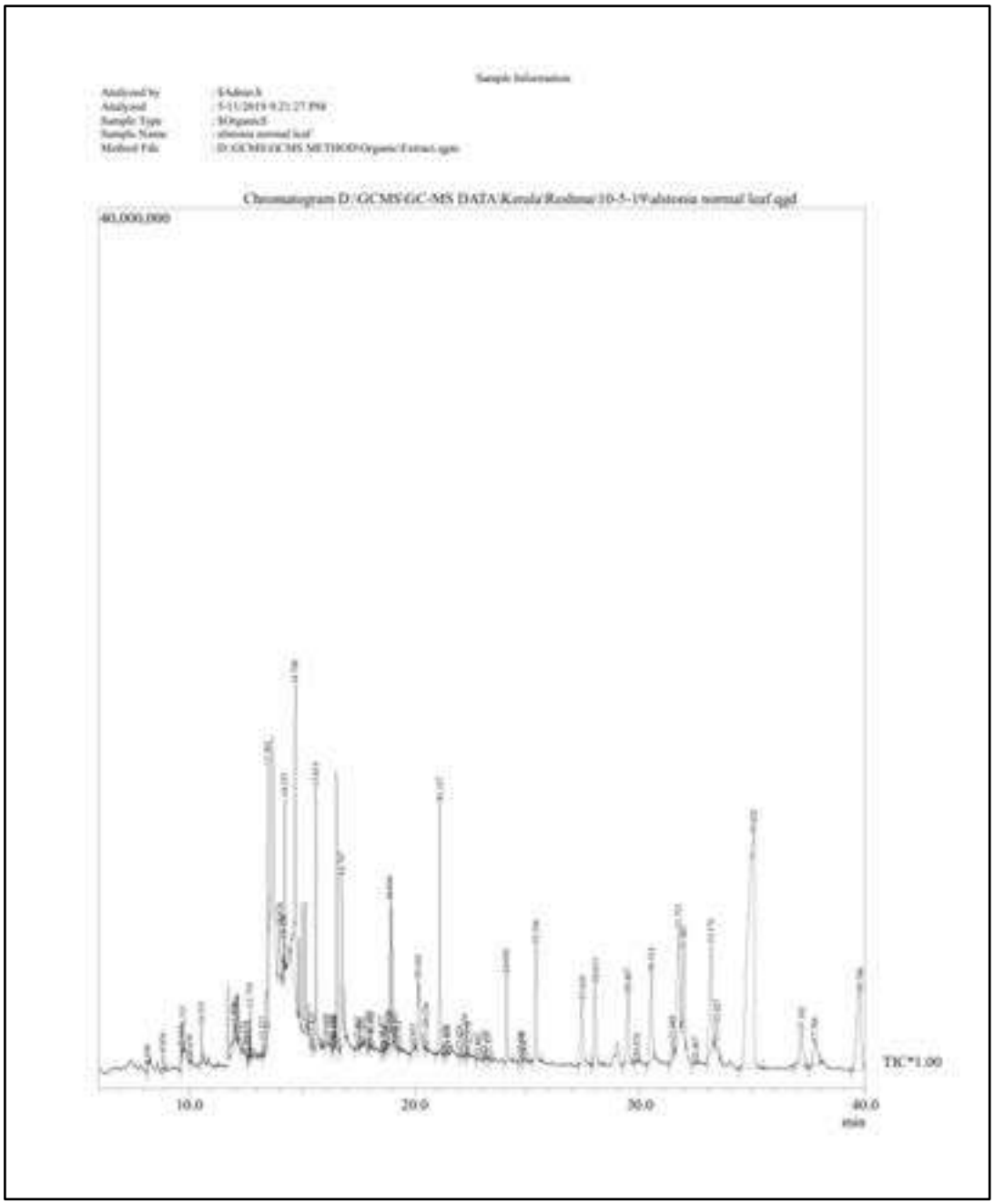


Plate 3.15: GC-MS chromatogram obtained through GC-MS analysis of the methanolic extract of *A. scholaris* normal leaves (AS-NL). Each peak represents a compound eluted at a specific retention time, indicating the presence of different phytoconstituents in the extract of *A. scholaris* normal leaf extracts, showing different peaks.

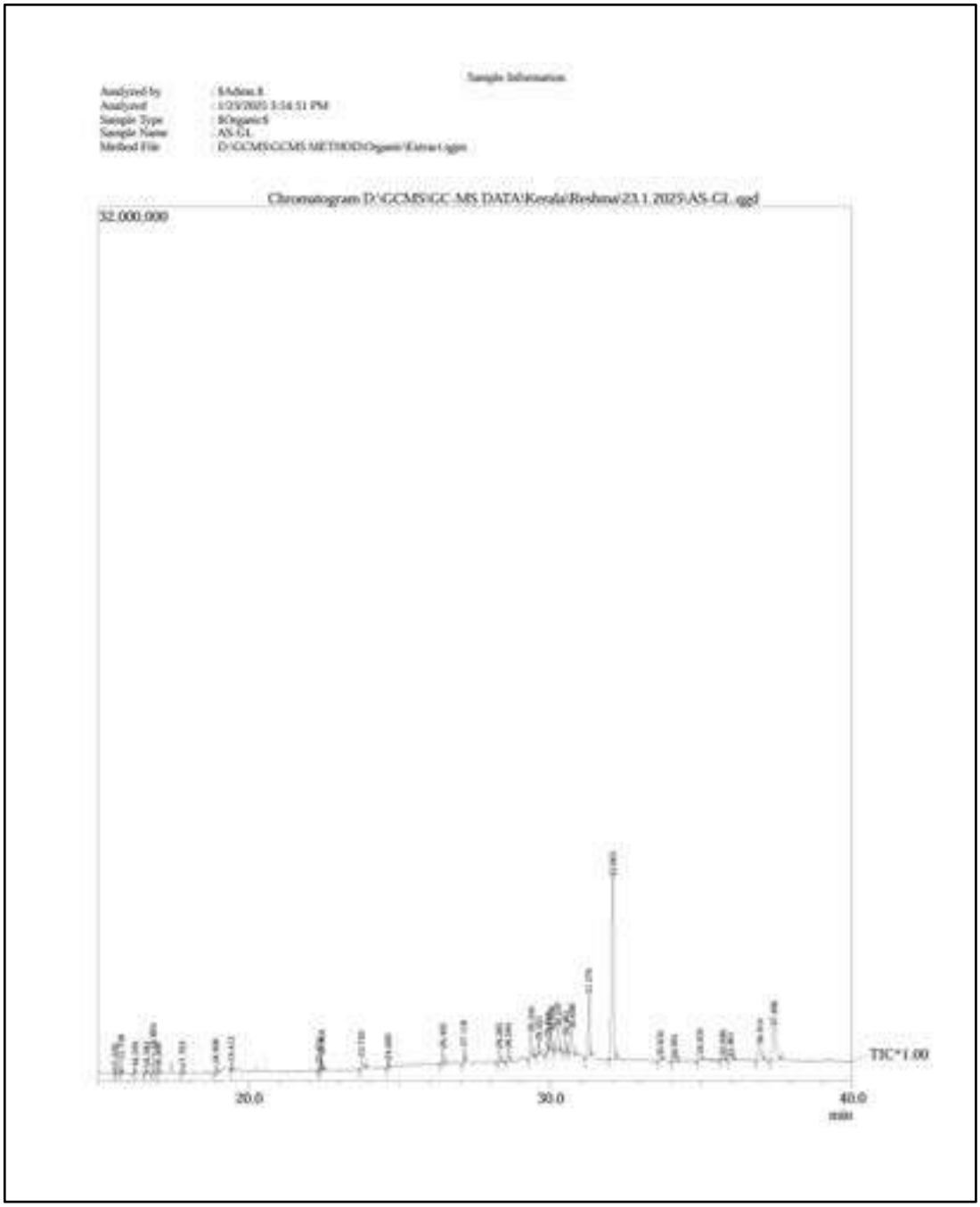


Plate 3.16: GC-MS profile of methanolic extract of gall leaf of *A. scholaris* (AS-GL). Each peak in this chromatogram corresponds to a bioactive compound separated based on its retention time, reflecting the metabolic diversity present in the sample.

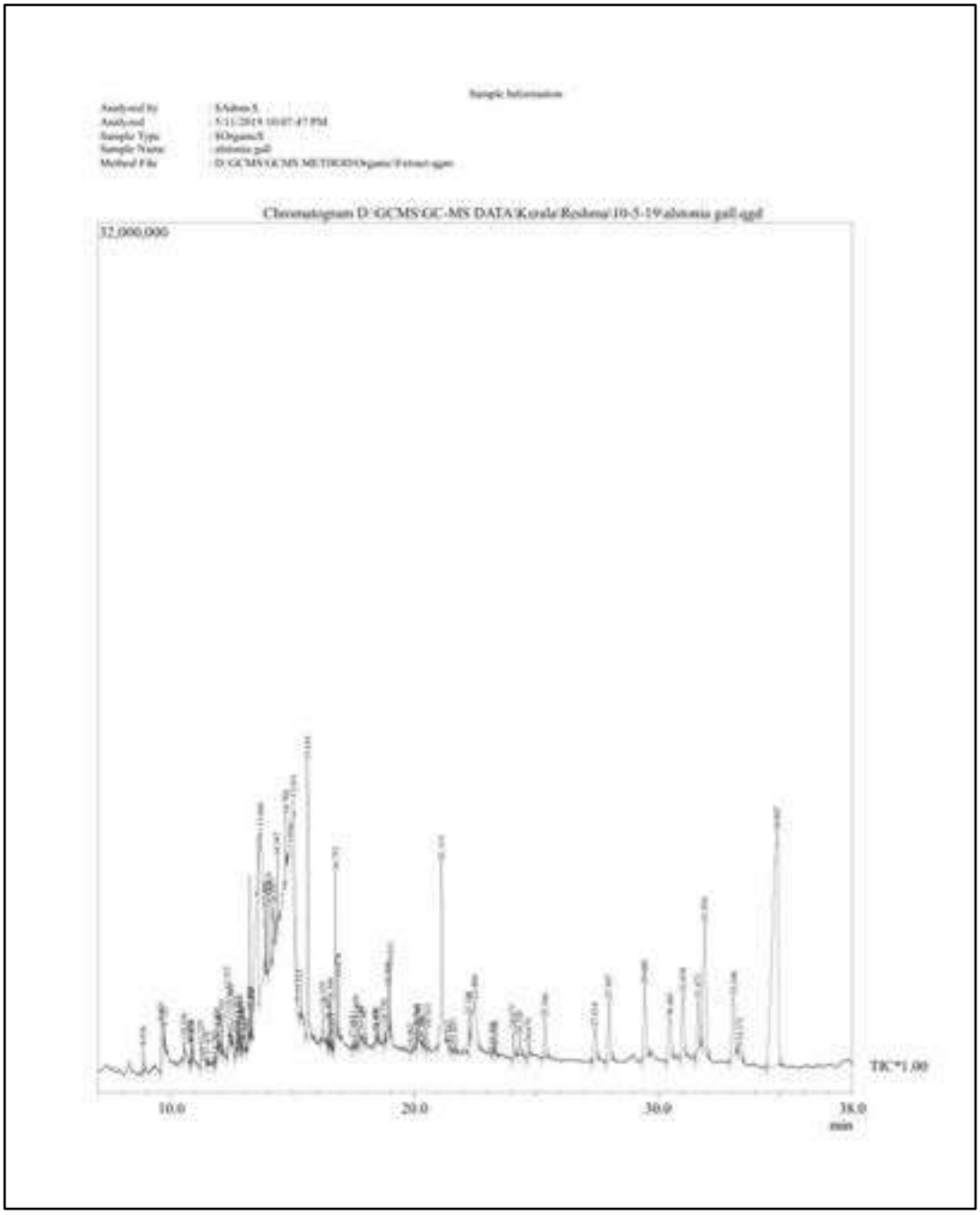


Plate 3.17: GC-MS chromatogram of *A. scholaris* gall (AS-GA) illustrates the separation of volatile constituents present in the methanolic extract of gall of *A. scholaris*. Each peak represents a distinct compound identified based on its retention time, highlighting the phytochemical complexity of the gall extract.

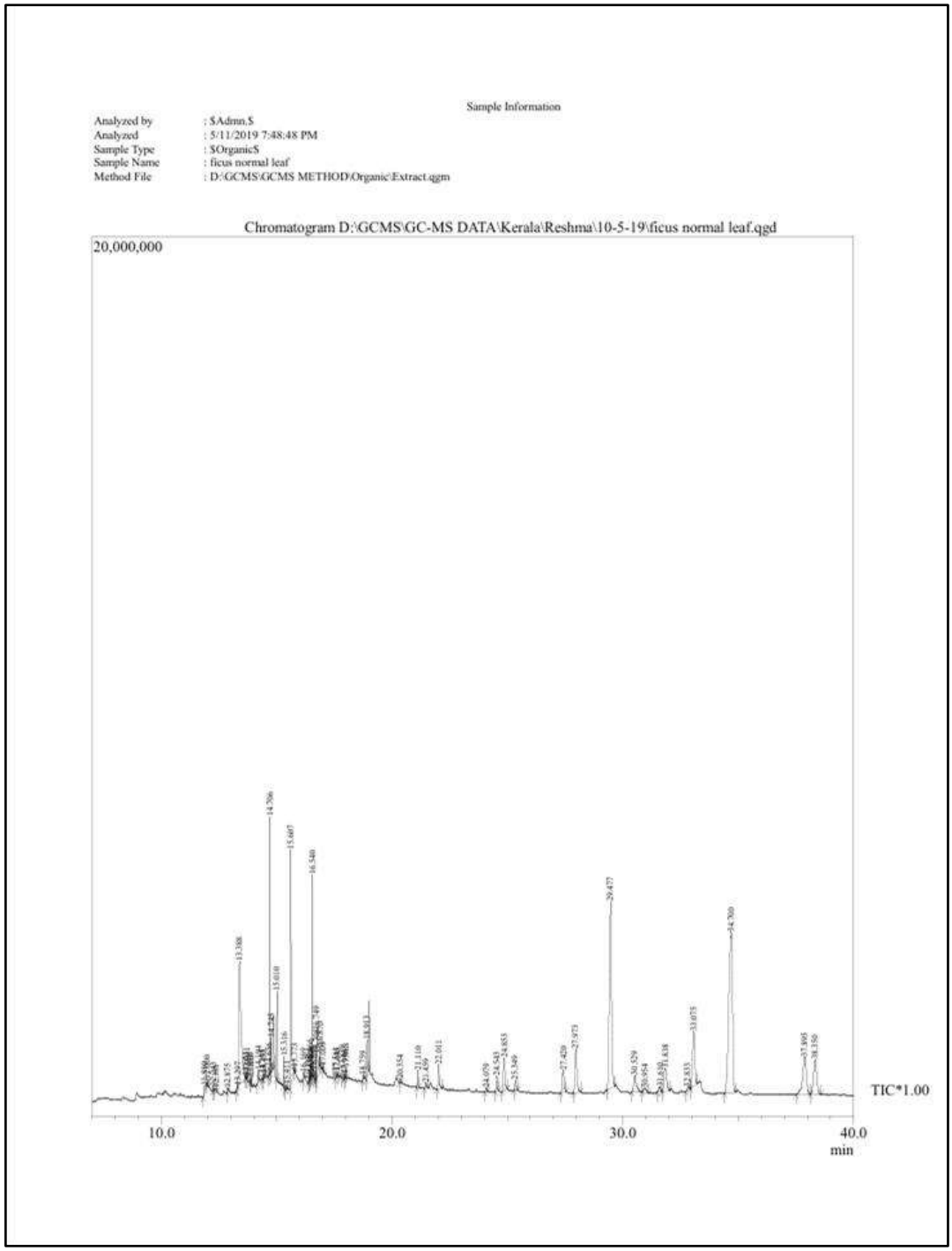


Plate 3.18: GC-MS chromatogram obtained through GC-MS analysis of the methanolic extract of normal leaf of *F. racemosa* (FR-NL). Each peak represents a compound eluted at a specific retention time, indicating the presence of different phytoconstituents in the extract.

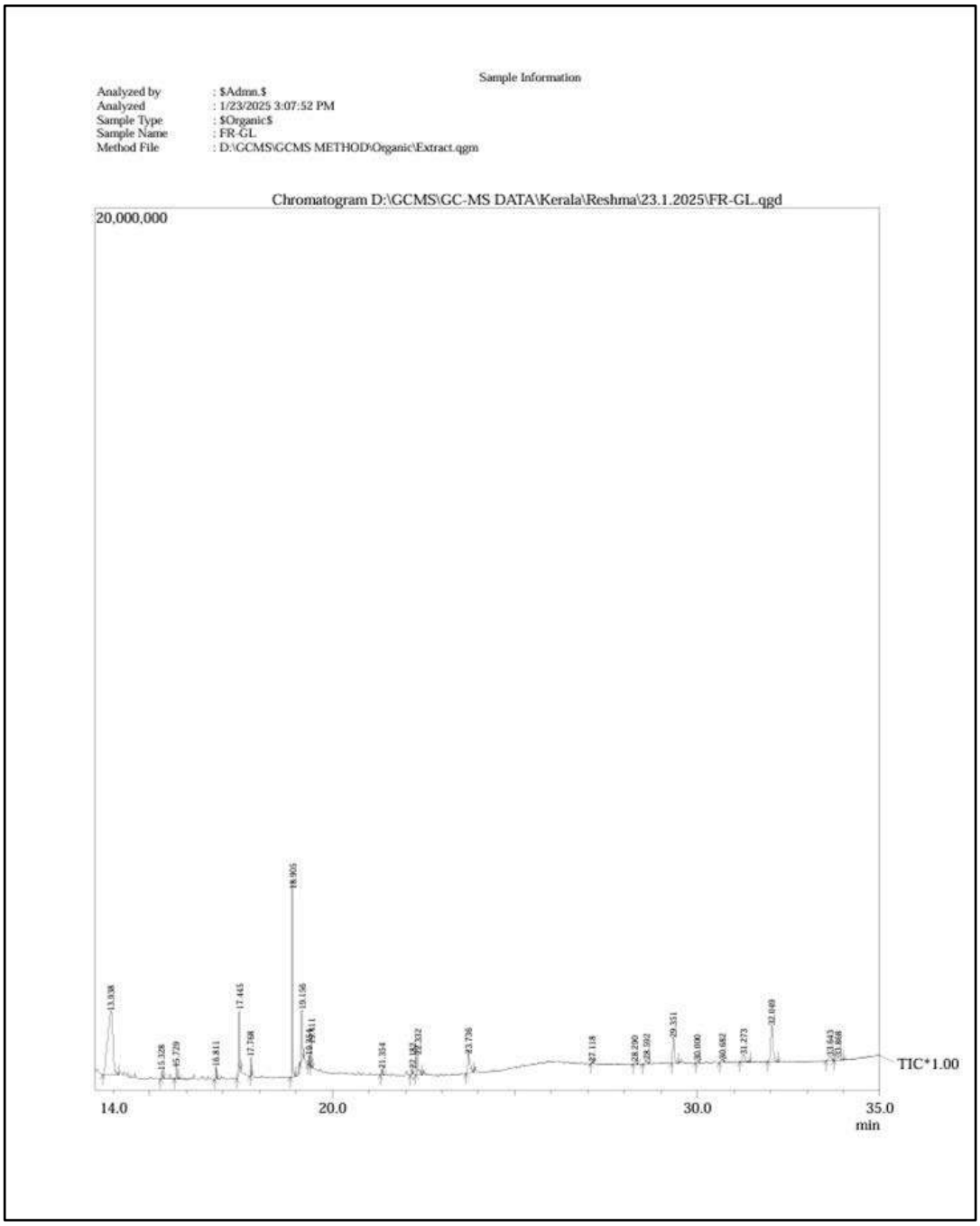


Plate 3.19: GC-MS profile of *F. racemosa* gall leaf (FR-GL) extract. Each peak in this chromatogram corresponds to a bioactive compound separated based on its retention time, reflecting the metabolic diversity present in the sample.

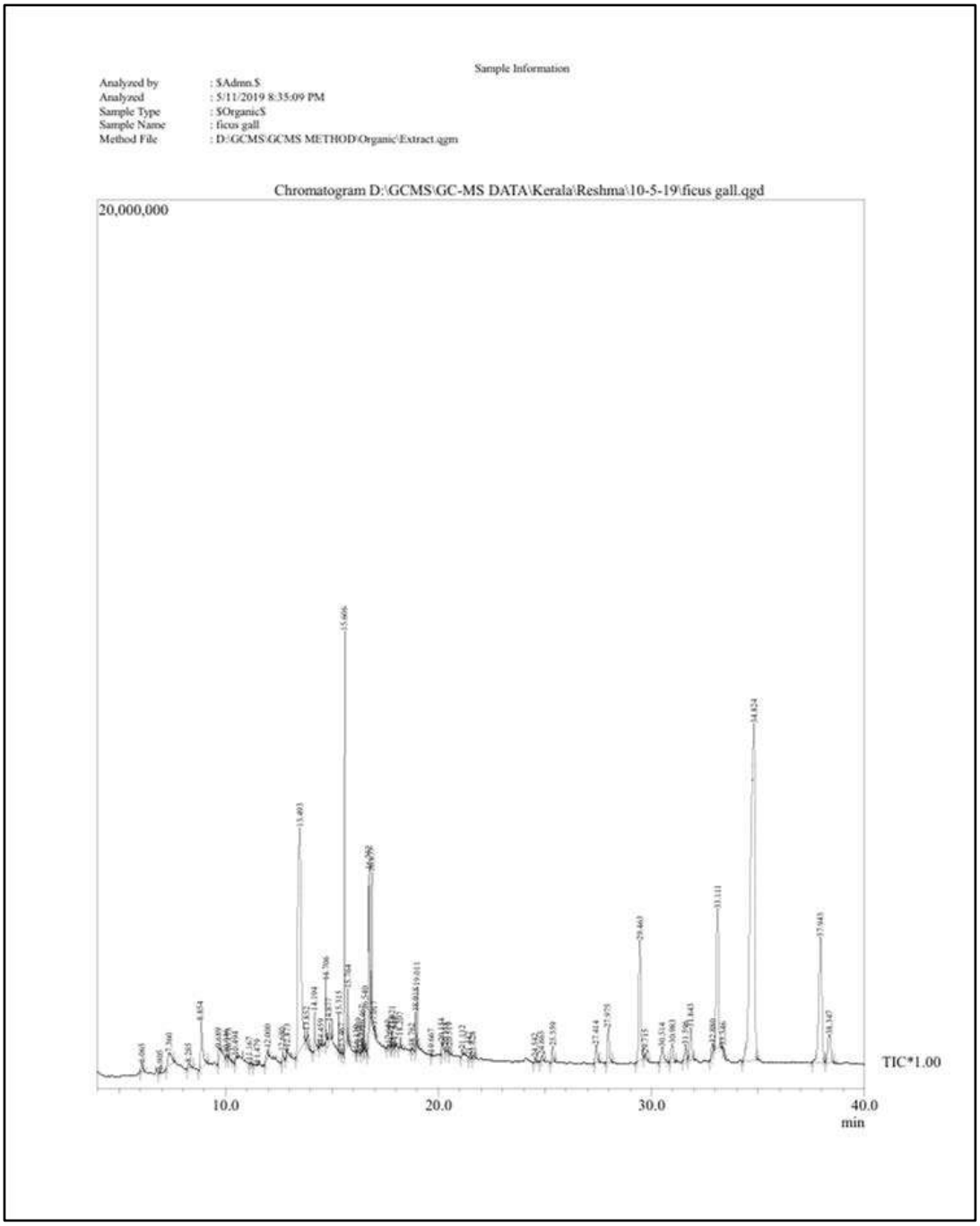


Plate 3.20: GC-MS chromatogram *F. racemosa* gall (FR-GA) extract illustrates the separation of volatile constituents present in the methanolic extract of *F. racemosa*. Each peak represents a distinct compound identified based on its retention time, highlighting the phytochemical complexity of the gall extract.

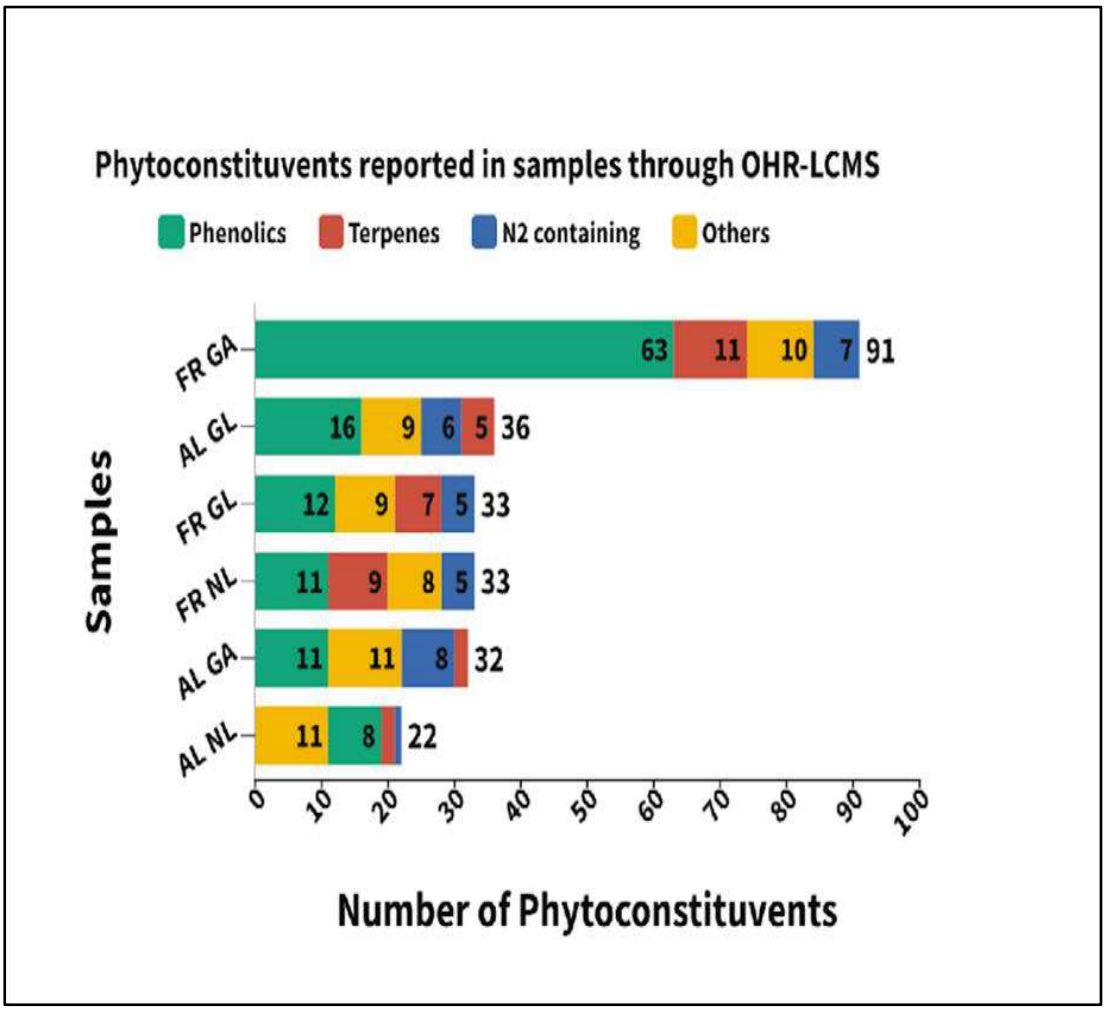


Plate 3.21: Bar diagram representing the number of different phytochemical compound classes identified in normal leaves, gall leaves and leaf galls of *A. scholaris* and *F. racemosa.*

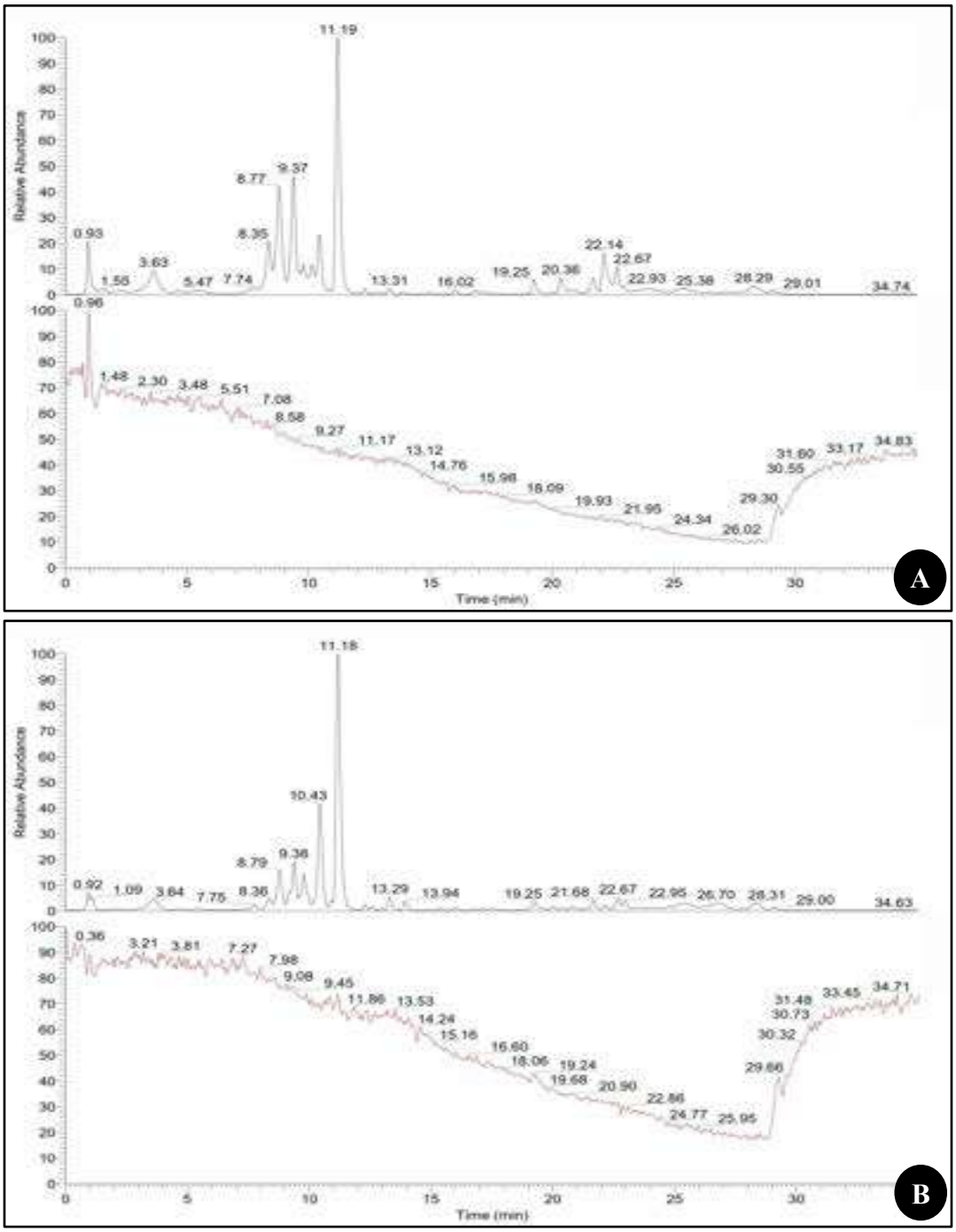


Plate 3.22: Figure A: OHR-LC-MS-Chromatogram of ethanolic extracts of AS-NL presents the metabolic profile of the AS-NL, revealing the presence of several bioactive compounds and Figure B: OHR-LC-MS Chromatogram of ethanolic extracts of AS-GL shows the chemical profile of the gall leaf, indicating the presence of a variety of secondary metabolites based on distinct retention time peaks.

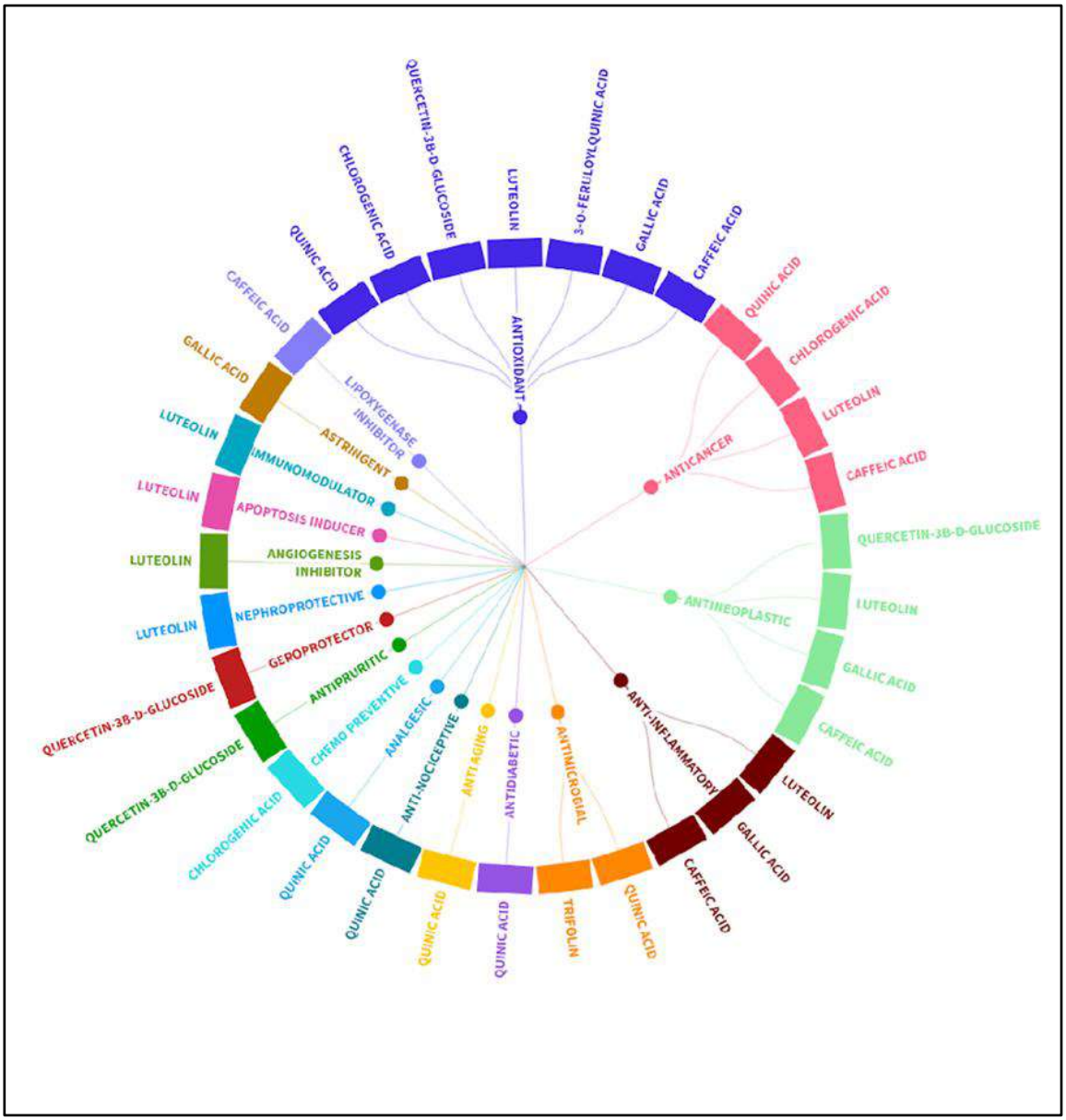


Plate 3.23: The diagram illustrates the diverse pharmacological properties of phenolic constituents present in AS-NL. These activities are represented with lines linking specific compounds to their known bioactivities, highlighting the potential therapeutic value of the identified phytochemicals.

extracted through OHR-LC-MS with molecular formula, chemical class and reported bioactivities. Plate 3.24 represents a sunburst diagram showing the phenolics compounds present in the AS-GL with their reported bioactivity.

Quantitative phytochemical analysis of *A. scholaris*-gall (AS-GA)

AS-GA yielded 32 peaks through OHR-LC-MS analysis. Of these chemical compounds 11 were different phenolic compounds, they include phenols, flavonoids and lignins. The gall also indicated the presence of terpenes, nitrogen containing compounds, fatty acids and amino acids. N-acetyldopamine, which is a reactive intermediate in sclerotization, was found in gall. It may act as sclerotizing agent of the insect cuticle (Karlson and Sekeris, 1962). Similar to AS-GL and AS-NL, gall also contained 12-oxo phytodienoic acid, a fatty acid. The chromatogram of gall is shown in Plate 3.25: Figure A. Table 3.8 represents the list of compounds detected in AS-GA through OHR-LC-MS, with molecular formula, chemical class and their bioactivities. Plate 3.26 represents the diagram showing the phenolics compounds present in the AS-GA with their reported bioactivity.

By analysing the results it has been found that 7 compounds were common to normal leaves, gall leaves and galls of *A. scholaris*, they are trifolin, ursolic acid, quercetin-3β-D-glucoside, 12,13-dihydroxy octadec-9-enoic acid, azelaic acid, gallic acid and 18-β-glycyrrhetinic acid. Out of reported chemical compounds 11 each were found exclusive to normal leaves and gall leaves and 9 were unique to galls. About 15 compounds were found to be shared between gall and associated tissues. Only 3 common compounds were present in normal leaves and gall leaves and only 1 compound shared between normal leaves and gall of *A. scholaris*.

Previous works reported lower carbohydrate levels in mature galls. Gall tissue showed no appreciable alterations in its protein and total amino acid composition. Compared to young ungalled leaves, mature galls exhibited higher lipid content. Among them, some lipids may serve as building blocks for the insect moulting hormone, ecdysteroid, while others may give structural role in cellular membranes of insects (Manna et al., 2018; Muhammad et al., 2023). The present work identified the presence of N-acetyldopamine from gall tissue, which is a reactive intermediate in sclerotization of insects

(Karlson and Sekeris, 1962). As gall develops phenol production of gall tissues also increases (Biswas et al., 2014; Khurshid et al., 2021). In this study, OHR-LC-MS analysis of normal leaf of *A. scholaris* yielded 8 phenolic compounds belonging to different categories such as flavonoids, phenols, lignin etc. In the case of gall leaf and gall, the total number of compounds identified was 36 and 32 respectively. Among them 16 phenolic compounds were reported from gall leaves and 11 phenolics found from gall. The majority of them found to have different bioactivities. Our observation also supports the previous finding of increase in the production of phenolic compounds as a result of gall induction.

Biswas and colleagues in 2014 found that during gall development, ROS were produced, which increased the production of plant hormones and polyphenols in gall tissues. This may lead to the disruption of some biochemical pathways and ultimately resulting in gall formation. Elicitors produced by insects activate the synthesis of different enzymes and some secondary metabolites which are important for resistance in plants. Different antioxidant enzymes (catalase and peroxidase) act as biochemical markers of foliar gall resistance in *A. scholaris* (Biswas et al., 2014). A monoterpene indole alkaloid, 2-hydroxy deacetyl akuammiline and other compounds such as ursolicacid, lupeol acetate, 17-nor-excelsinidine and 19, 20 E-vallesamine were isolated from methanolic and extracts of leaf galls of *A. scholaris* (Pandey et al., 2023). Phytochemical study of the gall discovered the presence of a nor-monoterpenoid indole alkaloid, alstorisine A, in both gall and gall leaves and not found in normal healthy leaves (Chen et al., 2016). Our work also detected compounds like trifolin, ursolic acid, quercetin-3β-D-glucoside, 12, 13- dihydroxy octadec-9-enoic acid, azelaic acid, gallic acid and 18-β-glycyrrhetinic acid from all the samples analysed.

Quantitative phytochemical analysis of *F. racemosa*-normal leaves (FR-NL)

The chromatogram of FR-NL shown in Plate 3.25: Figure B. Table 3.9 represents the list of compounds discovered with its molecular formula and bioactivity. Plate 3.27 represents a sunburst diagram showing the phenolics compounds present in the FR-NL with their reported biological activity.

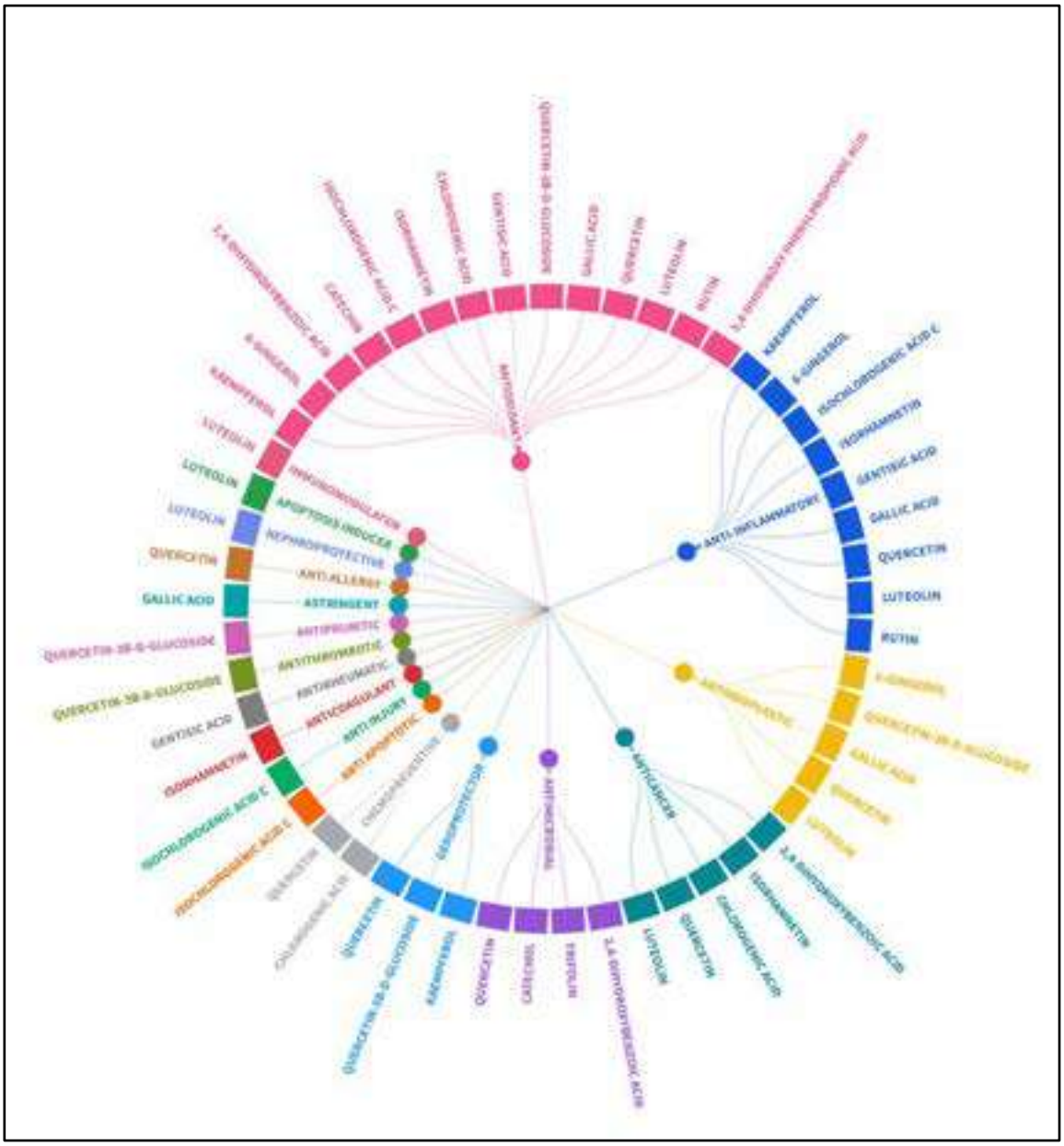


Plate 3.24: Schematic diagram illustrating the bioactivities of phenolic compounds identified in AS-GL. Each line links a specific phenolic compound to its corresponding biological activity.

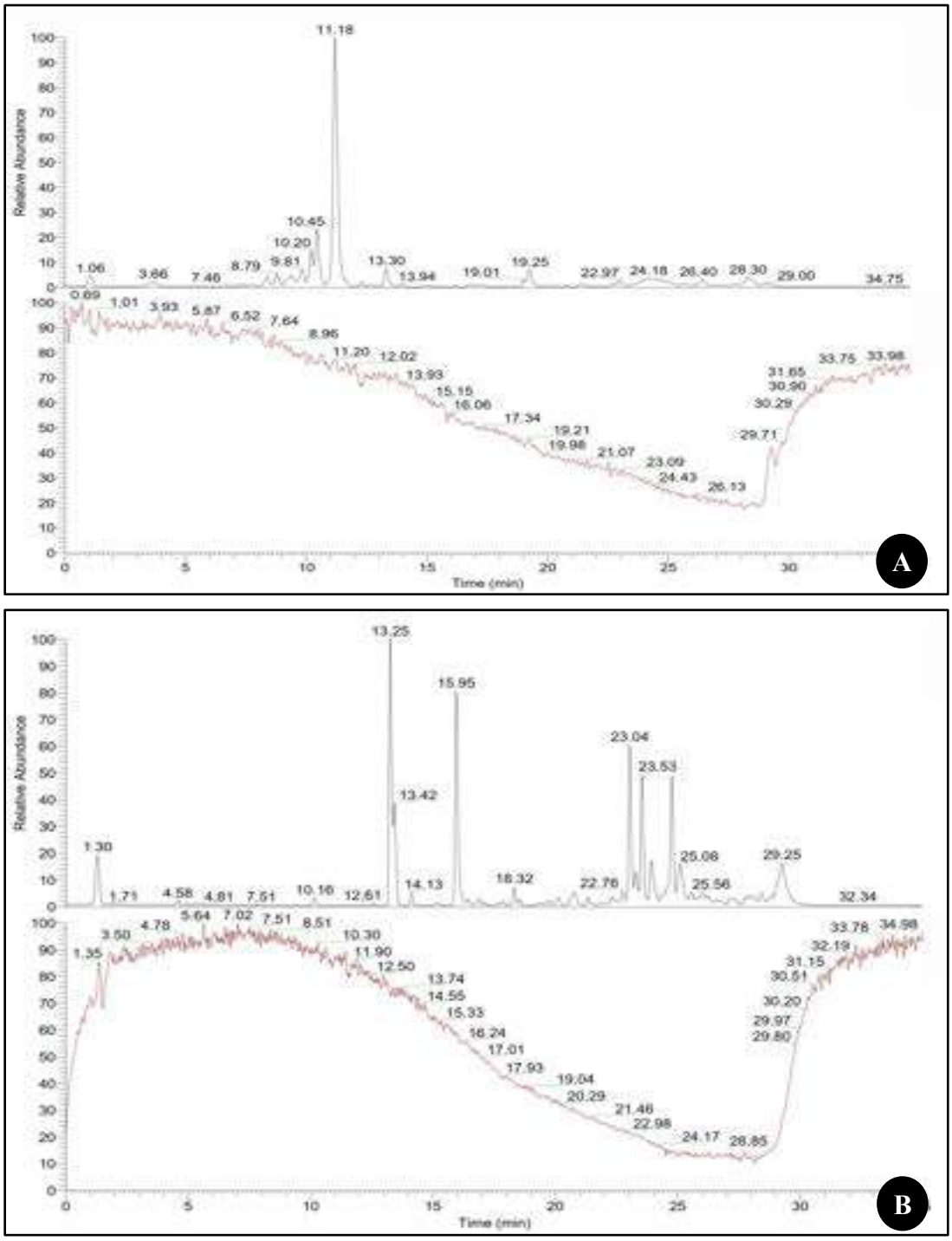


Plate 3.25: Figure A: Shows the chromatographic profile of AS-GA, characterized by several sharp and well-resolved peaks at different retention times (RT), indicating the presence of distinct secondary metabolites and Figure B: displays the OHR-LC-MS chromatogram of ethanolic extracts of FR-NL with distinct peaks corresponding to individual compounds.

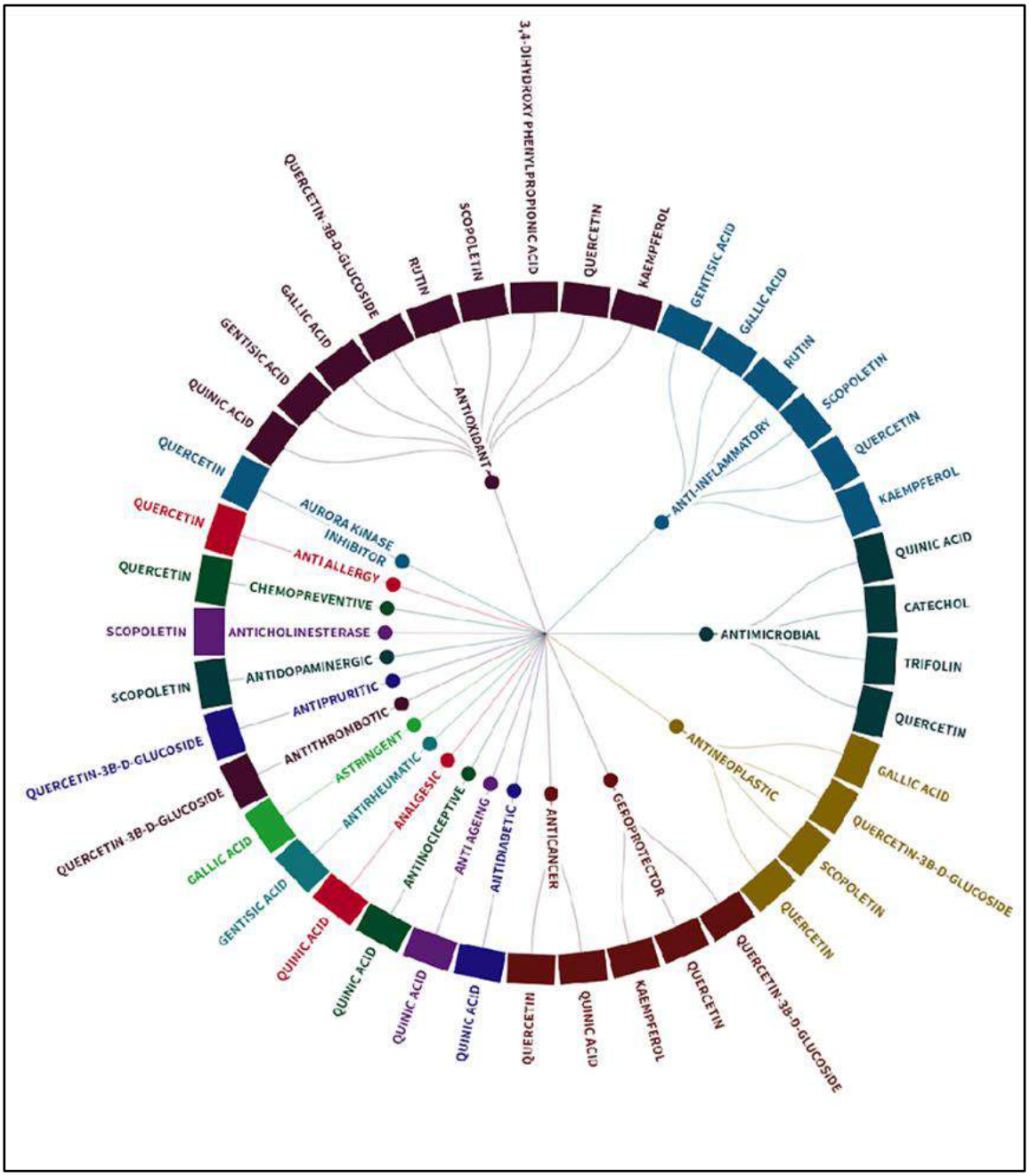


Plate 3.26: The diagram illustrates the pharmacological potential of phenolic constituents specifically present in the AS-GA. Compounds and their respective activities are connected by lines and many of the compounds have multiple bioactivity.

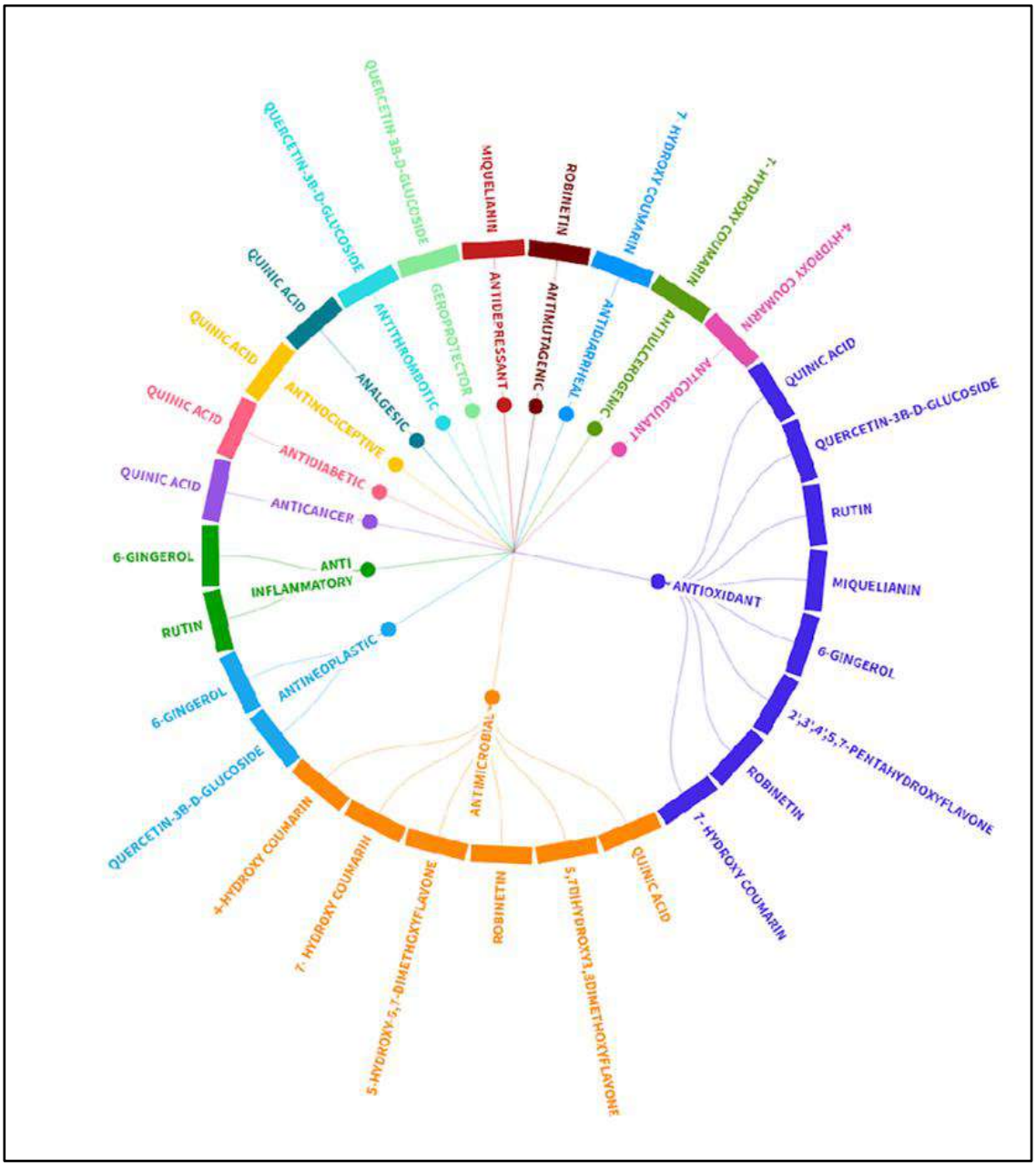


Plate 3.27: The diagram illustrates the diverse pharmacological properties of phenolic constituents present in FR-NL. These bioactivities are represented with lines linking specific compounds, highlighting the potential therapeutic value of the identified phytochemicals.

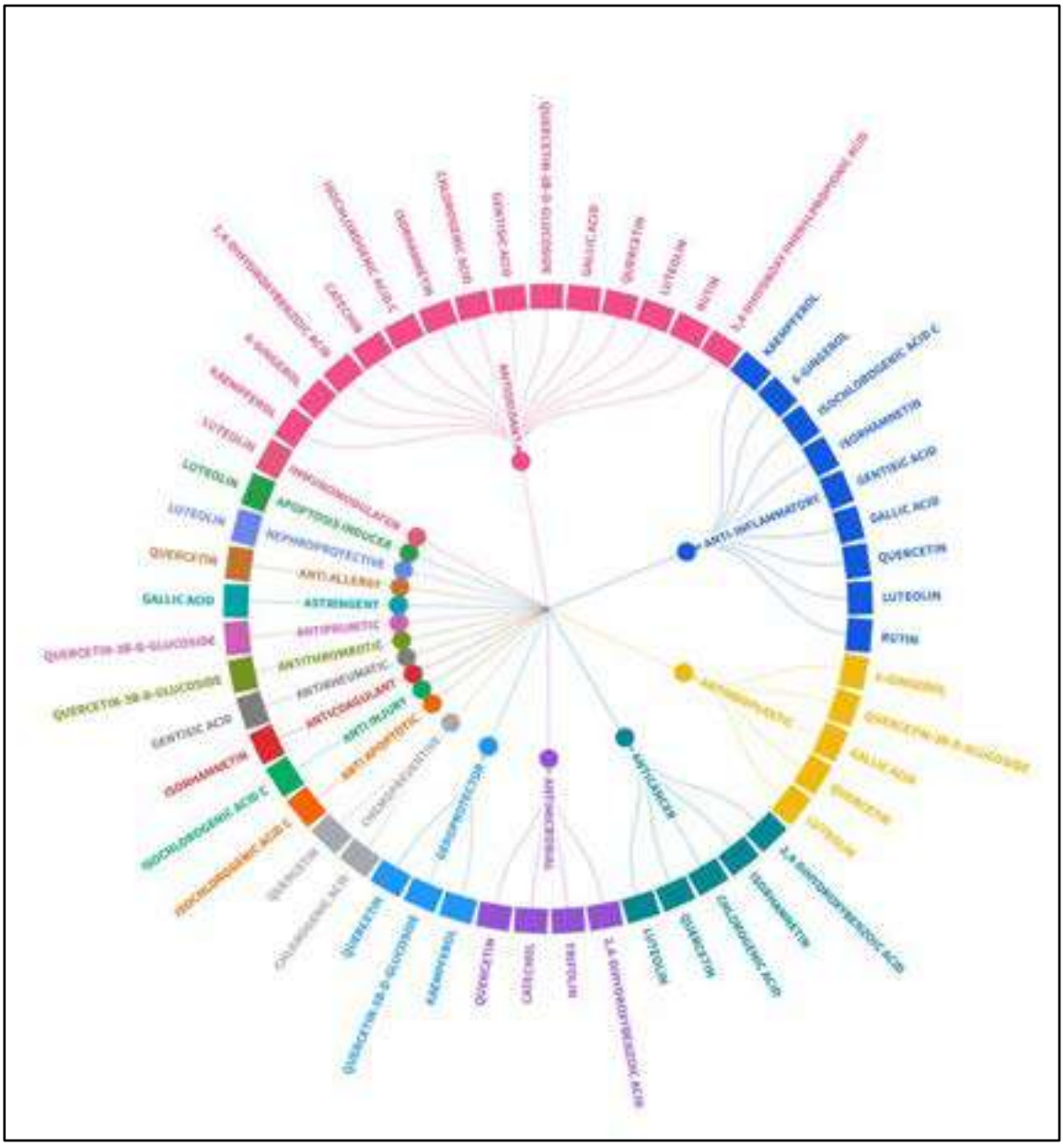


Plate 3.24: Schematic diagram illustrating the bioactivities of phenolic compounds identified in AS-GL. Each line links a specific phenolic compound to its corresponding biological activity.

OHR-LC-MS analysis of FR-NL detected 33 different phytochemical compounds of important pharmacological activities. Among these compounds, the majority of them are important phenolic compounds of different categories like phenols, flavonoids, flavonol, flavone and coumarins. The other compounds present in the normal leaf include various classes of terpenes (monoterpene, diterpene and sesquiterpene), alkaloids and fatty acids. The majority of the identified phenolic compounds have been found to possess antioxidant properties. 12- oxo phytodienoic acid, which was reported from AS-GL and AS-GA through our analysis, was also discovered in FR-NL and found to have roles in defense responses.

Quantitative phytochemical analysis of *F. racemosa*-gall leaves (FR-GL)

The chromatogram of FR-GL is displayed in Plate 3.28: Figure A. Plate 3.29 represents the diagram showing the phenolics compounds present in the FR-GL with their reported bioactivity (Table 3.10).

In the case of FR-GL, 33 components were discovered, 12 of which were bioactive phenolic compounds of different categories like phenols, flavonoids, flavonols, flavone and coumarins. It is also found to possess terpenes, alkaloids and fatty acids. 7-hydroxycoumarin, 4-hydroxycoumarin, 2′,3′,4′,5,7-pentahydroxyflavone, 5,7-dihydroxy-3,8-dimethoxyflavone, 12-oxo-phytodienoic acid, and several other compounds were common to both FR-NL and FR-GL.

Quantitative phytochemical analysis of *F. racemosa*-gall (FR-GA)

The chromatogram of gall is shown in Plate 3.28: Figure B. Table 3.11 represents the list of compounds discovered with its molecular formula and bioactivity. Plate 3.30 and Plate 3.31 together represent the sunburst diagrams showing the phenolics compounds present in the FR-GA with their reported bioactivity. Analysis revealed 91 compounds, including 63 phenolic compounds, 11 terpenes, 7 alkaloids and various fatty acids. The number of compounds found from FR-GA is thrice compared to FR-NL and FR-GL. Phenolic compounds detected belonged to different categories (phenols- 22, flavonoid-12, flavonol-5, flavone-10, isoflavone-5, isoflavonoid-1, coumarin-7, lignin-2 and anthocyanin-2). Other compounds identified from the analysis include terpenes, alkaloids and fatty acids.

Dhas and colleagues identified active phytoconstituents from aqueous leaf extracts of the *F. racemosa* through LC-MS analysis, they include citronellol, delcosine and amitenone (terpenoids), gamma-nonalactone and gallocatechin-4 beta-ol (flavonoids), phloretin (phenolics) etc. (Dhas et al., 2021). Terpenes, alkaloids, phenolic and fatty acids were identified from normal leaves of the plant through OHR-LC-MS analysis done during this work. All the samples of *F. racemosa* examined were shared 11 components, including quinic acid, quercetin-3β-D-glucoside, azelaic acid, rutin, robinetin, 7-hydroxycoumarin, 4-hydroxycoumarin etc. Among the 33 phytochemicals found, 16 were unique to normal leaves. 75 of the 91 compounds were peculiar to gall. About 18 of the 33 were exclusive to gall leaves. Quercetin-3β-D-glucoside and azelaic acid were found to be present in all the 3 samples of both plants analysed.

Higher levels of total phenols and ortho-dihydric phenols were discovered in the galls, compared to the gall leaves (Dsouza and Ravishankar, 2014). The same observation was found by Ushir and colleagues in 2015 (Ushir et al., 2015). Savitha and colleagues discovered variations in phenolic content among different stages of gall development such as young, medium, and mature; and reported that higher levels of phenols were present in young galls compared to medium and mature galls (Savitha et al., 2013). Eshwarappa and colleagues in 2014 viewed the presence of phenolics, flavonoids, phytosterols, terpenoids and reducing sugars in both aqueous and methanol extracts of leaf galls of *F. racemosa* (Eshwarappa et al., 2015). The presence of phytochemical substances in plant extracts responsible for defense mechanisms of the host plant against pathogens. Following psylloid herbivory, *A. scholaris* and *F. racemosa* both showed notable alterations in their biochemistry. From our study, a total of 33 components from different categories were detected by OHR-LC-MS analysis in the FR-GL, 12 of which were bioactive phenolic compounds. However, FR-GA showed an enormous increase in the amount of phytochemicals, gall extracts showed 91 peaks, including 63 phenolic compounds, 11 terpenes, 7 alkaloids and different fatty acids. Out of the 91 compounds detected in gall, 67 were unique to galls. This illustrates how significantly the phytochemistry of a plant is impacted by gall induction. Similar to earlier findings, this study supports the trend of increased levels of phenolic compounds in the gall and gall-associated tissues.

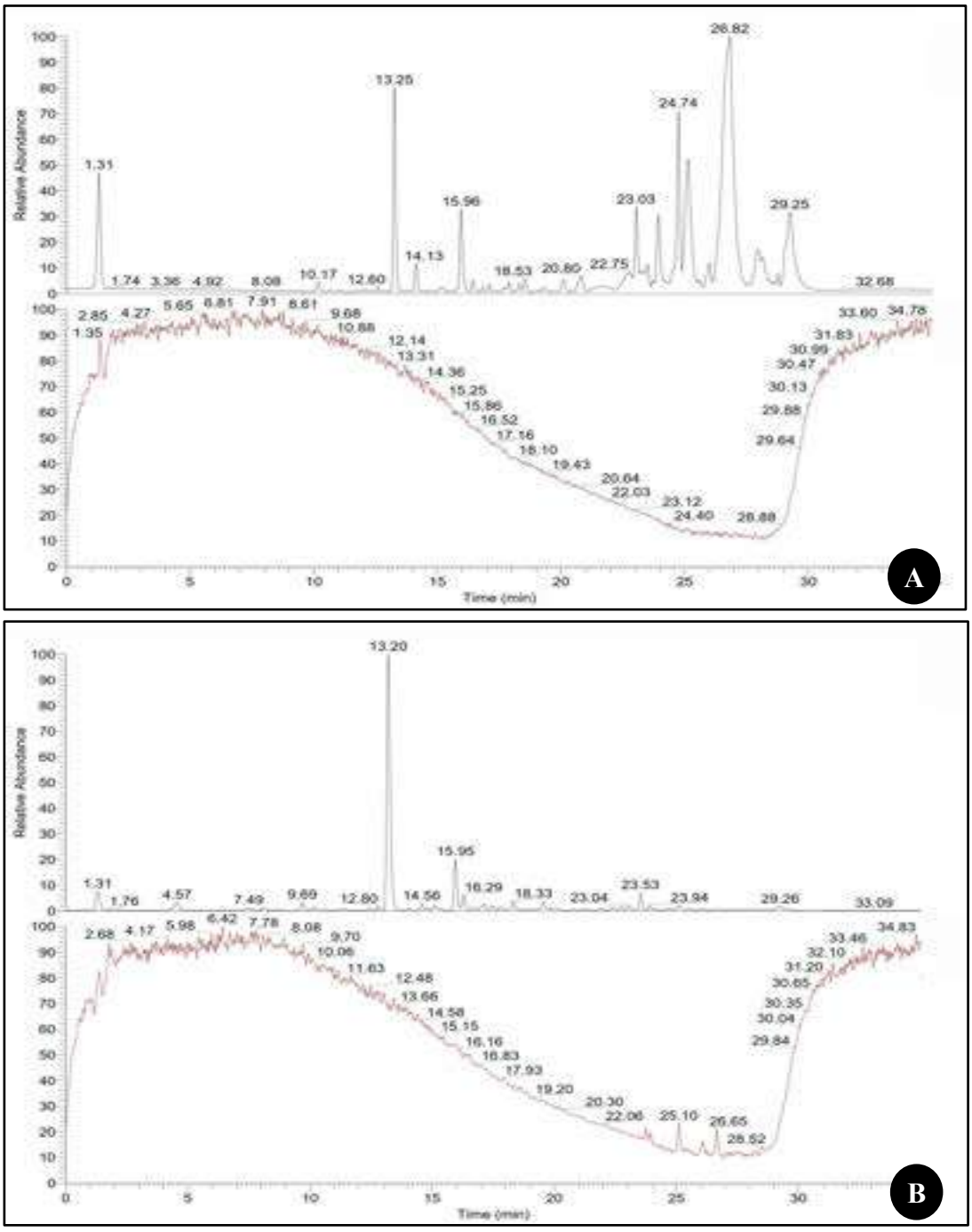


Plate 3.28: Figure A: OHR-LC-MS chromatogram of ethanolic extracts of FR-GL represents the metabolic profile of the FR-GL, revealing the presence of several bioactive compounds and Figure B: OHR-LC-MS chromatogram of ethanolic extracts of FR-GA shows the chemical profile of the gall, indicating the presence of a variety of secondary metabolites based on distinct retention time peaks.

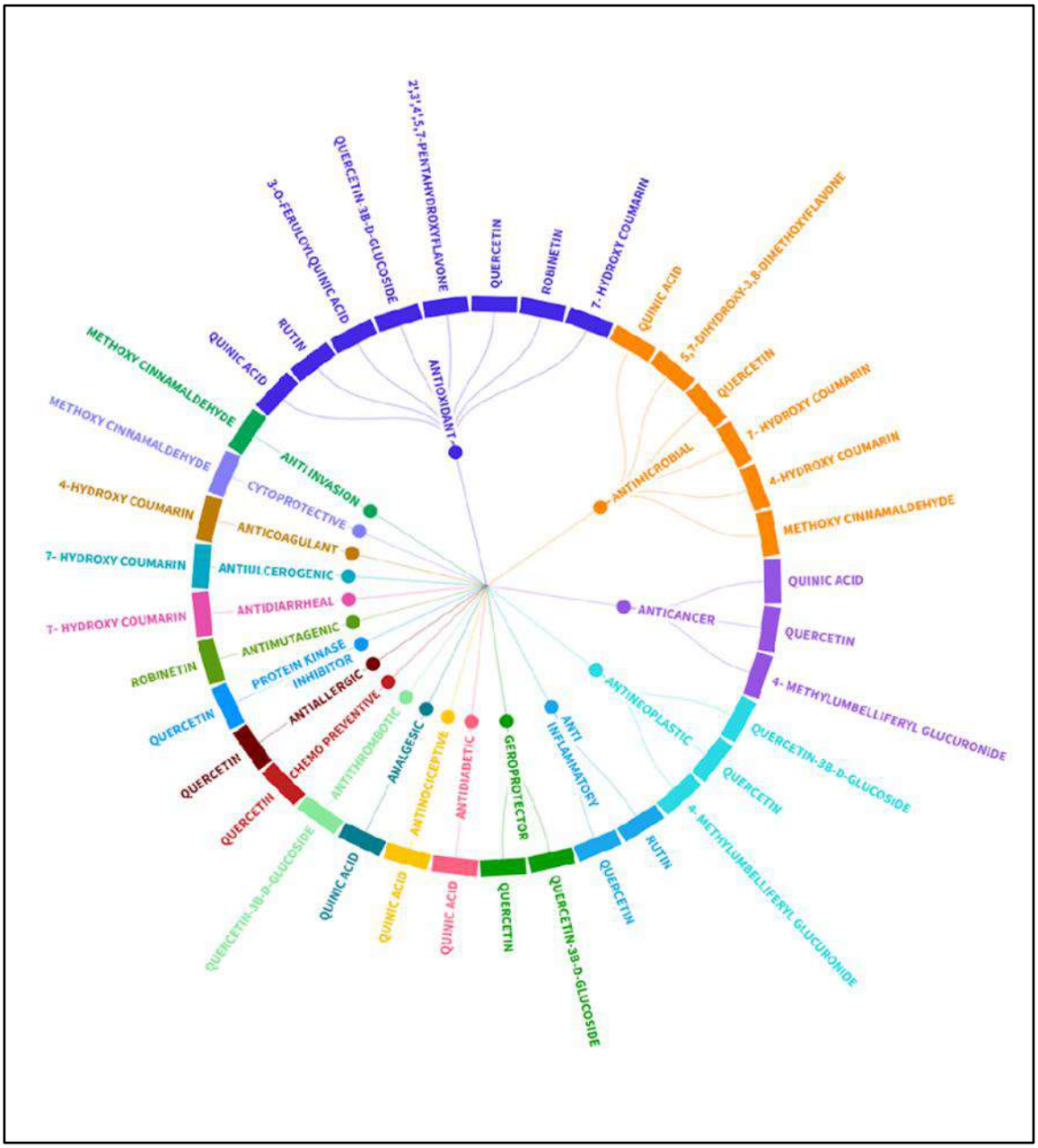


Plate 3.29: The schematic illustration represents the bioactivity of phenolic compounds found in the FR-GL. The lines in the diagram connect individual phenolic compounds to their respective bioactivities.

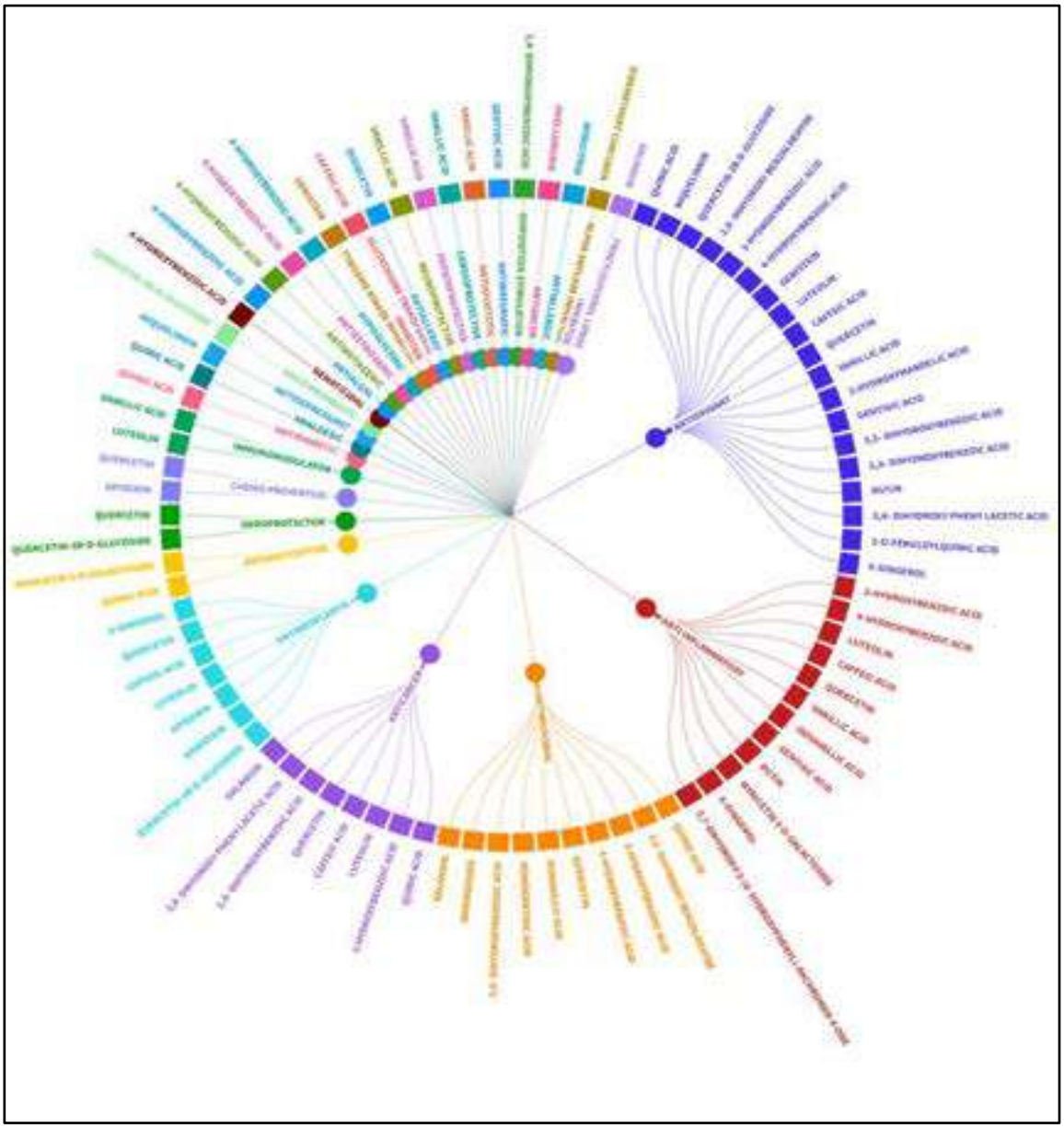


Plate 3.30: The diagram (part-1) illustrates the pharmacological relevance of phenolic constituents found in FR-GA. Each compound is connected to its corresponding biological activities through lines. This is part 1 of the complete diagram; the remaining phenolic compounds and their bioactivities are presented in Part 2.

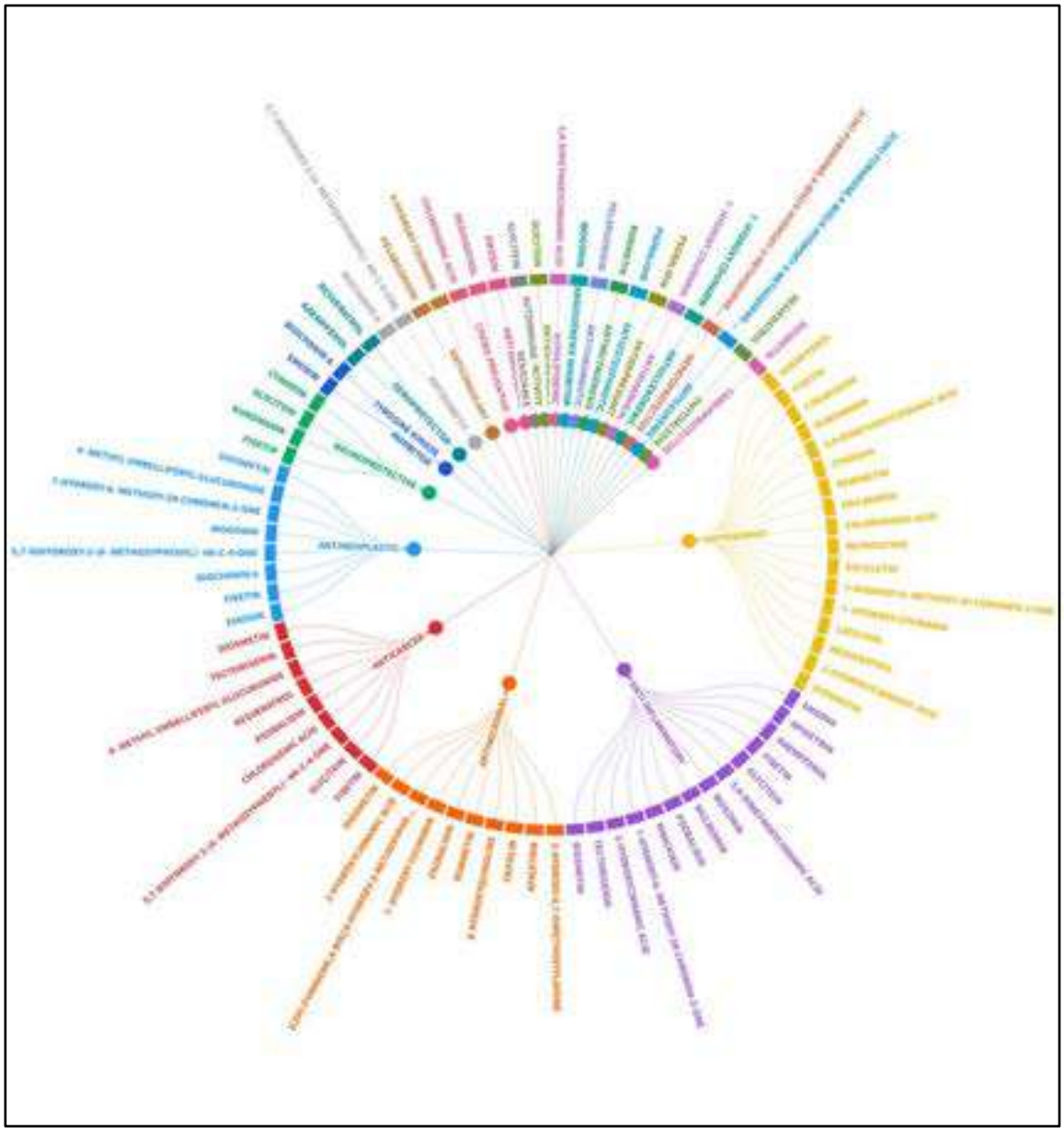


Plate: 3.31: The diagram (Part-2) is the continuation of bioactivities of phenolic compounds identified in FR-GA. This second part completes the illustration of bioactive phenolic compounds found in FR-GA. Together, both parts emphasize the complex and diverse bioactive profile of gall-derived phenolic compounds.

Table 3.6: List of compounds of AS-NL identified by OHR-LC-MS analysis with molecular formula, chemical class and reported bioactivities.

Sl. No.	Name of compound	Molecular formula	Class of compound	Reported bioactivity	Reference
1	Quinic acid	$C_7H_{12}O_6$	Phenolic compound	Antioxidant, antidiabetic, anticancer, antimicrobial, antiviral, aging protective, anti-nociceptive and analgesic effects.	(Benali et al., 2024)
2	Chlorogenic acid	$C_{16}H_{18}O_9$	Phenolic acids. Intermediate in the biosynthesis of lignin.	Potential antioxidant, chemo preventive and carcinogenic inhibitor.	(Kim et al., 2023)
3	Trifolin	$C_{21}H_{20}O_{11}$	Flavonoid	Antifungal agent.	(Kim et al., 2023)
4	Ursolic acid	$C_{30}H_{48}O_3$	Pentacyclic triterpenoid	Anti-inflammatory, antioxidant, antiviral, serum lipid-lowering and antineoplastic activities. Promotes apoptosis and inhibits cancer cell proliferation.	(Kim et al., 2023)
5	Quercetin-3β-D-glucoside	$C_{21}H_{20}O_{12}$	Flavonoid	Antineoplastic, antioxidant activity. Bone density conservation agent, an osteogenesis regulator, histamine antagonist, an antipruritic drug and a geroprotector.	(Kim et al., 2023)
6	12,13-Dihydroxy octadec-9-enoicacid	$C_{18}H_{34}O_4$	Long-chain fatty acid.	Prevention of obesity and metabolic diseases.	(Macêdo et al., 2022)
7	Luteolin	$C_{15}H_{10}O_6$	Tetrahydroxy flavone	Antioxidant, a free radical scavenger, an anti-inflammatory agent, anticancers, antineoplastic agent,	(Kim et al., 2023)

				nephroprotective agent, an angiogenesis inhibitor, apoptosis inducer and immunomodulator.	
8	Azelaic acid	$C_9 H_{16} O_4$	Fatty acid-Saturated dicarboxylic acid	Antibacterial, antineoplastic, antioxidant activity. Dermatologic drug.	(Kim et al., 2023)
9	16-Hydroxy hexadecanoic acid	$C_{16} H_{32} O_3$	Omega-hydroxy-long-chain fatty acid	A component of the plant cuticle. These apoplastic structures are important plant-environment interfaces that act as barriers limiting water and nutrient loss and protecting plants from radiation and pathogens.	(Kim et al., 2023)
10	Oleoyl-L-α-lysophosphatidic acid	$C_{21} H_{41} O_7 P$	Lysophosphatidic acid (LPA) species	Role in breast cancer metastasis.	(Nam et al., 2018)
11	3-O-Feruloylquinic acid	$C_{17} H_{20} O_9$	Phenolic compound	Antioxidant activity.	(Liu et al., 2021)
12	(3beta,9xi)-3-(beta-D-Glucopyranosyloxy)-14-hydroxycard-20(22)-enolide	$C_{29} H_{44} O_9$	Cardenolide glycoside.	Cardioprotective agent.	(Degtyarenko et al., 2007)
13	Methylmalonic acid	$C_4 H_6 O_4$	Fatty acid	Vital intermediate in the metabolism of fat and protein.	(Kim et al., 2023)
14	L-Phenylalanine	$C_9 H_{11} N O_2$	Essential aromatic amino acid nutraceutical, a micronutrient	Anti-inflammatory activity.	(Davis et al., 1968)
15	Gallic acid	$C_7 H_6 O_5$	Phenolic acid	Astringent, a cyclooxygenase 2 inhibitor. Antioxidant, antineoplastic, anti-inflammatory activities.	(Kahkeshani et al., 2019)

16	9-hydroxyoctadeca-10,12 dienoic acid	C_{18} H_{32} O_3	Fatty acid	Markers of oxidative stress.	(Vangaveti et al., 2010)
17	Coriolic acid	C_{18} H_{32} O_3	Fatty acid	Antibacterial and anti-inflammatory activity.	(Hanh et al., 2011; Mundt et al., 2003)
18	9,10-dihydroxy-12Z-octadecenoicacid	C_{18} H_{34} O_4	Polyunsaturated fatty acid	Antifungal activity.	(Christopher et al., 2023)
19	13S-hydroxy-9Z,11E, 15Z-octadecatrienoic acid	C_{18} H_{30} O_3	Fatty acid	Antiviral activity.	(More et al., 2022)
20	9(10)-Epoxy-12Z-octadecenoic acid	C_{18} H_{32} O_3	Medium-chain fatty acid	Chemical mediators in insects.	(Xu et al., 2014)
21	Caffeic acid	C_9 H_8 O_4	Caffeic acid is a hydroxycinnamic acid that is cinnamic acid and polyphenol	Glutathione transferase inhibitor, lipoxygenase inhibitor, histone deacetylase inhibitor, antioxidant, anti-inflammatory, anticancer and antineoplastic activities.	(Kim et al., 2023)
22	18-β-Glycyrrhetinic acid	C_{30} H_{46} O_4	Pentacyclic triterpenoid	Immunomodulator, antiviral, potential expectorant, anticancer, hepatoprotective, antioxidant and antiulcer activities.	(Hardy et al., 2012; Wang et al., 2018)

Table 3.7: List of compounds found in AS-GL through OHR-LC-MS analysis with molecular formula, chemical class and reported bioactivities.

Sl. No.	Name of compound	Molecular formula	Class of compound	Reported bioactivity	Reference
1.	Betaine	C_5 H_{11} N O_2	Amino acid derivative	Osmoprotectant, anti-inflammatory, antioxidant activities. Beneficial for treatment of obesity, diabetes, cancer and Alzheimer's disease.	(Guangfu et al., 2018)

2.	Palmitic acid	C_{16} H_{32} O_2	Long chain fatty acid	Anti-inflammatory, antioxidant, hypocholesterolemic, pesticide, nematicide, antiandrogenic, hemolytic, antipsychotic, antibacterial, antifungal and cytotoxic activities. 5-Alpha reductase inhibitor.	(Ortega and Campos, 2021)
3.	Methyl 2-[4-ethenyl -2,6-dihydroxy-3-(3-hydroxy prop -1-en-2-yl) -4-methylcyclohexyl] prop-2-enoate	C_{16} H_{24} O_5	Sesquiterpenoid.	Unknown activity.	-
4.	9-Oxo-10(E),12(E)-octadecadienoic acid	C_{18} H_{30} O_3	Fatty acid	Anticancer agent and potent PPAR α agonist to decrease triglyceride accumulation.	(Kim et al., 2011; Zhao et al., 2015)
5.	Kaempferol	C_{15} H_{10} O_6	Flavanol	Anti-inflammatory, antioxidant activity and geroprotector.	(Kim et al., 2023)
6.	Monolaurin	C_{15} H_{30} O_4	Fatty acid-monoglyceride	Antibacterial, antiviral, antifungal and immunomodulatory activity.	(Barker et al., 2019; Nitbani et al., 2022)
7.	12-Oxo phytodienoic acid	C_{18} H_{28} O_3	Fatty acid (intermediate in biosynthesis of jasmonic acid)	Defence responses, suppresses neuroinflammation and anti-inflammatory activities.	(Stelmach et al., 1998; Taki-Nakano et al., 2016)
8.	DL-Stachydrine	C_7 H_{13} N O_2	Proline derivative	Anti-obesity, anti-fibrotic, anticancer, neuroprotective, anti-inflammatory property and cardiovascular system protection properties.	(Cheng et al., 2020)
9.	6-Gingerol	C_{17} H_{26} O_4	Phenolics	Antineoplastic, antioxidant and anti-inflammatory activity.	(Kim et al., 2023)
10.	Betulin	C_{30} H_{50} O_2	Triterpenoid	Antiviral, analgesic, anti-carcinomic, anti-flu, cytotoxic, anti-inflammatory	(Bębenek et al., 2019; Kim et al., 2023)

				and antineoplastic activities.	
11.	2,4-Dihydroxy benzoic acid	$C_7 H_6 O_4$	Phenolic acid	Antioxidant, antimicrobial and antiproliferative activity.	(Popiołek et al., 2023)
12.	Nicotinic acid	$C_6 H_5 N O_2$	Alkaloid	Antidote and antilipemic drug. A vasodilator agent, nicotinamidase inhibitor and antihyperlipidemic activity.	(Kim et al., 2023)
13.	Catechin	$C_{15} H_{14} O_6$	Polyphenolic (flavonoid)	Antioxidant.	(Kim et al., 2023)
14.	3,4-Dihydroxy phenylpropionic acid	$C_9 H_{10} O_4$	Phenolic compound	Antioxidant property.	(Degtyarenko et al., 2007)
15.	Cinchonidine	$C_{19} H_{22} N_2 O$	Alkaloid	Antimalarial, anti-obesity, anticancer, antioxidant, anti-inflammatory and antimicrobial activities.	(Gurung and De, 2017)
16.	Gelsemine	$C_{20} H_{22} N_2 O_2$	Indole alkaloid	Anti-inflammatory, anticancer and antioxidant activities.	(Lin et al., 2015)
17.	Isochlorogenic acid C	$C_{25} H_{24} O_{12}$	Phenolic compound	Anti-inflammatory, antioxidant, anti-apoptotic, anti-injury and anti-hepatitis B virus effects.	(Liu et al., 2023; Lodise et al., 2019; Yin et al., 2018)
18.	Nicotinamide	$C_6 H_6 N_2 O$	Alkaloid	Antioxidant, neuroprotective, anti-inflammatory activities. Histone deacetylase inhibitor and geroprotector.	(Kim et al., 2023)
19.	Catharanthine	$C_{21} H_{24} N_2 O_2$	Monoterpenoid indole alkaloid	Anti-parasitic, vasodilatory, antimalarial, anti-cancer and antihypertensive activities.	(Gao et al., 2023)
20.	Isorhamnetin	$C_{16} H_{12} O_7$	Flavone	Tyrosinase inhibitor, anticoagulant, anticancer, anti-inflammatory, antioxidant activities. organ protection and prevention of obesity.	(Gong et al., 2020)
21.	Arecoline	$C_8 H_{13} N O_2$	Alkaloid	Anti-helminthic and anti-parasitic activity	(Liu et al., 2016)

22.	Chlorogenic acid	$C_{16}\ H_{18}\ O_9$	Phenolic acids (intermediate in the biosynthesis of lignin)	Antioxidant, chemo preventive and carcinogenic inhibitor activities.	(Kim et al., 2023)
23.	Gentisic acid	$C_7\ H_6\ O_4$	Phenolic compound	Anti-inflammatory, antirheumatic and antioxidant properties.	(Kim et al., 2023)
24.	Quercetin-3β-D-glucoside	$C_{21}\ H_{20}\ O_{12}$	Flavonoid	Antineoplastic, antioxidant and antithrombotic activity, bone density conservation agent, osteogenesis regulator, histamine antagonist, antipruritic drug and geroprotector.	(Kim et al., 2023)
25.	Trifolin	$C_{21}\ H_{20}\ O_{11}$	Flavonoid	Antifungal agent	(Kim et al., 2023)
26.	Azelaic acid	$C_9\ H_{16}\ O_4$	Fatty acid-Saturated dicarboxylic acid	Antibacterial, antineoplastic and antioxidant activity. Dermatologic drug.	(Kim et al., 2023)
27.	Gallic acid	$C_7\ H_6\ O_5$	Phenolic acid	Antioxidant, antineoplastic, anti-inflammatory activity. An astringent, a cyclooxygenase 2 inhibitor.	(Kahkeshani et al., 2019)
28.	Catechol	$C_6\ H_6\ O_2$	Phenolic compound	Antibacterial and antifungal effect.	(Kocaçalışkan et al., 2006)
29.	Quercetin	$C_{15}\ H_{10}\ O_7$	Flavanol	Antibacterial, anticancer, antineoplastic, antioxidant, chemopreventive, anti-inflammatory and anti-allergy activities. A protein kinase inhibitor, chelator, aurora kinase inhibitor and geroprotector.	(Kim et al., 2023)
30.	16-Hydroxy hexadecanoic acid	$C_{16}\ H_{32}\ O_3$	Omega-hydroxy-long-chain fatty acid (component of the plant cuticle)	Protecting plants from radiation and pathogens.	(Kim et al., 2023)

31.	12,13-Dihydroxy octadec-9-enoic acid	C_{18} H_{34} O_4	Long-chain fatty acid	Use in treatment of obesity and metabolic diseases.	(Macêdo et al., 2022)
32.	(3beta,9xi)-3-(beta-d glucopyranosyloxy)-14-hydroxycard 20(22)-enolide	C_{29} H_{44} O_9	Cardenolide glycoside	Cardioprotective agent.	(Degtyarenko et al., 2007)
33.	Luteolin	C_{15} H_{10} O_6	Tetra hydroxy flavone	Antioxidant, anti-inflammatory, anticancers, antineoplastic activities. Free radical scavenger, vascular endothelial growth factor receptor antagonist, nephroprotective agent, angiogenesis inhibitor, an apoptosis inducer and immunomodulator.	(Kim et al., 2023)
34.	Rutin	C_{27} H_{30} O_{16}	Flavanol glycoside (polyphenolic compound)	Antioxidant and anti-inflammatory activities.	(Kim et al., 2023)
35.	Ursolic acid	C_{30} H_{48} O_3	Triterpenoid	Anti-inflammatory, antioxidant, antiviral, serum lipid-lowering and antineoplastic activities. Promote apoptosis and inhibit cancer cell proliferation.	(Kim et al., 2023)
36.	18-β-Glycyrrhetinic acid	C_{30} H_{46} O_4	Triterpenoid	Immunomodulator, potential expectorant, gastrokinetic and antiulcer activities.	(Hardy et al., 2012; Wang et al., 2017b)

Table 3.8: List of compounds found in AS-GA through OHR-LC-MS analysis with molecular formula, chemical class and reported bioactivities.

Sl. No.	Name of compound	Molecular formula	Class of compound	Reported bioactivity	Reference
1.	Quinic acid	$C_7 H_{12} O_6$	Phenolic compound	Antioxidant, antidiabetic, anticancer, antimicrobial, antiviral, aging protective, anti-nociceptive and analgesic effects.	(Benali et al., 2024)
2.	Gentisic acid	$C_7 H_6 O_4$	Phenolic compound	Anti-inflammatory, antirheumatic and antioxidant properties.	(Kim et al., 2023)
3.	Catechol	$C_6 H_6 O_2$	Phenolic compound	Antibacterial and antifungal effect.	(Kocaçalışkan et al., 2006)
4.	Gallic acid	$C_7 H_6 O_5$	Phenolic compound	Astringent, a cyclooxygenase 2 inhibitor, antioxidant, antineoplastic and anti-inflammatory activities.	(Kahkeshani et al., 2019)
5.	12,13-Dihydroxy octadec-9-enoic acid	$C_{18} H_{34} O_4$	Long-chain fatty acid.	Used in the treatment of obesity and metabolic diseases.	(Macêdo et al., 2022)
6.	Ursolic acid	$C_{30} H_{48} O_3$	Triterpenoid	Anti-inflammatory, antioxidative, antiviral, lipid-lowering and antineoplastic activity. Promote apoptosis and inhibit cancer cell proliferation.	(Kim et al., 2023)
7.	Azelaic acid	$C_9 H_{16} O_4$	Fatty acid-saturated dicarboxylic acid	Antibacterial, antineoplastic, anti-inflammatory and antioxidant activity. Dermatologic drug.	(Kim et al., 2023)
8.	Quercetin-3β-D-glucoside	$C_{21} H_{20} O_{12}$	Flavonoid	Antineoplastic, antioxidant, antithrombotic, bone density conservation activity. Osteogenesis regulator, histamine antagonist, antipruritic drug and geroprotector.	(Kim et al., 2023)
9.	18-β-Glycyrrhetinic acid	$C_{30} H_{46} O_4$	Triterpenoid	Immunomodulator, antiulcer, potential expectorant and gastrokinetic activities.	(Hardy et al., 2012; Wang et al., 2017b)

10.	Rutin	C_{27} H_{30} O_{16}	Flavonol glycoside. (polyphenolic compound)	Antioxidant and anti-inflammatory activities.	(Kim et al., 2023)
11.	Trifolin	C_{21} H_{20} O_{11}	Flavonoid	Antifungal agent.	(Kim et al., 2023)
12.	Betaine	C_5 H_{11} N O_2	Amino acid derivative	Anti-inflammatory and anticancer activity.	(Guangfu et al., 2018)
13.	(S)-AL 8810	C_{24} H_{31} F O_4	Long-chain fatty acid	Anti -inflammatory and antioxidant activities.	(Sharif and Klimko, 2019)
14.	Rauwolscine	C_{21} H_{26} N_2 O_3	Alkaloid	Central nervous system stimulant, alpha-2-adrenergic blocking activity and anti-depressant	(Kim et al., 2023)
15.	Cinchonine	C_{19} H_{22} N_2 O	Alkaloid	Antimalarial and anti-diabetic activities.	(Boratyński et al., 2019)
16.	Palmitic acid	C_{16} H_{32} O_2	Long-chain fatty acid	Anti-inflammatory, antioxidant, hypocholesterolemic, pesticide, nematicide, antiandrogenic, hemolytic, 5-Alpha reductase inhibitor, antipsychotic, antibacterial, antifungal and cytotoxic activities.	(Ortega and Campos, 2021)
17.	Scopoletin	C_{10} H_8 O_4	Phenolic compound (coumarin)	Antineoplastic, antidopaminergic, antioxidant, anti-inflammatory and anticholinesterase effects.	(Kim et al., 2023)
18.	DL-Stachydrine	C_7 H_{13} N O_2	Proline derivative	Anti-obesity, anti-fibrotic, anticancer, neuroprotective, anti-inflammatory properties. Cardiovascular system protection.	(Cheng et al., 2020)
19.	3,4-Dihydroxy phenylpropionic acid	C_9 H_{10} O_4	Phenolic compound	Antioxidant properties.	(Degtyarenko et al., 2007)
20.	9-Oxo-10(E),12(E)-octadecadienoic acid	C_{18} H_{30} O_3	Fatty acid	Anticancer agent and potent PPAR α agonist to decrease triglyceride accumulation.	(Kim et al., 2011; Zhao et al., 2015)

21.	2-Oxindole	$C_8 H_7 NO$	Alkaloid	Antiproliferative property.	(Sharma et al., 2023)
22.	Monolaurin	$C_{15} H_{30} O_4$	Fatty acid-monoglyceride	Antibacterial, antiviral, antifungal and immunomodulatory activity.	(Barker et al., 2019; Nitbani et al., 2022)
23.	Gelsemine	$C_{20} H_{22} N_2 O_2$	Alkaloid	Anti-inflammatory, anticancer and antioxidant activities.	(Lin et al., 2015)
24.	Quercetin	$C_{15} H_{10} O_7$	Flavanol	Antibacterial, chemopreventive activity, anti-inflammatory, anti-allergy, anticancer, antioxidant, a protein kinase inhibitor and antineoplastic activities. A chelator, Aurora kinase inhibitor and geroprotector.	(Kim et al., 2023)
25.	Kaempferol	$C_{15} H_{10} O_6$	Flavanol	Anti-inflammatory and antioxidant activity, Geroprotector.	(Kim et al., 2023)
26.	Mitragynine	$C_{23} H_{30} N_2 O_4$	Monoterpenoid indole alkaloid.	Analgesic effects.	(Karunakaran et al., 2022)
27.	D-δ-Tocopherol	$C_{27} H_{46} O_2$	Vitamin E	Antioxidant activity.	(Kim et al., 2023)
28.	Arecoline	$C_8 H_{13} NO_2$	Alkaloid	Antihelmintic, ganglionic stimulant, parasympathomimetic activities and a vermifuge.	(Sharma and Anand, 1997)
29.	12-Oxo phytodienoi acid	$C_{18} H_{28} O_3$	Fatty acid (Intermediate in biosynthesis of jasmonic acid)	Defence responses and suppresses neuroinflammation. Anti-inflammatory compound.	(Stelmach et al., 1998; Taki-Nakano et al., 2016)
30.	Trigonelline	$C_7 H_7 N O_2$	Alkaloid	Hypoglycemic, hypolipidemic, neuroprotective, antimigraine, sedative, memory-improving, antibacterial, anti-diabetic, antiviral and anti-tumor activities.	(Zhou et al., 2012)
31.	Catharanthine	$C_{21} H_{24} N_2 O_2$	Monoterpenoid indole alkaloid	Anti-parasitic, vasodilatory, anticancer, antimalarial and antihypertensive activities.	(Kim et al., 2023)

32.	N-Acetyldopamine	C_{10} H_{13} N O_3	N-acetylated derivative of dopamine.	Reactive intermediate in sclerotization. Sclerotizing agent of the insect cuticle	(Karlson and Sekeris, 1962)

Table 3.9: List of compounds found in FR-NL identified by OHR-LC-MS analysis with molecular formula, chemical class and reported bioactivities.

Sl. No.	Name of compound	Molecular formula	Class of compound	Reported bioactivity	Reference
1.	Quinic acid	C_7 H_{12} O_6	Phenolic compound	Antioxidant, antidiabetic, anticancer, antimicrobial, antiviral, aging, protective, anti-nociceptive and analgesic effects.	(Benali et al., 2024)
2.	Quercetin-3β-D-glucoside	C_{21} H_{20} O_{12}	Flavonoid	Antineoplastic, antioxidant, antithrombotic, bone density conservation activity. Osteogenesis regulator, histamine antagonist, antipruritic drug and geroprotector.	(Kim et al., 2023)
3.	Rutin	C_{27} H_{30} O_{16}	Flavanol	Antioxidant and anti-inflammatory activities	(Kim et al., 2023)
4.	Azelaic acid	C_9 H_{16} O_4	Fatty acid	Antibacterial, antineoplastic, antioxidant activity. Dermatologic drug.	(Kim et al., 2023)
5.	[5-Hydroxy-3-(hydroxymethyl)-2-oxo-6-propan-2-ylcyclohex-3-en-1-yl] 3-methylbutanoate	C_{15} H_{24} O_5	Monoterpenoid	Unknown activity.	-
6.	13S-Hydroxy-9Z,11E,	C_{18} H_{30} O_3	Fatty acid	Antiviral activity.	(More et al., 2022)

	15Z-octadecatrienoic acid				
7.	Miquelianin	$C_{21}H_{18}O_{13}$	Flavonoid	Antioxidant and antidepressant activities.	(Kim et al., 2023)
8.	6-Gingerol	$C_{17}H_{26}O_4$	Phenolics	Antineoplastic, antioxidant and anti-inflammatory activity.	(Kim et al., 2023)
9.	Oleic acid	$C_{18}H_{34}O_2$	Fatty acid	Antioxidant activity.	(Kim et al., 2023)
10.	2',3',4',5,7-Pentahydroxyflavone	$C_{15}H_{10}O_7$	Flavone	Antioxidant activity.	(Zhang et al., 2011)
11.	Nicotinic acid	$C_6H_5NO_2$	Alkaloid	Antidote and antilipemic drug. A vasodilator agent, nicotinamidase inhibitor and antihyperlipidemic activity.	(Kim et al., 2023)
12.	5,7-Dihydroxy-3,8-dimethoxyflavone	$C_{17}H_{14}O_6$	Flavone	Fungitoxicity.	(Cotoras et al., 2011)
13.	Robinetin	$C_{15}H_{10}O_7$	Flavone	Antifungal, antiviral, antibacterial, anti-mutagenesis and antioxidant activity.	(Birt et al., 1986)
14.	Andrographolide	$C_{20}H_{30}O_5$	Diterpenoid	Anti-HIV, anti-inflammatory and antineoplastic.	(Kim et al., 2023)
15.	Arecoline	$C_8H_{13}NO_2$	Alkaloid	Anti-helminthic and anti-parasitic activity.	(Liu et al., 2016)
16.	Trigonelline	$C_7H_7NO_2$	Alkaloid	Hypoglycemic, hypolipidemic, neuroprotective, antimigraine, sedative, memory-improving, antibacterial, anti-diabetic, antiviral and anti-tumor activities.	(Zhou et al., 2012)
17.	10- Hydroxy camptothecin	$C_{20}H_{16}N_2O_5$	Alkaloid	Anticancer activity.	(Yuan-Gang et al., 2003)

18.	9-Hydroxy-10E,12Z-octadecadienoic acid	$C_{18}H_{32}O_3$	Fatty acid	Markers of lipid peroxidation and oxidation.	(Spiteller and Spiteller, 1997)
19.	Nootkatone	$C_{15}H_{22}O$	Sesquiterpenoid	Anti-inflammatory, anticancer, antibacterial, hepatoprotective, neuroprotective and cardioprotective activity. Insect repellent.	(Jha et al., 2022)
20.	9-Oxo-10(E),12(E)-octadecadienoic acid	$C_{18}H_{30}O_3$	Fatty acid	Anticancer agent and potent PPAR α agonist to decrease triglyceride accumulation.	(Kim et al., 2011; Zhao et al., 2015)
21.	5-(4-Carboxy-3-methylbutyl)-5,6,8atrimethyl-3-oxo3,4,4a, 5,6,7,8,8a octahydronaphthalene-1-carboxylic acid	$C_{20}H_{30}O_5$	Diterpenoid	Unknown	-
22.	Reserpine	$C_{33}H_{40}N_2O_9$	Alkaloids	Antihypertensive and anti- psychotic activities.	(Kim et al., 2023)
23.	12-Oxo phytodienoic acid	$C_{18}H_{28}O_3$	Fatty acid (biosynthesis of jasmonic acid)	Defence responses, suppresses neuroinflammation and anti-inflammatory activities.	(Stelmach et al., 1998; Taki-Nakano et al., 2016)
24.	5-Hydroxy-6,7-dimethoxyflavone	$C_{17}H_{14}O_5$	Flavonoid	Antiviral activities.	(Wu et al., 2010)
25.	7- Hydroxy coumarin	$C_9H_6O_3$	Coumarin	Antidiarrheal, antibacterial, antiulcerogenic effects.	(Cruz et al., 2020)
26.	4-Hydroxycoumarin	$C_9H_6O_3$	Coumarin	Antimicrobial and anti-coagulant activity.	(Završnik et al., 2008)
27.	Ouabain	$C_{29}H_{44}O_{12}$	Cardiac glycoside	Anticancer and antiviral activity.	(Carvalho et al., 2022; Du et al., 2021)

28.	Ilicic acid	$C_{15}H_{24}O_3$	Sesquiterpenoid	Anti-inflammatory activity	(Barrero et al., 2008)
29.	Zedoarondiol	$C_{15}H_{24}O_3$	Sesquiterpenoid.	Cytotoxic, anti-inflammatory and antioxidant activity.	(Anuchapreeda et al., 2018)
30.	Polygodial	$C_{15}H_{22}O_2$	Sesquiterpene	Antibacterial and antifungal activity. Antifeedant activity against aphids.	(Asakawa et al., 1988; Kubo et al., 2005)
31.	Zerumbone	$C_{15}H_{22}O$	Sesquiterpenoid.	Anti-inflammatory agent and glioma-associated oncogene inhibitor.	(Kim et al., 2023)
32.	Perillartine	$C_{10}H_{15}NO$	Monoterpene	Anti-obesity activity.	(Xiao et al., 2001)
33.	Palmitic acid	$C_{16}H_{32}O_2$	Fatty acid	Anti-inflammatory, antioxidant, hypocholesterolemic, pesticide, nematicide, antiandrogenic, hemolytic, antipsychotic, antibacterial, antifungal and cytotoxic activities. 5-Alpha reductase inhibitor.	(Ortega and Campos, 2021)

Table 3.10: List of compounds found in FR-GL identified by OHR-LC-MS analysis with molecular formula, chemical class and reported bioactivities.

Sl. No.	Name of compound	Molecular formula	Class of compound	Reported bioactivity	Reference
1.	Quinic acid	$C_7H_{12}O_6$	Phenolic compound	Antioxidant, antidiabetic, anticancer, antimicrobial, antiviral, aging protective, anti-nociceptive and analgesic effects.	(Benali et al., 2024)
2.	Quercetin-3β-D-glucoside	$C_{21}H_{20}O_{12}$	Flavonoid	Antineoplastic, antioxidant, antithrombotic, bone density conservation activity. Osteogenesis regulator, histamine antagonist, antipruritic drug	(Kim et al., 2023)

				and geroprotector.	
3.	Rutin	$C_{27}H_{30}O_{16}$	Flavanol	Antioxidant and anti-inflammatory activities.	(Kim et al., 2023)
4.	Azelaic acid	$C_9H_{16}O_4$	Fatty acid	Antibacterial, antineoplastic, antioxidant activity. Dermatologic drug.	(Kim et al., 2023)
5.	3-O-Feruloyl quinic acid	$C_{17}H_{20}O_9$	Phenolic compound	Antioxidant activity.	(Liu et al., 2021)
6.	2',3',4',5,7-Penta hydroxyflavone	$C_{15}H_{10}O_7$	Flavone	Antioxidant activity.	(Zhang et al., 2011)
7.	5,7-Dihydroxy-3,8-di methoxyflavone	$C_{17}H_{14}O_6$	Flavone	Fungitoxicity.	(Cotoras et al., 2011)
8.	Quercetin	$C_{15}H_{10}O_7$	Flavanol	Antibacterial, chemopreventive, anti-inflammatory, anti-allergy, anticancer, antioxidant, a protein kinase inhibitor, antineoplastic activities. A chelator, Aurora kinase inhibitor and geroprotector.	(Kim et al., 2023)
9.	Robinetin	$C_{15}H_{10}O_7$	Flavone	Anti-mutagenic, antiradical and anti-promotion properties.	(Birt et al., 1986)
10.	Arecoline	$C_8H_{13}NO_2$	Alkaloid	Anti-helminthic, antiparasitic activity.	(Liu et al., 2016)
11.	Trigonelline	$C_7H_7NO_2$	Alkaloid	Hypoglycemic, hypolipidemic, neuroprotective, antimigraine, sedative, memory-improving, antibacterial, anti- diabetic, antiviral and anti-tumor activities.	(Zhou et al., 2012)
12.	Nicotinic acid	$C_6H_5NO_2$	Alkaloid	Antidote and antilipemic drug. A vasodilator agent, nicotinamidase inhibitor and antihyperlipidemic activity.	(Kim et al., 2023)

13.	Andrographolide	C_{20} H_{30} O_5	Diterpenoid	Anti-HIV, anti-inflammatory and antineoplastic activities.	(Kim et al., 2023)
14.	10- Hydroxy camptothecin	C_{20} H_{16} N_2 O_5	Alkaloid	Anti-tumor activity.	(Xiao et al., 2001)
15.	Nootkatone	C_{15} H_{22} O	Sesquiterpenoid	Anti-inflammatory, anticancer, antibacterial, hepatoprotective, neuroprotective and cardioprotective activity. Insect repellent.	(Jha et al., 2022)
16.	12-Oxo phytodienoic acid	C_{18} H_{28} O_3	Fatty acid (intermediate in biosynthesis of jasmonic acid)	Defence responses and suppresses neuroinflammation. Anti-inflammatory compound.	(Stelmach et al., 1998; Taki-Nakano et al., 2016)
17.	Palmitic acid	C_{16} H_{32} O_2	Fatty acid	Anti-inflammatory, antioxidant, hypocholesterolemic, pesticide, nematicide, antiandrogenic, hemolytic, antipsychotic, antibacterial, antifungal and cytotoxic activities. 5-Alpha reductase inhibitor.	(Ortega and Campos, 2021)
18.	5-(4-Carboxy-3-methylbutyl) -5,6,8a trimethyl -3- oxo 3,4,4a,5,6,7,8,8a octa hydro naphthane-1-carboxylic acid	C_{20} H_{30} O_5	Diterpenoid	Unknown activity	-
19.	7- Hydroxy coumarin	C_9 H_6 O_3	Coumarin	Antidiarrheal, antibacterial, antiulcerogenic effects and antioxidant capacity.	(Cruz et al., 2020)
20.	4- Hydroxy coumarin	C_9 H_6 O_3	Coumarin	Antimicrobial and anti-coagulant activity.	(Završnik et al., 2008)

21.	15-OxoEDE	$C_{20}H_{34}O_3$	Fatty acid	Unknown activity.	-
22.	(±)13-HpODE	$C_{18}H_{32}O_4$	Fatty acid	Unknown activity.	-
23.	Ilicic acid	$C_{15}H_{24}O_3$	Sesquiterpenoid.	Anti-inflammatory activity.	(Barrero et al., 2008)
24.	Tetranor-12(R)-HETE	$C_{16}H_{26}O_3$	Fatty acid	Unknown activity.	-
25.	Zedoarondiol	$C_{15}H_{24}O_3$	Sesquiterpenoid.	Cytotoxic, anti-inflammatory and antioxidant activity.	(Anuchapreeda et al., 2018)
26.	4- Methyl umbelliferyl glucuronide	$C_{16}H_{16}O_9$	Coumarin	Anti-tumourigenic and antineoplastic agent.	(Kim et al., 2023)
27.	9-Hydroxy-10E,12Z-octadecadienoic acid	$C_{18}H_{32}O_3$	Fatty acid	Markers of lipid peroxidation and oxidation.	(Spiteller and Spiteller, 1997)
28.	13S-Hydroxy-9Z,11E, 15Z-octadecatrienoic acid	$C_{18}H_{30}O_3$	Fatty acid	Antiviral activity.	(More et al., 2022)
29.	9-Oxo-10(E),12(E)-octadecadienoic acid	$C_{18}H_{30}O_3$	Fatty acid	Anticancer agent and potent PPAR α agonist to decrease triglyceride accumulation.	(Kim et al., 2011; Zhao et al., 2015)
30.	Reserpine	$C_{33}H_{40}N_2O_9$	Alkaloids	Antihypertensive and antipsychotic activities.	(Kim et al., 2023)
31.	Zerumbone	$C_{15}H_{22}O$	Sesquiterpenoid.	Anti-inflammatory agent and glioma-associated oncogene inhibitor.	(Kim et al., 2023)
32.	Methoxy cinnamaldehyde	$C_{10}H_{10}O_2$	Flavonoid	Cytoprotective, antimicrobial and anti-invasion activity.	(Van et al., 2022)
33.	Perillartine	$C_{10}H_{15}NO$	Monoterpene	Anti-obesity activity.	(Xiao et al., 2001)

Table 3.11: List of compounds found in FR-GA identified by OHR-LC-MS analysis with molecular formula, chemical class and reported bioactivities.

Sl. No.	Name of compound	Molecular formula	Class of compound	Reported bioactivity	Reference
1.	2,5- Dihydroxy benzaldehyde	$C_7 H_6 O_3$	Phenolic compound	Antibacterial, antifungal and antioxidant activity. Tyrosine kinase inhibitor.	(Sahin et al., 2013)
2.	3-Hydroxybenzoic acid	$C_7 H_6 O_3$	Phenolic compound	Antimicrobial, anti-inflammatory, anticancer and antioxidant.	(Satpute et al., 2018)
3.	4-Hydroxybenzoic acid	$C_7 H_6 O_3$	Phenolic compound	Antioxidant, estrogenic, antimicrobial, antialgal, antimutagenic, antiestrogenic, hypoglycemic and anti-inflammatory activities.	(Chaudhary et al., 2013; Chong et al., 2009)
4.	Quinic acid	$C_7 H_{12} O_6$	Phenolic compound	Antioxidant, antidiabetic, anticancer, antimicrobial, antiviral, anti-nociceptive and analgesic effects.	(Benali et al., 2024)
5.	Azelaic acid	$C_9 H_{16} O_4$	Fatty acid	Antibacterial, antineoplastic and antioxidant activity. Dermatologic drug.	(Kim et al., 2023)
6.	Miquelianin	$C_{21} H_{18} O_{13}$	Flavanol	Antioxidant and antidepressant activities.	(Kim et al., 2023)
7.	Quercetin-3β-D-glucoside	$C_{21} H_{20} O_{12}$	Flavonoid	Antineoplastic, antioxidant, antithrombotic, bone density conservation activity. Osteogenesis regulator, histamine antagonist, antipruritic drug and geroprotector.	(Kim et al., 2023)
8.	Genistein	$C_{15} H_{10} O_5$	Isoflavone	Antineoplastic agent, a tyrosine kinase inhibitor, DNA topoisomerase inhibitor, phytoestrogen and antioxidant	(Kim et al., 2023)

				activities.	
9.	Apigenin	$C_{15}H_{10}O_5$	Flavone	Antineoplastic and chemo-preventive agent. Inhibits the expression of involucrin.	(Kim et al., 2023)
10.	Luteolin	$C_{15}H_{10}O_6$	Flavone	Antioxidant, anti-inflammatory, antineoplastic, nephroprotective, angiogenesis, anticancer, apoptosis inducer and immunomodulating activities..	(Kim et al., 2023)
11.	Caffeic acid	$C_9H_8O_4$	Phenolic compound	Glutathione transferase inhibitor, lipoxygenase inhibitor, histone deacetylase inhibitor, antioxidant, anti-inflammatory, anticancer and antineoplastic activities.	(Kim et al., 2023)
12.	Quercetin	$C_{15}H_{10}O_7$	Flavanol	Antibacterial, chemopreventive, anti-inflammatory, anti-allergy, anticancer, antioxidant, a protein kinase inhibitor, antineoplastic activities. A chelator, Aurora kinase inhibitor and geroprotector.	(Kim et al., 2023)
13.	9,10-Dihydroxy12Z-octadecenoic acid	$C_{18}H_{34}O_4$	Fatty acid	Cytotoxic activity.	(Christopher et al., 2023; Hildreth et al., 2020)
14.	Vanillic acid	$C_8H_8O_4$	Phenolic compound	Antioxidant, anti-inflammatory, immuno-stimulating, neuroprotective, hepatoprotective, cardioprotective and antiapoptotic activities.	(Sharma et al., 2020)
15.	3-Hydroxymandelic acid	$C_8H_8O_4$	Phenolic compound	Antioxidant properties.	(Parcheta et al., 2022)
16.	Isovanillic acid	$C_8H_8O_4$	Phenolic compound	Anti-inflammatory and antibacterial activity.	(Kim et al., 2023)

17.	Homogentisic acid	$C_8 H_8 O_4$	Phenolic compound	Antibacterial activity.	(Nguyen et al., 2018)
18.	Gentisic acid	$C_7 H_6 O_4$	Phenolic compound	Anti-inflammatory, antirheumatic and antioxidant properties.	(Kim et al., 2023)
19.	2,4- Dihydroxy benzoic acid	$C_7 H_6 O_4$	Flavonoid	Anticancer, antiparasite, antibacterial properties and oviposition stimulation in insects.	(Riddick et al., 2019)
20.	3,5- Dihydroxy benzoic acid	$C_7 H_6 O_4$	Phenolic compound	Antioxidant and antimicrobial activities.	(Kalinowska et al., 2021)
21.	2,3- Dihydroxy benzoic acid	$C_7 H_6 O_4$	Phenolic compound	Antiradical activity.	(Kalinowska et al., 2021)
22.	Rutin	$C_{27} H_{30} O_{16}$	Flavanol	Antioxidant and anti- inflammatory activities.	(Kim et al., 2023)
23.	Homovanillic acid	$C_9 H_{10} O_4$	Fatty acid	Anti-inflammatory agent and marker for metabolic stress.	(Putra et al., 2019)
24.	1,3,7-Trimethyl luricacid	$C_8 H_{10} N_4 O_3$	Alkaloid	Antioxidant activities.	(Bhat et al., 2001)
25.	3-Isopropylmalic acid	$C_7 H_{12} O_5$	Fatty acid	Antioxidant and antimicrobial activities.	(Ricciutelli et al., 2020)
26.	3,4- Dihydroxy phenylacetic acid	$C_8 H_8 O_4$	Phenolic compound	Antioxidant and cytotoxic activities.	(Samsonowicz et al., 2018; Tang et al., 2016)
27.	Myricetin 3-O-galactoside	$C_{21} H_{20} O_{13}$	Flavone	Antinociceptive and anti-inflammatory effects.	(Oliveira et al., 2015)
28.	3-O-Feruloyl quinic acid	$C_{17} H_{20} O_9$	Phenolic compound	Antioxidant activity.	(Liu et al., 2021)
29.	6-Gingerol	$C_{17} H_{26} O_4$	Phenolic compound	Antineoplastic, antioxidant and anti-inflammatory activity.	(Kim et al., 2023)
30.	Phellamurin	$C_{26} H_{30} O_{11}$	Flavonoid	Anti-tumor activity.	(Zhang et al., 2019)
31.	Myricitrin	$C_{21} H_{20} O_{12}$	Flavone	Anti-allergic agent.	(Kim et al., 2023)
32.	Linoleic Acid	$C_{18} H_{32} O_2$	Fatty acid	Anticancer and anti-obesity.	(Pariza et al., 1999)

33.	10-Nitrooleate	$C_{18}H_{33}NO_4$	Fatty acid	Antioxidant and anti-inflammatory actions.	(Narala et al., 2022)
34.	Naringoside	$C_{27}H_{32}O_{14}$	Flavonoid	Antiviral activity.	(Faisal et al., 2021)
35.	Oleic acid	$C_{18}H_{34}O_2$	Fatty acid	Anticancer, Used as an herbicide, insecticide and fungicide.	(Kim et al., 2023)
36.	Didemethoxy curcumin	$C_{19}H_{16}O_4$	Phenolic compound	Alpha-amylase inhibitor.	(Kim et al., 2023)
37.	5,7-Dihydroxy-3-(4-hydroxyphenyl)-4H chromen-4-one	$C_{15}H_{10}O_5$	Flavonoid	Anti-inflammatory activity.	(Gomes et al., 2012)
38.	Galangin	$C_{15}H_{10}O_5$	Flavanols	Growth inhibitor of breast tumor cells, antimicrobial agent, triacylglycerol lipase inhibitor.	(Kim et al., 2023)
39.	Emodin	$C_{15}H_{10}O_5$	Phenolic compound	Role as a tyrosine kinase inhibitor, an antineoplastic agent, anti-inflammatory, signalling, antibiotic, muscle building and anti-angiogenic functions.	(Kim et al., 2023)
40.	5-Hydroxy-6,7-dimethoxyflavone	$C_{17}H_{14}O_5$	Flavonoid	Antiviral activity.	(Wu et al., 2010)
41.	Apigetrin	$C_{21}H_{20}O_{10}$	Flavone	Anti-inflammatory and antibacterial activity.	(Kim et al., 2023)
42.	Kaempferol	$C_{15}H_{10}O_6$	Flavanol	Anti-inflammatory, antioxidant activity and geroprotector.	(Kim et al., 2023)
43.	Fisetin	$C_{15}H_{10}O_6$	Flavonoid	Potential antioxidant, neuroprotective, anti-inflammatory, antineoplastic, anticancer, senolytic and longevity promoting activities.	(Kim et al., 2023)

44.	Cynaroside	$C_{21}H_{20}O_{11}$	Flavone	Antioxidant activity.	(Kim et al., 2023)
45.	Kuromanin	$C_{21}H_{20}O_{11}$	Flavonoids (anthocyanin)	Antioxidant and neuroprotective activity.	(Winter et al., 2017)
46.	Andrographolide	$C_{20}H_{30}O_5$	Diterpene	Anti-HIV, anti-inflammatory and antineoplastic activities.	(Kim et al., 2023)
47.	Biochanin A	$C_{16}H_{12}O_5$	Isoflavones	Fatty acid amide hydrolase inhibitor, antidiabetic agents, a tyrosine kinase inhibitor and an antineoplastic agent.	(Kim et al., 2023)
48.	Glycitein	$C_{16}H_{12}O_5$	Isoflavone	Anticancer, anti-inflammatory, neuroprotective, protective effects against bone loss, cardiovascular diseases, autoimmune diseases and estrogenic activities.	(Shimoda and Hamada, 2010)
49.	3,4-Dimethoxy cinnamic acid	$C_{11}H_{12}O_4$	Phenolic compound	Antioxidant, anti-inflammatory and hypolipidemic activities.	(Panagiotis et al., 2023)
50.	5,7-Dihydroxy-3-(4-methoxyphenyl)-4H-chromen-4-one	$C_{16}H_{12}O_5$	Isoflavone	Anticarcinogenic, antidiabetic and antineoplastic properties.	(Kim et al., 2023)
51.	Wogonin	$C_{16}H_{12}O_5$	Flavone	Anti-inflammatory, cyclooxygenase 2 inhibitor, an antineoplastic and angiogenesis inhibitor activities.	(Kim et al., 2023)
52.	Trifolin	$C_{21}H_{20}O_{11}$	Flavonoid	Antifungal agent.	(Kim et al., 2023)
53.	Pelargonidin	$C_{15}H_{10}O_5$	Flavonoid	Anticoagulant, antithrombotic activity, improving blood circulation.	(Ku et al., 2016)
54.	8-Hydroxyquinoline	C_9H_7NO	Phenolic compound	Antibacterial, antifungal and pesticide properties.	(Kim et al., 2023)
55.	Cyanidin	$C_{15}H_{10}O_6$	Flavonoids (anthocyanin)	Neuroprotective agent and antioxidant activities.	(Kim et al., 2023)
56.	Robinetin	$C_{15}H_{10}O_7$	Flavone	Antifungal, antiviral, antibacterial, anti-mutagenesis and antioxidant	(Birt et al., 1986)

				activity.	
57.	Arecoline	$C_8 H_{13} N O_2$	Alkaloid	Anti-helminthic and anti-parasitic activity.	(Liu et al., 2016)
58.	Mulberrin	$C_{25} H_{26} O_6$	Flavone	Antioxidative and anti-inflammatory properties.	(Ye et al., 2022)
59.	Trigonelline	$C_7 H_7 N O_2$	Alkaloid	Hypoglycemic, hypolipidemic, neuroprotective, antimigraine, sedative, memory-improving, antibacterial, anti-diabetic, antiviral and anti-tumor activities.	(Zhou et al., 2012)
60.	Cafestol	$C_{20} H_{28} O_3$	Diterpene	Apoptosis inducer, a hypoglycemic agent, an angiogenesis inhibitor, an antineoplastic agent, an antioxidant and anti-inflammatory agent.	(Kim et al., 2023)
61.	Chlorogenic acid	$C_{16} H_{18} O_9$	Lignin.	Potential antioxidant, chemo preventive and carcinogenic inhibitor.	(Kim et al., 2023)
62.	Reynoutrin	$C_{20} H_{18} O_{11}$	Flavonoid	Antioxidant and free radical-scavenging activity.	(Kim et al., 2023)
63.	Psoralidin	$C_{20} H_{16} O_5$	Coumarin	Anticancer, anti-osteoporotic, anti-inflammatory, anti-vitiligo, antibacterial, antiviral, and antidepressant-like effects.	(Sharifi-Rad et al., 2020)
64.	Maackiain	$C_{16} H_{12} O_5$	Isoflavonoid	Antipyretic and anti-inflammatory effects.	(Huh et al., 2020)
65.	Esculetin	$C_9 H_6 O_4$	Coumarin	Antioxidant activity.	(Kim et al., 2023)
66.	7-Hydroxy-6-methoxy -2H chromen-2-one	$C_{10} H_8 O_4$	Coumarin	Antineoplastic, antidopaminergic, antioxidant, anti-inflammatory and anticholinesterase effects.	(Kim et al., 2023)
67.	Nootkatone	$C_{15} H_{22} O$	Sesquiterpenoid	Anti-inflammatory, anticancer, antibacterial, hepatoprotective, neuroprotective and cardioprotective. Insect repellent.	(Jha et al., 2022)

68.	18 alpha-Glycyrrhetic acid	$C_{30}H_{46}O_4$	Triterpenoid	Antiasthmatic, hepatoprotective, anticancer, nephroprotective, antidiabetic, antileishmanial, antiviral, antibacterial, anti-psoriasis, anti-osteoporosis, antiepileptic, antiarrhythmic and anti-inflammatory activities.	(Shinu et al., 2023)
69.	12-Oxo phytodienoic acid	$C_{18}H_{28}O_3$	Fatty acid (intermediate in biosynthesis of jasmonic acid)	Defence responses, suppresses neuroinflammation and anti-inflammatory activities.	(Stelmach et al., 1998; Taki-Nakano et al., 2016)
70.	10- Hydroxy camptothecin	$C_{20}H_{16}N_2O_5$	Alkaloid	Anticancer bioactivity.	(Yuan-Gang et al., 2003)
71.	Palmitic acid	$C_{16}H_{32}O_2$	Fatty acid	Anti-inflammatory, antioxidant, hypocholesterolemic, pesticide, nematicide, antiandrogenic, hemolytic, antipsychotic, antibacterial, antifungal and cytotoxic activities. 5-Alpha reductase inhibitor.	(Ortega and Campos, 2021)
72.	7- Hydroxy coumarin	$C_9H_6O_3$	Coumarin	Antidiarrheal, anti-bacterial and antiulcerogenic effects	(Cruz et al., 2020)
73.	4-Hydroxycoumarin	$C_9H_6O_3$	Coumarin	Antimicrobial activity and anti-coagulant activity.	(Završnik et al., 2008)
74.	Nicotinic acid	$C_6H_5NO_2$	Alkaloid	Antidote and antilipemic drug. A vasodilator agent, nicotinamidase inhibitor and antihyperlipidemic activity.	(Kim et al., 2023)
75.	Catechin	$C_{15}H_{14}O_6$	Flavonoid	Antioxidant activities.	(Kim et al., 2023)
76.	3-[4-Methyl-1-(2-methyl propanoyl) - 3-	$C_{15}H_{24}O_4$	Monoterpene	Antiviral and antidiabetic properties.	(Kim et al., 2023)

	oxocyclohexyl] butanoic acid				
77.	2-[(2R,4aR,8R,8aR)- 8-Hydroxy-4a,8-dimethyldecahydronaphthal en-2-yl] prop-2- enoic acid	$C_{15}H_{24}O_3$	Sesquiterpenoid	Anti-inflammatory activity.	(Kim et al., 2023)
78.	Cinchonidine	$C_{19}H_{22}N_2O$	Alkaloid	Anti-malarial, anti-obesity, anticancer, antioxidant, anti-inflammatory and antimicrobial activity.	(Gurung and De, 2017)
79.	Pulegone	$C_{10}H_{16}O$	Monoterpene	Insecticidal, antifeedant, antiparasitical, pesticidal and insect repellent properties.	(Božović and Ragno, 2017)
80.	Reserpine	$C_{33}H_{40}N_2O_9$	Alkaloids	Antihypertensive and antipsychotic activities.	(Kim et al., 2023)
81.	Artemisinin	$C_{15}H_{22}O_5$	Sesquiterpenoid	Antimalarial activity.	(Kim et al., 2023)
82.	2(3H)-Furanone,4-bis [(4-hydroxy-3-methoxy phenyl)methyl]	$C_{20}H_{22}O_7$	Phenolic compound	Hepatoprotective, antileukemic and antifungal activity.	(Yao et al., 2021)
83.	Polygodial	$C_{15}H_{22}O_2$	Sesquiterpenoid	Antibacterial and antifungal activity. Antifeedant activity against aphids.	(Asakawa et al., 1988; Kubo et al., 2005)
84.	Resveratrol	$C_{14}H_{12}O_3$	Lignin	Phytoalexin, an oncogene inhibitor, a geroprotector, antioxidant and has potential chemopreventive activities.	(Kim et al., 2023)
85.	4-Methylumbelliferyl glucuronide	$C_{16}H_{16}O_9$	Coumarins	Anti-tumorigenic and antineoplastic agent.	(Kim et al., 2023)

86.	2-Hydroxycinnamic acid	$C_9 H_8 O_3$	Phenolic compound	Anti-inflammatory, antioxidant and antimicrobial activities.	(Sova and Saso, 2020)
87.	Ursolic acid	$C_{30} H_{48} O_3$	Triterpenoid	Anti-inflammatory, antioxidant, antiviral, anticancer, serum lipid-lowering and antineoplastic activities.	(Kim et al., 2023)
88.	Tectorigenin	$C_{16} H_{12} O_6$	Isoflavone	Anti-inflammatory and anticancer activity.	(Kim et al., 2023)
89.	Diosmetin	$C_{16} H_{12} O_6$	Flavone	Anticancer, antimicrobial, antineoplastic, cardioprotective, antioxidant, oestrogenic and anti-inflammatory activities.	(Kim et al., 2023)
90.	Zerumbone	$C_{15} H_{22} O$	Sesquiterpenoid	Anti-inflammatory agent and a glioma-associated oncogene inhibitor.	(Kim et al., 2023)
91.	Monolaurin	$C_{15} H_{30} O_4$	Fatty acid	Antibacterial, antiviral, antifungal and immunomodulatory activity.	(Barker et al., 2019; Nitbani et al., 2022)

3.3.3. Other preliminary quantitative analysis

Estimation of chlorophyll content

Fresh samples of normal leaves, gall leaves and galls of *A. scholaris* and *F. racemosa* were examined for the estimation of amounts of chlorophyll a, chlorophyll b, total chlorophyll and carotenoids. The findings were displayed in Table 3.12. The graphical representation of the results of both plants was shown in Plate 3.32: Figure A and B, Plate 3.33: Figure A and B. The gall leaves were found to possess higher chlorophyll content *i.e.* chlorophyll a, chlorophyll b and total chlorophyll than normal leaves of *A. scholaris*. But gall showed much less amount of all pigments than normal leaf and gall leaf. The same observation was found in the case of *F. racemosa*. But carotenoid showed a different pattern, *i.e.* higher in normal leaf, followed by gall leaf and then gall. When it comes to *F. racemosa*, gall leaves appear to have the greatest amount of pigments compared to normal leaf and gall. It was discovered that gall leaves had significantly higher levels of carotenoids than normal leaves. All pigments are substantially less abundant in gall, however among them carotenoids were somewhat more abundant in *F. racemosa* gall than the other tissues.

From these analyses of chlorophyll pigment content of the two plants, it is clear that the gall leaves are rich in photosynthetic pigments compared to normal leaf and gall. Insect induced biochemical and physiological modifications lead to the development of gall, so they are meant for providing shelter and nutrition for the developing insects. Gall tissues were found to contain nutritive cells which act as nutritive sources for the insects. It is clear that gall are not photosynthetically efficient, they are completely developed for the protection and nourishment of the inducer itself. So, they may act as a sink for photoassimilates. Then the surrounding tissue of gall, *i.e.* gall leaves, acts as a source of these photosynthetic products. The chlorophyll content estimation report from these two plants also correlated with the above findings as gall leaf is a richer source of pigments than normal leaf and gall. Gall contained only a trace amount of photosynthetic pigments. Due to the presence of higher pigment content, gall leaves showed a high photosynthetic rate; as a result more sugars are synthesized in gall leaves

and they accumulate in the gall. The adaptive manipulation of insects may be the reason behind increase in chlorophyll content in gall leaves compared to gall and normal leaves.

Several previous studies have demonstrated that the chlorophyll content of galls decreases as the galls mature (Albert et al., 2011; Biswas et al., 2014; Hossain et al., 2017). The net chlorophyll content of fresh ungalled *A. scholaris* leaves was much higher than that of galled leaves. Mesophyll redifferentiation and chloroplast disappearance are the causes of reduction of chlorophyll (Albert et al., 2011; Manna et al., 2018). Ivanova and colleagues in 2022 found that the loss of carotenoids and chlorophylls in the galled regions of the leaf surface caused the amount of pigment per leaf to decrease. The amounts of carotenoid and chlorophyll in the uninfected sections of infected leaves stayed consistent and were comparable to those seen in uninfected leaves of the same species. Thus, they found that a gall mite infection that lowers the chlorophyll content might result in a dramatic reduction in photosynthesis and productivity that is proportionate to the severity of the illness (Ivanova et al., 2022). Kot and colleagues also discovered that the presence of galls on *Quercus robur* infected by Cynipidae species caused a significant decrease of chlorophyll a, b and carotenoids contents (Kot et al., 2018). Huang and associates examined the chlorophyll biosynthesis of five different insect-induced galls made from the leaves of three host plants. They discovered that insect-induced gall may cause the infected leaf to produce fewer photosynthetic pigments, which lowers photosynthetic activity. This allows the plant system to manufacture more chemicals to protect the insects in the gall (Huang et al., 2014).

Table 3.12. Result of quantitative estimation of chlorophyll content in normal leaves, gall leaves and galls of *A. scholaris* and *F. racemosa.*

Plant	Sample	Chlorophyll a (µg/mg)	Chlorophyll b (µg/mg)	Total chlorophyll (µg/mg)	Carotenoids (µg/mg)
A. scholaris	Normal	4257.9 ± 60.3	2730.9 ± 19.7	6974.9 ± 22.2	5284.8 ± 24.2

	leaf				
	Gall leaf	4426.5 ± 19.9	4584.03 ± 98.9	8989.1 ± 60.6	1296.1 ± 28.6
	Gall	1620.49 ± 58.2	824.7 ± 45.8	2440.8 ± 94.9	833.2 ± 28.9
F. racemosa	Normal leaf	3588.1 ± 87.8	826.89 ± 46.7	5392.8 ± 98.1	4097.7 ± 34.2
	Gall leaf	4246.7 ± 20.5	2465.9 ± 46.6	6318.9 ± 49.2	7054.9 ± 67.2
	Gall	493.15± 10.9	249.5 ± 10.6	1368.16 ± 60.4	2618.8 ± 86.2

Each value represents mean ± standard deviation (n = 3)

Estimation of phenylalanine ammonia-lyase (PAL) enzyme activity

The results of PAL enzyme activity analysis in the normal leaves, gall leaves and galls of both plants are displayed in Table 3.13. The analysis of PAL enzyme activity showed a comparable pattern in both plants. The findings made it evident that galls had the highest level of enzyme activity, followed by gall leaves. Gall and gall-associated tissues exhibited higher levels of enzyme activity than normal healthy tissues. In contrast to *A. scholaris*, the *F. racemosa* gall exhibited greater PAL enzyme activity.

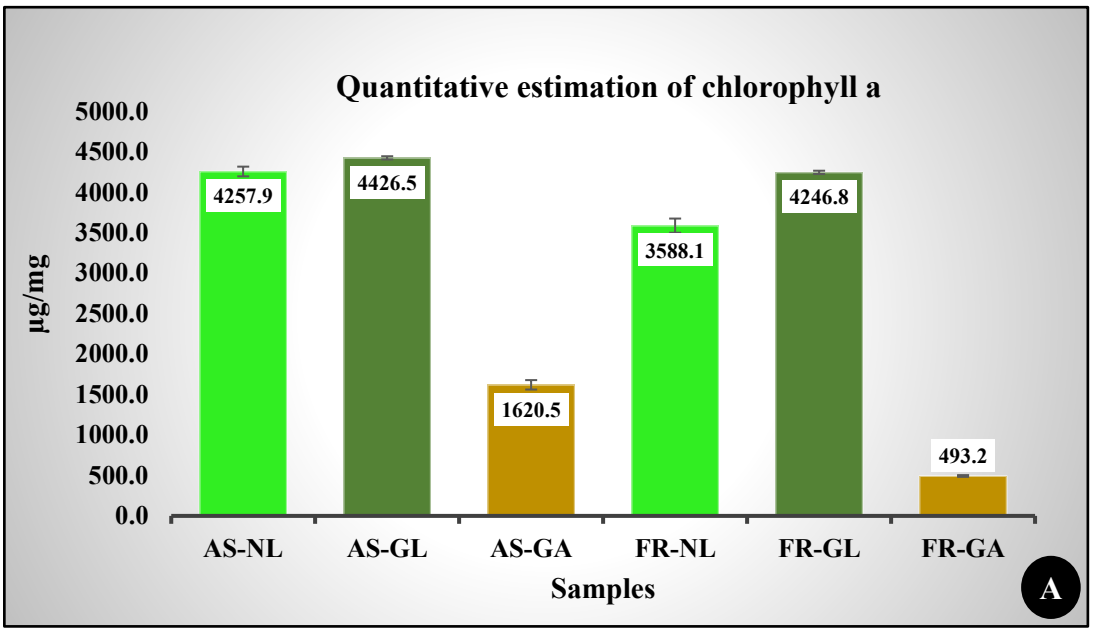
Since PAL is a crucial enzyme in the phenylpropanoid pathway, there is a strong correlation between the quantity of phenolic compounds produced and the PAL enzyme activity. Thus, the previously mentioned result will clearly demonstrate the infection with gall inducers has been associated with the increased PAL enzyme activity. Gall will emerge as a result of the inducer attack and this reaction will trigger several defense mechanisms. Activating the production of defensive phytochemicals is one of those mechanisms. Thus, compared to normal tissues, gall and gall leaves have increased enzyme activity. Plate 3.34 displays the results of PAL enzyme activity analysis graphically.

Table 3.13. Result of quantitative estimation of PAL enzyme activity in normal leaves, gall leaves and galls of *A. scholaris* and *F. racemosa*.

Name of plant	Sample	Enzyme activity (unit/ mL)
A. scholaris	Normal leaf	0.02900 ± 0.0017
	Gall leaf	0.1724 ± 0.019
	Gall	0.3261 ± 0.00664
F. racemosa	Normal leaf	0.02295 ± 0.0019
	Gall leaf	0.4549 ± 0.0025
	Gall	0.5937 ± 0.0269

Each value represents mean ± standard deviation (n = 3)

Medda and colleagues in 2020 demonstrated the correlation between phenolic chemicals and PAL enzyme. The PAL enzyme activity of myrtle (*Myrtus communis* L.) leaves and berries was measured. PAL enzyme was found to have a negative association with total polyphenols in berries, as well as a positive correlation with flavonoids and anthocyanins (Medda et al., 2020). The phenolic content of galls has been found to rise during gall formation. A strong relationship was discovered between the development of gall tissues and phenol production in *A. scholaris* (Biswas et al., 2014; Khurshid et al., 2021). Concentration of phenolic compounds was reported in higher quantities in *F. racemosa* galls compared to leaves (Dsouza and Ravishankar, 2014; Savitha et al., 2013). Our findings also support the previous results of higher PAL enzyme activity in gall and gall associated tissues.



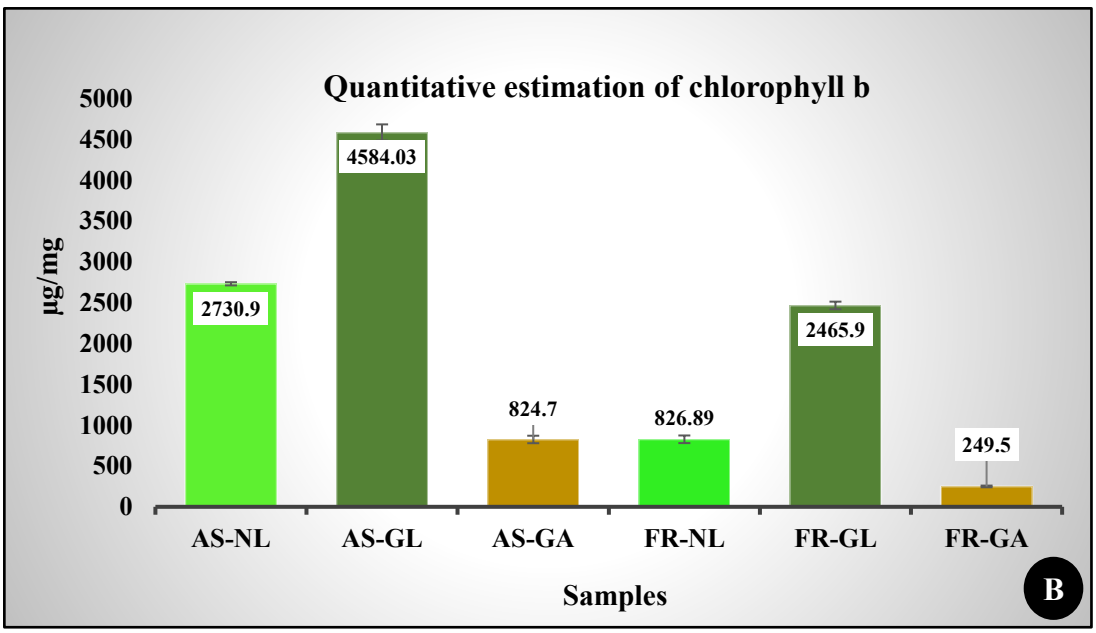
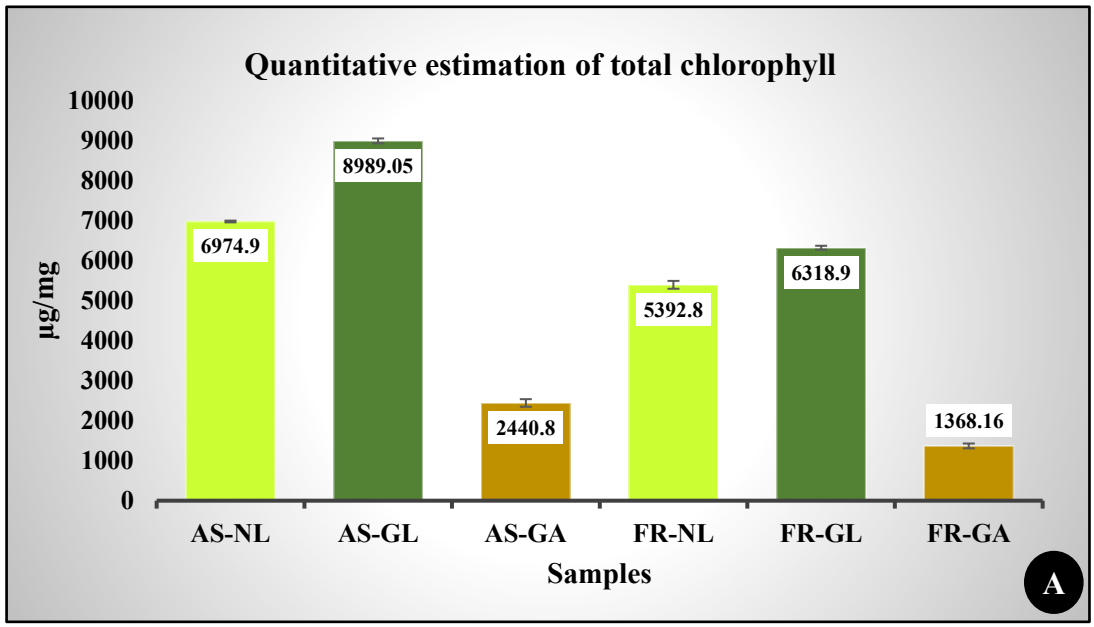


Plate 3.32: Figure A: Results of quantitative estimation of chlorophyll a in normal leaves, gall leaves and galls of *A. scholaris* and *F. racemosa* and Figure B: Results of quantitative estimation of chlorophyll b in normal leaves, gall leaves and leaf galls of *A. scholaris* and *F. racemosa.*



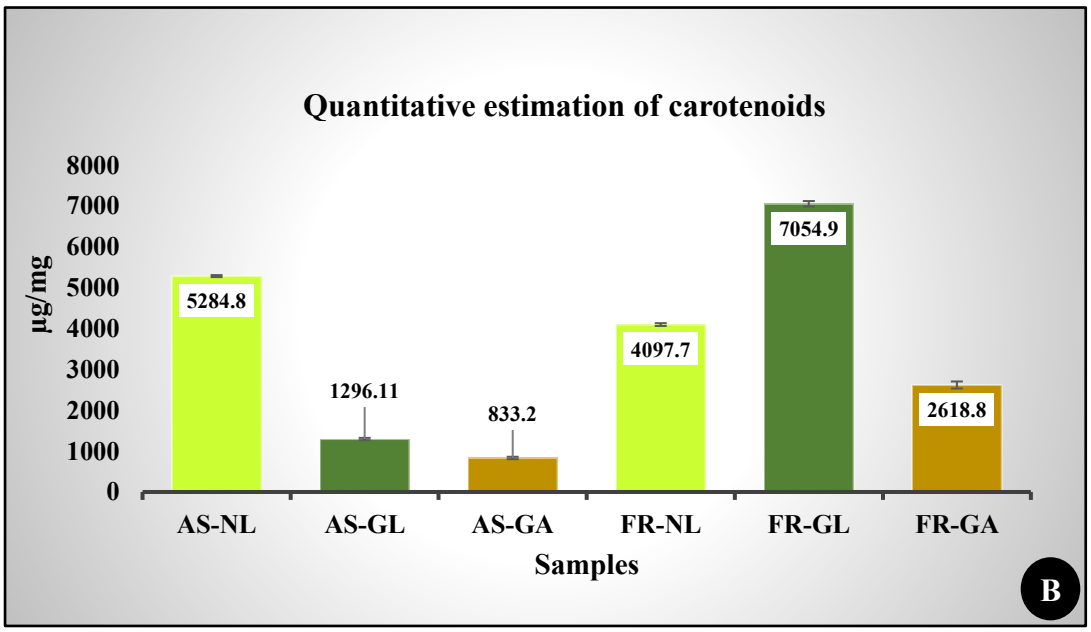


Plate 3.33: Figure A: Results of quantitative estimation of total chlorophyll in normal leaves, gall leaves and leaf galls of *A. scholaris* and *F. racemosa* and Figure B: Results of quantitative estimation of carotenoids in normal leaves, gall leaves and leaf galls of *A. scholaris* and *F. racemosa.*

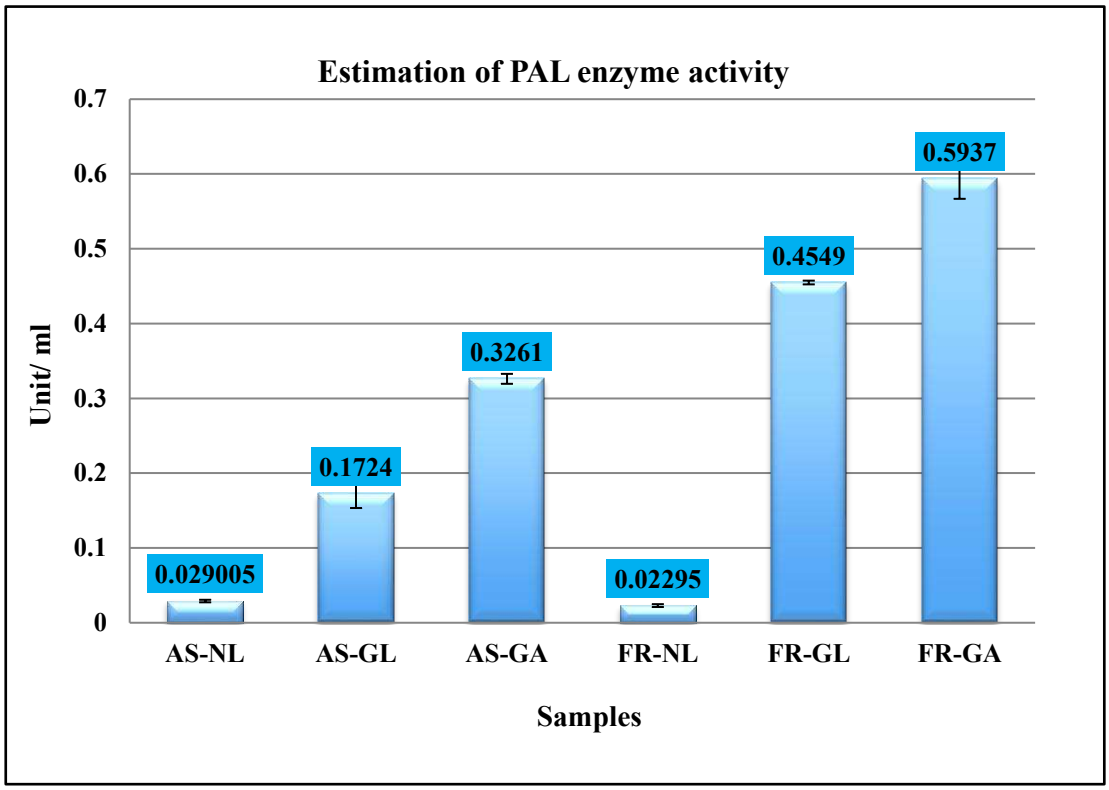


Plate 3.34: The bar graph represents quantitative estimation of PAL enzyme activity in three tissue types - normal leaves, gall leaves and leaf galls of *A. scholaris* and *F. racemosa*. Error bars represent the standard deviation of three replicates (n = 3).

In summary, this study demonstrates that gall formation significantly alters the phytochemical profile of *A. scholaris* and *F. racemosa*, with galled tissues exhibiting higher diversity and concentrations of secondary metabolites, particularly phenolic compounds. These changes likely serve as a defense mechanism against the gall-inducing insects, highlighting the intricate interplay between plant biochemistry and herbivore interactions.

3.4. CONCLUSIONS

In conclusion, this study investigated the impact of gall-inducing psyllids on *A. scholaris* and *F. racemosa*, two plants known to develop leaf galls. By examining the transverse sections, we can identify the alterations in anatomy generated due to gall formation and it was concluded that galls are totally deformed entities. In addition to serving as a source of nourishment, galls also provide a habitat for developing insects. The qualitative phytochemical examination of *A. scholaris* normal leaves, gall leaves and galls revealed that most of the secondary metabolic components including phenolics, alkaloids, flavonoids, saponins and coumarins, had been detected in gall extracts. The GC-MS analysis of methanolic extracts of both plants recognized different phytoconstituents of pharmacological importance. Additionally, it was found that gall and gall-associated tissues possess more phytochemical compounds than normal leaves and each of these compounds is expressed in high concentration in galls. The number of common compounds in normal leaves, gall leaves and galls were lower than the number of unique compounds in each sample.

OHR-LC-MS analysis discovered that compared to normal leaves, the phytochemical content of plant galls were distinct. More phytoconstituents were discovered in gall and gall leaves, and there was also substantial variation in the phenolic contents of gall. It was discovered gall tissues resulting from infection by inducing insects have a significant uniqueness. Based on these findings, it can be stated that gall were morphologically, anatomically and biochemically distinct and unique structures, which have been produced due to insect-induced alteration in the tissues that they affect. These results also corroborate earlier research showing that infected tissues produce more phytochemical substances, particularly phenolics, as a defensive mechanism. The upcoming chapters will shed insight on the molecular basis of

differential phytoconstituent expression as well as provide a better understanding of the gall inducer-host plant interaction.

This study provides a detailed examination of the anatomical and phytochemical alterations induced by gall formation in *A. scholaris* and *F. racemosa*. The results demonstrate that galls are not merely pathological structures but are sites of intense metabolic activity, leading to the accumulation of bioactive compounds with potential therapeutic applications. The findings support the hypothesis that gall induction enhances the production of secondary metabolites, thereby increasing the medicinal value of these plants.

CHAPTER 4 - BIOACTIVITY ANALYSIS OF NORMAL LEAVES, GALL LEAVES AND LEAF GALL EXTRACTS

4.1. INTRODUCTION

This chapter deals with the bioactivities of normal leaves, gall leaves and galls of *Alstonia scholaris* and *Ficus racemosa*. The previous chapter provided the details of phytochemicals present in the different samples of both plants. It is well established that the secondary metabolites present in plants are the basis of their medicinal properties and thus biological activities. The leaf galls induced by insects are rich in phenolic compounds, as demonstrated in Chapter 3, so here in this chapter the bioactivities such as antimicrobial, antioxidant and cytotoxic activities of normal leaves, gall leaves and gall of *A. scholaris* and *F. racemosa* were analyzed to see if gall induction has any positive effect on the bioactive properties of the tissues being infected.

Plants have been used for therapeutic purposes for thousands of years. Initially known as herbalism, the use of plants to treat diseases has evolved into modern phytotherapy, defined as the application of plants or their extracts for therapeutic and medical purposes to treat illnesses and enhance human health. According to the World Health Organization (WHO), approximately 80% of the global population relies on herbal medicines for primary healthcare, utilizing either plant extracts or their active principles (Farnsworth et al., 1987; Houghton, 1995). Indian Vedas outline the use of plants in medicine to treat a variety of ailments. The traditional medicinal systems of India, including Ayurveda, Siddha, Unani and Naturopathy, employ over 7,500 plant species, establishing the country as a global leader in phytopharmacology (Shakya, 2016).

Plants can be used in medicine in various forms, including crude preparations, refined extracts, which are partially purified or rationalized extracts having a few defined components or as single phytochemical species. There are three ways for use of plants in therapeutic fields, they are direct, semi-synthetic and total synthetic systems. In a direct system, the parts of plants that are applied straight in treatments. Semi-synthetic is the

Reshma K Ramesh. Comparative phytochemical analysis and gene expression profiling of leaf galls on selected medicinal plants. Thesis 2025. Govt. Victoria college, Palakkad, University of Calicut.

application of fundamental chemical components that are plant-derived but have been modified to have better qualities including increased efficacy and reduced toxicity. The plants with elements that are not being therapeutically beneficial, but can be converted into medicinal agents through different chemical or microbiological means. In the total synthetic system, natural molecules from plants can also be used as templates to create new compounds with enhanced activity, a higher therapeutic index or fewer side effects (Fowler, 2006; Houghton, 1995).

Many significant pharmaceuticals used in modern healthcare contain 'active elements' from traditional medicines. They still remain the recommended medications for specific ailments. So some of the bioactive compounds from plants can be directly used in medication (Houghton, 1995). Plant metabolites are divided into primary metabolites (e.g., glucose, proteins and lipids) essential for growth and development and secondary metabolites (e.g., phenolics, terpenes, alkaloids) that mediate ecological interactions like defense against herbivores and pathogens. Secondary metabolites are the primary source of therapeutic properties of plants, exhibiting bioactivities such as antioxidant, antimicrobial and cytotoxic effects. Plants synthesize secondary metabolites from primary metabolites through different metabolic pathways. They play no direct role in growth and reproduction, although they do participate in ecological processes such as defense mechanisms and pigment production (Shakya, 2016).

Phytochemicals and bioactivity

A medicinal plant is defined as a plant having chemicals with therapeutic value or containing precursors for chemo-pharmaceutical production. Phytochemical compounds present in medicinal plants possess immense therapeutic properties. The mixtures of secondary products found in plants usually produce the beneficial therapeutic effects of plants (Jain et al., 2019). These therapeutic effects typically arise from combinations of secondary products that exhibit a wide range of bioactivities including antioxidant, antidiabetic, antiviral, antibacterial, anticancer, neuroprotective, antimutagenic, antiallergic and anti-inflammatory properties (Soetan and Aiyelaagbe, 2009). Bioactivity possessed by different phytochemical compounds present in normal leaf, gall leaf and galls of both

selected plants were detailed in chapter 3. In this chapter, the experimental approaches used to analyze the antioxidant, antibacterial, antifungal and cytotoxic activities of these extracts are presented, based on the hypothesis that elevated concentrations of secondary metabolites in gall tissues enhance their bioactivity.

4.1.1. Antioxidant activity

Antioxidants are bioactive substances that inhibit oxidation by neutralizing free radicals, thereby mitigating oxidative damage associated with diseases such as cancer, cardiovascular disorders and neurodegenerative conditions. Medicinal plant-derived bioactive chemicals, particularly polyphenols like flavonoids and phenolic acids, exhibit potent antioxidant properties by scavenging peroxyl radicals, alkyl peroxyl radicals, superoxide, hydroxyl radicals and nitric oxide across various chemical oxidation systems, often by transferring a hydrogen atom from an aromatic hydroxyl group to the free radical. Phenolic substances, including phenols, phenolic acids, flavonoids, anthocyanins and tannins, are well-known for their strong antioxidant capabilities (Altemimi et al., 2017). Antioxidant activity can be assessed using methods such as DPPH free radical scavenging assay, ABTS (2,2'-azino-bis (3-ethyl benzothiazoline -6- sulphonic acid) assay, superoxide scavenging, lipid peroxidation, β-carotene bleaching, nitric oxide scavenging, reducing power, iron chelation, hydroxyl radical scavenging and FRAP (ferric reducing antioxidant power) assay (Alam et al., 2013; Altemimi et al., 2017; Shen et al., 2010).

4.1.2. Antimicrobial activity

Medicinal plants possess notable antibacterial and antifungal properties attributed to their diverse secondary metabolites. Phenolic compounds such as flavonoids and tannins, along with alkaloids and saponins, demonstrate potent antibacterial effects against both Gram-positive and Gram-negative bacteria by disrupting bacterial cell membranes or inhibiting critical enzymes essential for growth and metabolism. Certain bioactive substances also enhance the defense mechanisms of the body by modulating immune responses to combat infections. Similar to antibacterial substances, they may work by enhancing the immune system, disrupting fungal cells and inhibiting essential enzymes etc. (Dar et al., 2023).

4.1.3. Cytotoxic activity

Cytotoxicity refers to the potency of a chemical to cause changes in cellular functioning, negatively impact structures or essential processes and ultimately lead to cell death, often through DNA damage or inhibition of the cell cycle (Kmail et al., 2015). The use of herbal remedies for cancer treatment spans centuries and plant-derived bioactive secondary metabolites, such as vincristine and paclitaxel, are extensively investigated for their cytotoxic potential with fewer adverse effects compared to synthetic drugs (Mansour et al., 2022). The MTT assay, utilizing 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl tetrazolium Bromide, is a common colorimetric technique for assessing cell viability by measuring the activity of mitochondrial dehydrogenase, which converts the yellow MTT dye into insoluble purple formazan crystals in viable cells (Kmail et al., 2015; Mansour et al., 2022).

4.1.4. Anti-inflammatory activity

When tissues in the body get injured by an infection, physical trauma, hazardous substances or microbes like bacteria and viruses, they naturally react with inflammation to protect them. Redness, heat, swelling (edema), discomfort and loss of function are its main characteristic symptoms. Inflammation is a complicated bodily response that frequently results in pain and involves modifications to cellular membranes, protein denaturation and vascular permeability. The egg albumin denaturation assay is one of the techniques used to evaluate the anti-inflammatory properties of plant extracts. This *in vitro* technique is widely utilized, fast and reliable. It functions on the principle that denaturation of proteins can cause inflammation; hence, substances that prevent denaturation are thought to have anti-inflammatory properties. In this study selected extracts were used for protein denaturation assay for assessing its anti-inflammatory property (Chandra et al., 2012; Mirke et al., 2020; Vinchurkar et al., 2014).

The current application of plant-derived medications merely touches the surface of their vast potential. The biological and chemical diversity in plants, coupled with novel developments in plant biotechnology, molecular biology and genetic engineering, can provide innovative medicinal molecules for disease treatment in the future (Fowler, 2006). In

this context, the present study aims to evaluate the bioactivity of insect-induced plant tissues to elucidate their significance and therapeutic potential.

4.2. METHODOLOGY

4.2.1. Antioxidant activity analysis

Reducing power method

Different concentrations (20, 40, 60, 80 and 100 µg/mL of water, hexane and ethanol) of extracts were prepared. To 1 mL of extracts added 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% Potassium ferricyanide. The resulting mixture was incubated at 50 °C for 20 min, followed by the addition of 2.5 mL of 10% Trichloro acetic acid. The mixture was centrifuged at 5000 rpm for 10 min to collect the upper layer of the solution (2.5 mL), mixed with distilled water (2.5 mL) and 0.5 mL of 0.1% ferric chloride. The absorbance was then measured at 700 nm against a blank. Each reaction was done in triplicate. The increase in absorbance indicates the reducing power of the extracts (Alam et al., 2013; Jayaprakasha et al., 2001).

Reducing power method based on the principle of increases in the absorbance of the reaction mixtures. Increase in the absorbance indicates an increase in antioxidant activity. Antioxidant compounds cause the reduction of ferric form to the ferrous form because of their reductive capabilities. The Prussian blue coloured complex is formed by adding ferric chloride. Therefore, the reduction can be determined by measuring the formation of Prussian blue at 700 nm absorbance (Alam et al., 2013; Jayaprakasha et al., 2001).

DPPH free radical scavenging activity

The free radical scavenging activity of the extracts was measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH). 0.1 mM solution of DPPH in ethanol was prepared. 2.96 mL of DPPH solution was added to various concentrations of different samples. The sample solution was made up to 40 µL by adding Dimethyl sulfoxide (DMSO) to 2.5, 5, 7.5 and 10 µL of sample solutions taken from the stock solution of concentration 100 mg/mL to make different dilutions of the sample (0.25, 0.5, 0.75 and 1 mg/mL). The mixture was shaken

vigorously and allowed to stand for 30 min at room temperature, absorbance was measured at 517 nm using a UV-Visible spectrophotometer (Alam et al., 2013; Shen et al., 2010). Ascorbic acid was used as reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. All the tests were performed in triplicate mean value was calculated and the percentage radical scavenging activity was calculated by using the following equation;

$$\% RSA = Abs\ Control - Abs\ Sample / Abs\ Control \times 100$$

'RSA' is radical scavenging activity, 'Abs control' is the absorbance of the control reaction (containing all reagents except the test sample) and 'Abs sample' is the absorbance of the plant extracts.

4.2.2. Antibacterial activity analysis

Bacterial strains and growth conditions

Various cultures of human pathogenic Gram-positive and Gram-negative bacteria were selected for antibacterial assay. They are *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (MCMB 310), *Escherichia coli* (ATCC 35218) and *Pseudomonas aeruginosa* (MCMB 407). The cultures were obtained from Agharkar Research Institute, Pune, Maharashtra.

Nutrient broth preparation

Nutrient broth was prepared by dissolving 13 g of nutrient broth powder (HiMedia) in 1000 mL of distilled water. The mixture was stirred thoroughly until completely dissolved. The solution was then transferred to a clean conical flask and sterilized by autoclaving at 121 °C for 15 min. A loop full test organism was inoculated on nutrient broth and incubated for 24 h at 37 ± 1ºC and maintained in sterile condition.

Screening for antibacterial properties by agar well diffusion method

Nutrient agar was prepared by dissolving 28 g of nutrient agar powder (HiMedia) in 1000 mL of distilled water. The mixture was stirred thoroughly until fully dissolved. The

medium was then sterilized by autoclaving at 121 °C for 15 min. After sterilization, the molten medium was poured into sterile petri dishes and allowed to solidify at room temperature.

The culture plates were prepared by pouring 20 mL of sterile nutrient agar. After the solidification of the medium, the inoculum was swabbed uniformly over the entire agar surface using a sterile cotton swab and allowed to dry for 5 min. A sterile cork borer (8.5 mm) was used to make wells in each plate. These plates were labeled and 10 µL, 20 µL and 30 µL of each plant extracts were added aseptically into the well using a micropipette and the plates were then maintained at room temperature for 2 h to allow the diffusion of the solution into the medium. Then the plates were incubated for 24 h at 37 ºC for microbial growth to occur. For each bacterial strain, pure solvent was used as negative control. Streptomycin was used as positive control. Each test was repeated three times and the antibacterial activity was expressed as the mean diameter of the inhibition zones (mm) produced by each plant extracts when compared to the control (Mandal et al., 2000b; Misra et al., 2011).

4.2.3. Antifungal activity analysis

Fungal strains and growth conditions

Candida albicans (NFCCI 5123) and *Aspergillus niger* (MTCC1344) were standard fungal strains collected from Biovent Innovations, Department of Biotechnology, University of Kerala, Trivandrum. The fungal strains were cultured in sterile potato dextrose agar (PDA) at 37 °C for 48 h and the strains were also maintained as glycerol stocks.

PDA medium-preparation

Fresh potatoes (200 g) were washed thoroughly to remove surface dirt, peeled, and cut into small slices. The sliced potatoes were boiled in 1000 mL of distilled water for 20-25 min using a hot plate. The extract was filtered through muslin cloth to obtain the potato infusion.

To the potato infusion, 20 g of dextrose and 15 g of agar were added. The mixture was stirred using a magnetic stirrer and heated until all components were completely

dissolved. The final volume was adjusted to 1000 mL with distilled water. The medium was transferred to a conical flask, closed with a cotton plug and sterilized by autoclaving at 121 °C (15 psi) for 20 min.

Antifungal assay of *C. albicans* by agar-well diffusion method

Agar-well diffusion method was conducted to evaluate the antifungal activities of healthy leaves, gall leaves and galls of both plants against *C. albicans*. All the three extracts (water, hexane and ethanol) of plant samples were used for the analysis. *C. albicans* colonies that were stored on PDA agar slant were transferred to PDA broth and incubated at 35 °C for 3 days for complete growth. Prior to inoculation, 20 mL of PDA medium was poured into petri dishes and kept for solidification. The organisms were inoculated uniformly with sterile swabs on the surface of the solidified PDA plate. After inoculation, 8 mm diameter wells were made by using a sterile cork borer.

For primary screening of activity, 100 mg/mL stock solution was made for aqueous, hexane and ethanol extracts. From that stock solution 20 µL and 40 µL dilutions taken for screening (2 mg and 4 mg per well respectively) was added into the respective wells using a micropipette. After inoculation, the plates were incubated for 48 h at 37 °C. The results were measured as zone of inhibition in mm and the results were photographically recorded. The extracts that gave positive results were only selected for further analysis. These extracts were tested with different concentrations and each test was done in triplicates. Zone of inhibition was measured in triplicates and SD was calculated. A standard antifungal drug, Itraconazole 20 µL, 40 µL and 60 µL from 50 mg/mL stock, was used as a positive control (Gizaw et al., 2022; Raj et al., 2021; Sales et al., 2016).

Antifungal assay of *A. niger* by food poisoning method

The crude extracts of ethanol were used for antifungal analysis against *A. niger*. A standard antifungal drug, Itraconazole 60 µL from 50 mg/mL stock (3 mg), was used as a positive control. Here preliminary screening was conducted with different dilutions of ethanolic extracts.

The food poisoning approach was used to demonstrate antifungal activity against *A. niger*. Different dilutions of plant extracts were mixed with PDA media and added to sterile petri plates. A mycelial disc measuring 6 mm was then positioned in the middle of the plate after solidification of medium. Every treatment was carried out three times. The plates were incubated at 30 ± 2 °C for seven days. The growth of the fungal colony was measured on alternate days and percentage inhibition was calculated by using the following formula;

$$PGI = (AC - AT)/AC \times 100$$

Where 'AC' is the average mycelial growth of the control, 'AT' is the average mycelial growth of the treatment with plant extract and 'PGI' is the percentage growth inhibition. Values were given as the mean ± standard deviation (SD) of tests performed in triplicates (Chakrapani et al., 2020; Rafiq et al., 2022; Schoss et al., 2022).

4.2.4. Cytotoxic activity analysis

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay is performed to evaluate the cytotoxicity of different extracts of *A. scholaris* and *F. racemosa*. MTT in the living cells generate purple formazan crystals. The amount of formazan produced is determined by measuring the absorbance. The cytotoxicity studies were done at Stem cell and Regenerative Biology lab, School of Biotechnology, Amrita Vishwavidyapeetham, Vallikavu, Kollam, Kerala.

Cell culture

The extract was tested for cytotoxicity on 3T3 cells. The 3T3 cell line is derived from mouse embryonic fibroblasts. Freshly harvested cells were used for assay. Seeded an appropriate quantity of the cells in a cell culture plate for the assay and incubated at 37 °C with 5% CO_2. Allow the cells to grow for up to 24 h, or until confluence occurs. Harvested the cells and used them for the experiment.

Assay procedure

The harvested cells were cultured using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were grown in 96-well plates at a density of 5×10^3 cells and maintained for 24 h at 37 °C with 5% CO_2. After 24 h the medium is replaced by DMEM containing 10% FBS and different amounts of plant extracts. Test concentrations of extracts of *A. scholaris* and *F. racemosa* were used to screen cytotoxic activity initially. The extracts that showed lower cell viability percentages were selected for further evaluation of cytotoxic effects at different dilutions (50 mg/mL - 500 mg/mL).

Cells treated with dimethyl sulfoxide (DMSO) and phosphate buffered saline (PBS) served as negative controls and cells treated with Triton X-100 served as positive control. After an additional 24 h incubation at 37 °C with 5% CO_2, the medium was replaced with 90 µL of DMEM and 10 µL of MTT reagent (3-(4,5- dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide) per well. The plates were incubated for 4 h at 37 °C with 5% CO_2 to allow the reduction of MTT to formazan crystals by metabolically active cells. Then, 100 µL of solubilisation solution was added to each well to dissolve the formazan crystals and the plate was placed on a shaker for 30-45 min.

Absorbance was measured at 570 nm with a reference wavelength of 630 nm using a microplate reader. The average values of 570 nm absorbance values of the control wells was subtracted from the average values of 570 nm absorbance values of corresponding experimental wells to calculate relative cell viability (Abdoli et al., 2021; Kmail et al., 2015). All the experiments were done in triplicates and mean OD values were taken for calculation. The cell viability was calculated using the following formula;

$$Percentage\ cell\ viability = (AS/AC) \times 100$$

Where, AS = absorbance of extract treated cells and AC = absorbance of control cells

4.2.5. Anti-inflammatory activity analysis

The anti-inflammatory activity of the plant extract was determined *in vitro* by assessing its inhibition of egg albumin (protein) denaturation.

Assay procedure

A reaction mixture was prepared by mixing 0.2 mL of a 1-2% egg albumin solution (obtained from fresh hens eggs), 2 mL of the sample extract and 2.8 mL of phosphate-buffered saline (pH 7.4), resulting in a total volume of 5 ml. Samples were prepared using five dilutions of 1, 2, 3, 4 and 5 µg/µL. A total volume of 5 mL of the control was created by combining 2 mL of distilled water, 0.2 mL of 1-2% egg albumin solution and 2.8 mL of phosphate-buffered saline. Diclofenac sodium was used as a standard drug at a final concentration of 50 mg/ml. The reaction mixtures were incubated at 37 ± 2 °C for 30 min and subsequently heated in a water bath at 70 ± 2 °C for 15 min. After cooling, their absorbance was measured at 660 nm. The assay was performed in triplicate. The percentage inhibition of protein denaturation was calculated using the following formula;

$$Percentage\ inhibition = (Abs\ Control - Abs\ sample) \times 100/Abs\ control$$

Statistical analysis

For each treatment or sample type, three independent replicates (n = 3) were used to generate all experimental data in order to validate the reliability and repeatability of the results obtained. The mean ± standard deviation (SD) was used to express the data. One-way analysis of variance (ANOVA) was used to compare the overall differences among group means using Python 3.10 software. Significant variations in the measured parameters between the tissue types might be identified with this approach. Tukey's honestly significant difference (HSD) post-hoc test was used to identify the particular group pairs that differed significantly from one another where the ANOVA displayed significant variation ($p < 0.05$). Prior to analysis, all statistical presumptions, such as homogeneity of variances and normality were verified. Throughout the study, $p < 0.05$ was deemed statistically significant.

4.3. RESULTS AND DISCUSSION

4.3.1. Results of antioxidant activity analysis

Reducing power method:

The reducing power of aqueous, hexane and ethanolic extracts from normal leaves, gall leaves and galls of *F. racemosa* and *A. scholaris* was analyzed to assess their antioxidant activity. The reducing power assay measures the ability of extracts to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), with higher absorbance at 700 nm indicating greater antioxidant potential. Ascorbic acid was used as the positive control, tested at five concentrations (20, 40, 60, 80, 100 µg/mL). Results of reducing power analysis of 100 µg/mL aqueous, hexane and ethanolic extracts of *A. scholaris* normal leaves (AS-NL), *A. scholaris* gall leaves (AS-GL) and *A. scholaris* galls (AS-GA) are shown in Plate 4.1: Figure A-I. The photographs of the results of reducing power method of different extracts of *F. racemosa* normal leaves (FR-NL), *F. racemosa* gall leaves (FR-GL) and *F. racemosa* galls (FR-GA) are shown in Plate 4.2: Figure A-I.

Reducing power activity of aqueous extracts

Plate 4.3: Figure A is the graphical representation of the dose-dependent reducing power of aqueous extracts for both plants, highlighting superior activity of AS-GL. Table 4.1 shows the percentage radical scavenging activity of aqueous extracts of both *A. scholaris* and *F. racemosa.* Among aqueous extracts, FR-GL and AS-GL exhibited higher reducing power than normal leaf and gall extracts. Specifically, AS-GL at 100 µg/mL showed the highest activity among all aqueous extracts. *A. scholaris* extracts generally outperformed *F. racemosa* extracts in aqueous solvent, with AS-NL, AS-GL and AS-GA also showing moderate activity. In contrast, FR-NL and FR-GA had low reducing power, each below 25% at 100 µg/mL, while FR-GL exhibited the highest activity among *F. racemosa* samples (25.4 ± 0.85%). The %RSA of *A. scholaris* and *F. racemosa* gall and gall leaves tissues were significant at the level p value < 0.05.

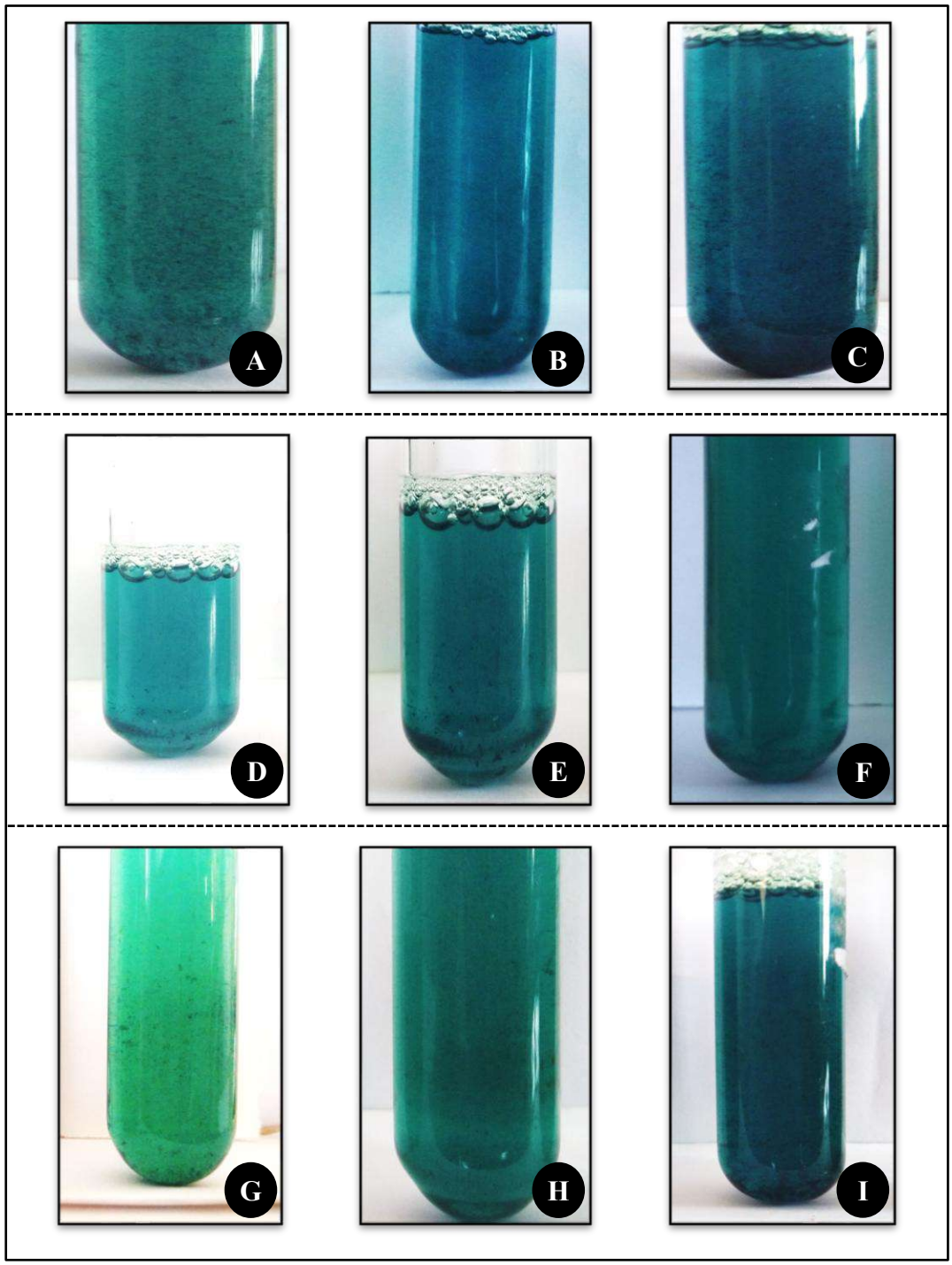
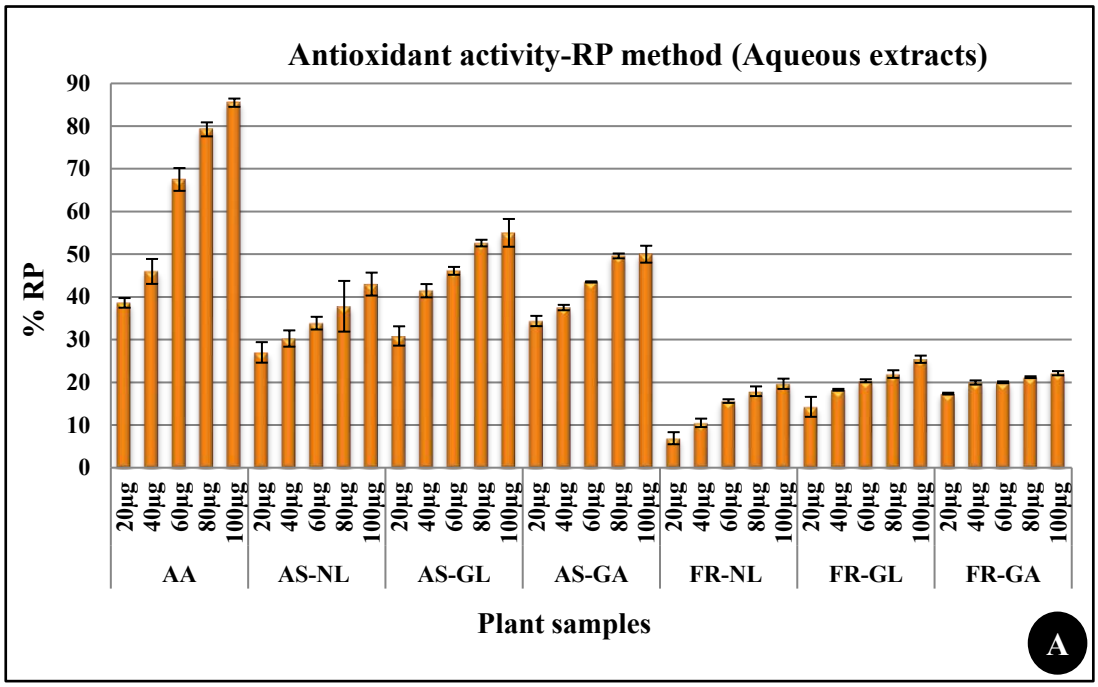


Plate 4.1: Results of reducing power analysis of *A. scholaris* extracts (100 µg/ml). Figure A-C: shows the reducing power analysis result of aqueous extracts of AS-NL, AS-GL and AS-GA, Figure D-F: represents the result of hexane extracts of AS-NL, AS-GL and AS-GA and Figure G-I: shows the result of ethanol extracts of AS-NL, AS-GL and AS-GA.



Plate 4.2: Results of reducing power analysis of *F. racemosa* extracts (100 µg/ml). Figure A-C: shows the reducing power of aqueous extracts of FR-NL, FR-GL and FR-GA; Figure D-F: shows reducing power activity of hexane extracts of FR-NL, FR-GL and FR-GA and Figure G-I: shows the result of ethanol extracts of FR-NL, FR-GL and FR-GA.



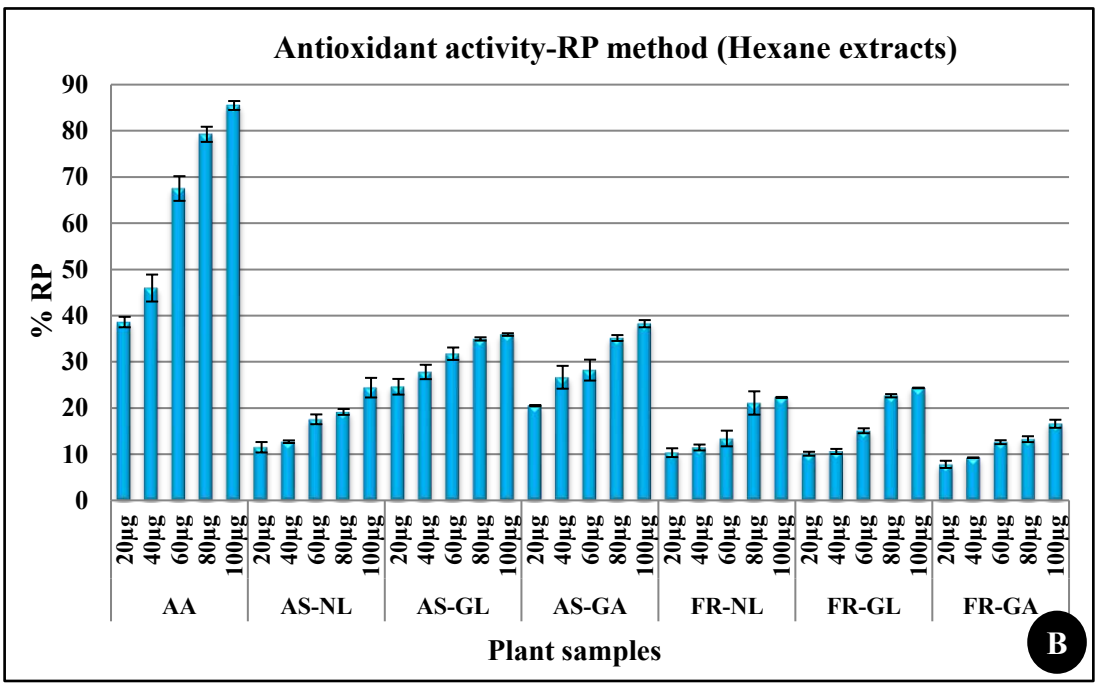


Plate 4.3: Figure A: Graphical representation of antioxidant activity analysis results of aqueous extracts of AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA using reducing power method. Ascorbic acid taken as positive control. Each bar represents reducing power of the sample with SD and Figure B: Graphical representation of results of antioxidant activity analysis of hexane extracts of AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA using RP method. Ascorbic acid taken as positive control. Each bar represents reducing power of the sample with SD.

Table 4.1: The percentage radicle scavenging activity of aqueous extracts of different samples of *A. scholaris* and *F. racemosa* determined using RP method.

Sl. No.	Name of sample (Aqueous extracts)	Dilutions of sample used (µg/mL)	%RSA
1.	AS-NL	20	$27 \pm 2.40^{+}$
2.		40	$30.25 \pm 1.91^{+}$
3.		60	$33.85 \pm 1.48^{+}$
4.		80	$37.8 \pm 5.94^{+}$
5.		100	$43 \pm 2.69^{+}$
6.	AS-GL	20	$30.85 \pm 2.26^{*}$
7.		40	$41.45 \pm 1.56^{*}$
8.		60	$46.1 \pm 0.92^{*}$
9.		80	$52.6 \pm 0.78^{*}$
10.		100	$55.0 \pm 3.25^{*}$
11.	AS-GA	20	$34.35 \pm 1.20^{*}$
12.		40	$37.5 \pm 0.64^{*}$
13.		60	$43.5 \pm 0.14^{*}$
14.		80	$49.6 \pm 0.57^{*}$
15.		100	$50.0 \pm 1.98^{*}$
16.	FR-NL	20	$6.9 \pm 1.41^{++}$
17.		40	$10.5 \pm 0.99^{++}$
18.		60	$15.6 \pm 0.42^{++}$
19.		80	$17.9 \pm 1.13^{++}$
20.		100	$19.65 \pm 1.20^{++}$
21.	FR-GL	20	$14.25 \pm 2.33^{++*}$
22.		40	$18.25 \pm 0.21^{++*}$

23.		60	$20.35 \pm 0.35^{++*}$
24.		80	$21.93 \pm 0.89^{++*}$
25.		100	$25.4 \pm 0.85^{++*}$
26.	FR-GA	20	$17.35 \pm 0.21^{++*}$
27.		40	$19.95 \pm 0.49^{++*}$
28.		60	$20.0 \pm 0.21^{++*}$
29.		80	$21.2 \pm 0.21^{++*}$
30.		100	$22.13 \pm 0.49^{++*}$
31.	Ascorbic acid	20	38.6 ± 1.13
32.		40	45.97 ± 2.92
33.		60	67.51 ± 2.67
34.		80	79.24 ± 1.64
35.		100	85.48 ± 0.97

Each value of % RSA represents mean ± standard deviation (n = 3). ++ indicates values significantly different from ascorbic acid (control) at the corresponding concentration at the level $p < 0.001$. + indicates values significantly different from ascorbic acid (control) at the corresponding concentration p-value < 0.05). *indicates values significantly different from normal leaf extract of each plant at the corresponding concentration ($p < 0.05$).

Reducing power activity of hexane extracts

Plate 4.3: Figure B illustrates the reducing power of hexane extracts, emphasizing superior performance of *A. scholaris.* The results of antioxidant activity of different samples of *A. scholaris* and *F. racemosa* hexane extracts shown in Table 4.2. Hexane extracts of normal leaves, gall leaves and galls were less active than aqueous extracts overall, but *A. scholaris* samples consistently showed higher reducing power than *F. racemosa* samples. The plant samples such as AS-GL and AS-GA exhibited significantly higher reducing power when compared with AS-NL (p-value < 0.05), with AS-GA at 100 µg/mL demonstrating the

highest activity (38.25 ± 0.78%) among hexane extracts. For *F. racemosa*, no clear pattern emerged. FR-GL at 100 µg/mL showed the highest activity among *F. racemosa* samples, same as observed in aqueous extracts, followed by FR-NL, while FR-GA had the lowest reducing power, possibly due to lower solubility of active phenolic compounds in hexane.

Table 4.2: The percentage radicle scavenging activity of hexane extracts of different samples of *A. scholaris* and *F. racemosa* determined using RP method.

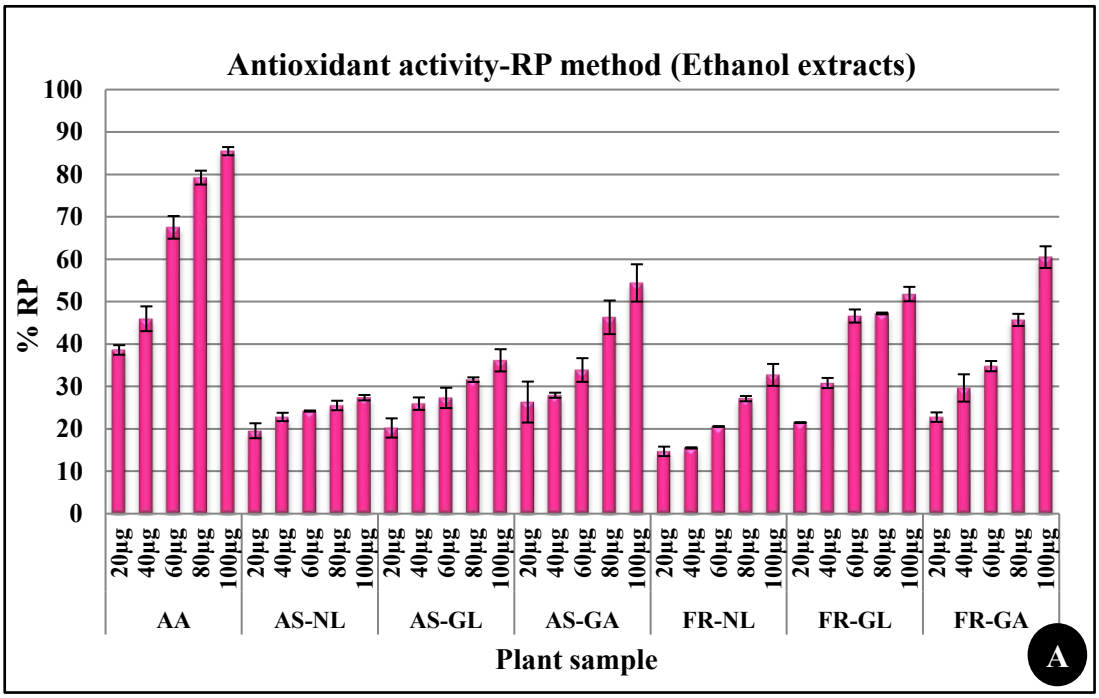
Sl. No.	Name of sample (Hexane extracts)	Dilutions of sample used (µg/mL)	%RSA
1.	AS-NL	20	$11.5 \pm 1.13^{++}$
2.		40	$12.7 \pm 0.28^{++}$
3.		60	$17.55 \pm 1.06^{++}$
4.		80	$19.15 \pm 0.64^{++}$
5.		100	$24.4 \pm 2.12^{++}$
6.	AS-GL	20	$24.6 \pm 1.70^{+*}$
7.		40	$27.8 \pm 1.56^{+*}$
8.		60	$31.75 \pm 1.34^{+*}$
9.		80	$34.95 \pm 0.35^{+*}$
10.		100	$35.9 \pm 0.28^{+*}$
11.	AS-GA	20	$20.5 \pm 0.14^{+*}$
12.		40	$26.65 \pm 2.47^{+*}$
13.		60	$28.2 \pm 2.26^{+*}$
14.		80	$35.15 \pm 0.64^{+*}$
15.		100	$38.25 \pm 0.78^{+*}$
16.	FR-NL	20	$10.33 \pm 0.95^{++}$
17.		40	$11.45 \pm 0.64^{++}$
18.		60	$13.4 \pm 1.70^{++}$
19.		80	$21.09 \pm 2.52^{++}$

20.		100	$22.28 \pm 0.11^{++}$
21.	FR-GL	20	$10.1 \pm 0.42^{++*}$
22.		40	$10.59 \pm 0.55^{++*}$
23.		60	$15.08 \pm 0.53^{++*}$
24.		80	$22.65 \pm 0.35^{++*}$
25.		100	$24.35 \pm 0.06^{++*}$
26.	FR-GA	20	$7.8 \pm 0.78^{++}$
27.		40	$9.24 \pm 0.06^{++}$
28.		60	$12.6 \pm 0.42^{++}$
29.		80	$13.25 \pm 0.64^{++}$
30.		100	$16.59 \pm 0.87^{++}$
31.	Ascorbic acid	20	38.6 ± 1.13
32.		40	45.97 ± 2.92
33.		60	67.51 ± 2.67
34.		80	79.24 ± 1.64
35.		100	85.48 ± 0.97

Each value of %RSA represents mean ± standard deviation (n = 3). ++ indicates values significantly different from ascorbic acid (control) at the corresponding concentration at the level $p < 0.001$. + indicates values significantly different from ascorbic acid (control) at the corresponding concentration p-value <0.05). *indicates values significantly different from normal leaf extract of each plant at the corresponding concentration ($p < 0.05$).

Reducing power activity of ethanolic extracts

The results of antioxidant activity of different samples of *A. scholaris* and *F. racemosa* ethanolic extracts shown in Table 4.3. Plate 4.4: Figure A graphically represents the reducing power of ethanolic extracts, highlighting the dominance of FR-GL and AS-GA. Ethanolic extracts demonstrated the highest reducing power across all other solvents, reflecting the efficacy of ethanol in extracting bioactive phenolic compounds. FR-GA and



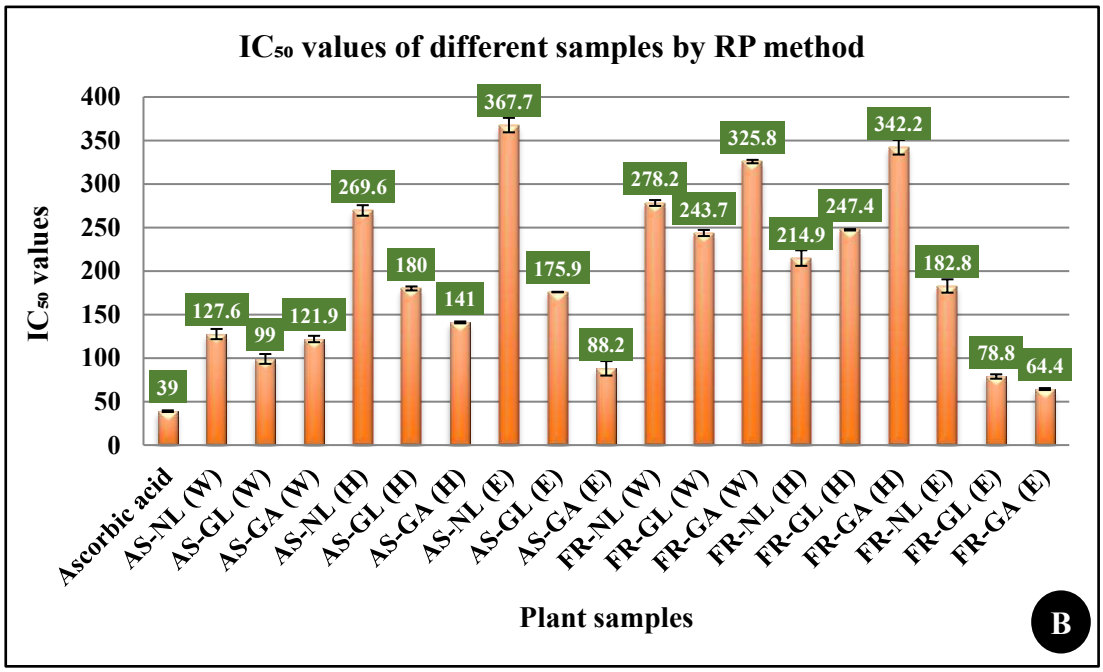


Plate 4.4 : Figure A: Graphical representation of results of antioxidant activity analysis of ethanolic extracts of AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA using RP method. Ascorbic acid taken as positive control. Each bar represents reducing power of the sample with standard deviation and Figure B: IC_{50} values obtained by RP assay using aqueous, hexane and ethanolic extracts of different samples of *A. scholaris* and *F. racemosa*.

FR-GL exhibited the highest activities, with percentage radical scavenging activity of 60.49% and 51.77% at 100 µg/mL, respectively, significantly outperforming FR-NL ($p < 0.05$). A similar trend was observed in AS-GA at 100 µg/mL showed the highest reducing power (54.41%), followed by AS-GL (36.16%), which was notably lower than AS-GA but still higher than AS-NL. When comparing all extracts, ethanolic extracts had the highest reducing power, followed by aqueous extracts, while hexane extracts showed the lowest activity.

Table 4.3: The percentage radicle scavenging activity of ethanolic extracts of different samples of *A. scholaris* and *F. racemosa* evaluated through RP method.

Sl. No.	Name of sample (Ethanolic extracts)	Dilutions of sample used (µg/mL)	%RSA
1.	AS-NL	20	$19.55 \pm 1.77^{++}$
2.		40	$22.8 \pm 0.99^{++}$
3.		60	$24.2 \pm 0.13^{++}$
4.		80	$25.51 \pm 1.12^{++}$
5.		100	$27.35 \pm 0.64^{++}$
6.	AS-GL	20	$20.2 \pm 2.27+^{*}$
7.		40	$25.94 \pm 1.47^{+*}$
8.		60	$27.3 \pm 2.40^{+*}$
9.		80	$31.59 \pm 0.55^{+*}$
10.		100	$36.16 \pm 2.62^{+*}$
11.	AS-GA	20	$26.32 \pm 4.83^{+*}$
12.		40	$27.93 \pm 0.60^{+*}$
13.		60	$33.88 \pm 2.79^{+*}$
14.		80	$46.3 \pm 3.96^{+*}$
15.		100	$54.41 \pm 4.40^{+*}$
16.	FR-NL	20	$14.69 \pm 1.12^{+}$

17.		40	$15.5 \pm 0.14^{+}$
18.		60	$20.54 \pm 0.06^{+}$
19.		80	$27.12 \pm 0.62^{+}$
20.		100	$32.72 \pm 2.59^{+}$
21.	FR-GL	20	$21.47 \pm 0.04^{+*}$
22.		40	$30.80 \pm 1.20^{+*}$
23.		60	$40.61 \pm 1.55^{+*}$
24.		80	$47.21 \pm 0.21^{+*}$
25.		100	$51.77 \pm 1.70^{+*}$
26.	FR-GA	20	$22.76 \pm 1.12^{*}$
27.		40	$29.64 \pm 3.22^{*}$
28.		60	$34.79 \pm 1.21^{*}$
29.		80	$45.69 \pm 1.44^{*}$
30.		100	$60.49 \pm 2.56^{*}$
31.	Ascorbic acid	20	38.6 ± 1.13
32.		40	45.97 ± 2.92
33.		60	67.51 ± 2.67
34.		80	79.24 ± 1.64
35.		100	85.48 ± 0.97

Each value of %RSA represents mean ± standard deviation (n = 3). + indicates values significantly different ($p < 0.05$) from ascorbic acid (control) at the corresponding concentration. ++ indicates values significantly different from ascorbic acid (control) at the corresponding concentration ($p < 0.001$).* indicates values significantly different from normal leaves of each plant at the corresponding concentration ($p < 0.05$).

Evaluation of antioxidant potency based on IC_{50} values

To quantify antioxidant potency, IC_{50} values (the concentration required to achieve 50% reducing power) were calculated (Table 4.4 and Plate 4.4: Figure B). Ethanolic extracts of FR-GL and FR-GA exhibited the lowest IC_{50} values (78.8 ± 2.53 µg/mL and 66.4 ± 1.13 µg/mL, respectively), indicating high potency. For *A. scholaris*, the ethanolic extract of galls had the lowest IC_{50} (88.2 ± 8.33 µg/mL), followed by AS-GL (175.9 ± 0.27 µg/mL). Aqueous extracts showed moderate IC_{50} values (e.g., AS-GL: 99 ± 5.66 µg/mL), while hexane extracts had the highest IC_{50} values (e.g., AS-NL: 269.6 ± 6.05 µg/mL), reflecting lower potency. Ascorbic acid, the positive control, had an IC_{50} of 39 ± 0.95 µg/mL, serving as a benchmark for comparison.

ANOVA confirmed significant differences in %RSA among normal leaf extract to gall leaf and gall extracts of *A. scholaris* and *F. racemosa*. Then Tukey's HSD tests were done to find the significant levels of each sample. All the extracts of *A. scholaris* showed significance at $p < 0.05$ against normal leaf extracts. In the case of *F. racemosa*, except Fr-GA hexane extract, all other extracts showed $p < 0.05$ against FR-NL. When the ascorbic acid (standard) at the corresponding concentration tested for significance against different samples and found that except AS-GL and AS-GA (aqueous extracts) and ethanolic extract of AS-GA, all other extracts showed significance at the level $p < 0.05$. In the case of *F. racemosa*, all other samples and extracts, except hexane exhibited $p < 0.05$. According to Tukey's HSD tests, all of the *F. racemosa* extracts were noticeably more effective than those of *A. scholaris* ($p < 0.05$). The greater antioxidant capacity of galls compared to normal tissues is indicated by the higher IC_{50} values of normal leaf and gall leaf extracts across *A. scholaris*. In *F. racemosa*, the gall-leaf extract is more potent than the normal leaf and gall extract, as indicated by its lower IC_{50} values.

Table 4.4: IC_{50} values of *A. scholaris* and *F. racemosa* extracts of normal leaves, gall leaves and leaf galls analysed through RP method

Sl. No.	Plant sample	IC_{50} (µg/mL)
1	AS-NL (Aqueous)	127.6 ± 5.87
2	AS-GL (Aqueous)	99 ± 5.66
3	AS-GA (Aqueous)	121.9 ± 3.67
4	AS-NL (Hexane)	269.6 ± 6.05
5	AS-GL (Hexane)	180 ± 2.29
6	AS-GA (Hexane)	141 ± 1.08
7	AS-NL (Ethanol)	367.7 ± 8.33
8	AS-GL (Ethanol)	175.9 ± 0.27
9	AS-GA (Ethanol)	88.2 ± 8.33
10	FR-NL (Aqueous)	278.2 ± 3.37
11	FR-GL (Aqueous)	243.7 ± 3.53
12	FR-GA (Aqueous)	325.8 ± 1.97
13	FR-NL (Hexane)	214.9 ± 8.94
14	FR-GL (Hexane)	247.4 ± 0.80
15	FR-GA (Hexane)	342.2 ± 8.38
16	FR-NL (Ethanol)	182.8 ± 7.67
17	FR-GL (Ethanol)	78.8 ± 2.53
18	FR-GA (Ethanol)	66.4 ± 1.13
19	Ascorbic acid (AA)	39 ± 0.95

Each value represents mean ± Standard deviation (n = 3)

Results of DPPH free radical scavenging assay:

The DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay evaluated the antioxidant activity of aqueous, hexane and ethanolic extracts of normal leaves, gall leaves and galls from *A. scholaris* and *F. racemosa*. The assay measures the ability of extracts to neutralize free radicals, with percentage radical scavenging activity (%RSA) calculated. Results are presented below by solvent type, comparing AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA.

Plate 4.5 showing the results of DPPH free radical scavenging activity of ethanol, aqueous and hexane extracts of AS-NL, AS-GL and AS-GA in different dilutions of extracts (0.25, 0.5, 0.75 and 1 mg/ml). Plate 4.6 showing the free radical scavenging activity of ethanol, aqueous and hexane extracts of FR-NL, FR-GL and FR-GA in different dilutions of extracts.

DPPH free radical scavenging activity of aqueous extracts

Table 4.5 shows the radical scavenging activity percentage exhibited by different samples of *A. scholaris* and *F. racemosa* aqueous extracts. Plate 4.7: Figure A illustrates the dose-dependent %RSA of aqueous extracts of both plants, with error bars representing standard deviations. Aqueous extracts of *A. scholaris* showed higher %RSA than *F. racemosa* across all samples. For AS-GL and AS-GA at 1 mg/mL exhibited comparable and high %RSA (42.8% and 43.7%, respectively), significantly outperforming AS-NL (13.18%, $p < 0.001$). In contrast, *F. racemosa* aqueous extracts displayed low %RSA, with FR-GA at 1 mg/mL achieving the highest activity (30.03%), followed by FR-NL (19.54%) and FR-GL (13.03%). The superior performance of *A. scholaris* aqueous extracts suggests greater water solubility of active compounds, likely phenolics in *A. scholaris* compared to *F. racemosa*.

Table 4.5: Radical scavenging activity percentage (%RSA) of aqueous extracts of different samples of *A. scholaris* and *F. racemosa* compared with Ascorbic acid (standard) at different concentrations through the DPPH method.

Sl. No.	Name of sample (Aqueous extract)	Dilutions of sample used (mg/mL)	%RSA
1.	AS-NL	0.25	$10.82 \pm 0.88^{++}$
2.		0.5	$11.27 \pm 0.71^{++}$
3.		0.75	$12.52 \pm 0.38^{++}$
4.		1	$13.18 \pm 0.52^{++}$
5.	AS-GL	0.25	$40.62 \pm 0.28^{++**}$
6.		0.5	$41.60 \pm 0.33^{++**}$
7.		0.75	$42.54 \pm 0.19^{++**}$
8.		1	$42.82 \pm 0.37^{++**}$
9.	AS-GA	0.25	$41.07 \pm 0.44^{++**}$
10.		0.5	$41.87 \pm 0.11^{++**}$
11.		0.75	$43.02 \pm 0.07^{++**}$
12.		1	$43.75 \pm 0.34^{++**}$
13.	FR-NL	0.25	$17.61 \pm 0.45^{++}$
14.		0.5	$18.12 \pm 0.10^{++}$
15.		0.75	$18.99 \pm 0.25^{++}$
16.		1	$19.54 \pm 0.10^{++}$
17.	FR-GL	0.25	$8.91 \pm 1.20^{++*}$
18.		0.5	$11.39 \pm 0.31^{++*}$
19.		0.75	$12.01 \pm 0.40^{++*}$
20.		1	$13.03 \pm 0.30^{++*}$

21.	FR-GA	0.25	$18.45 \pm 0.23^{++*}$
22.		0.5	$23.25 \pm 0.41^{++*}$
23.		0.75	$26.64 \pm 0.42^{++*}$
24.		1	$30.03 \pm 0.42^{++*}$
25.	Ascorbic acid	0.025	47.70 ± 0.03
26.		0.05	47.84 ± 0.06
27.		0.075	47.95 ± 0.07
28.		0.1	48.21 ± 0.06

Each value of %RSA represents mean ± standard deviation (n = 3). ++ indicates values significantly different from ascorbic acid (control) at the corresponding concentration ($p < 0.001$). * indicates values significantly different from normal leaves of each plant at the corresponding concentration ($p < 0.05$). ** indicates values significantly different from the normal leaf of each plant at the corresponding concentration ($p < 0.001$).

DPPH free radical scavenging activity of hexane extracts

The results of antioxidant activity of different samples of *A. scholaris* and *F. racemosa* hexane extracts determined using DPPH assay is shown in Table 4.6. Plate 4.7: Figure B represents the %RSA of hexane extracts, highlighting superior activity of FR-GA. Hexane extracts of *F. racemosa* generally outperformed *A. scholaris*, with FR-GA at 1 mg/mL showing the highest %RSA (37.18%), followed by FR-GL (35.13%) and FR-NL (29.54%). For *A. scholaris*, a sequential increase in %RSA was observed from AS-NL (18.38%) to AS-GL (21.34%) to AS-GA (25.12%) at 1 mg/mL, indicating higher activity in gall tissues and the differences were significant at $p < 0.05$. The lower %RSA of *A. scholaris* hexane extracts compared to *F. racemosa* suggests that the active compounds of *F. racemosa* are more effectively extracted in non-polar solvents.

Table 4.6: The percentage radicle scavenging activity of hexane extracts of different samples of *A. scholaris* and *F. racemosa* analysed through DPPH method.

Sl. No.	Name of sample (Hexane extract)	Dilutions of sample used (mg/mL)	%RSA
1.	AS-NL	0.25	$15.61 \pm 0.28^{++}$
2.		0.5	$16.56 \pm 0.00^{++}$
3.		0.75	$17.3 \pm 0.09^{++}$
4.		1	$18.38 \pm 0.15^{++}$
5.	AS-GL	0.25	$19.23 \pm 0.22^{++}$
6.		0.5	$19.81 \pm 0.20^{++}$
7.		0.75	$20.44 \pm 0.05^{++}$
8.		1	$21.34 \pm 0.20^{++}$
9.	AS-GA	0.25	$20.41 \pm 0.15^{++**}$
10.		0.5	$21.87 \pm 0.19^{++**}$
11.		0.75	$23.23 \pm 0.01^{++**}$
12.		1	$25.12 \pm 0.26^{++**}$
13.	FR-NL	0.25	$27.59 \pm 0.45^{++}$
14.		0.5	$28.43 \pm 0.13^{++}$
15.		0.75	$29.12 \pm 0.49^{++}$
16.		1	$29.54 \pm 0.61^{++}$
17.	FR-GL	0.25	$28.73 \pm 0.40^{++*}$
18.		0.5	$30.8 \pm 0.41^{++*}$
19.		0.75	$32.96 \pm 0.17^{++*}$

20.		1	$35.13 \pm 0.17^{++*}$
21.	FR-GA	0.25	$35.77 \pm 0.29^{++**}$
22.		0.5	$36.26 \pm 0.29^{++**}$
23.		0.75	$36.8 \pm 0.09^{++**}$
24.		1	$37.18 \pm 0.09^{++**}$
25.	Ascorbic acid	0.025	47.70 ± 0.03
26.		0.05	47.84 ± 0.06
27.		0.075	47.95 ± 0.07
28.		0.1	48.21 ± 0.06

Each value of %RSA represents mean ± standard deviation (n = 3). ++ indicates values significantly different from ascorbic acid at the corresponding concentration ($p < 0.001$). * indicates values significantly different from normal leaves at corresponding concentration ($p < 0.05$). ** indicates values significantly different from the normal leaf of each plant at the particular concentration ($p < 0.001$).

DPPH free radical scavenging activity of ethanolic extracts

The results of antioxidant activity of different samples of *A. scholaris* and *F. racemosa* ethanolic extracts shown in Table 4.7. Plate 4.8: Figure A illustrates the %RSA of ethanolic extracts, emphasizing the dominance of gall leaves and galls. Ethanolic extracts exhibited the highest %RSA across both plants, reflecting the efficacy of ethanol in extracting bioactive phenolics. FR-GL and FR-GA at 1 mg/mL showed comparable and high %RSA (43.24% and 44.53%, respectively), significantly outperforming FR-NL (33.99%) with p-value < 0.05. Similarly, AS-GL and AS-GA at 1 mg/mL achieved 44.9% and 46.68% RSA%, respectively, surpassing AS-NL (31.04%). The near-equivalent %RSA of *F. racemosa* and *A. scholaris* ethanolic extracts from gall tissues suggests presence of potent antioxidants.

Table 4.7: The percentage radicle scavenging activity of ethanolic extracts of different samples of *A. scholaris* and *F. racemosa* analysed through DPPH method.

Sl. No.	Name of sample (Ethanol)	Dilutions of sample used (mg/mL)	%RSA
1.	AS-NL	0.25	$26.56 \pm 0.39^{++}$
2.		0.5	$27.96 \pm 0.02^{++}$
3.		0.75	$30.56 \pm 0.06^{++}$
4.		1	$31.04 \pm 0.17^{++}$
5.	AS-GL	0.25	$41.74 \pm 0.14^{+**}$
6.		0.5	$42.47 \pm 0.22^{+**}$
7.		0.75	$43.92 \pm 0.39^{+**}$
8.		1	$44.91 \pm 0.18^{+**}$
9.	AS-GA	0.25	$42.07 \pm 0.15^{**}$
10.		0.5	$44.86 \pm 0.26^{**}$
11.		0.75	$45.67 \pm 0.03^{**}$
12.		1	$46.68 \pm 0.21^{**}$
13.	FR-NL	0.25	$31.03 \pm 0.62^{++}$
14.		0.5	$33.21 \pm 0.33^{++}$
15.		0.75	$33.61 \pm 0.24^{++}$
16.		1	$33.99 \pm 0.36^{++}$
17.	FR-GL	0.25	$31.50 \pm 0.69^{+*}$
18.		0.5	$36.91 \pm 0.61^{+*}$
19.		0.75	$41.78 \pm 0.37^{+*}$
20.		1	$43.24 \pm 0.15^{+*}$
21.	FR-GA	0.25	$35.85 \pm 0.17^{+*}$
22.		0.5	$37.70 \pm 066^{+*}$

23.		0.75	$41.52 \pm 0.60^{+*}$
24.		1	$44.53 \pm 0.30^{+*}$
25.	Ascorbic acid	0.025	47.70 ± 0.03
26.		0.05	47.84 ± 0.06
27.		0.075	47.95 ± 0.07
28.		0.1	48.21 ± 0.06

Each value of %RSA represents mean ± standard deviation (n = 3). + indicates values significantly different from ascorbic acid (standard) at the corresponding concentration ($p < 0.05$). ++ indicates values significantly different from ascorbic acid at the particular concentration ($p < 0.001$). * indicates values significantly different from normal leaves of the particular plant at the corresponding concentration ($p < 0.05$). ** indicates values significantly different from the normal leaf extract of each plant at the corresponding concentration ($p < 0.001$).

Evaluation of antioxidant potency based on IC_{50} values

To quantify antioxidant potency, IC_{50} values were calculated (Table 4.8, Plate 4.8: Figure B). Ethanolic extracts of FR-GL exhibited the lowest IC_{50} (1.35 ± 0.02 mg/mL), followed by FR-GA (1.47 ± 0.05 mg/mL) and AS-GA (1.51 ± 0.03 mg/mL), indicating high potency. AS-GL ethanolic extracts had an IC_{50} of 2.18 ± 0.23 mg/mL. Aqueous extracts of FR-NL showed the highest IC_{50} (12.70 ± 2.49 mg/mL), reflecting low potency, followed by AS-NL aqueous extracts (12.22 ± 1.75 mg/mL) and FR-NL hexane extracts (10.34 ± 5.64 mg/mL). Ascorbic acid, the positive control, had an IC_{50} of 0.38 ± 0.03 mg/mL. These results confirm that ethanolic extracts of gall associated tissues have the highest antioxidant capability.

ANOVA confirmed significant differences in %RSA among normal leaf extract to gall leaf and gall extracts of *A. scholaris* and *F. racemosa*. Tukey's HSD tests revealed that all the extracts of *F. racemosa* significantly more potent than *A. scholaris* ($p < 0.05$). In the case of *F. racemosa,* except FR-GA hexane extract ($p < 0.001$); all other extracts showed p <

0.05 when compared with FR-NL. When the ascorbic acid (standard) at the corresponding concentration tested for significance against different samples and found that except ethanolic extracts of AS-GA, all other samples and extracts exhibited $p < 0.05$.

Table 4.8: IC_{50} values of normal leaves, gall leaves and leaf galls of *A. scholaris* and *F. racemosa* extracts determined using DPPH method.

Sl. No.	Plant sample	IC_{50} (mg/mL)
1.	AS-NL (Aqueous)	12.22 ± 1.75
2.	AS-GL (Aqueous)	3.31 ± 0.03
3.	AS-GA (Aqueous)	2.69 ± 0.05
4.	AS-NL (Hexane)	9.86 ± 1.2
5.	AS-GL (Hexane)	11.57 ± 1.37
6.	AS-GA (Hexane)	5.05 ± 0.40
7.	AS-NL (Ethanol)	3.91 ± 0.35
8.	AS-GL (Ethanol)	2.18 ± 0.23
9.	AS-GA (Ethanol)	1.51 ± 0.03
10.	FR-NL (Aqueous)	12.70 ± 1.49
11.	FR-GL (Aqueous)	8.23 ± 1.42
12.	FR-GA (Aqueous)	2.66 ± 0.03
13.	FR-NL (Hexane)	10.34 ± 1.64
14.	FR-GL (Hexane)	2.22 ± 0.10
15.	FR-GA (Hexane)	8.04 ± 1.33
16.	FR-NL (Ethanol)	5.23 ± 0.34
17.	FR-GL (Ethanol)	1.35 ± 0.02
18.	FR-GA (Ethanol)	1.47 ± 0.05
19.	Ascorbic Acid	0.38 ± 0.03

Each value represents mean ± standard deviation (n = 3)

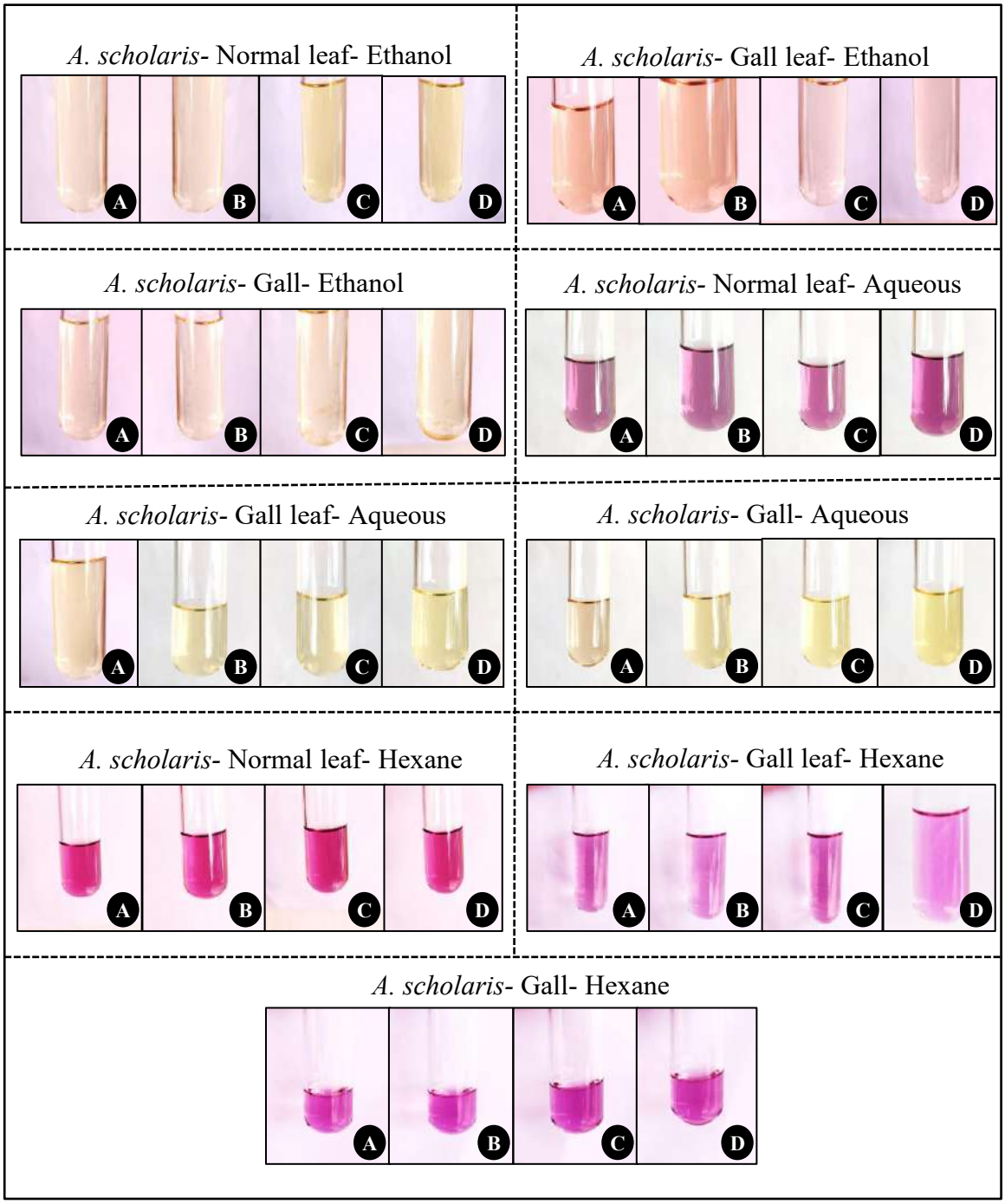


Plate 4.5: Photographs showing the results of DPPH radical scavenging activity of ethanol, aqueous and hexane extracts of *A. scholaris* -normal leaf, gall leaf and gall in different dilutions of extracts (A: 2.5 µL, B: 5 µL, C: 7.5 µL and D: 10 µL).

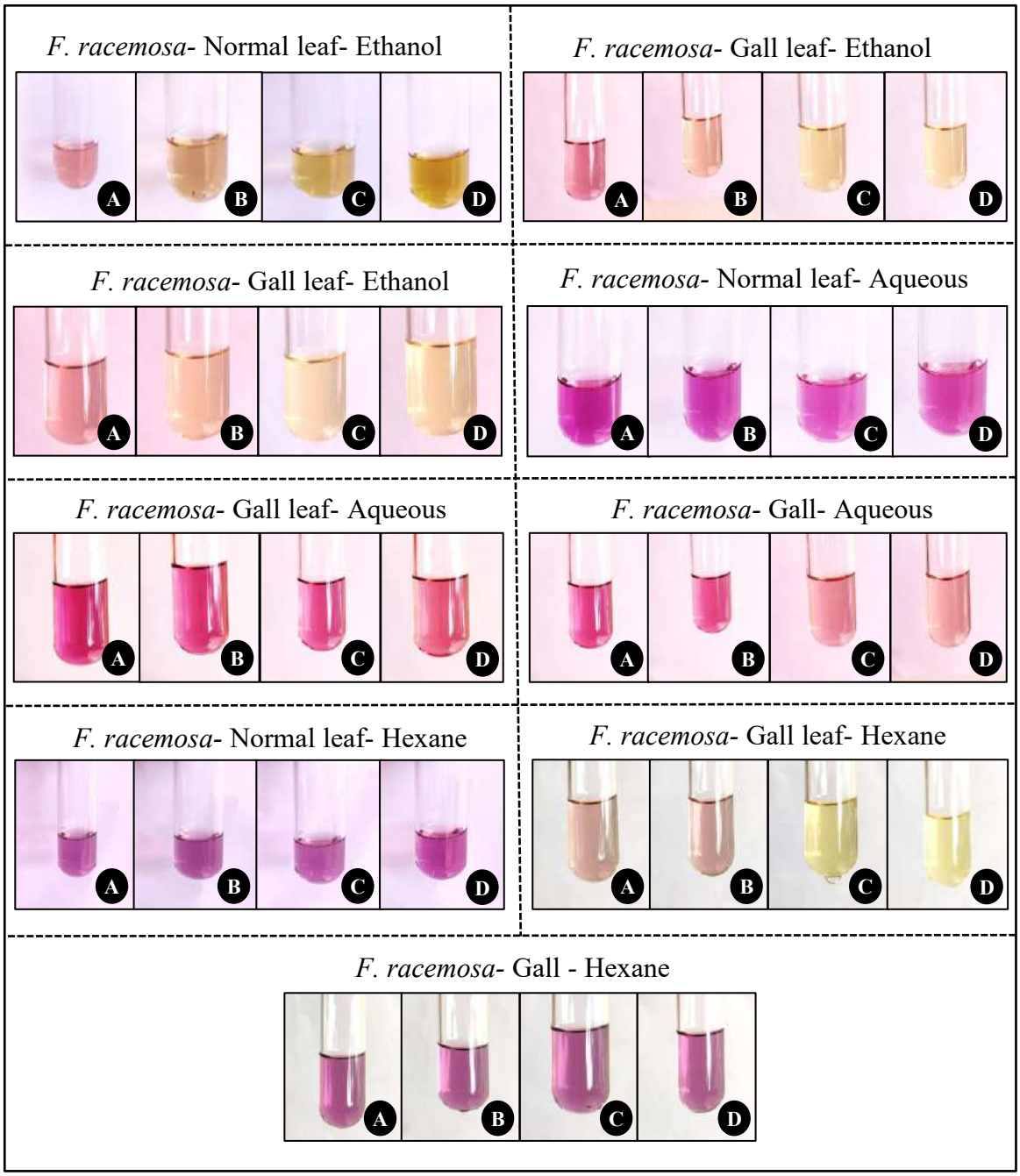
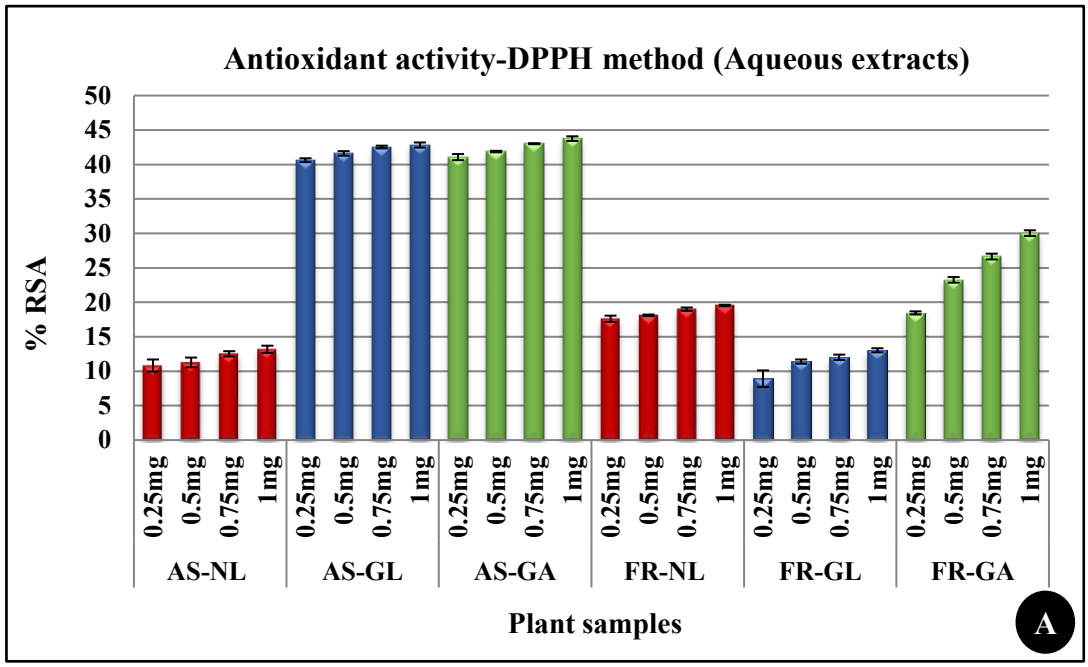


Plate 4.6: Photographs showing the results of DPPH radical scavenging activity of ethanol, aqueous and hexane extracts of *F. racemosa*- normal leaf, gall leaf and gall in different dilutions of extracts (A: 2.5 µL, B: 5 µL, C: 7.5 µL and D: 10 µL).



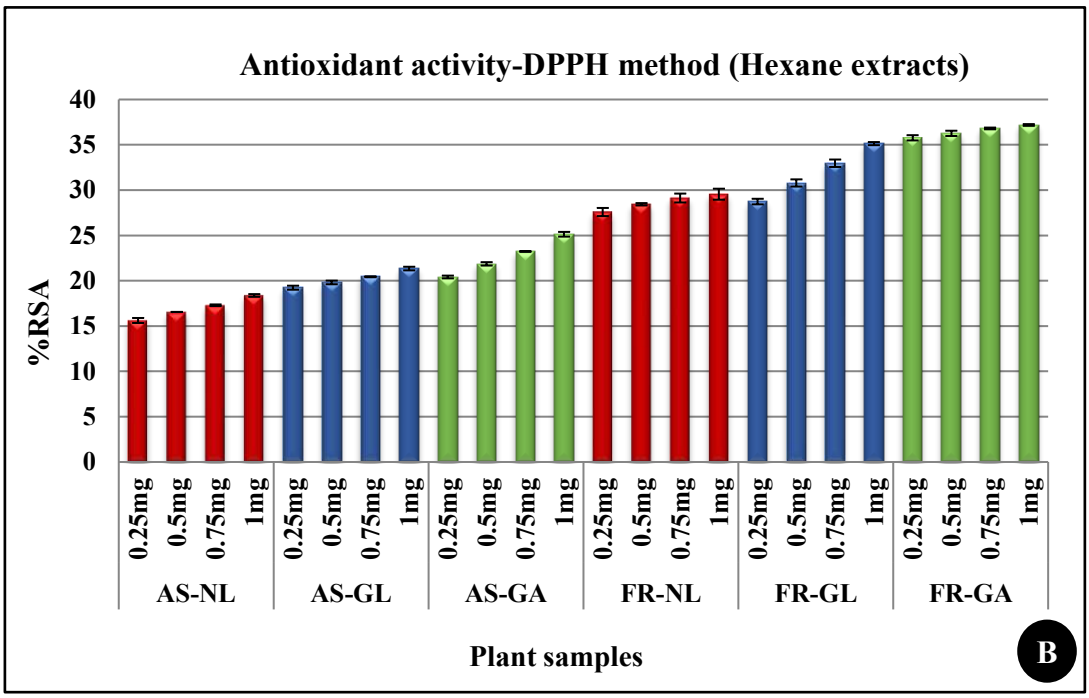
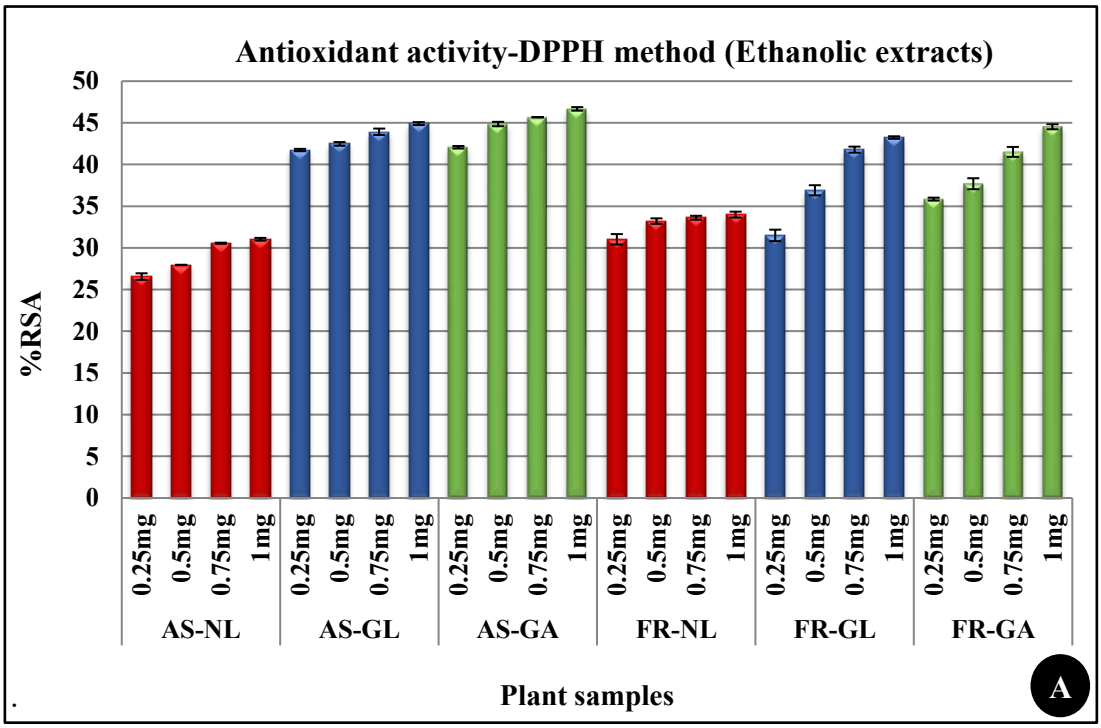


Plate 4.7: Figure A: Graphical representation of results of DPPH antioxidant activity analysis of aqueous extracts of AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA. Each bar represents the mean % RSA of a sample with standard deviation and Figure B: Graphical representation of results of antioxidant activity of hexane extracts by DPPH method. Each bar represents the mean % RSA of a sample with standard deviation.



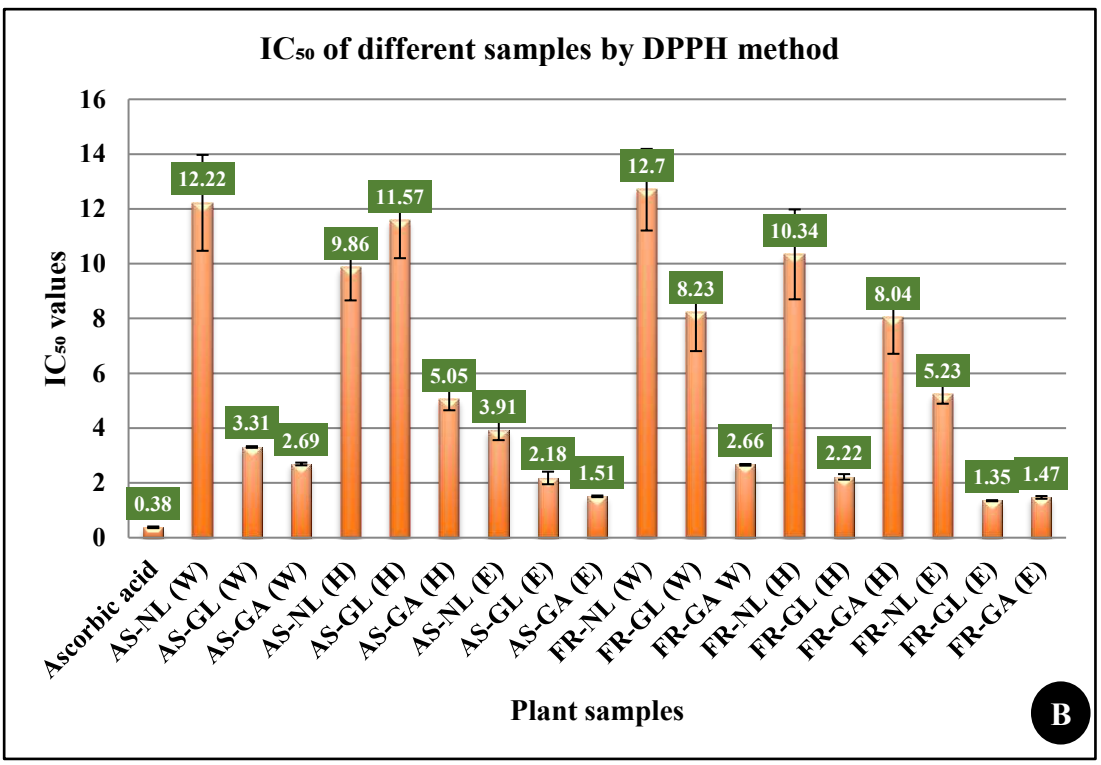


Plate 4.8: Figure A: Graphical representation of antioxidant activity of ethanolic extracts of AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA. Each bar represents the mean %RSA of a sample with standard deviation and Figure B: IC_{50} values of different samples of *A. scholaris* and *F. racemosa* determined using DPPH radical scavenging assay.

Ethanolic extracts of AS-GA exhibited the highest antioxidant activity through this study, with an IC_{50} of 1.95 ± 0.65 mg/mL, followed by AS-GL (2.04 ± 0.43 mg/mL) and AS-NL (3.64 ± 0.74 mg/mL). Ganjewala and Gupta reported that methanol extracts of *A. scholaris* leaves, follicles and latex scavenged DPPH free radicals, with leaf extracts showing 70-80% activity (Ganjewala and Gupta, 2013). Bopche and colleagues (2023) found a concentration-dependent antioxidant activity in *A. scholaris* methanolic leaf extracts, with an IC_{50} of 77.67 µg/mL compared to 26.72 µg/mL for ascorbic acid (Bopche et al., 2023). Antony and colleagues in 2011 reported an IC_{50} of 2.83 mg/mL for *A. scholaris* aqueous leaf extracts, higher than our ethanolic gall extracts, indicating greater potency (Antony et al., 2011). Shanmugapriya and Jayanthi found IC_{50} values of 60.9 µg/mL for *A. scholaris* methanolic crude extracts and 41.64 µg/mL for purified samples (Shanmugapriya and Jayanthi, 2019). Arulmozhi and colleagues (2010) noted that dichloromethane and ethyl acetate fractions of *A. scholaris* ethanolic leaf extracts were effective DPPH scavengers (Arulmozhi et al., 2010). In this study, the reducing power (RP) method confirmed that ethanolic extracts had the highest inhibitory action, with galls showing the greatest activity.

Previous studies compared the antioxidant properties of *F. racemosa* bark and leaves. At a concentration of 200 µg/mL, *F. racemosa* leaves had a scavenging activity of 48.55 ± 2.670%, while bark had a scavenging activity of 73.46 ± 1.455% (Sultana et al., 2014). Since FR-NL, FR-GL and FR-GA have different phytochemical profiles, the antioxidant qualities of these samples were examined in the current study. Previous research revealed that an ethanolic extract of *F. racemosa* leaves produced a considerable antioxidant capability. It was revealed using a number of techniques, including H_2O_2 scavenging activity assay, FRAP assay etc. (Yadav et al., 2019). In this work, the RP method and DPPH assay were performed on aqueous, hexane and ethanolic extracts of different *F. racemosa* samples and it was discovered that the ethanolic extracts had the highest levels of antioxidant activity.

Sumi and colleagues (2016) reported an IC_{50} of 10.29 µg/mL for *F. racemosa* methanolic leaf extracts by DPPH assay and in case of reducing power assay IC_{50} of 45.564 µg/mL (Sumi et al., 2016). According to the RP assay, the reducing capacity of various samples varied with dose and the gall ethanolic extracts had the highest antioxidant activity when compared to other extracts. Based on a study by Bagyalakshmi and colleagues, toluene

extracts of *F. racemosa* leaves had the strongest antioxidant activity, with an IC_{50} of 2.35 ± 0.41 µg/mL and an inhibition percentage of 88.5%. Nearly the same percentages of inhibition were displayed by the ethanol and ethyl acetate extracts (54% and 56%, respectively) (Bagyalakshmi et al., 2019). Londhe and colleagues reported an IC_{50} of 47.96 µg/mL for *F. racemosa* ethanolic leaf extracts, with increasing reducing power at higher concentrations (Londhe et al., 2012). Through DPPH assay, the present work discovered that the FR-GL ethanolic extracts possessed significant antioxidant activity with an IC_{50} value of 1.32 ± 0.11 mg/mL and FR-GA extracts displayed an IC_{50} of 1.38 ± 0.18 mg/mL. Since ascorbic acid had an IC_{50} of 0.38 ± 0.09 mg/mL, it may be concluded that FR-GA and FR-GL showed substantial activity when compared to ascorbic acid.

4.3.2. Results of antibacterial activity analysis

The antibacterial activity of crude aqueous, hexane and ethanolic extracts prepared from normal leaves, gall leaves and galls of *A. scholaris* and *F. racemosa* were evaluated against four pathogenic bacteria: *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*. Preliminary screening tested three dilutions (10 µL, 20 µl and 30 µL) from a 200 mg/mL stock concentration. Aqueous and hexane extracts of all samples from both plants showed no activity against any of the four bacteria at these dilutions. Ethanolic extracts, however, exhibited activity against *S. aureus*, *B. subtilis* and *E. coli*, but not against *P. aeruginosa*, across various dilutions. Consequently, further analysis focused on ethanolic extracts at four dilutions: 10 µL (2000 µg), 20 µL (4000 µg), 30 µL (6000 µg) and 40 µL (8000 µg). Streptomycin (10 µg/disc) served as the positive control. Plate 4.9 shows the antibacterial activity of controls (streptomycin- positive control and ethanol-negative control) against *B. subtilis* (Figure A), *E. coli* (Figure B), *P. aeruginosa* (Figure C) and *S. aureus* (Figure D).

Antibacterial activity of *A. scholaris*

Antibacterial analysis performed against ethanolic extracts of *A. scholaris* is shown in Plate 4.10: Figure A shows the zone of inhibition produced by AS-NL, AS-GL and AS-GA against *B. subtilis*, Figure B, is the image showing the zone of inhibition produced by AS-NL, AS-GL and AS-GA against *E.coli* and Figure C represents the antibacterial activity of

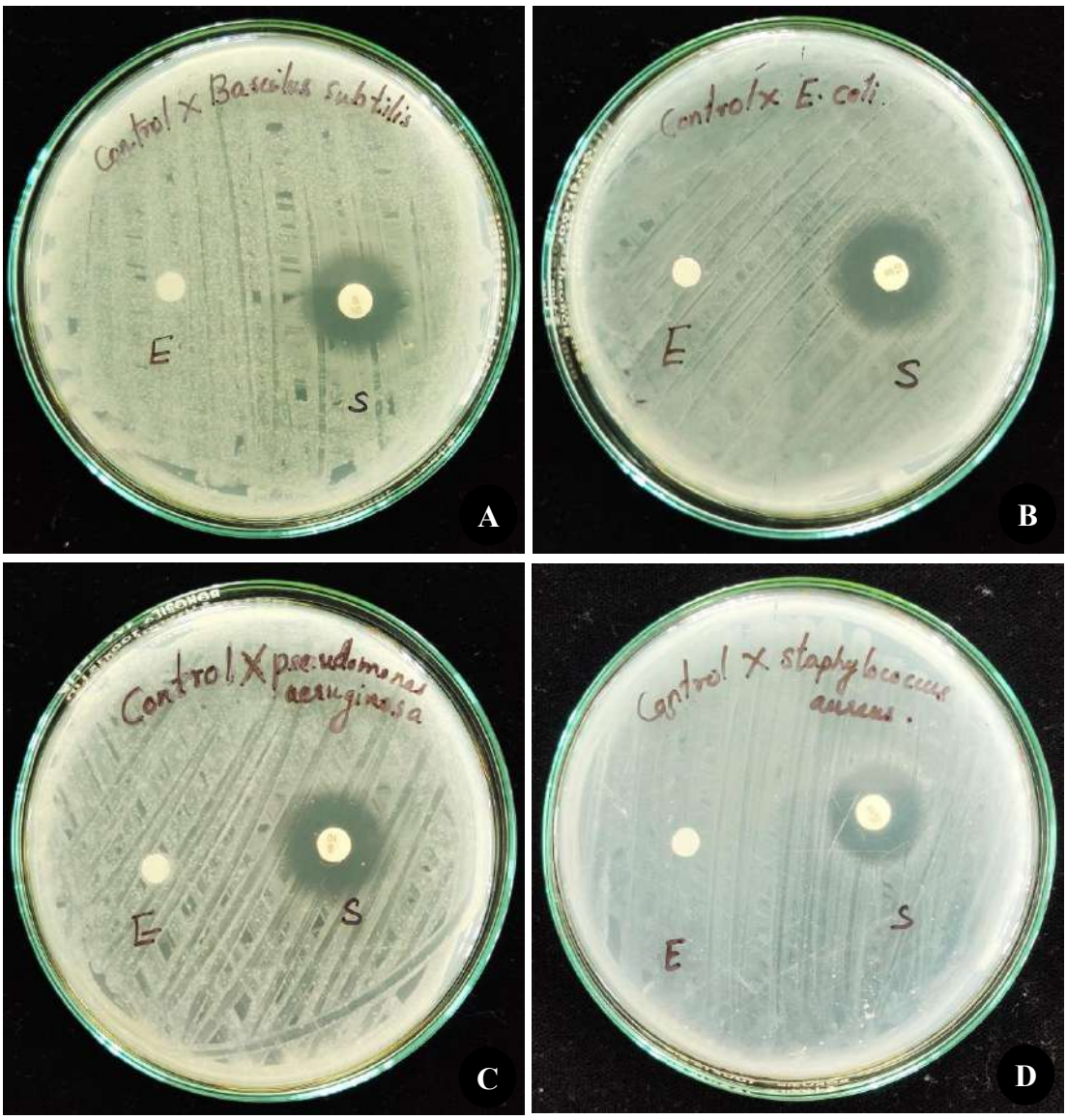


Plate 4.9: Antibacterial analysis of controls. The activity of ethanol (negative control) and streptomycin (positive control) was tested against four tested bacterial strains. Figure A: *Bacillus subtilis*, Figure B: *Escherichia coli*, Figure C: *Pseudomonas aeruginosa* and Figure D: *Staphylococcus aureus*. The figures demonstrate streptomycin exhibiting standard antibacterial activity, while ethanol shows no inhibition of bacterial growth.

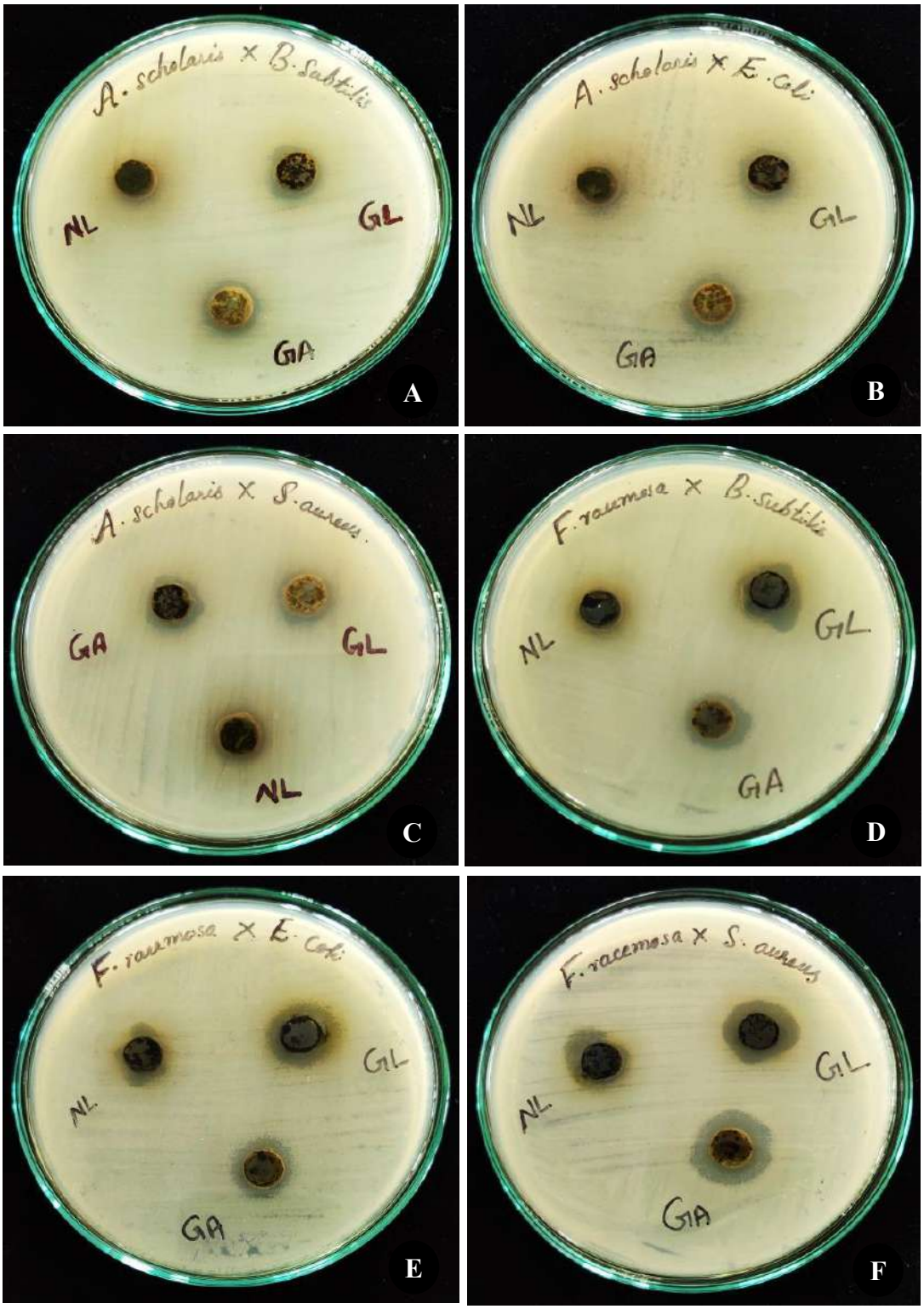


Plate 4.10: Antibacterial activity of ethanolic extracts of *A. scholaris* and *F. racemosa*. Figure A-C: Zones of inhibition produced by AS-NL, AS-GL and AS-GA against *B. subtilis, E. coli* and *S. aureus*, respectively and Figure D-F: Zones of inhibition of FR-NL, FR-GL and FR-GA against *B. subtilis, E. coli* and *S. aureus,* respectively.

AS-NL, AS-GL and AS-GA against *S. aureus*. The results of antibacterial activity analysis against *E. coli*, *B. subtilis* and *S.aureus* with the average of inhibition zone (mm) produced with standard deviation (SD) is represented in Table 4.9. The antibacterial activity of an antibacterial drug (streptomycin) was shown in Table 4.11.

Ethanolic extracts of AS-NL, AS-GL and AS-GA showed varying antibacterial activity against *S. aureus*, *B. subtilis* and *E. coli*. AS-NL at 10 µL (2000 µg) exhibited no activity against any bacteria, while at 20 µL and above, it was active against *E. coli* (e.g., 8.66 ± 0.7 mm at 20 µL) and *B. subtilis* (e.g., 12.25 ± 0.25 mm at 40 µL), but not *S. aureus*. AS-GL at 10 µL showed activity only against *B. subtilis* (10 ± 0.50 mm), but displayed concentration-dependent activity against *B. subtilis* across all dilutions, peaking at 40 µL (13.5 ± 1.0 mm). AS-GL also showed activity against *E. coli* at 30 µL (10.25 ±0.25 mm) and 40 µL (12.66 ± 0.29 mm) and against *S. aureus* at 40 µL (10.5 ± 0.5 mm). AS-GA was inactive at 10 µL but showed activity against *E. coli* and *B. subtilis* at 20 µL and above, with the highest inhibition zone against *E. coli* at 40 µL (14.33 ± 0.29 mm). AS-GA also exhibited activity against *S. aureus* at 40 µL (14.16 ± 0.58 mm). Overall, AS-GA at 40 µL demonstrated the highest activity among *A. scholaris* extracts, particularly against *E. coli*.

Table 4.9: Antibacterial activity of AS-NL, AS-GL and AS-GA with the average zone of inhibition (mm) produced with SD calculated in 4 different dilutions (10µL, 20µL, 30µL and 40µL).

Sample	Bacteria	10 µL (mm)	20 µL (mm)	30 µL (mm)	40 µL (mm)
AS-NL	*E. coli*	-	$8.66 \pm 0.7^{++}$	$9.33 \pm 0.29^{++}$	$10.5 \pm 0.50^{++}$
	B. subtilis	-	$10 \pm 0.5^{++}$	$11.25 \pm 0.25^{++}$	$12.25 \pm 0.25^{++}$
	S.aureus	-	-	-	-
AS-GL	*E. coli*	-	-	$10.25 \pm 0.25^{++*}$	$12.66 \pm 0.29^{++**}$

	B. subtilis	$10 \pm 0.5^{++**}$	$11 \pm 0.5^{++}$	$12 \pm 0.5^{++}$	$13.5 \pm 1.0^{++}$
	S.aureus	-	-	-	$10.5 \pm 0.5^{++**}$
AS-GA	*E. coli*	-	$9 \pm 0.1^{++*}$	$11.16 \pm 0.29^{++**}$	$14.33 \pm 0.29^{++**}$
	B. subtilis	-	$10.08 \pm 0.52^{++}$	$12.33 \pm 1.26^{++}$	$13.5 \pm 0.50^{++}$
	S.aureus	-	-	-	$14.16 \pm 0.58^{++**}$

Values represent mean ± standard deviation (n = 3). ++ indicates values significantly different from Streptomycin (standard antibacterial drug) at the level of $p < 0.001$. * indicates values significantly different from the normal leaf extract of *A. scholaris* at the corresponding concentration at $p < 0.05$. ** indicates values significantly different ($p < 0.001$) from the normal leaf of *A. scholaris* at the corresponding concentration.

Antibacterial activity of *F. racemosa*

Antibacterial analysis performed against ethanolic extracts of *F. racemosa* (Plate 4.10: Figure D) produced zones of inhibition against *B. subtilis*. Plate 4.10: Figure E represents the photograph showing the zone of inhibition produced by FR-NL, FR-GL and FR-GA against *E. coli* and Figure F shows the activity of FR-NL, FR-GL and FR-GA against *S. aureus.* The results of antibacterial activity analysis of *F. racemosa* extracts against *E. coli*, *B. subtilis* and *S. aureus* with the average of inhibition zone (mm) produced with standard deviation (SD) is represented in Table 4.10. The antibacterial activity of an antibacterial drug, the control (streptomycin) was shown in Table 4.11. The results of all extracts and samples of both plants were represented as heat map in Plate 4.11.

Ethanolic extracts of FR-NL, FR-GL and FR-GA exhibited concentration-dependent antibacterial activity against *S. aureus*, *B. subtilis* and *E. coli*. FR-NL showed the lowest activity, with no inhibition at 10 µL, activity against *B. subtilis* at 20 µL (9.1 ± 0.1 mm) and 30 µL (10.25 ± 0.25 mm) and activity against both *B. subtilis* (11 ± 1.0 mm) and *S. aureus* (11.5 ± 0.50 mm) at 40 µL. FR-GL was highly active, showing activity against *E. coli* (9.25 ± 0.25 mm) and *S. aureus* (11.5 ± 0.87 mm) at 10 µL and against all three bacteria at higher dilutions. At 40 µL, FR-GL produced the highest inhibition zones: 17.25 ± 0.25 mm (*E. coli*),

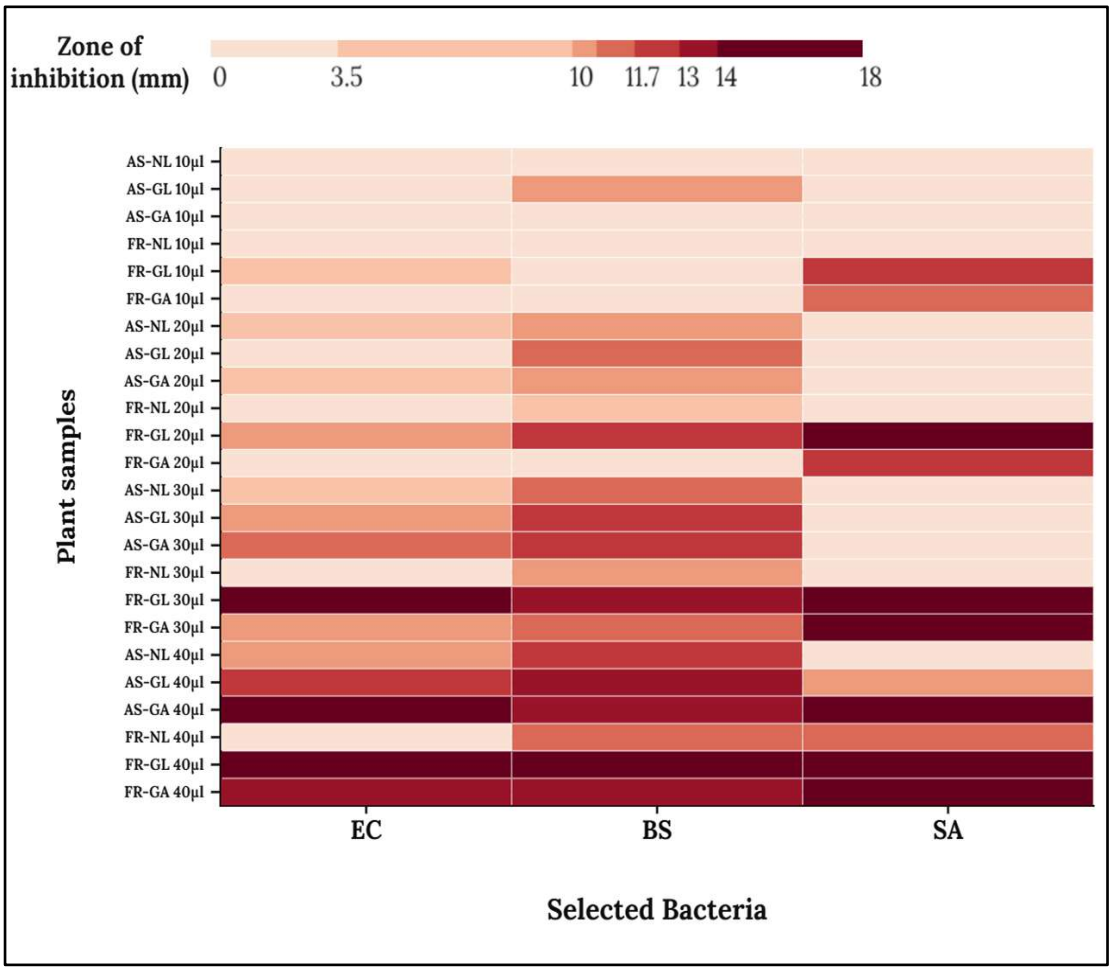


Plate 4.11: Heat maps showing results of antibacterial analysis: The results of antibacterial analysis of AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA ethanolic extracts in 4 different dilutions with the zone of inhibition (mm) produced. Light colours indicates no activity and the increase of darkness of colours indicates increase in inhibition zone against *Staphylococcus aureus* (SA), *Bacillus subtilis* (BS) and *Escherichia coli* (EC).

15.66 ± 0.29 mm (*B. subtilis*) and 17.16 ± 0.58 mm (*S. aureus*). FR-GA showed activity against *S. aureus* at 10 µL (10.83 ± 0.29 mm), but required higher concentrations for *E. coli* and *B. subtilis* (e.g., 10.25 ± 1.25 mm for *E. coli* at 30 µL). At 40 µL, FR-GA produced inhibition zones of 17.16 ± 0.14 mm (*S. aureus*), 13.25 ± 0.25 mm (*E. coli*) and 13.83 ± 1.26 mm (*B. subtilis*), which were lower than FR-GL zones for *E. coli* and *B. subtilis*. FR-GL at 40 µL exhibited the highest antibacterial activity among all samples and dilutions.

Table 4.10: Antibacterial activity of FR-NL, FR-GL and FR-GA with the average zone of inhibition (mm) produced with SD calculated in 4 different dilutions (10 µL, 20 µL, 30 µL and 40 µL).

Sample	Bacteria	10 µL (mm)	20 µL (mm)	30 µL (mm)	40 µL (mm)
FR-NL	*E. coli*	-	-	-	-
	B. subtilis	-	$9.1 \pm 0.1^{++}$	$10.25 \pm 0.25^{++}$	$11 \pm 1.0^{++}$
	S. aureus	-	-	-	$11.5 \pm 0.50^{++}$
FR-GL	*E. coli*	$9.25 \pm 0.25^{++**}$	$10.5 \pm 0.5^{++**}$	$14 \pm 0.5^{++**}$	$17.25 \pm 0.25^{++**}$
	B. subtilis	-	$12.67 \pm 0.29^{++**}$	$13 \pm 0.5^{++**}$	$15.66 \pm 0.29^{++*}$
	S. aureus	$11.5 \pm 0.87^{++**}$	$14 \pm 1.0^{++**}$	$15.83 \pm 0.29^{++**}$	$17.16 \pm 0.58^{++**}$
FR-GA	*E. coli*	-	-	$10.25 \pm 1.25^{++**}$	$13.25 \pm 0.25^{++**}$
	B. subtilis	-	-	$10.66 \pm 0.29^{++}$	$13.83 \pm 1.26^{++*}$
	S. aureus	$10.83 \pm 0.3^{++**}$	$12.33 \pm 0.29^{++**}$	$16.33 \pm 0.29^{++**}$	$17.16 \pm 0.14^{++**}$

Values represent mean ± standard deviation (n = 3). ++ indicates values significantly different from Streptomycin (standard) at the level of $p < 0.001$. * indicates values significantly different from the normal leaf extract of *F. racemosa* at the corresponding concentration at $p < 0.05$. ** indicates values significantly different ($p < 0.001$) from the normal leaves of *F. racemosa* at the corresponding concentration.

Table 4.11: The average zone of inhibition produced by streptomycin (+ve control) against all tested organisms with SD.

Control	Concentration	Bacteria	Average inhibition zone (mm)
Streptomycin disc	10µg	*E. coli*	21 ± 1.00
	10µg	*B. subtilis*	22.25 ± 0.75
	10µg	*S. aureus*	24.16± 0.29

ANOVA was done for finding the statistical significance of the obtained results and confirmed significant differences in inhibition zones among extracts and dilutions ($p < 0.05$). Tukey's HSD tests for comparing both plants it is found that, FR-GL at 40 µL (8000 µg) consistently produced the largest inhibition zones against *S. aureus* (17.16 ± 0.58 mm), *E. coli* (17.25 ± 0.25 mm) and *B. subtilis* (15.66 ± 0.29 mm), outperforming *A. scholaris* extracts ($p < 0.05$). AS-GA at 40 µL was the most active among extracts of *A. scholaris*, particularly against *E. coli* (14.33 ± 0.29 mm). Tukey's HSD tests indicated that FR-GL ($p < 0.05$) and FR-GA ($p < 0.05$) at 40 µL were significantly more potent than other samples. Streptomycin (positive control), produced inhibition zones of 21 ± 1.00 mm (*E. coli*), 22.25 ± 0.75 mm (*B. subtilis*) and 24.16 ± 0.29 mm (*S. aureus*), serving as a benchmark.

The current investigation found that only ethanolic extracts of *A. scholaris* effective against *S. aureus, B. subtilis* and *E. coli*, but not *P. aeruginosa*. In this study, AS-GA ethanolic extracts produced inhibition zones of 14.33 ± 0.29 mm against *E. coli* and 14.16 ± 0.58 mm against *S. aureus,* while AS-NL showed no activity against *S. aureus* at any tested dose. Khyade and colleagues (2009) reported that methanol extracts *A. scholaris* of leaves exhibited broad-spectrum antibacterial activity against Gram-positive and Gram-negative bacteria, while petroleum ether extracts showed no inhibition, aligning with our findings for ethanolic extracts (Khyade et al., 2009). Khan and colleagues (2003) evaluated *A. scholaris* crude methanolic extracts and fractions (petrol, dichloromethane, ethyl acetate and butanol) and found that butanol fractions were highly active against bacteria, but no fractions inhibited the tested fungus (Khan et al., 2003). Misra and colleagues noted that methanol and

isopropanol extracts of *A. scholaris* leaves were active against *S. aureus*, supporting the efficacy of polar solvents (Misra et al., 2011).

Bonvicini and colleagues found that ethanolic extracts of *A. scholaris* exhibited antimicrobial activity against reference strains and clinical isolates, including methicillin-resistant *S. aureus* (Bonvicini et al., 2014). Bagheri and colleagues (2020) reported that methanol and isopropanol extracts of *A. scholaris* leaves showed stronger activity against Gram-positive bacteria than Gram-negative ones (Bagheri et al., 2020). From the present study it was clear that different extracts of *A. scholaris* were active against both Gram-positive and Gram-negative bacteria. Labaran and colleagues (2024) observed large inhibition zones against *S. aureus* and *E. coli* with *A. scholaris* leaf extracts, noting greater susceptibility of *S. aureus* (Labaran et al., 2024). The crude ethanolic extracts of *A. scholaris* leaves produced inhibition zones of 14 mm against *S. aureus* and 13 mm against *E. coli*, with petroleum ether showing no activity (Ara et al., 2020). Mushtaq and colleagues in 2023 reported that *S. aureus* growth was suppressed with increasing concentrations of *A. scholaris* leaf extracts (Mushtaq et al., 2023), a trend observed in our study as antibacterial activity increased from 10 µL to 40 µL.

In the preliminary screening, the petroleum ether extract of *F. racemosa* leaves was discovered to be more active than the others against *S. aureus*, *B. subtilis*, *P. aeruginosa* and *E. coli* in earlier investigations. As a result, it was subjected to further examination with different concentrations. The concentration of 350 mg/mL was shown to be the most efficient against *S. aureus* (Mandal et al., 2000b). Similar to that, in order to identify the best solvent, three extracts were first screened in this study. Ethanolic extracts were found to be the most efficient against the tested organisms based on that assessment. Further analysis was conducted using various ethanolic extract dilutions. Thilagavathi and Kathiravan (2017) reported minimal antibacterial activity of *F. racemosa* ethanolic leaf extracts, with moderate efficacy against *S. aureus* and *E. coli* but no activity against *P. aeruginosa*, consistent with our findings (Thilagavathi and Kathiravan, 2017). The present investigation also came to the same conclusion that there was no activity against *P. aeruginosa.*

Kumari and colleagues evaluated the antibacterial efficacy of *F. racemosa* leaf and gall extracts in 2020. The Dichloromethane fraction of crude extract inhibited *E. coli* and *P. aeruginosa* with zone of inhibition of 19.71 ± 0.31 mm and 19.75 ± 0.11 mm, respectively. The crude extract of *F. racemosa* leaf and galls with ethyl acetate showed the highest inhibition with 19.50 ± 0.19 mm zone. Hexane fraction of crude extract produced a zone of inhibition of 18.50 ± 0.23 against *S. aureus* (Kumari et al., 2020). A study conducted by Hosain and colleagues revealed that methanolic extract of *F. racemosa* leaves has poor activity against Gram-positive and Gram-negative bacteria. The extracts produced a zone of inhibition of 8 mm for *S. aureus* and 9 mm for *B. subtilis* (Hosain et al., 2011). The current investigation came to the same conclusion that normal leaves of *F. racemosa* were less effective at inhibiting bacterial growth. At low concentrations, ethanolic extracts of FR-NL did not suppress bacterial growth, however at higher concentrations; they formed 11 mm and 11.5 mm zones of inhibition against *B. subtilis* and *S. aureus*. There is no effect found on *E. coli* proliferation.

Bagyalakshmi and colleagues (2019) found that toluene extracts of *F. racemosa* leaves inhibited *Staphylococcus* spp., while methanolic and aqueous extracts were ineffective against various bacteria (Bagyalakshmi et al., 2019). Mpala and colleagues in 2019 reported no antibacterial efficacy of *F. racemosa* methanolic and aqueous leaf extracts (Mpala et al., 2019). In contrast, our results showed that ethanolic extracts of FR-GL and FR-GA were highly effective, with FR-NL also exhibiting some activity. Phytochemical analysis revealed high concentrations of phenolic and flavonoid components in *F. racemosa* leaves and galls, likely responsible for the observed bioactivity.

4.3.3. Results of antifungal activity analysis

Antifungal assay of *C. albicans* by agar-well diffusion method:

The antifungal properties of various extracts from *A. scholaris* and *F. racemosa* were evaluated against *C. albicans* using the agar-well diffusion method. A stock solution of 100 mg/mL was used to prepare extracts of normal leaves, gall leaves and galls. For initial screening, hexane, ethanolic and aqueous extracts were tested at 20 µL and 40 µL. Plates showing a zone of inhibition were considered positive, while those without were considered

negative. Itraconazole, an antifungal agent used as positive control for the assay. Table 4.12 shows antifungal activity against *C.albicans* by all the extracts of ethanol in different concentrations with the zone of inhibition in mm and IC_{50} values of the extracts. Plate 4.12: Figure A shows the zone of inhibition of positive control Itraconazole and Figure B represents a photograph of plate showing the activity of negative control. Hexane and aqueous extracts of all samples of *A. scholaris* produced negative results. Plate 4.12: Figure C shows the anticandidal activity of AS-NL, AS-GL and AS-GA ethanolic extracts, Figure D shows the activity of hexane extracts, no activity obtained for AS-NL, AS-GL and AS-GA hexane extracts and Figure E represents the activity of aqueous extracts of different samples of *A. scholaris*. In the case of *A. scholaris* only ethanolic extracts showed antifungal activity. In the case of *F. racemosa* hexane and aqueous extracts have no activity against *C. albicans* (Plate 4.12: Figure G and Figure H respectively). Only ethanolic extracts exhibited anticandidal activity, consequently ethanolic extracts were selected for further analysis at different dilutions prepared from a 150 mg/mL stock solution. Representative photographs showing the antifungal activity of ethanolic extracts of *F. racemosa* were shown in Plate 4.12: Figure F.

The results of the analysis showed that every extract exhibited concentration-dependent effectiveness against *C. albicans*. Highest anticandidal activity was possessed by FR-GL in 60 µL dilution, it showed a zone of inhibition of 13.2 ± 0.14 mm, followed by AS-GA (60 µL) with zone of inhibition of 12.45 ± 0.21 mm. The inhibition zone produced by FR-GA (60 µL) and AS-GL (60 µL) was 9.35 ± 0.35 mm and 9.05 ± 0.35 mm, respectively. AS-NL and FR-NL extracts had the least amount of efficacy against *C. albicans* in a 60 µL dilution. After comparing all of the dilutions, it was discovered that FR-GL exhibited the greatest activity, directly proportional with the extract concentrations. The results of antifungal activity against *C. albicans* were compared with the Itraconazole (60 µL), which showed 24.5 ± 2.8 mm inhibition zone.

FR-GL exhibits comparable antifungal activity at higher concentrations of extracts. IC_{50} values were calculated for all extracts. FR-GL had the lowest IC_{50} (0.534 mL), indicating the highest potency, followed by AS-GA (0.548 mL). FR-NL had the highest IC_{50} (3.051 mL), indicating the lowest potency. The IC_{50} value of Itraconazole (0.178 mL) was significantly lower, reflecting its superior efficacy. Plate 4.13: Figure A shows the graphical representation of the antifungal activity of ethanol extracts of different samples of *A. scholaris* and *F. racemosa* against *C. albicans.*

The statistical analysis confirmed significant differences in anticandidal activity ($p < 0.05$). All the dilutions of all three extracts showed a significant difference from the control, Itraconazole. Tukey's HSD test revealed that FR-GL at 60 µL produced significantly larger inhibition zones than all other extracts ($p < 0.001$), followed by AS-GA. AS-GA at 60 µL was significantly more effective than AS-GL and AS-NL ($p < 0.001$). FR-GA and AS-GL showed similar activity at 60 µL, with significant difference ($p < 0.05$). FR-NL and AS-NL exhibited the lowest activity, significantly lower than AS-GA ($p < 0.001$). These results confirm that FR-GL is the most potent extract, followed closely by AS-GA.

Table 4.12: Results of antifungal activity of ethanolic extracts against *C. albicans* using AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA along with antifungal drug Itraconazole. Zone of inhibition represented in mm with SD. IC_{50} values of all the samples presented in mm.

Sl. No.	Name of sample	Inhibition zone (mm) of 20 µL dilution	Inhibition zone (mm) of 40 µL dilution	Inhibition zone (mm) of 60 µL dilution	IC_{50} (mL)
1	AS-NL	$7.25 \pm 0.07^{++}$	$7.6 \pm 0.42^{++}$	$8.125 \pm 0.25^{++}$	1.973
2	AS-GL	$8.1 \pm 0.28^{++**}$	$8.85 \pm 0.35^{++**}$	$9.05 \pm 0.35^{++**}$	1.784
3	AS-GA	$9.4 \pm 0.14^{++**}$	$11.7 \pm 0.57^{++**}$	$12.45 \pm 0.21^{++**}$	0.548
4	FR-NL	$8.15 \pm 0.07^{++}$	$8.45 \pm 0.21^{++}$	$8.7 \pm 0.28^{++}$	3.051

5	FR-GL	$11.225 \pm 0.11^{++**}$	$12.55 \pm 0.35^{++**}$	$13.2 \pm 0.14^{++**}$	0.534
6	FR-GA	$8.25 \pm 0.35^{++}$	$9.257 \pm 0.11^{++*}$	$9.35 \pm 0.35^{++*}$	1.532
7	Itraconazole	16.01 ± 1.8	21.39 ± 1.06	24.5 ± 2.8	0.178

Values represent mean ± standard deviation (n = 3). ++ indicates values significantly different from Itraconazole (+ve control) at the level of $p < 0.001$. * indicates values significantly different from the normal leaf extract of the particular plant at the corresponding concentration at $p < 0.05$. ** indicates values significantly different ($p < 0.001$) from the normal leaf extract of the corresponding plant at the respective concentration.

Antifungal assay of *A. niger* by food poisoning method

Table 4.13 shows the percentage growth inhibition produced by different ethanolic extracts of *A. scholaris* and *F. racemosa* against *A. niger* along with positive control Itraconazole. Antifungal activity of ethanolic extracts of *A. scholaris* and *F. racemosa* against *A. niger* is shown in Plate 4.14. The Figure A is the photograph showing the growth of hyphae in PDA medium with Itraconazole, Figure B shows the growth of *A. niger* hyphae in medium with ethanol, Figure C is the growth of hyphae in medium with AS-NL extract, Figure D shows the growth of hyphae in medium with AS-GL ethanolic extract, Figure E represents the growth of hyphae in medium with AS-GA extract, here maximum inhibition in growth can be seen with a growth inhibition percentage of 84.89 ± 1.22. Figure F and G shows the growth of hyphae in medium with FR-NL extract and FR-GL extract respectively. Among all the extracts tested the highest inhibition of growth exhibited by FR-GL extract, with growth inhibition percentage of 90.41 ± 2.20. Figure H shows the growth of hyphae in medium with FR-GA extract. Plate 4.13: Figure B represents a bar diagram showing the average growth inhibition percentages of all extracts with standard deviation.

All other extracts except ethanol have no activity against all the tested microorganisms, so only ethanolic extracts of *A. scholaris* and *F. racemosa* were selected to analyse antifungal activity against *A. niger*. All the three samples of the both plants were used to screen the antifungal activity by food poisoning method. The study is limited only to preliminary screening with 2 concentrations. 100 mg/mL stock solutions of all extracts were

prepared. 1 mL from the stock is used for first screening and doesn't show any inhibition of mycelial growth. Then concentration increased upto 500 mg and activity was found. The average mycelial growth of treated plates was compared with control. The results indicate that lower concentration of all the extracts have no action against *A. niger*. Here antifungal drug Itraconazole (50 mg/mL stock concentration) was also tested for activity against *A. niger*.

Table 4.13: Percentage growth inhibition against *A. niger* shown by ethanolic extracts of different samples of *A. scholaris* and *F. racemosa* along with SD.

Sl. No.	Name of sample	Growth inhibition percentage
1.	Control (Itraconazole)	93.47 ± 0.90
2.	AS-NL	$10.15 \pm 1.60^{++}$
3.	AS-GL	$16.80 \pm 4.53^{++*}$
4.	AS-GA	$84.89 \pm 1.22^{+**}$
5.	FR-NL	$17.09 \pm 4.98^{++}$
6.	FR-GL	$90.41 \pm 2.20^{+**}$
7.	FR-GA	$74.91 \pm 2.31^{++**}$

Each value of growth inhibition percentage represents mean ± standard deviation (n = 3). + indicates values significantly different from Itraconazole (+ve control) at the level of $p < 0.05$. ++ indicates values significantly different from Itraconazole ($p < 0.001$). * indicates values significantly different from normal leaf samples of a particular plant ($p < 0.05$). ** indicates values significantly different from the normal leaf of the corresponding plant at the level $p < 0.001$.

The statistical analysis revealed that all the extracts of *A. scholaris* and *F. racemosa* exhibited significant antifungal activity when compared to the control drug, Itraconazole ($p < 0.05$). Among the tested extracts, the ethanolic extract of gall leaves of *F. racemosa* (FR-GL) demonstrated the highest inhibitory effect against *A. niger*, showing 90.41 ± 2.20% growth

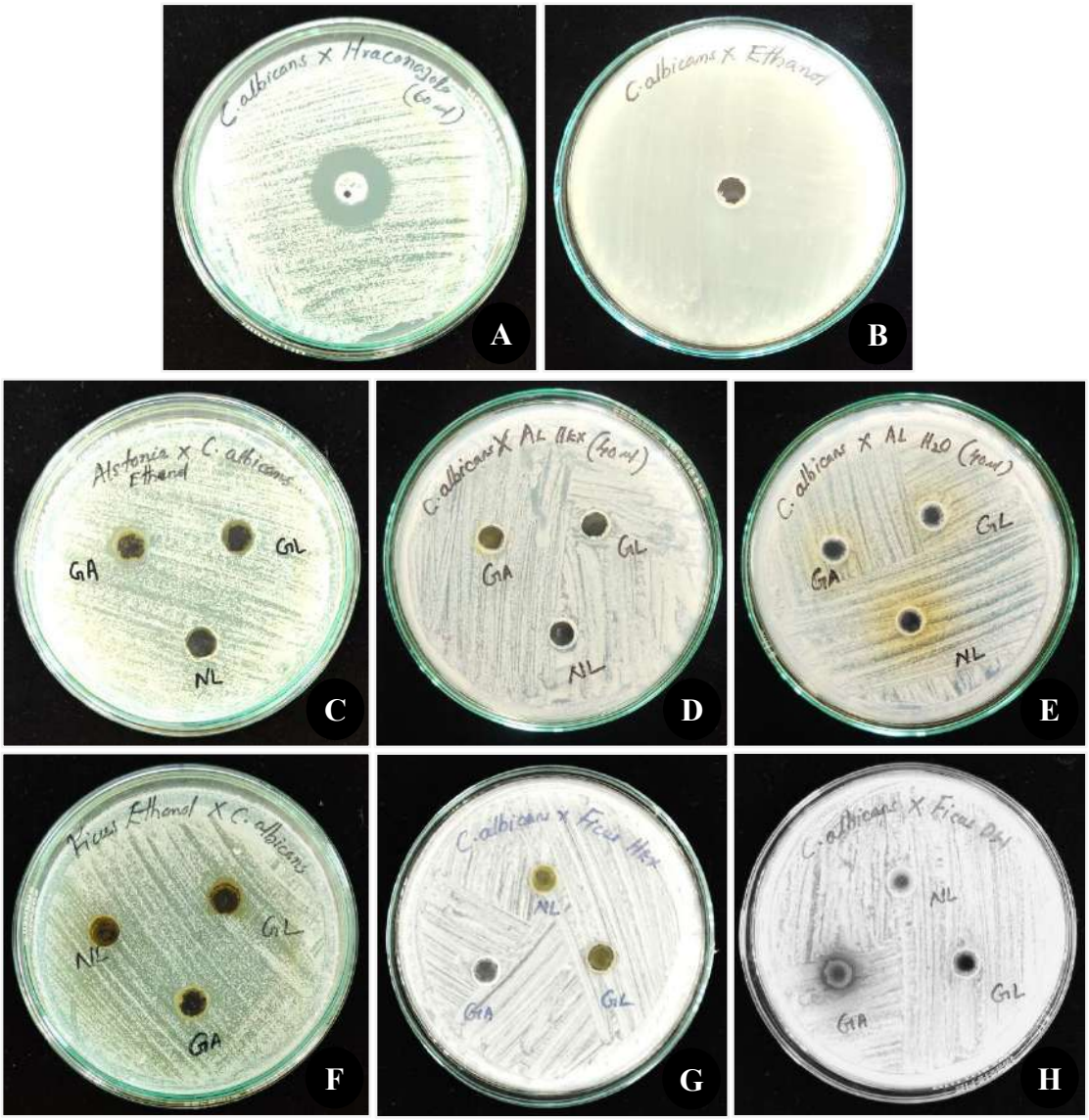


Plate 4.12: Anticandidal activity of *A. scholaris* and *F. racemosa*. Figure A: positive control (Itraconazole), Figure B: negative control, Figure C: activity of AS-NL, AS-GL and AS-GA ethanolic extracts, Figure D: no activity for AS-NL, AS-GL and AS-GA hexane extracts, Figure E: AS-NL, AS-GL and AS-GA aqueous extracts (no activity), Figure F: zone of inhibition of FR-NL, FR-GL and FR-GA ethanolic extracts, Figure G: FR-NL, FR-GL and FR-GA hexane extracts (no activity) and Figure H: no activity for FR-NL, FR-GL and FR-GA aqueous extracts.

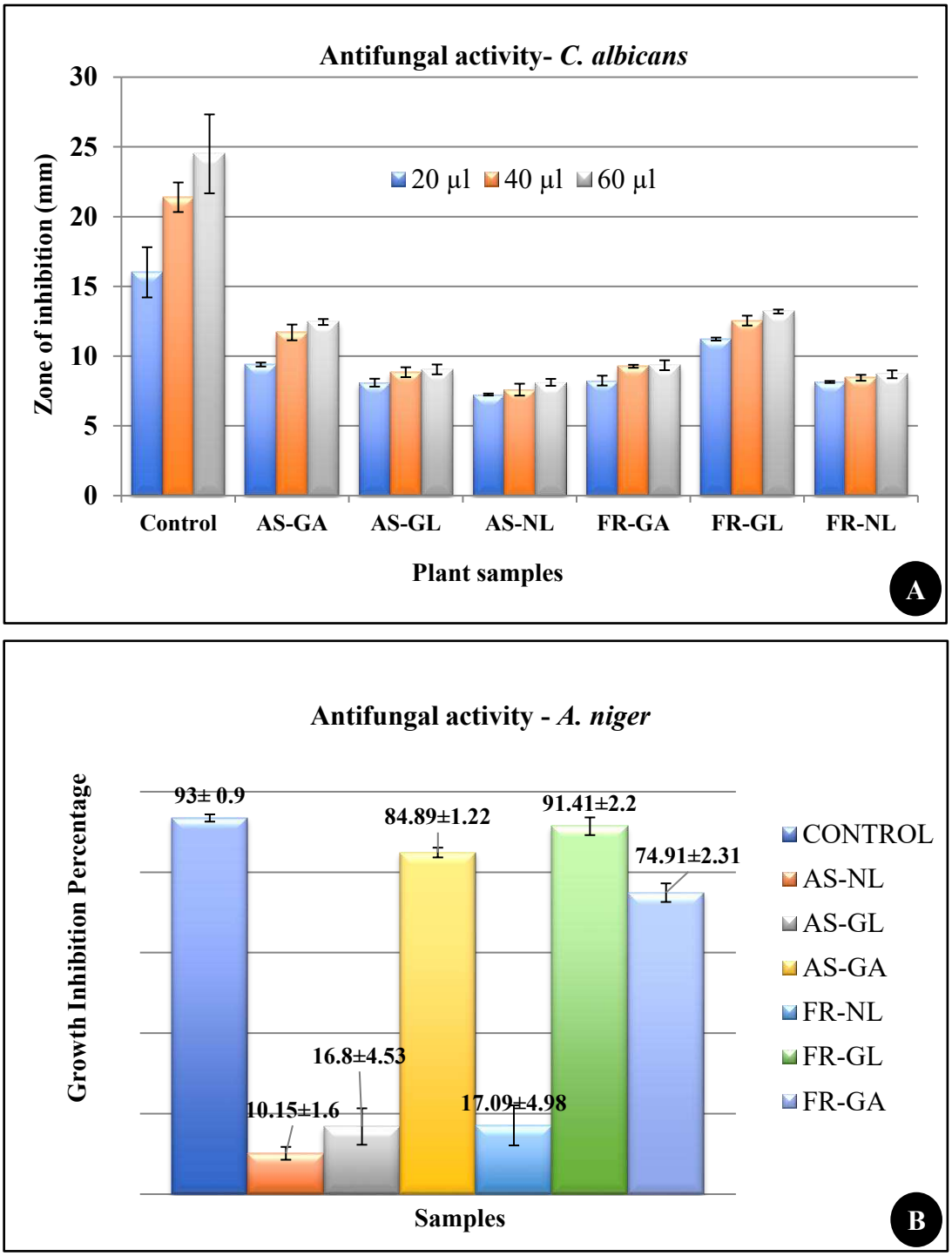


Plate 4.13: Graphical representation of antifungal activity. Figure A: Antifungal activity of ethanolic extracts of *A. scholaris* and *F. racemosa* against *C. albicans*. The X-axis represents different plant samples and positive control, while the Y-axis shows inhibition zones (mm) and Figure B: Percentage growth inhibition of ethanolic extracts *of A. scholaris* and *F. racemosa* against *A. niger*, with Itraconazole as the positive control. The X-axis represents different samples and the Y-axis shows growth inhibition (%).

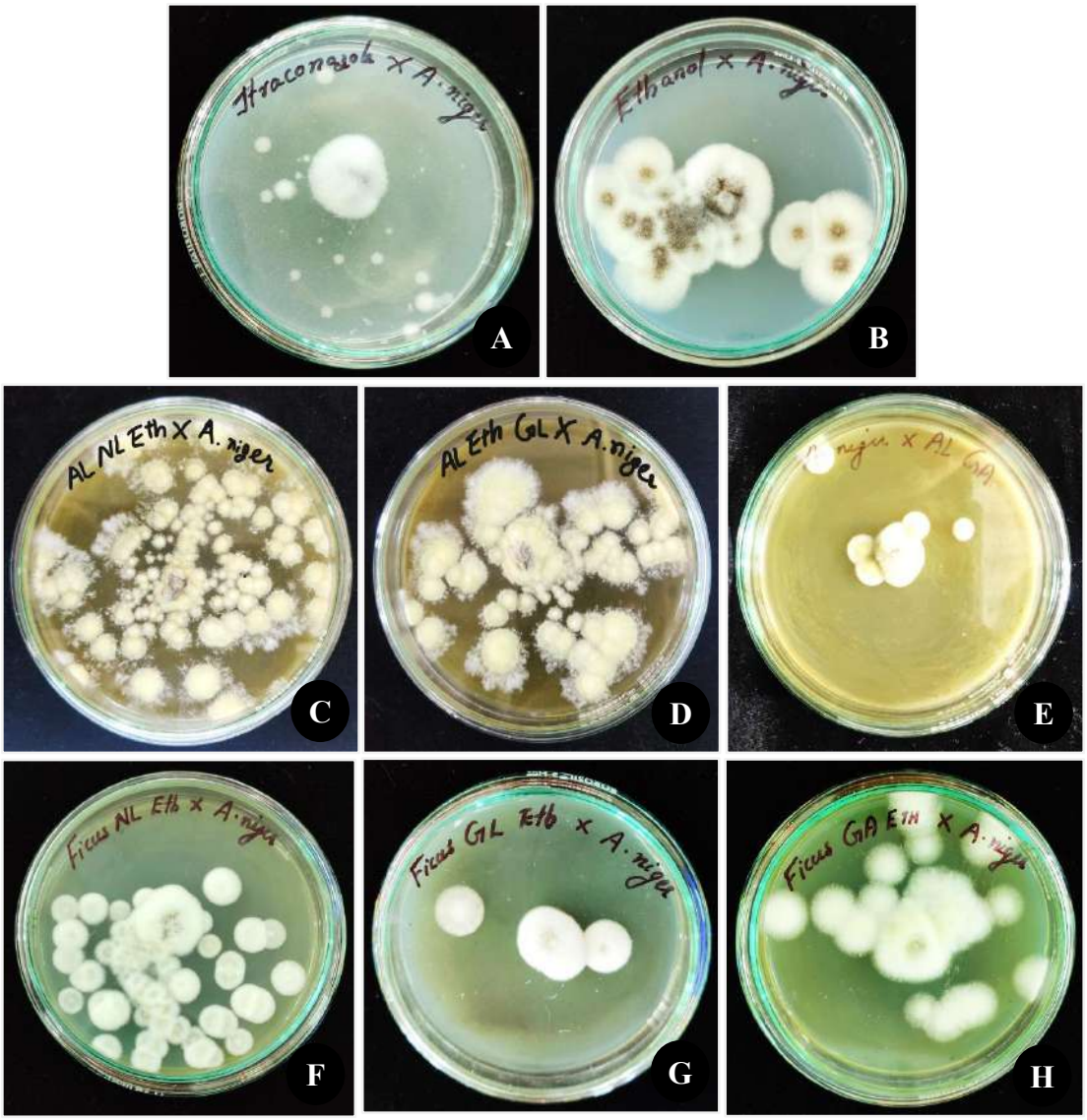


Plate 4.14: Antifungal activity of ethanolic extracts of *A. scholaris* and *F. racemosa* against *A. niger.* Figure A: Growth of hyphae in medium with Itraconazole, Figure B: Growth of hyphae in medium with ethanol, Figure C: Growth of hyphae AS-NL extract, Figure D: Growth in medium with AS-GL extract, Figure E: Growth of hyphae in AS-GA extract, Figure F: Growth of hyphae with FR-NL extract, Figure G: Growth in medium with FR-GL extract and Figure H: Growth of hyphae in medium with FR-GA extract.

inhibition. This was followed by the gall extract of *A. scholaris* (AS-GA), which exhibited 84.89 ± 1.22% inhibition. Statistical evaluation indicated that all extracts, except AS-GL, showed highly significant antifungal activity ($p < 0.001$), while AS-GL also exhibited a significant level of inhibition ($p < 0.05$). These results suggest that gall-associated tissues, particularly in *F. racemosa*, possess more potent antifungal constituents compared to normal leaf extracts.

Bonvicini and colleagues found that ethanolic extracts of *A. scholaris* exhibited antifungal activity against *C. albicans* (Bonvicini et al., 2014). The isopropanol extracts of *A. scholaris* leaves are highly effective against pathogenic fungi (Bagheri et al., 2020). Another study proved that at all doses, the ethanolic leaf extract of *F. racemosa* exhibited moderate activity against *Rhizopus* and no activity against *A. flavus* (Thilagavathi and Kathiravan, 2017). In the present investigation, FR-GL at 60 µL dilution demonstrated a 13.2 ± 0.14 mm zone of inhibition against *C. albicans,* while AS-GA at 60µL demonstrated 12.45 ± 0.21 mm zone of inhibition. FR-GL found to inhibit *A. niger* growth by 9%. In 2023, Mishra and colleagues investigated the bactericidal properties and found the methanol extract showed the greatest activity with zone of inhibition against *S. aureus* (23.42 mm), *C. albicans* (26.74 mm) and *A. niger* (25.78 mm) (Mishra et al., 2023).

4.3.4. Results of cytotoxic activity analysis

Preliminary screening for cytotoxic assay

The cytotoxic activity of aqueous, hexane and ethanolic extracts (250 mg/mL) from *A. scholaris* and *F. racemosa* was evaluated using the MTT assay on 3T3 cell lines. For AS-GA ethanolic extracts showed the highest cytotoxic activity, with a cell viability of 37.36%, significantly lower than other AS-NL and AS-GL (Plate 4. 15: Figure A). For FR-GL ethanolic extracts demonstrated the highest cytotoxicity, with a cell viability of 35.62% (Plate 4. 15: Fig B). The preliminary screening results were shown in Table 4.14. Initial screening determined cell viability percentages, revealing that ethanolic extracts of galls AS-GA and FR-GL exhibited the highest cytotoxicity.

For finding statistical significance, ANOVA was conducted to assess differences in cell viability across concentrations with a significance level ($p < 0.05$). Tukey's HSD post hoc test was conducted to compare cell viability percentages between normal leaves, gall leaves and galls samples for *A. scholaris* and *F. racemosa* across aqueous, ethanol and hexane extracts. Significant differences ($p < 0.05$) were observed in aqueous extract and ethanolic extracts of both plants and also in hexane extract of *F. racemosa*. No significant differences were found in hexane extract of *A. scholaris*.

Table 4.14: Percentage cell viability of aqueous, ethanolic and hexane extracts of *A. scholaris* and *F. racemosa*.

Sl. No.	Extract (250 mg/mL)	Name of sample	Percentage of cell viability
1.	Aqueous	AS-NL	87.84 ± 3.97
2		AS-GL	74.51 ± 3.09*
3.		AS-GA	68.22 ± 5.30*
4.		FR-NL	54.72 ± 2.46
5.		FR-GL	90.37 ± 8.46**
6.		FR-GA	83.47 ± 3.93**
7.	Ethanol	AS-NL	75.50 ± 0.62
8.		AS-GL	80.71 ± 2.73
9.		AS-GA	37.36 ± 1.43**
10.		FR-NL	45.07 ± 5.60
11.		FR-GL	35.62 ± 2.32*
12.		FR-GA	66.55 ± 4.80*
13.	Hexane	AS-NL	67.81 ± 6.71

14.		AS-GL	68.59 ± 1.13
15.		AS-GA	77.23 ± 1.79*
16.		FR-NL	84.88 ± 4.54
17.		FR-GL	51.40 ± 2.00**
18.		FR-GA	64.33 ± 5.44*

Each value of percentage of cell viability represents mean ± standard deviation (n = 3). * indicates the value of percentage of cell viability, which is significantly different from percentage of cell viability of the normal leaf samples of the particular plant ($p < 0.05$). ** indicates the value of percentage of cell viability, which is significantly different from percentage of cell viability of the normal leaf samples of the particular plant at the level of significance $p < 0.001$.

Concentration-dependent MTT assay

Based on the preliminary results, ethanolic extracts of AS-GA and FR-GL were selected for concentration-dependent MTT analysis. 3T3 cells treated with DMSO served as controls, while cells treated with test solutions at concentrations of 50, 100, 200, 300 and 400 mg/mL were analyzed. Absorbance was measured at 570 nm and 630 nm using a microplate reader and cell viability percentages were calculated (Table 4.15). The MTT assay results showed that cell viability decreased as extract concentration increased, indicating a concentration-dependent increase in cytotoxicity. Plate 4.16: Figure A is the image of untreated 3T3 cells used for cytotoxicity assay, Figure B shows the microscopic photograph of 3T3 cells after treatment, showing the formation of formazan crystals indicating metabolically active cells and Figure C is the photograph of MTT treated 3T3 cells after addition of solubilisation reagent showing the developed color intensity used for viability measurement. Representation of a culture plate showing formazan crystals represented in Plate 4.17: Figure A. The percentage cell viability of each concentration of both extracts was represented graphically in Plate 4.17: Figure B.

The viability percentage of each dilution of extracts (50 mg/ml, 100 mg/ml, 200 mg/ml, 300 mg/ml and 400 mg/ml) were calculated. It showed that as the concentration increases the cytotoxic effect of extract also increases. 50 mg/mL ethanolic extracts of AS-GA showed 80 ± 0.65% of cell viability and the same dilutions of FR-GL ethanol extracts showed maximum cell viability of 97.71 ± 2.54%. For dilutions 100 mg/mL and 200 mg/mL of extracts of *A. scholaris* showed 49.44 ± 2.91% and 40.89 ± 2.69% cell viability respectively. 34.15 ± 4.14% and 15.60 ± 5.8% cell viability was shown by 300 mg/mL and 400 mg/mL of extracts. In the case of FR-GL ethanol extracts, 100 mg/mL and 200 mg/mL dilutions displayed 89.43 ± 2.11% and 79.27 ± 3.94% cell viability respectively. The percentage cell viability lowers upto 9.90 ± 5.15% for concentration 400 mg/ml. IC_{50} value was calculated for ethanolic extracts of AS-GA (IC_{50}-170.6 ± 4.72 mg/mL). The ethanolic extracts of FR-GL exhibited an IC_{50} value of 258.8 ± 8.47 mg/mL.

Table 4.15: Concentration dependent cell viability assay results of AL-GA and FR-GL showing the cell viability percentage of 50-500 mg/mL of sample concentrations.

Sl. No.	Concentration of sample (mg/mL)	AL-GA (Percentage of cell viability)	FR-GL Percentage of cell viability)	Triton X-100 (control) (Percentage of cell viability)
1.	50 mg/mL	$80.00 \pm 0.65^{++}$	$97.71 \pm 2.54^{++}$	0.00
2.	100 mg/mL	$49.44 \pm 2.91^{++}$	$89.43 \pm 2.11^{++}$	0.00
3.	200 mg/mL	$40.89 \pm 2.69^{++}$	$79.27 \pm 3.94^{++}$	0.00
4.	300 mg/mL	$34.15 \pm 4.14^{++}$	$27.83 \pm 0.31^{++}$	0.00
5.	400 mg/mL	$15.60 \pm 5.80^{++}$	$9.40 \pm 5.15^{++}$	0.00

Each value of percentage cell viability represents mean ± SD (n = 3). ++ indicates values significantly different from Triton X-100 (+ve control) at the level of $p < 0.001$.

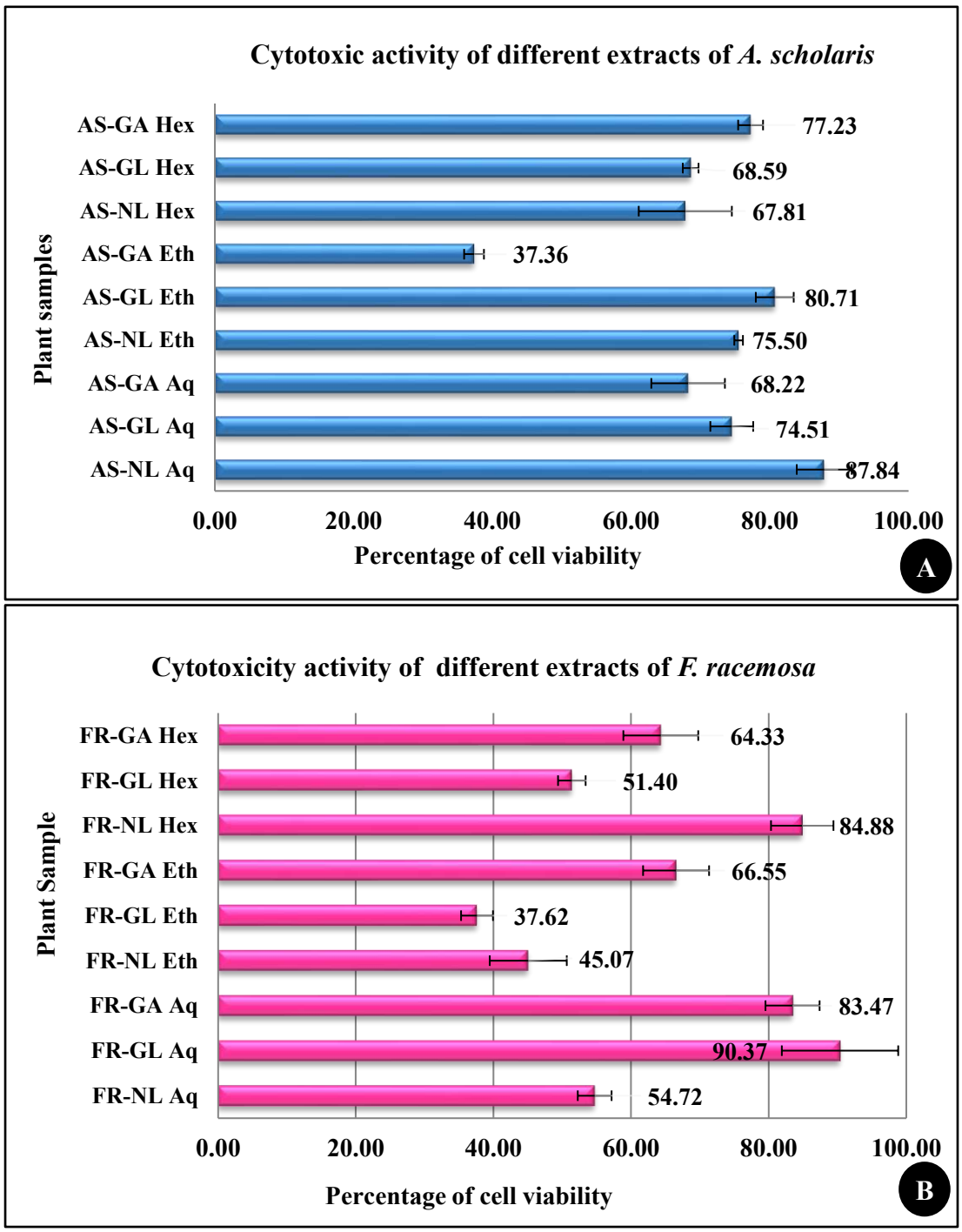


Plate 4.15: Figure A: Cell viability percentage of *A. scholaris* extracts- AS-NL, AS-GL and AS-GA showing mean values with standard deviation, indicating their effect on cell viability and Figure B: Cytotoxic activity of *F. racemosa* extracts-FR-NL, FR-GL and FR-GA represented as cell viability percentage with standard deviation

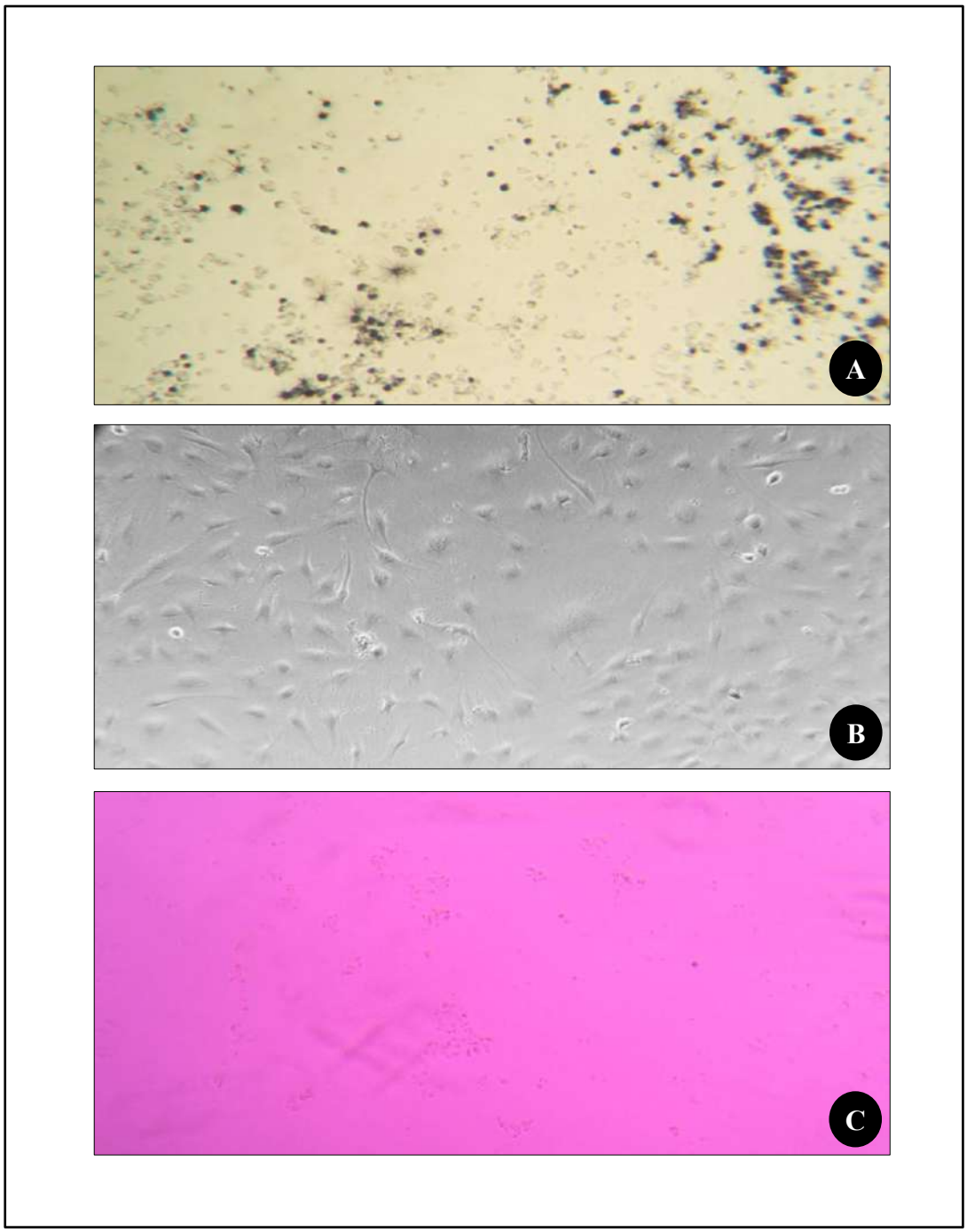
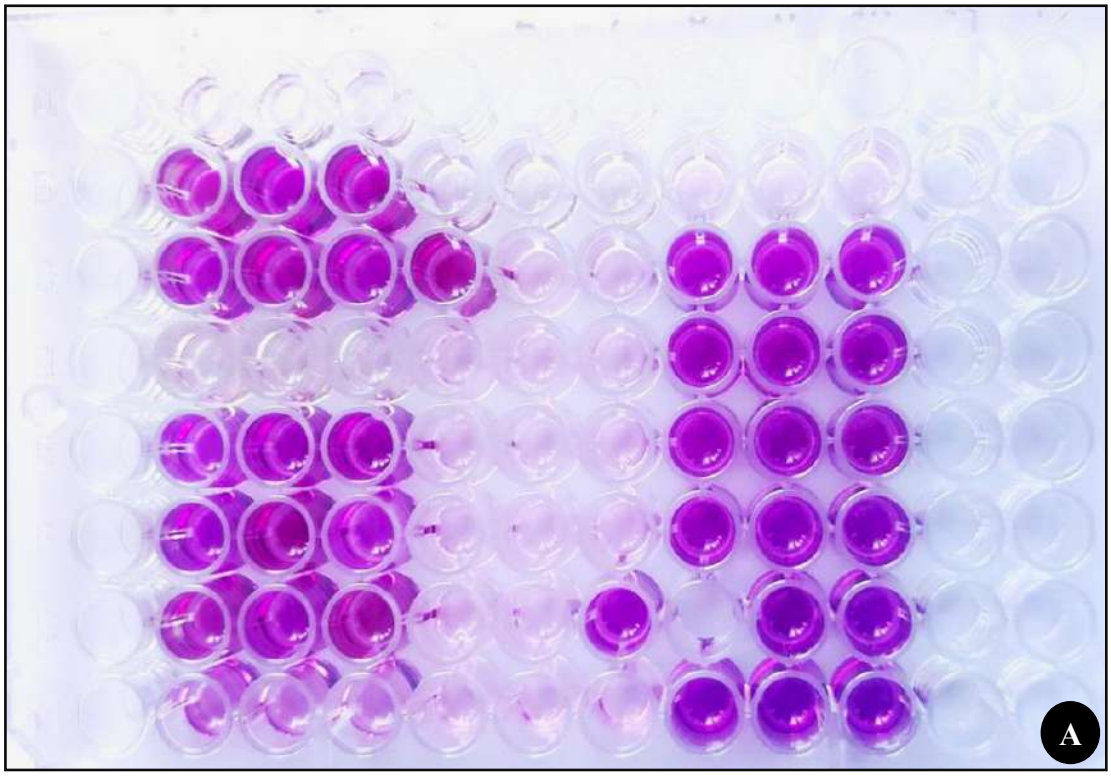


Plate 4.16: Microscopic representation of 3T3 cells. Figure A: Image of untreated 3T3 cells used for cytotoxicity assay, Figure B: Image of 3T3 cells after treatment, showing the formation of formazan crystals indicating metabolically active cells and Figure C: Photograph of MTT-treated 3T3 cells after addition of solubilisation reagent showing the developed color intensity used for viability measurement.



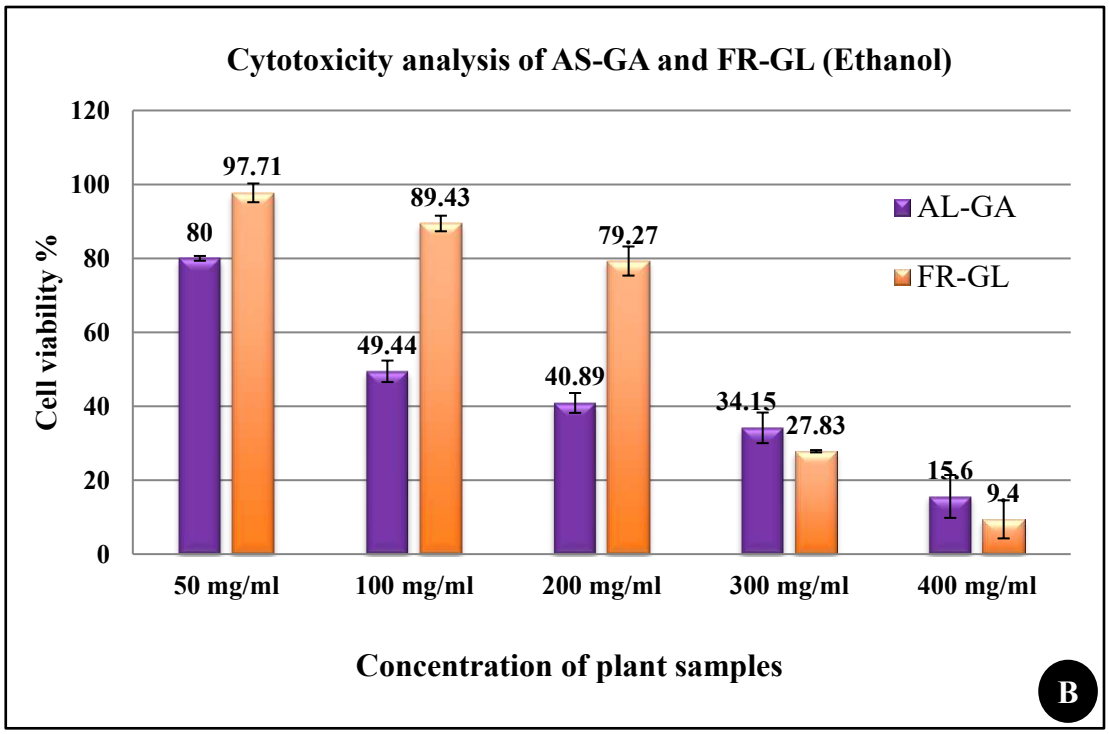


Plate 4.17: Figure A: shows the representation of a culture plate having purple formazan crystals and Figure B: The graph showing the results of cell viability percentage of etanolic extracts of *A. scholaris* gall (AS-GA) and *F. racemosa* gall leaf (FR-GL) using MTT assay.

Comparing the two extracts, AS-GA exhibited higher cytotoxicity than FR-GL at lower concentrations (50-200 mg/mL). At 50 mg/mL, AS-GA had a cell viability of 80.00 ± 0.65% compared to FR-GL 97.71 ± 2.54%. At 100 mg/mL, AS-GA cell viability (49.44 ± 2.91%) was significantly lower than FR-GL (89.43 ± 2.11%). However, at higher concentrations (400 mg/mL), both extracts showed comparable cytotoxicity, with cell viabilities of 15.60 ± 5.80% (AS-GA) and 9.40 ± 5.15% (FR-GL). The steeper decline in cell viability for AS-GA at lower concentrations suggests greater potency, corroborated by its lower IC_{50} value. From the results it was discovered that compared to normal leaves of the selected plants the cytotoxicity was higher in the gall associated portions. In the case of AS-GA, it displayed a higher cytotoxic effect than other parts, but in the case of *F. racemosa,* the leaf portion in which gall resides *i.e.* gall leaves showed maximum cytotoxicity. From these observations it can be found that gall induction on the leaves of both selected plants will result in greater content of bioactive chemicals in those portions of plants. These bioactive compounds may result in the different biological activities shown by the plants.

The statistical analysis confirmed significant differences in cytotoxic activity ($p < 0.001$). Tukey's HSD post-hoc test indicated that cell viability at 50 mg/mL and 100 mg/mL differed significantly between AS-GA and FR-GL ($p < 0.001$), with AS-GA showing greater cytotoxicity. All the tested concentrations showed significant cytotoxicity at $p < 0.001$. The concentration-dependent decrease in cell viability was statistically significant for both extracts ($p < 0.001$).

In 2023, Shanmugapriya and coworkers used the MTT assay to examine whether *A. scholaris* leaf extract induced apoptosis in the breast cancer cell lines MCF-7 and MDA-MB 231. They reported that in breast cancer cell lines, treatment with *A. scholaris* extract significantly increased the rate of apoptosis (Shanmugapriya et al., 2023). Itam and colleagues employed the brine shrimp lethality test method in their study to examine the cytotoxic activities of *A. scholaris* leaf extract. They discover that the death rate of *Artemia salina* larvae rises in concordance with the extract concentration. With an IC_{50} value of 883.28 µg/mL, methanol extract exhibited highest cytotoxicity, followed by ethyl acetate extract with IC_{50} 954.72 µg/mL (Itam et al., 2018). Sarkar and colleagues reported an LC_{50} of 61.85 µg/mL for *A. scholaris* leaf extracts against brine shrimp (Sarkar et al., 2021). The

MTT technique was used in this study to assess the cell viability of 3T3 cell lines in different samples of aqueous, hexane and ethanol extracts. Based on the initial screening conducted, ethanolic extracts of AS-GA exhibit much higher cytotoxic activity than aqueous and hexane extracts. Concentration-dependent cytotoxic action was demonstrated by gall ethanolic extracts.

In 2016, Bagheri and associates found that *A. scholaris* hexane extracts caused 100% cell death in Dalton's lymphoma ascites cells at 500 µg/mL, while methanolic extracts lacked cytotoxicity. However, at lower concentrations, its cytotoxicity was less pronounced in the aqueous extracts of *A. scholaris* leaves, benzene and isopropanol exhibited minimal action at the higher dosage (Bagheri et al., 2016). Ethyl acetate fraction of *A. scholaris* had the highest cytotoxicity activity against brine shrimp with LD_{50} values 944.730 ppm after 6 h treatment (Ara et al., 2020). Based on the current investigation, the highest cytotoxicity was detected at 500 mg/mL of AS-GA, with cell viability of -4.80 ± 2.66% and an IC_{50} of 170.6 ± 4.72 mg/mL. In an earlier investigation, the cytotoxicity was also examined on a mammalian cell line and it was discovered that at the tested dilutions, the extracts had no effect on the proliferation of mammalian cells (Bonvicini et al., 2014). The present study does not give information regarding the cytotoxic activity of extracts in cancer or mammalian cell lines. The results of this experiment can only be interpreted as a basic cell viability assessment of extracts.

Gorla and Shankar (2016) analysed *F. racemosa* ethanolic and hexane extracts for cytotoxicity with XTT assay resulted in moderate anticancer activities against A549 and MDA-MB-435S cell lines. At a dosage of 100 µg/mL, *F. racemosa* hexane extract inhibited cell growth more effectively than ethanol extracts (Gorla and Shankar, 2016). A study published in 2021 disclosed anticancer properties of various parts of *F. racemosa*. MTT analysis of the anticancer effects on DLA tumour cells revealed a concentration-dependent antitumorigenic effect (Poongothai and Annapoorani, 2021). In 2011, Hosain and colleagues investigated the cytotoxic potential of *F. racemosa* leaf extracts. LC_{50} Value of methanol extract of *F. racemosa* leaves was 0.467 µg/mL which indicates significant activity (Hosain et al., 2011). In this experiment, gall leaves ethanolic extracts were further examined at five different concentrations. The outcomes indicated that the cytotoxic efficiency increased with

increase in concentration of extracts. Cell viability at a 400 mg/mL concentration of FR-GL is 9.40%. The IC_{50} value for FR-GL was 258.8 ± 8.47 mg/mL.

4.3.5. Results of anti-inflammatory activity analysis

Anti-inflammatory activity of FR-GL extracts were analysed using protein denaturation inhibition assay in five different dilutions of sample.The results were shown in Table 4.16. The extract (FR-GL) exhibited dose-dependent inhibition of protein denaturation, indicating notable anti-inflammatory potential. At the highest concentration (5 µg/µL), the extract showed 34.33% protection, compared to 80.23% by the standard drug, Diclofenac sodium. These results suggest that FR-GL possesses moderate anti-inflammatory activity. The photographs of the results were shown in Plate 4.18: Figure A- Figure G.

Table 4.16: Anti-inflammatory activity of five different dilutions of FR-GL extracts, using protein denaturation inhibition assay.

Sl. No.	Dilutions of FR-GL extract (µg/µL)	Percentage of protection
1	1	17.37 ± 0.03**
2	2	22.21 ± 0.47**
3	3	28.04 ± 0.64**
4	4	31.63 ± 1.75**
5	5	34.33 ± 0.30**
6	Diclofenac sodium (standard)	80.23 ± 0.20

Each value of percentage of protection represents mean ± standard deviation (n = 3). ** indicates values of percentage of protection, that significantly different from the percentage of protection value of standard - Diclofenac sodium at $p < 0.001$.

Statistical analysis (ANOVA) followed by Tukey's HSD post hoc test revealed a significant ($p < 0.001$) concentration-dependent increase in inhibition of protein denaturation from 17.37 ± 0.03% at 1 µg/µL to 34.33 ± 0.30% at 5 µg/µL. All dilutions of FR-GL extract showed significantly lower inhibition of protein denaturation compared to the standard Diclofenac sodium (80.23 ± 0.20%).

The present findings corroborate earlier reports highlighting the anti-inflammatory potential of *F. racemosa*. The *in vitro* assays provide supporting evidence for the anti-inflammatory efficacy of *F. racemosa*. The bark extracts showed a concentration-dependent inhibition of protein denaturation, a mechanism closely associated with the prevention of inflammatory processes. Interestingly, hot water extracts were more effective than cold water extracts, and their inhibitory potential even surpassed that of standard reference drugs such as Prednisolone and Ibuprofen at lower concentrations (Dharmadeva et al., 2018). This highlights the role of extraction methods in maximizing bioactive compound availability and effectiveness. In our study, the FR-GL extract at a concentration of 5 µg/µL provided 34.33% protection against protein denaturation, although the standard Diclofenac sodium exhibited a much higher protection rate of 80.23%.

In addition to *in vitro* studies, the carrageenan, serotonin, histamine and dextran-induced rat paw edema models, leaf extracts at 200 and 400 mg/kg demonstrated significant anti-inflammatory activity, with the maximum inhibition observed at 400 mg/kg (30.4 - 33.9% reduction in paw edema after 3 h) (Mandal et al., 2000a). These outcomes indicate that *F. racemosa* possesses broad-spectrum activity against different mediators of inflammation, suggesting both antihistaminic and antiserotonergic properties in addition to its inhibitory effect on carrageenan-induced edema. Similarly, intraperitoneal administration of the extract at a lower dose (20 mg/100 g) was also reported to suppress carrageenan-induced acute inflammation, with notable effects at later phases (3^{rd}, 5^{th} and 7^{th} h), thus confirming the consistency of its anti-inflammatory action across different experimental models (Mehta et al., 2012). This suggests that *F. racemosa* exhibits measurable anti-inflammatory activity.

In this study a preliminary analysis was conducted to evaluate the antibacterial, antifungal, antioxidant and cytotoxic activities of aqueous, hexane and ethanolic extracts from *A. scholaris* and *F. racemosa*. The results demonstrated that ethanolic extracts were the most effective across all tested activities, particularly those from AS-GA and FR-GL. So, FR-GL extracts were analysed by separation of different compounds using TLC (Chapter 5). The same sample is used for detection of anti-inflammatory activity.

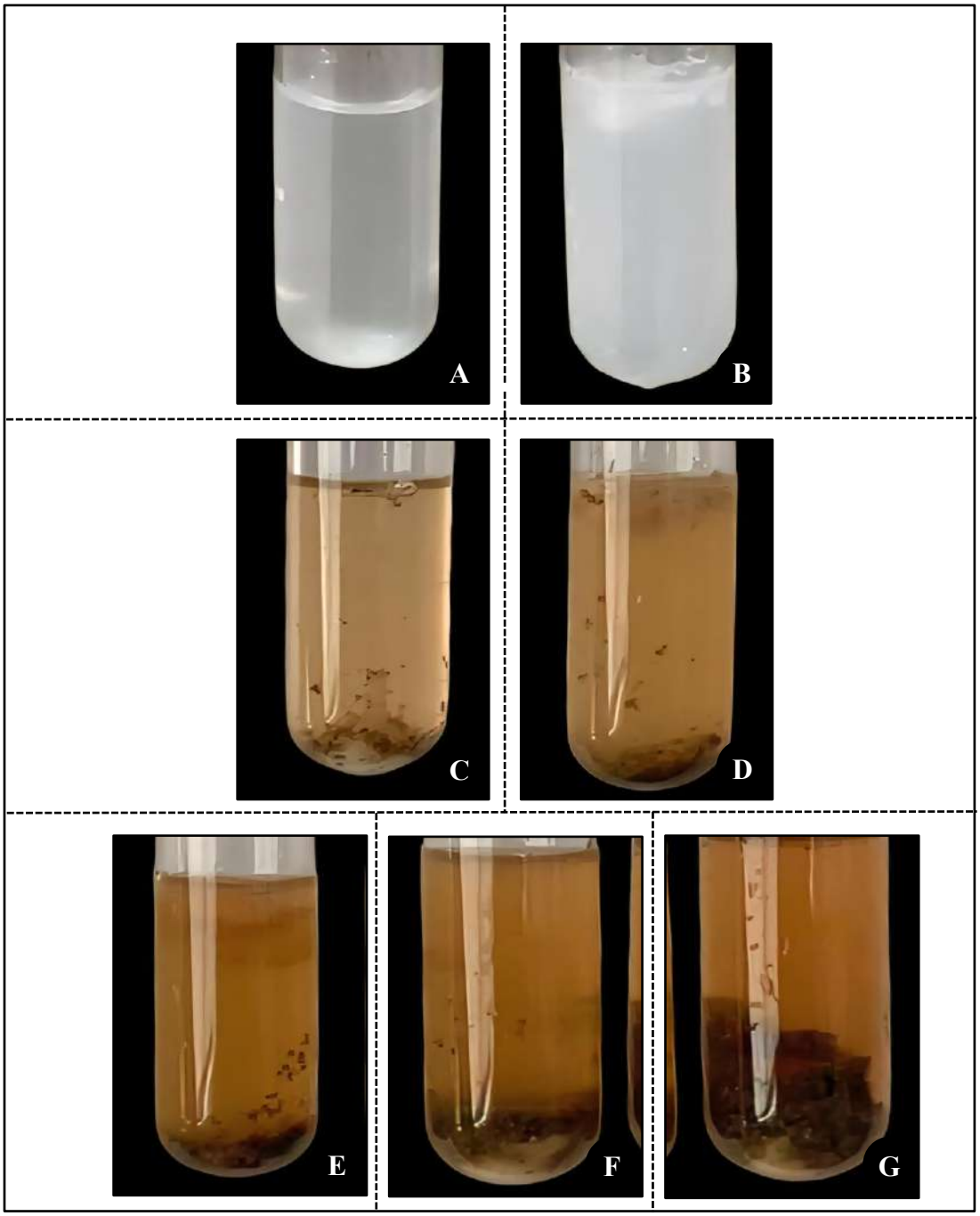


Plate 4.18: Anti-inflammatory activity analysis by protein denaturation inhibition assay. Figure A: Control assay showing untreated protein sample, Figure B: Activity of standard drug (Diclofenac sodium) showing inhibition of protein denaturation and Figure C-G: Anti-inflammatory activity of *F. racemosa* gall leaf (FR-GL) ethanolic extract at concentrations of 1, 2, 3, 4 and 5 µg/µL, respectively, showing progressive inhibition of protein denaturation with increasing extract concentration.

4.4. CONCLUSIONS

According to the studies of the bioactive properties of the tissues of *A. scholaris* and *F. racemosa*, the gall and tissues related to galls exhibit increased activity. Bioactivity of plant extracts is due to the presence of various phytochemical components present in them. As mentioned in the preceding chapter, galls and gall leaves have substantially more secondary metabolites than normal leaves. There is another finding that the bioactivity may vary between various extracts, which could be explained by variable degrees of solubility of the active components in the solvents utilized. The antimicrobial properties of extracts may be related to the presence of antimicrobial components including alkaloids, flavonoids, saponins, tannins and phenolics. Many bioactive phytoconstituents may be present in the plant samples, which could explain the reducing power ability of extracts. As mentioned above, galls and gall leaves extracts contain greater quantity and number of bioactive substances, such as phenols, flavonoids, tannins, etc., which are tumour inhibitors, scavengers of free radicals, inducers of apoptosis and promoters of cell differentiation. The FR-GL extract possessed greater activity than all other samples in terms of antioxidant, antibacterial, antifungal and cytotoxic activities. FR-GL was tested and proved to possess moderate anti-inflammatory activity. Gall and gall associated tissues of both plants have higher activity than healthy leaves. So, it can be concluded that gall or gall associated tissues have much greater phytoconstituents and thus significant bioactivity. Additionally in future exploring the ecological roles of gall-induced bioactivity and comparing these plants with other gall-forming species could generalize the findings, contributing to phytopharmacology and sustainable drug development.

CHAPTER 5 - ISOLATION OF BIOACTIVE FRACTIONS RESPONSIBLE FOR POTENT BIOLOGICAL ACTIVITY

5.1. INTRODUCTION

Chromatography is used for separation of a mixture into its components and allows more accurate study of each compound instead of the whole mixture. It is used in industries like pharmaceuticals, biotechnology and clinical research (Kaale et al., 2011). Thin-layer chromatography (TLC) is a widely used chromatographic technique for the analysis of plant materials, separation and identification of medicinal plants and their extracts. TLC offers advantages such as low cost, ease of operation, rapid analysis and minimal sample preparation (Bele and Khale, 2011; Cimpoiu, 2006).

TLC operates on the principle of differential distribution of compounds between a stationary phase and mobile phase. The stationary phase consists of a thin layer of adsorbent material, made up from silica gel, cellulose or aluminum oxide, coated onto glass, plastic or aluminum sheet plate. The mobile phase is selected based on the properties of the compounds to be separated. When the mobile phase moves upward through capillary action, compounds partition between the stationary and mobile phases based on their relative affinities. This will result in separation of components (Ahamed et al., 2017; Bele and Khale, 2011). The retention factor (Rf) value is calculated as the ratio of the distance traveled by a compound to the distance traveled by the solvent front. The polar compounds interact more strongly with a polar stationary phase like silica gel, resulting in lower Rf values, while less polar compounds move higher up the plate, yielding higher Rf values. Factors influencing Rf values include nature of adsorbent, nature of mobile phase, temperature conditions, layer thickness, nature of developing tank, sample mass and also based on the type of chromatographic technique (Bele and Khale, 2011; Stoddard et al., 2007).

TLC allows multiple samples to be evaluated on a single plate, supports flexible selection of mobile and stationary phases and enables multiple detection methods and specific derivatization. TLC fingerprints, based on Rf values and spot colors, facilitate compound identification. These fingerprints can be captured as images, aiding in result

Reshma K Ramesh. Comparative phytochemical analysis and gene expression profiling of leaf galls on selected medicinal plants. Thesis 2025. Govt. Victoria college, Palakkad, University of Calicut.

communication and documentation (Cimpoiu, 2006). TLC can be used to monitor the reaction progression, identification of the components of the mixture and the determination of the purity of the mixture. It is used for checking the identity and purity of drugs. It also helps find any adulterations or substitutions in a simple and accurate way (Ahamed et al., 2017; Asante et al., 2016; Porika et al., 2014).

Preparative thin-layer chromatography (PTLC) is a method used to purify and isolate larger amounts of compounds for synthesis, testing or analysis. It processes larger sample sizes (10-1000 mg) to obtain sufficient purified material. It uses stationary phase layers of 0.5-2 mm or more thickness to handle increased sample volumes. It also uses larger plates (20 x 20 cm or bigger) to accommodate more sample volume and simplify collection after separation (Rabel and Sherma, 2017; Sherma and Fried, 1987). This technique is commonly applied in pharmaceutical and organic chemistry labs to purify compounds to get large amounts of pure material. PTLC is used to isolate pure compounds for further analysis such as gas chromatography, analytical TLC, high-performance liquid chromatography (HPLC), visible spectrometry, fluorescence spectrometry, infrared (IR) spectrometry, ultraviolet (UV) spectrometry, nuclear magnetic resonance (NMR) spectrometry (^{1}H and ^{13}C) etc. Multiple rounds of PTLC may be needed to achieve sufficient compound purity for tasks like structure elucidation (Rabel and Sherma, 2017; Sherma and Fried, 1987).

Nuclear Magnetic Resonance (NMR) spectroscopy is a fundamental analytical technique widely used for the structural elucidation of natural products. It provides detailed insights into molecular structure, stereochemistry and dynamics through the interaction of atomic nuclei with an external magnetic field. The measurement of metabolites can be effectively done using NMR spectroscopy. In plant metabolomics, NMR aids in identifying and measuring different plant metabolites present in mixtures. NMR-based metabolomics is developing rapidly and is becoming a common method because it is reliable, fast and needs very little sample preparation. It can also be used for studying samples directly in living systems (*in vivo*). One-dimensional proton (^{1}H) NMR is the most commonly used NMR because it gives highly reproducible results, making it suitable for creating shared databases. However, in the case of complex mixtures, additional two-dimensional NMR techniques are often employed to resolve overlapping signals and obtain clearer spectral data. These

advanced methods enable accurate identification of individual metabolites within complex mixtures. The resulting chemical shift values and signal patterns are then compared with reference databases such as Biological Magnetic Resonance Data Bank for metabolite identification. Using advanced two-dimensional NMR techniques, it can identify both known and unknown compounds in complex mixtures. It also offers the advantages of quick sample preparation and less time-consuming. Another major benefit of NMR is that it is non-destructive, meaning the same sample can be used for further studies after the analysis (Brennan, 2014; Cvetkovic et al., 2020; Halabalaki et al., 2014). NMR-based metabolomics mainly uses proton detection because it provides high sensitivity. Although ^{13}C NMR offers valuable structural information with broader spectral separation and clear signals, it is less commonly used due to its low natural abundance and sensitivity. NMR spectroscopy plays a central role in natural products chemistry, serving as a basic technique for structural elucidation, dynamic studies and metabolite identification (Brennan, 2014; Clendinen et al., 2014).

Here in this chapter, various chromatographic and spectroscopic techniques such as TLC, PTLC and NMR were employed to isolate, purify and characterize the bioactive fractions from the selected plant extracts. These methods helped in identifying the specific compounds responsible for the potent biological activities observed in the study.

5.2. METHODOLOGY

5.2.1. Selection of most bioactive plant sample

Bioactivity analyses (antioxidant, antibacterial, antifungal and cytotoxicity assays), were performed on samples AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA. The data obtained from these analyses were subjected to statistical evaluation. Based on the results of bioactivity assays and statistical analyses, the sample exhibiting the highest bioactivity was selected for further fractionation and characterization. The selection criteria for the potent bioactive sample included: (i) the lowest IC_{50} value in antioxidant assays, indicating higher free radical scavenging efficiency; (ii) maximum zone of inhibition in antibacterial and antifungal assays; (iii) highest percentage inhibition in cytotoxicity assays and (iv) finally statistically significant differences compared to control and other samples.

5.2.2. Optimization of chromatographic conditions of the targeted plant extracts

Preliminary TLC analysis was done for selected plant sample using pre-coated silica plates. To determine suitable solvents for the separation of compounds in the selected sample, trial TLC analysis was performed using pre-coated silica gel plates 60 F_{254} (Merck). TLC silica plates on aluminum (20 × 20 cm) were cut into smaller strips (2 × 10 cm) and employed for the TLC separation. The selected plant extracts were applied onto pre-coated TLC plates using capillary tubes and developed in a TLC chamber using various solvent systems (mobile phases). The developed TLC plates were air-dried and examined under visible light and ultraviolet (UV) light at both 254 nm and 366 nm. The solvent system that achieved optimal separation was selected for preparative TLC analysis (Bele and Khale, 2011; Jesionek et al., 2015).

5.2.3. Preparative TLC analysis for separation of compounds

Preparative TLC analysis was done for separation of compounds from the selected sample. The following are the different steps for performing preparative TLC (Hostettmann et al., 1998; Iborra et al., 1992; Kagan and Flythe, 2014; Sherma and Fried, 1987).

(i) Plate preparation

Clean the TLC plates by developing it in one or more polar, neutral solvents, in order to move adsorbed contaminants away from the zone of development. A slurry of 25 g of silica gel G and 75 mL distilled water is stirred in a beaker with a glass rod for 5 min to assure complete mixing and poured on to a pre cleaned 20 × 20 cm glass plate. While holding the plate in one hand, it is tilted to spread the layer evenly over the whole plate. The plate is then placed on a level surface at ~25 °C for 2 h before placing it overnight in a storage rack to dry slowly by spontaneous evaporation. The plate is activated at 130 °C for 2 h and cooled slowly to ~25 °C. The edges are scraped straight with a spatula and the plate is ready for sample application. The adsorbent thickness is made to 2 mm (Hostettmann et al., 1998; Iborra et al., 1992; Kagan and Flythe, 2014; Sherma and Fried, 1987).

(ii) Sample application

Fine tipped capillaries were used to apply the concentrated solutions of the extracts to be resolved in a band. The sample is applied 1 cm above the application line.

(iii) Solvent selection

Solvent selection for preparative TLC was done on the basis of separation obtained in TLC plate 60 F_{254}. Different solvent systems were tried and the best resolution was obtained for Hexane: Acetone (8:2).

(iv) Plate development

The plates were developed by an ascending technique inside the glass TLC developing chambers. Inserted the plates into the chamber in a way that the tops lean against the chamber walls. The chamber was covered and let the solvent move up the plate by capillary action. When solvent reached the top of the plates, removed plates from the chamber and arranged in a standing position within the fume hood until solvent had evaporated (Kagan and Flythe, 2014).

(v) Component detection and isolation

The components separated were detected by using a UV chamber in visible, long and short UV range. The movement of the sample was monitored to find its retention factor (Rf). Each band was measured and expressed as Rf using the following formula;

$$Retension\ factor\ (Rf) = \frac{Distance\ travel\ by\ solute}{Distance\ travel\ by\ solvent}$$

The bands located were removed by scraping off with a spatula. Solvents were added and the adsorbent was separated by filtration and centrifugation (Hostettmann et al., 1998; Iborra et al., 1992; Kagan and Flythe, 2014; Sherma and Fried, 1987).

5.2.4. Antibacterial assay of separated fractions using disc diffusion method

To examine the antibacterial activity of components of the most bioactive crude extract, disc diffusion method was used. TLC separated and purified fractions were evaluated for antibacterial activity against Gram-positive and Gram-negative bacteria using two concentrations of sample (20 µL and 80 µL). Filter paper discs infused with each fraction were placed on agar plates inoculated with the bacteria. After incubation, the zone of inhibition was measured to determine the antibacterial activity of each fraction. Ethyl acetate (solvent used in the extraction), was used as negative control. Discs with ethyl acetate

showed no inhibition, verifying that the antibacterial activity originated from the fractions, not from the solvent (Sbhatu and Abraha, 2020; Subramaniam et al., 2020).

5.2.5. Characterization of most active fractions using NMR technique

Proton (^{1}H) NMR were done for identification of the compound that showed the highest antibacterial activity. The structure, dynamics, reaction state and chemical environment of molecules will all be clearly demonstrated through this method. The intra-molecular magnetic field surrounding an atom in a molecule alters the resonance frequency. This gives data on the electronic structure and individual functional groups of the compound. NMR analysis was done using the equipment Bruker Avance 400 MHz FT-NMR Spectrometer at Central Laboratory for Instrumentation and Facilitation, University of Kerala, Karivattom Campus Trivandrum.

5.3. RESULTS AND DISCUSSION

5.3.1. Selection of most bioactive sample

All the extracts of *A. sholaris* and *F. racemosa* were evaluated for their antioxidant potential using the RP and DPPH free radical scavenging assays. The obtained results from both assays were statistically analyzed to compare the antioxidant efficiency among the samples. Selection of the most active extracts was based on the following criteria: (i) percentage radical scavenging activity (ii) lowest IC_{50} values in both RP and DPPH assays, indicating higher antioxidant potency; (iii) consistent performance across both assays and (iv) statistically significant difference ($p < 0.05$) compared to the other samples. The highest antioxidant activity was observed in FR-GL (ethanol) and FR-GA (ethanol) based on these parameters.

Based on the antibacterial screening, the selection of the most active sample was carried out by considering the zone of inhibition (mm) against three bacterial strains (*E. coli*, *B. subtilis* and *S. aureus*). Among the samples of *A. scholaris*, AS-GA ethanolic extracts exhibited the largest inhibition zones across all tested bacteria (*E. coli*: 14.33 mm; *B. subtilis*: 13.5 mm; *S. aureus*: 14.16 mm) and was therefore considered the most active among samples of *A. scholaris*. In the case of *F. racemosa*, FR-GL (ethanol) showed the highest antibacterial

activity with maximum zones of inhibition recorded for *E. coli* (17.25 mm), *B. subtilis* (15.66 mm) and *S. aureus* (17.16 mm), surpassing both FR-NL and FR-GA extracts. Comparing the most active extracts of both plants, FR-GL demonstrated the overall strongest antibacterial potential, indicating it as the most potent sample. Similarly, in antifungal assays, AS-GA demonstrated substantial activity against *C. albicans* and *A. niger*, with maximum inhibition zones of 12.45 mm (IC_{50} - 0.548 mL) and 84.89% growth inhibition respectively. FR-GL again outperformed all other extracts, showing inhibition zones of 13.2 mm against *C. albicans* (IC_{50} - 0.534 mL), 90.41% growth inhibition against *A. niger*, closely approaching the standard antifungal drug, Itraconazole. Overall, across both antibacterial and antifungal assays, FR-GL emerged as the most potent extract.

The cytotoxic potential of aqueous, ethanolic and hexane extracts of *A. scholaris* and *F. racemosa* was evaluated based on percentage cell viability. Among the *A. scholaris* extracts, AS-GA showed significant cytotoxicity, particularly in the ethanolic extract, with a cell viability of 37.36%. In the case of *F. racemosa*, FR-GL demonstrated the highest cytotoxic effect overall, with the ethanolic extract exhibiting the lowest cell viability of 35.62%. In concentration dependent cytotoxic assays using FR-GL (ethanol) and AS-GA (ethanol), FR-GL showed a more gradual reduction in cell viability from lower concentrations (97.71 ± 2.54% at 50 mg/mL) to higher concentration (79.27 ± 3.94% at 200 mg/mL). In AS-GA (80.00 ± 0.65% at 50 mg/mL and 40.89 ± 2.69% at 200 mg/mL), while both reached comparable cytotoxicity at 400 mg/mL (9.40 ± 5.15% for FR-GL and 15.6 ± 5.8% for AS-GA). Overall, FR-GL shows the highest cytotoxic potential, effectively lowering cell viability at concentrations relevant for biological activity.

FR-GL demonstrated consistently higher activity across all tested assays, and statistical analysis verified its significant potency ($p < 0.05$), making it the preferred extract for subsequent fractionation and purification.

5.3.2. Optimization of chromatographic conditions of FR-GL plant extracts

The objective of TLC using pre-coated silica plates was to optimize a solvent system to effectively separate the most bioactive sample (FR-GL) into multiple compounds. Through systematic trials of various solvent systems, an optimal mobile phase of hexane : acetone in

an 8:2 ratio was determined, which successfully resolved seven distinct compounds. Plate 5.1: Figure A represents TLC analysis of FR-GL on pre-coated silica gel plate, shown under visible light, Figure B shows the TLC image under UV 254 nm and Figure C represents TLC analysis of FR-GL on pre-coated silica gel plate visible under UV 365 nm.

5.3.3. Preparative TLC analysis

The separation of FR-GL extract was done on plate prepared using silica gel G and water, shown in Plate 5.1: Figure D. The TLC analysis of FR-GL extract in hexane: acetone (8:2) resulted in seven bands. For each band Rf values vere calculated. The following Table 5.1 shows the Rf values of different bands separated from the FR-GL extract. The plant extract spotted in a plate and develops in a chamber saturated with solvent shown in Plate 5.1: Figure E and the plant extract separated into seven bands inside the chamber shown in Figure F. TLC plate images under visible light, under UV (254 nm) and under UV (365nm) were shown in Plate 5.2: Figure A, Figure B and Figure C respectively. The separated bands were scraped off (Plate 5.3: Figure A) and dissolved using ethyl acetate. Then the filtered samples were stored in glass containers for future use. The seven samples separated and collected were shown in Plate 5.3: Figure B.

Table 5.1: The retention factor values of the bands produced through preparative TLC analysis of FR-GL extract.

Sl. No.	Band number	Retention factor
1	Band 1	0.05
2	Band 2	0.07
3	Band 3	0.13
4	Band 4	0.19
5	Band 5	0.23
6	Band 6	0.27
7	Band 7	0.37

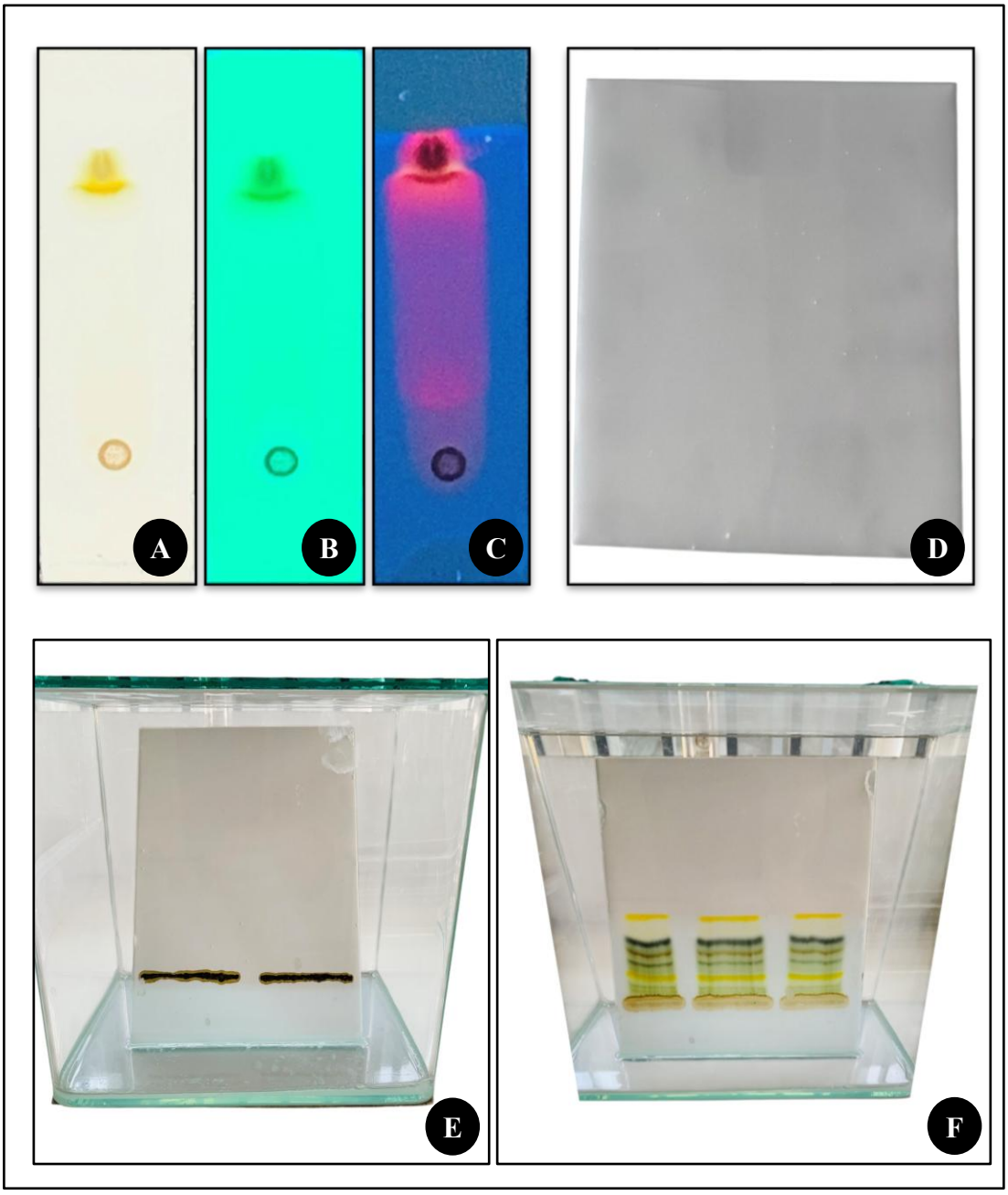


Plate 5.1: Figure A: TLC analysis of FR-GL using pre-coated silica gel plate, shown under visible light, Figure B: Pre-coated silica gel plate for separation of FR-GL under UV 254 nm, Figure C: TLC on pre-coated silica gel plate visible under UV 365 nm, Figure D: TLC plate prepared using silica gel G and water is drying at room temperature, Figure E: FR-GL extracts spotted in the plate and developing in chamber saturated with solvent and Figure F: Separation of the extract into distinct bands inside the chromatographic chamber.

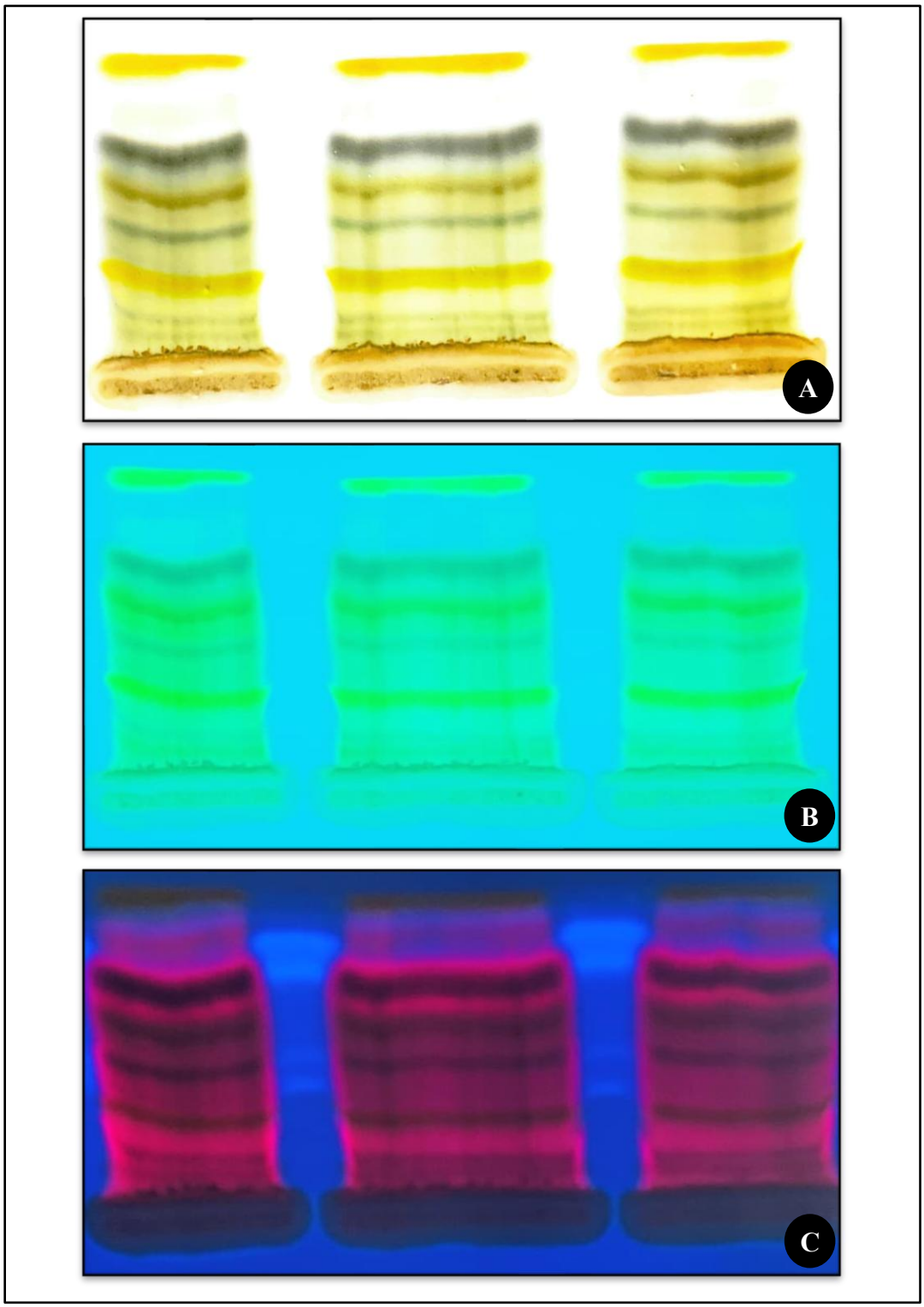


Plate 5.2: Figure A: TLC separated plate images under visible light, Fig B: TLC plate showing separated bands under UV light of 254 nm and Figure C: TLC separated plate images under UV light (365nm).



Plate 5.3: Figure A: Scraping off and separation of compounds from the silica gel plate and Figure B: Seven distinct compounds isolated from FR-GL extract, labeled as R1–R7.

Kumar and colleagues performed HPTLC analysis of *F. racemosa* leaves and resolved bands for quercetin and kaempferol (Kumar et al., 2019). TLC of leaf extract in ethyl acetate and acetone reports three spots for various phytochemicals (Ganatra et al., 2012). FR-GL extract analysed by TLC in the present work produced 7 distinct bands for various phytochemicals. *F. racemosa* stem bark were analysed by TLC technique in reported work and found that stem bark and small branches have similar phytochemical profile (Verma et al., 2015). Stem bark was also analysed by Solanki and Bhavsar in 2015 proved that alcoholic fraction were rich with highly polar compounds such as polyphenols, flavonoids, alkaloids, etc., than aqueous extract (Solanki and Bhavsar, 2017). In 2021, TLC was performed on fruit extract of *F. racemosa* separated 3 different compounds (Anand et al., 2021).

5.3.4. Results of antibacterial activity assay of separated and purified fractions

As mentioned in the previous section, preparative thin-layer chromatography yielded seven distinct bands, designated as compounds C1, C2, C3, C4, C5, C6 and C7. These compounds were tested for antibacterial activity using the disc diffusion method against *S. aureus*, *B. subtilis* and *E. coli* at two concentrations (20 µL and 80 µL), with ethyl acetate as the negative control.

Against *S. aureus*, only compound C1 was active, producing an inhibition zone of 10 mm at 20 µL, with no increase in activity at 80 µL (10 mm), suggesting a plateau in efficacy. The resulted plate images showing the activity were represented in Plate 5.4: Figure A and Figure B represent the activity of 20 µL and 80 µL respectively. Against *E. coli*, compounds C5 and C7 were active. At 20 µL, C5 produced an inhibition zone of 11 mm (Figure C), which increased to 15 mm at 80 µL (Figure D), indicating concentration-dependent activity. C7 produced an inhibition zone of 11.5 mm at 20 µL (Figure C), with a slight increase to 12 mm at 80 µL (Figure E), suggesting limited concentration dependence. Compounds C2, C3, C4 and C6 showed no activity against any of the tested bacteria at either concentration. None of the compounds (C1-C7) exhibited activity against *B. subtilis* at either concentration, producing no inhibition zones (Figure F).

The results were summarized in Table 5.2, indicating that C1, C5 and C7 are the primary active compounds in FR-GL ethanolic extract, with selective activity against *S. aureus* and *E. coli*. The highest zone of inhibition was produced by the compound C5 (15 mm), so this fraction of FR-GL was selected for identification through NMR technology. The selective activity of these compounds suggests that FR-GL antibacterial efficacy is driven by specific phenolic or flavonoid components, warranting further chemical characterization. The lack of replicate measurements due to limited extract quantities and time constraints is a limitation and future studies should include triplicate analyses to confirm reproducibility. Plate 5.5: Figure A shows the heat map of the results of antibacterial activity of different fractions obtained.

No previous studies have reported the bioactivity of fractions from galls or other parts of *F. racemosa*. In contrast, the bioactive fractions of stem bark extract of *Ficus ovata* are reported to exhibit notable antimicrobial potential against a range of fungal and bacterial species. The crude extract and fractions 2 and 4, showed broad-spectrum activity, while other fractions displayed more selective effects, highlighting the differential distribution of bioactive constituents within the extract (Kuete *et al.*, 2009). Kuete and colleagues in 2011 did vacuum flash chromatography of *Ficus polita* roots and yielded five fractions (FPR1-FPR5). Among these, fractions FPR1 and FPR2 exhibited broad-spectrum antimicrobial activity, effectively inhibiting the growth of all eight tested microorganisms, including *Salmonella typhi*, *E. coli* and *C. albicans* (Kuete *et al.*, 2011). Here, this study separated 7 distinct compounds from the gall leaves of the *F. racemosa* plant and among the compound 3 were active against the tested bacteria.

Table 5.2: Antibacterial activity analysis of the separated products of FR-GL, showing zone of inhibition in mm produced by 2 different dilutions of compound.

Name of bacteria tested	Compound	Zone of inhibition (mm) of 20 µL sample	Zone of inhibition (mm) of 80 µL sample
Bacillus subtilis	C1	No activity	No activity
	C2	No activity	No activity
	C3	No activity	No activity

	C4	No activity	No activity
	C5	No activity	No activity
	C6	No activity	No activity
	C7	No activity	No activity
Staphylococcus aureus	C1	10	10
	C2	No activity	No activity
	C3	No activity	No activity
	C4	No activity	No activity
	C5	No activity	No activity
	C6	No activity	No activity
	C7	No activity	No activity
Escherichia coli	C1	No activity	No activity
	C2	No activity	No activity
	C3	No activity	No activity
	C4	No activity	No activity
	C5	11	15
	C6	No activity	No activity
	C7	11.5	12

5.3.5. Results of Nuclear magnetic resonance (NMR) spectrometry analysis

The selected bioactive fraction (C5) was subjected to proton nuclear magnetic resonance (^{1}H NMR) spectroscopic analysis to identify the functional groups and structural features of the compound. The sample was dissolved in methanol and analyzed at 400 MHz. The obtained spectrum (Plate 5.5: Figure B) displayed several smaller peaks between δ 3.3-3.9 ppm, attributed to trace impurities. The minor signals observed in the δ 1.0-2.8 ppm region suggest the presence of aliphatic protons, while weak peaks between δ 7.0-7.7 ppm indicate possible aromatic protons environment. However, absence of distinct, well-resolved

peaks characteristic of a single compound implies that the fraction likely contains a mixture of components or impurities rather than a pure metabolite. Therefore, the NMR data were insufficient for definitive structure elucidation. Further purification and complementary analyses are required for the identification of the active component.

5.4. CONCLUSIONS

Optimized TLC conditions for FR-GL using pre-coated silica plates and then preparative TLC was done. The sample was separated into of seven different compounds using preparative TLC. The seperated fractions were purified and subjected to antibacterial assays to identify the most active fraction. Among these, the fraction labeled as C5 exhibited the highest antibacterial activity against the selected bacterial strains. The C5 fraction was subsequently analyzed using ^{1}H NMR spectroscopy and due to the absence of distinct, well-resolved peaks characteristic of a single compound implies that the fraction likely contains a mixture of components or impurities rather than a pure metabolite, but a mixture of structurally related components. Therefore, the NMR data were insufficient for definitive structure elucidation.

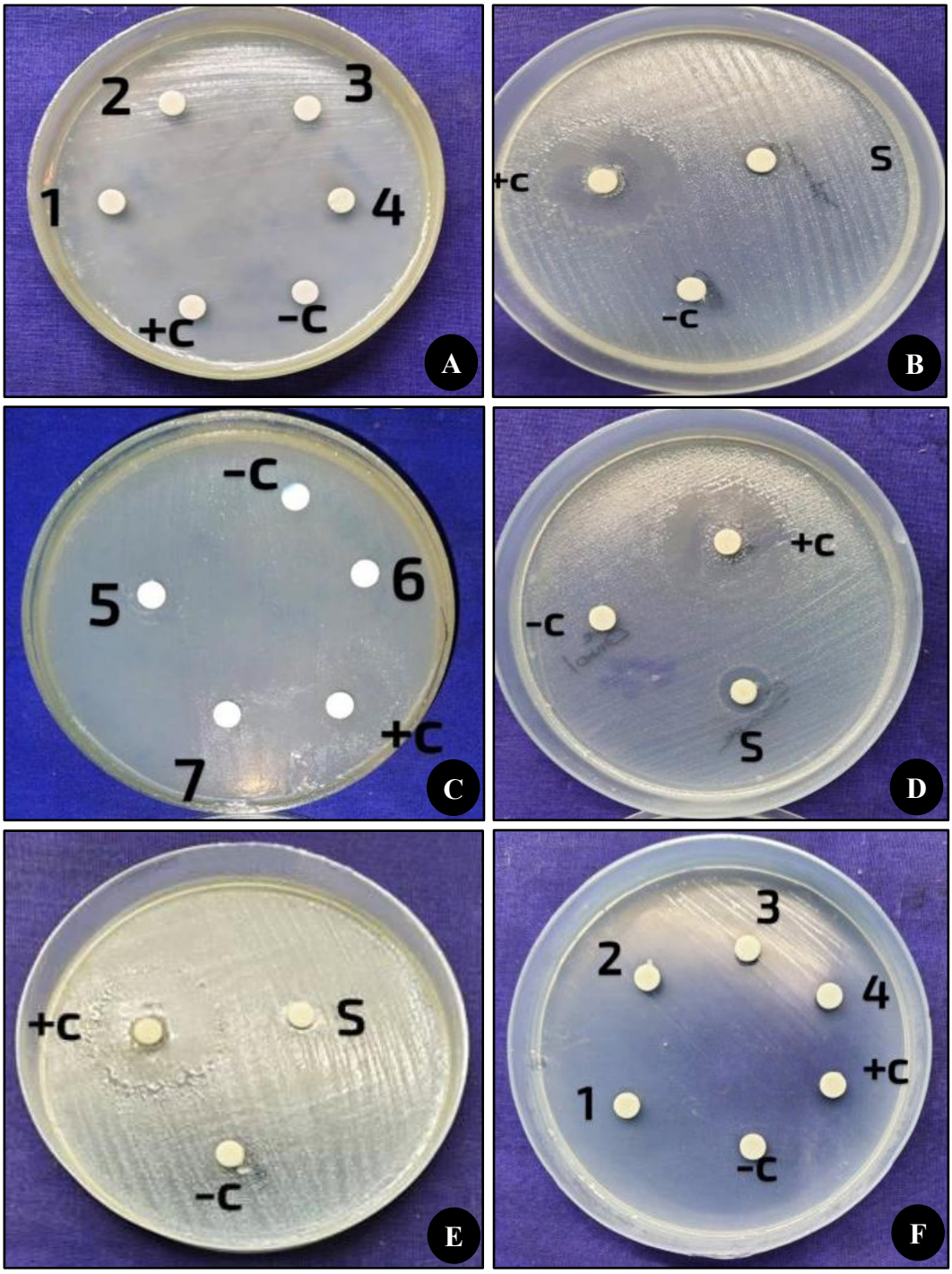


Plate 5.4: Antibacterial activity of TLC separated products: Figure A: Antibacterial activity of C1 (20 µL) against *S. aureus*, Figure B: Zone of inhibition produced by C1 (80 µL) sample (S) against *S. aureus*, Figure C: Antibacterial activity shown by C5 (20 µL) and C7 (20 µL) against *E.coli*, Figure D: Zone of inhibition produced by C5 (80 µL) sample (S) against *E. coli* along with positive and negative controls, Figure E: Antibacterial activity shown by C7 (80 µL) sample (S) against *E. coli* and Figure F: No activity detected by any TLC separated compounds against *B. subtilis*.

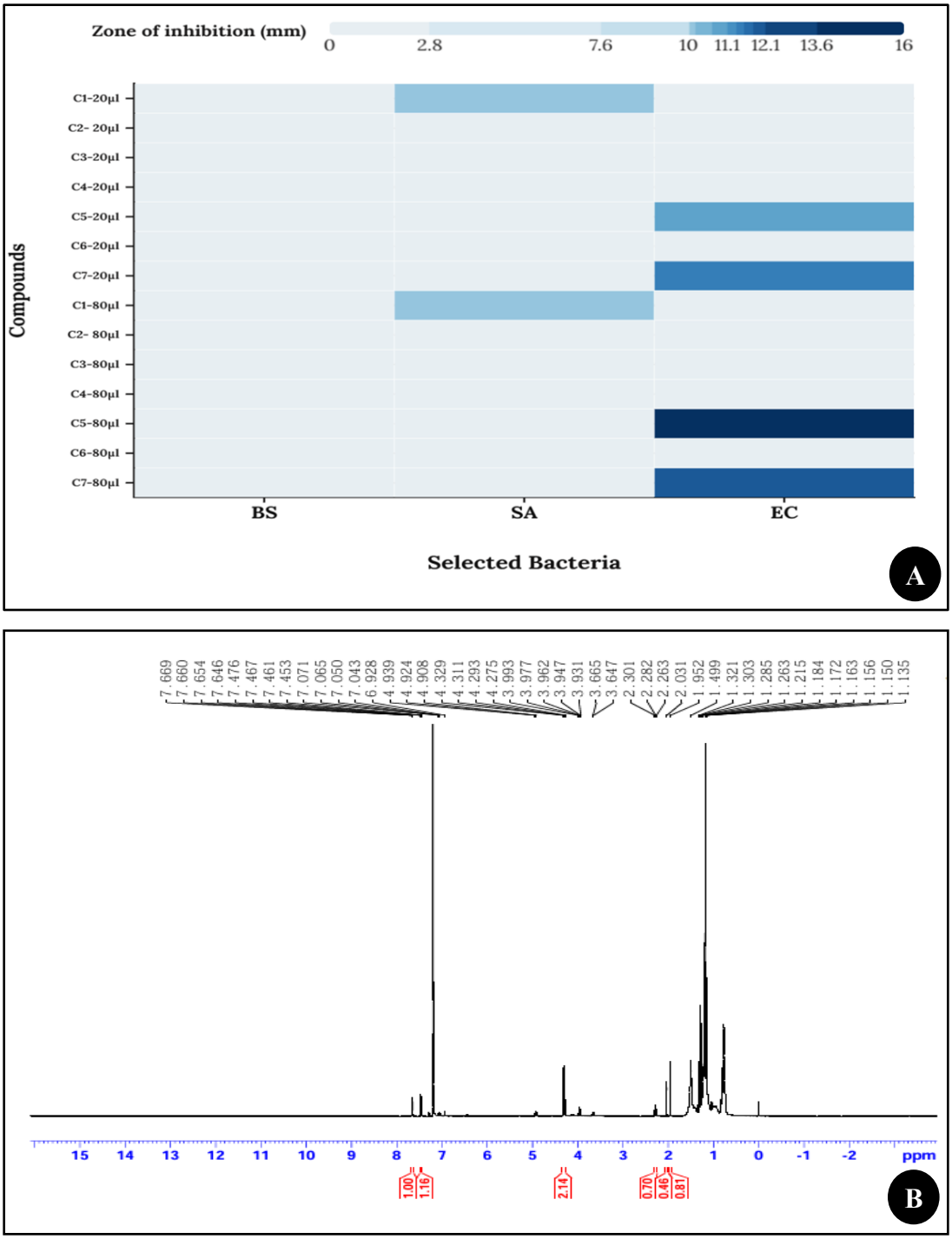


Plate 5.5: Figure A: Heat map representing the antibacterial activity of seven fractions separated from FR-GL ethanolic extract using preparative TLC technique (BS- *B. subtilis,* SA- *S. aureus* and EC- *E. coli*) and Figure B: The 1H NMR spectrum obtained from analysis of the compound C5 in methanol.

CHAPTER 6 - EXPRESSION PROFILING OF GENES INVOLVED IN THE SECONDARY METABOLIC PATHWAY FROM DIFFERENT SAMPLES

6.1. INTRODUCTION

Secondary metabolic pathway in plants helps to adapt to environmental stresses by producing bioactive compounds, critical for stress response and defense. This chapter examines gene expression changes in *Alstonia scholaris* and *Ficus racemosa* due to gall induction by *Pauropsylla* species, focusing on selected genes. Using quantitative real-time PCR (qRT-PCR), the gene expression is compared between normal leaves, galled leaves and galls to test the hypothesis that gall induction changes the expression of genes, enhancing phenolic biosynthesis. This study is to elucidating the molecular mechanisms, to addresses gaps in understanding gall-induced metabolism in medicinal plants, offering insights for phytopharmacological applications (Mir et al., 2015).

Secondary metabolites are chemical compounds that are not involved directly in the primary metabolic processes of plants, but they have function in defense systems, pollinator attraction and interactions with other species. Chapter 3 explains the use of these phytochemical compounds for mankind as they possess various biologically significant properties. The major classes of secondary metabolites are terpenoids, phenolics and nitrogen-containing compounds, which include alkaloids, cyanogenic glycosides and glucosinolates. Each category of secondary metabolites is synthesised through distinct biosynthetic pathways. They also function in the interaction of the plant with its environment. Here in this chapter, we will be looking deep into the pathways involved in this secondary metabolic production. The present work mainly focused on the production of phenolic compounds as these are found to be expressed as major compounds after gall induction by insects. It means phenolic compounds may be the important defensive compound produced by the plants as defence against the action of the gall inducers (Abrahamson et al., 1991; Hartley, 1998; Taiz and Zeiger, 2010).

Reshma K Ramesh. Comparative phytochemical analysis and gene expression profiling of leaf galls on selected medicinal plants. Thesis 2025. Govt. Victoria college, Palakkad, University of Calicut.

6.1.1. Secondary metabolites and plant defence

Secondary metabolites are pivotal in enabling plants to withstand biotic and abiotic stresses, with phenolic compounds playing a central role in defense against insect-induced gall formation in *A. scholaris* and *F. racemosa.* Biotic stresses, such as infection or attack by other organism and abiotic stresses, including UV radiation and drought will trigger the biosynthesis of secondary metabolites, regulated by intricate phytohormone-mediated signaling pathways (Akula and Ravishankar, 2011; Ashraf et al., 2018; Saddique et al., 2018). Phenolics are synthesized primarily through the shikimic acid and phenylpropanoid pathways, encompass flavonoids, lignin, phenolic acids, stilbenes and tannins, which confer antioxidant, antibacterial and insect-repellent properties (Sharma et al., 2019; Taiz and Zeiger, 2010). This study hypothesizes that gall induction enhances phenolic production, fortifying plant resistance and yielding bioactive compounds with therapeutic potential, as evidenced in phytochemical analysis in Chapter 3.

Plants employ both constitutive and induced defense mechanisms to counter biotic stressors like insects and pathogens. Induced defenses include systemic acquired resistance (SAR), induced systemic resistance (ISR) and hypersensitive responses (HR). Induced defese involve cellular changes such as cell wall lignification, reactive oxygen species (ROS) generation and accumulation of pathogenesis-related (PR) proteins (Kortekamp, 2006). These responses rely on transcriptional activation of defense genes, encoding enzymes, transcription factors and proteinase inhibitors that enhance resistance (Boava et al., 2011; Duan et al., 2014). In *A. scholaris* and *F. racemosa*, gall induction by *Pauropsylla* likely upregulates phenolic biosynthesis, with flavonoids acting as phytoalexins to deter insect feeding and lignin reinforcing gall tissues against further invasion. The upregulation of phenolic pathways is critical, as these compounds not only limit insect damage but also contribute to plant-microbe interactions, facilitating communication and symbiosis under stress (Mohammadi et al., 2012).

Beyond their role in defense, the significance of phenolics extends to phytopharmacology, as gall tissues rich in flavonoids and phenolic acids found to exhibit antimicrobial and antioxidant activities (Chapter 4). By focusing on gall-induced phenolic

accumulation, this study addresses a gap in understanding how medicinal plants respond to specific biotic stressors, offering insights into metabolic engineering for enhanced bioactive compound production. This chapter reveals the molecular mechanisms behind the defense response and their relation to secondary metabolites that enhance plant resilience and therapeutic potential.

6.1.2. Secondary metabolic pathways and important genes

The shikimic acid and phenylpropanoid pathways are two essential pathways for phenolic biosynthesis which is critical for plant defense against biotic stresses like attack by gall inducer. The shikimic acid pathway, a seven-step process, produces chorismate, a precursor to aromatic amino acids (phenylalanine, tyrosine and tryptophan) that feed into secondary metabolism (Mir et al., 2015).

The pathway begins with 3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS), which catalyzes the condensation of phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate to form 3-Deoxy-D-arabino-heptulosonate-7-phosphate (DAHP). In the next step, 3-dehydroquinic acid (DHQ) is produced by cycling DAHP after eliminating its phosphate. This cyclization is catalysed by 3-dehydroquinate synthase (DHQS). DHQ is converted to 3-dehydroshikimic acid (DHS) by the enzyme 3-dehydroquinate dehydratase. A reduction reaction involving DHS and nicotinamide adenine dinucleotide phosphate (NADPH) produces shikimic acid. In the next step, shikimic acid is activated with adenosine triphosphate (ATP) to form shikimic acid 3-phosphate (S3P). The enzyme catalyzes this reaction is shikimate kinase (SK). PEP is then added to S3P to produce 5-enolpyruvyl shikimic acid 3-phosphate (EPSP). The conversion is catalysed by the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Chorismate synthase (CS) catalyses the seventh and final step in the shikimic acid pathway, *i.e.*, conversion of EPSP to chorismate. Chorismate mutase catalyses the reaction of chorismate to prephenate, the first committed step in phenylalanine and tyrosine biosynthesis. The pathway products are the precursors of an array of natural products (Mir et al., 2015; Ralph et al., 2006b).

The phenylpropanoid pathway transforms phenylalanine into diverse phenolics, including flavonoids and lignins, vital for gall-induced defense. Phenylalanine ammonia-

lyase (PAL) initiates the pathway by converting phenylalanine to trans-cinnamic acid, a critical step regulating carbon flux from primary to secondary metabolism (Dae and Hwang, 2014). The trans-cinnamic acid is hydroxylated to 4-coumarate by the action of cinnamic acid 4-hydroxylase (C4H) activity. Then 4-coumarate is converted to 4-coumaroyl-CoA by 4-coumarate-CoA ligase (4CL). The synthesis of flavonoids, which begins with the generation of chalcones, is initiated by the incorporation of 4-coumaroyl-CoA into the flavonoid biosynthesis pathway. Chalcone synthase (CHS) combines one molecule of 4-coumaroyl-CoA with three molecules of malonyl-CoA to form naringenin chalcone. The chalcone is converted into a flavanone by the enzyme chalcone-flavanone isomerase (CHI) (Keykha et al., 2016).

The pathway split into various branches from these centre intermediates, each of which produces a distinct class of flavonoids. Flavanones are then subsequently transformed by flavanone 3-hydroxylase (FHT) and by dihydroflavonol 4-reductase (DFR) to leucoanthocyanidins (flavanols), which are the precursors of both anthocyanidins and flavan-3-ols (Hua et al., 2013; Lillo et al., 2008). DAHPS, EPSPS, CS, PAL, CHI and DFR were selected for this study due to their regulatory roles in channeling carbon toward phenolics, hypothesized to be upregulated in galls induced by *Pauropsylla* species. The shikimic acid and phenylpropanoid pathway is shown in Plate 6.1 and 6.2 respectively.

6.1.3. Gene expression profiling

Gene expression is the process through which DNA is transcribed into RNA and subsequently translated into proteins, it forms the basis of all biological functions and represents the central dogma of molecular biology. Differential gene expression enables cells with identical genetic information to perform specialized roles, shaping tissue-specific phenotypes and organismal responses to environmental stimuli, such as gall induction in *A. scholaris* and *F. racemosa* (Busch and Lohmann, 2007). In this study, gene expression profiling is employed to investigate how *Pauropsylla*-induced galls alter the expression of six genes (DAHPS, EPSPS, CS, PAL, CHI and DFR) in the shikimic acid and phenylpropanoid pathways, hypothesizing that gall formation upregulates these genes to enhance phenolic biosynthesis, as detailed in the Chapter 3.

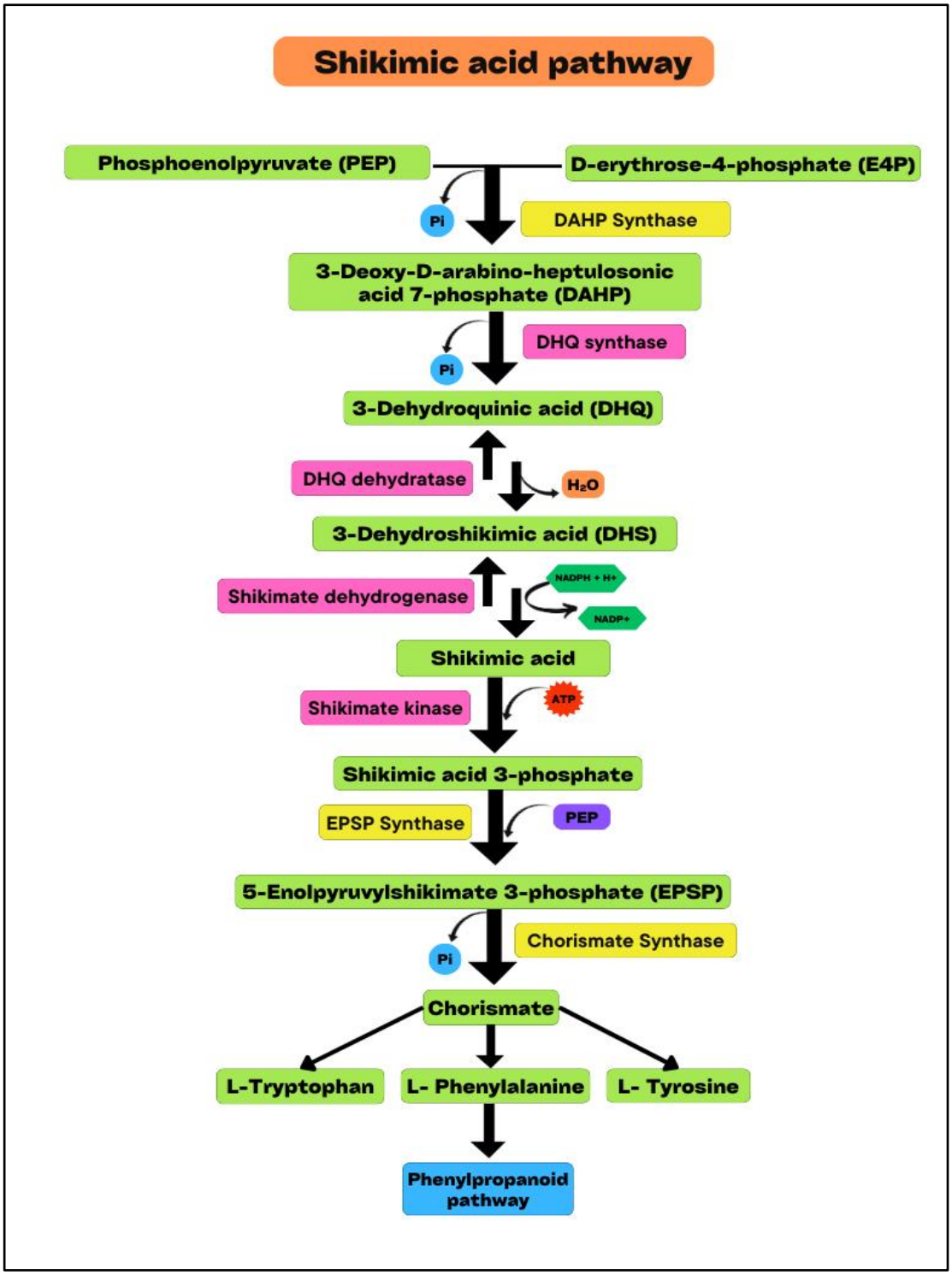


Plate 6.1: Diagrammatic representation of shikimic acid pathway with important genes and pathway products. Genes selected for the present expression analysis are shown in yellow boxes (Liu et al., 2014).

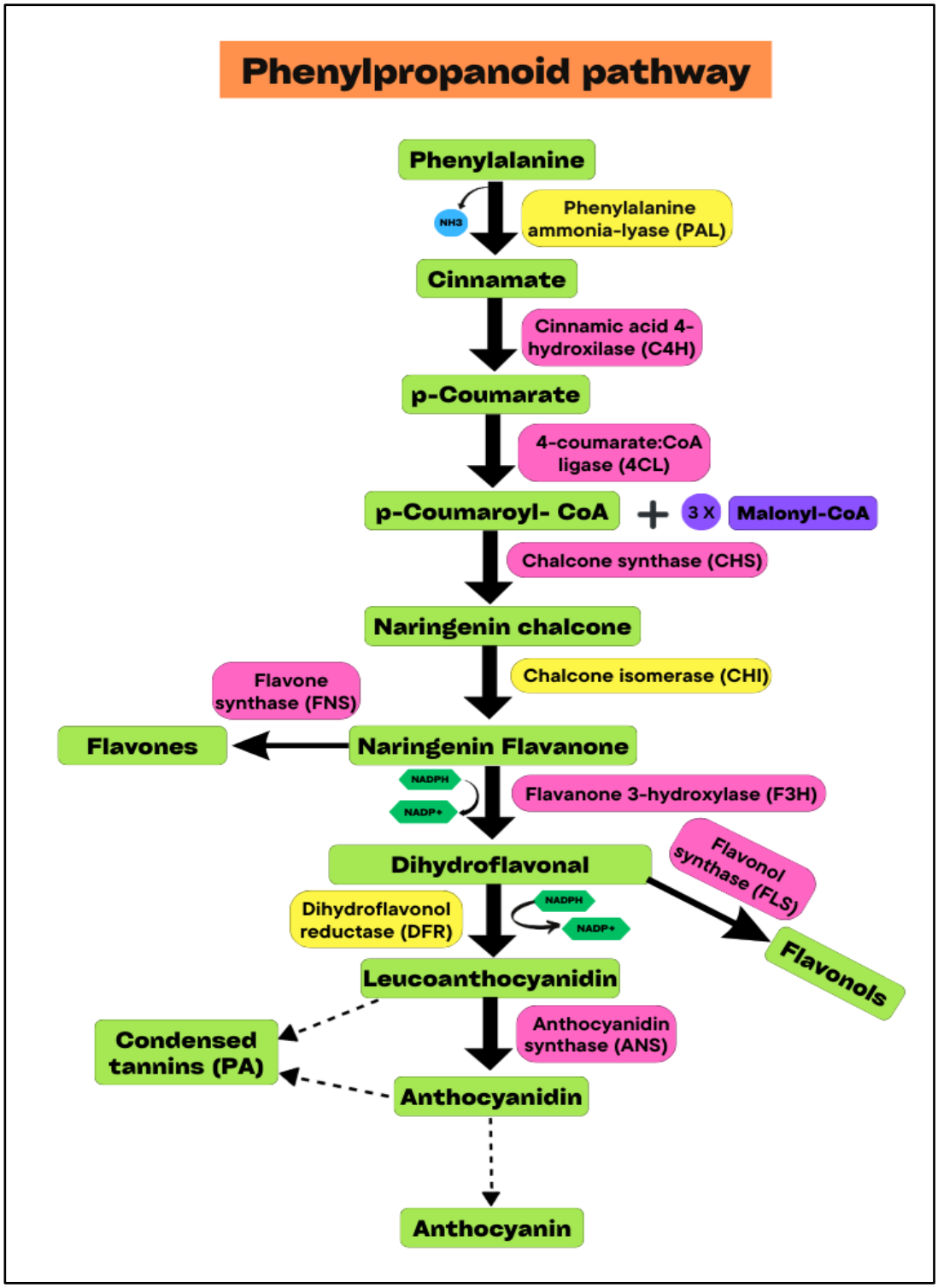


Plate 6.2: Diagrammatic representation of phenylpropanoid pathway. Genes selected for the present expression analysis are shown in yellow boxes. Dotted line arrow represents multiple steps (Ali et al., 2011; Choi et al., 2021).

Different techniques can be used for gene expression profiling. Northern Blotting, Reverse Transcription PCR, *In Situ* Hybridization are conventional methods for expression profiling. The foundation for our current knowledge of gene regulation and expression was established based on these gene expression profiling techniques (Bachman, 2013; Dozois et al., 1997; Drea et al., 2005; Kumlauf, 1991). Depending on their underlying technology, expression profiling techniques fall into three categories: PCR based, sequencing based and hybridization-based approaches. Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) is a PCR based method, which allows simultaneous profiling of gene expression for a limited number of genes. Serial analysis of gene expression (SAGE) is one of the sequencing-based expression analysis methods. Nowadays RNA sequencing technologies using next generation sequencing (NGS) methods are widely employed for profiling. It analyses transcriptomes with high speed and accuracy. It is a powerful tool for measurement which is practical and affordable (Matsumura et al., 2010; Yamamoto et al., 2001).

Microarrays gene expression uses hybridization as a basic principle. Microarrays consist of DNA probes that are attached to a solid support that hybridise with complementary RNA or cDNA samples. The hybridisation of nucleic acid leads to the emission of signals as fluorescence. The signal intensity is directly proportional to the level of expression. Microarray helps in the measurement of a large number of genes simultaneously. It can be used for analysis of known genes and transcripts. Signal saturation at the high end and background at the low-end limit signal measurement (Galbraith, 2006; Jain, 2012; Seki et al., 2010).

The choice of qRT-PCR reflects its ability to quantify subtle expression changes in response to *Pauropsylla*-induced stress, offering insights into how gall formation drives phenolic accumulation. By profiling DAHPS, EPSPS, CS, PAL, CHI and DFR, this study addresses gaps in understanding molecular mechanisms underlying gall-induced metabolism in medicinal plants for elucidating plant defense strategies and informing phytopharmacological applications, such as developing gall-derived therapeutic compounds.

Quantitative real-timePCR

Quantitative real-time PCR (qRT-PCR) is a molecular technique for analyzing gene expression. It works on the polymerase chain reaction (PCR), which amplifies DNA using primers and a thermostable Taq polymerase from *Thermus aquaticus*. qRT-PCR enables precise, real-time quantification of mRNA levels (Erlich, 1989; Zhu et al., 2020). Unlike traditional PCR, qRT-PCR monitors amplification through fluorescent reporters. It uses threshold cycle (Ct), the point where fluorescence exceeds background-indicating mRNA abundance for expression analysis. Lower Ct values reflect higher gene expression, making qRT-PCR ideal for detecting small changes in response to biotic stress. Three steps make up the qRT-PCR process: first, RNA is converted to cDNA using reverse transcriptase enzyme; second, the cDNA is amplified using PCR and third, is the quantification of amplified products (Derveaux et al., 2010; Jozefczuk and Adjaye, 2011).

It has high sensitivity, speed and accuracy, make it ideal method for the present study. It targets a small gene set (less than 10) to investigate gall-induced upregulation in normal leaves, galled leaves and galls. qRT-PCR has the ability to compare expression across tissues under experimental conditions, such as biotic stress and has established it as a cornerstone for plant molecular studies (Chen et al., 2009). For instance, qRT-PCR has revealed gene expression changes in plants under insect herbivory, supporting its application to use in the expression analysis of gall induction systems.

By elucidating how gall induction alters secondary metabolism, qRT-PCR provides insights into plant defense mechanisms and their pharmacological potential. This targeted approach not only advances our understanding of plant-insect interactions but also informs strategies for metabolic engineering and stress-resistant crop breeding (Chen et al., 2009). Detailed study of differential gene expression might lead to a better understanding of plant physiology. It will also help to uncover the physiological and molecular processes associated with plant interactions.

6.2. METHODOLOGY

6.2.1. Plant material collection and storage

Healthy leaves and gall possessing leaves were collected from both *A. scholaris* and *F. racemosa* plants with care to avoid contamination with RNases. The galls were separated from gall leaves immediately after collection. All the collected samples were stored in liquid nitrogen soon after collection. The samples were stored at -80 °C in a deep freezer until RNA extraction (Bilgin et al., 2009).

6.2.2. Extraction of total RNA

To extract RNA from the selected plant samples, various RNA extraction techniques were done. They include the Guanidine thiocyanate (GTC) method, Lithium chloride precipitation, Trizol method, modified Trizol method and modified method from Chan and colleagues (Chan et al., 2007). However, due to the increased concentrations of secondary compounds in the selected plant tissues, none of these approaches were able to isolate pure RNA from the plant tissues. Phenolic chemicals prevent pure RNA from being isolated from the tissues. Latex content in the tissue is also intercepted in the isolation of pure RNA. Using various doses of polyvinylpyrrolidone (PVP), we modified the methods in order to reduce the interference of phenolics in RNA extraction. There observed a little impact (Johnson, 2023; Shu et al., 2014; Vennapusa et al., 2020).

Due to the inability of above-mentioned methods to isolate RNA, we used Phytolyze Reagent (Origin) following the manufacturer protocol for RNA extraction and it worked in the tissues selected. The total RNA was isolated from normal leaves, gall leaves and gall of both plants using Phytolyze Reagent. It is a quick and convenient reagent for use in the simultaneous isolation of RNA from plants. Phytolyze Reagent performs well with large or small amounts of plant tissues. Prior to isolation of RNA, mortars and pestles were autoclaved at a temperature of 121 °C and 15 psi for 30-45 min. Mortar and pestle were stored at -20 °C overnight before use. Eppendorf tubes, tips, forceps etc. were also double autoclaved to remove any RNAse contamination.

RNA extraction procedure

Total RNA was extracted from plant tissues using Phytolyze Reagent following a standard protocol. Fresh plant samples were first frozen in liquid nitrogen and finely ground using a pre-cooled mortar and pestle. Approximately 100 mg of the powdered tissue was transferred into a 1.5 mL microcentrifuge tube. To this, 700 µL of Phytolyze Reagent and 20 µL of β-mercaptoethanol were added, followed by vortexing for 10-20 seconds to ensure complete homogenization. The mixture was then incubated at 65 °C for 30 min, with intermittent vortexing.

After incubation, 700 µL of chloroform was added to the lysate, vortexed and centrifuged at 12,000 rpm (~13,400 ×g) for 10 min. The upper aqueous phase was carefully transferred to a new RNase-free tube, avoiding the interphase. For RNA precipitation, 700 µL of ice-cold isopropanol was added to the supernatant, mixed by gentle inversion and incubated at room temperature for 5-10 min. The mixture was then centrifuged at 12,000 rpm for 10 min at 2-8 °C to pellet the RNA.

The resulting RNA pellet was washed with 75% ethanol (1 mL per 700 µL of Phytolyze Reagent used), vortexed and centrifuged again at 12,000 rpm for 5 min at 2-8 °C. The pellet was then briefly air-dried for 5-10 min to remove residual ethanol, taking care not to overdry. The RNA was resuspended in an appropriate volume of RNase-free water by gentle pipetting, followed by incubation at 55-60 °C for 10-15 min to enhance solubilization. To eliminate any contaminating genomic DNA, the resuspended RNA was subsequently treated with DNase I.

Evaluation of RNA quality

For each sample, RNA concentration was determined using a UV-visible spectrophotometer (Eppendorf, Thermomixer Comfort) by measuring absorbance at 260 nm. The 260:280 optical density (OD) ratio of RNA was checked to find the quality of RNA (Vennapusa et al., 2020).

Agarose gel electrophoresis to check purity and integrity of RNA

1.2% agarose gel was prepared by dissolving 1.2 g agarose in 100 mL of 1× Tris acetate EDTA (TAE) buffer. The mixture was heated in a microwave oven until the agarose

was completely dissolved. After cooling down to 50 °C, 2 µL of ethidium bromide (EtBr) (10 mg/mL) was added to the melted agarose, mixed well. The gel was added into a casting tray and a comb was placed in order to form wells. After the gel was solidified, it was submerged in a 1× TAE buffer within the electrophoresis tank. An aliquot of 8 µL RNA was mixed with the 2 µL 10× RNA loading dye and placed into each well. 10× RNA loading dye was prepared by mixing 3.9 mL Glycerol, 0.5 mL 10% SDS, 0.2 mL 0.5 M EDTA, 25 mg Bromophenol Blue and 25 mg Xylene Cyanol. The mixture was made up to 10 mL with RNase-free water.

The electrophoresis was run at 60 volts for 45 min or till dye front reached to 3/4th part of the gel (Armstrong and Schulz, 2015; Sambrook and Russell, 2001; Vennapusa et al., 2020). After that, a UV transilluminator was used to view the RNA bands and a gel image was taken as record.

6.2.3. Complementary DNA (cDNA) synthesis

Isolated RNA, which is treated with DNase I and then subjected to reverse transcription PCR reaction using a commercial kit (PrimeScript™ 1st Strand cDNA Synthesis Kit, TAKARA) according to the manufacturer's instructions.

Procedure of cDNA synthesis

The reaction was set up by mixing 5 µL RNA, 0.5 µL oligo dT primer, and 12 µL nuclease-free water, followed by incubation at 65 °C for 5 min and immediate chilling on ice for 2 min. Subsequently, 5 µL 5× reaction buffer, 1 µL 10 mM dNTPs, 0.5 µL RNase inhibitor (RNase I) and 1 µL reverse transcriptase enzyme were added to the vial. The mixture was incubated at 42 °C for 60 min in a Gradient Master Cycler (HIMEDIA Wee 32) and then heated at 70 °C for 10 min, after which the cDNA was collected. Then it was diluted into 1:10 ratio with sterile double distilled water and stored at -80 °C to use for PCR reactions (Sambrook and Russell, 2001).

Genes selected for the study

Initially, a literature search was done and identified 10 genes from both the phenylpropanoid and shikimic acid pathways for gene expression analysis. Following an

initial screening, six genes were selected for further investigation. Table 6.1 presents the genes selected for the current expression profiling analysis.

Table 6.1: List of selected genes with their reaction substrates and products

Sl. No.	Name of gene	Substrates for the reaction	Product of the reaction
1	3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS)	Phosphoenolpyruvate (PEP) and Erythrose-4-phosphate (E4P)	3-Deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP) and Phosphate
2	5-Enolpyruvylshikimate 3-phosphate synthase (EPSPS)	Shikimic acid 3-phosphate (S3P) and Phosphoenolpyruvate (PEP)	5-Enolpyruvylshikimate 3-phosphate (EPSP) and phosphate
3	Chorismate synthase (CS)	5-Enolpyruvylshikimate 3-phosphate (EPSP)	Chorismate and phosphate
4	Phenylalanine ammonia lyase (PAL)	Phenylalanine	Trans-cinnamic acid and ammonia
5	Chalcone-flavanone isomerase (CHI)	Chalcone	Flavanone
6	Dihydroflavonol 4-reductase (DFR)	Dihydroflavanone	Leucoanthocyanidins

6.2.4. Primer design and synthesis

Due to the lack of genomic sequences for *A. scholaris* and *F. racemosa* in the NCBI GenBank, the gene specific primers for real time amplification were designed by using blast results of similar accessions selected from NCBI. The accessions selected and used were listed in Table 6.2. Conserved regions of each gene were identified from blast results and the primers were designed using conserved sequences. The best set of primers was identified based on the region to be amplified. FAST PCR primer design software (version 6.7) was used for this purpose. The parameters considered for primer design were primer size of 19-22 bp, Tm of 55-62 ºC, GC% of 50-60%, product size of 100-300 bp and rest of the parameters

were set to default (Li et al., 2011). The designed primer sequences were given to manufacture for synthesis (Sigma, Bangalore, India).

Table 6.2: Gene accessions used for primer design, with their accession numbers and plant species.

Sl. No.	Name of selected genes	Plant species	Accession no.
1	DAHP synthase (DAHPS)	*Hevea brasiliensis*	XM_021786494.1
		Mangifera indica	XM_044652266.1
		Citrus sinensis	XM_006472513.3
		Pistacia vera	XM_031395831.1
2	EPSP synthase (EPSPS)	*Solanum tuberosum*	XM_006347816.2
		Capsicum annuum	XM_016719734.2
		Nicotiana tabacum	NM_001325913.1
		Coffea arabica	XM_027250652.1
3	Chorismate synthase (CS)	*Manihot esculenta*	XM_021770219.2
		Ricinus communis	XM_015725629.2
		Mangifera indica	XM_044651584.1
4	Phenylalanine ammonia-lyase (PAL)	*Manihot esculenta*	XM_021766727.2
		Hevea brasiliensis	XM_021804780.1
		Ricinus communis	XM_002519475.3
		Mangifera indica	XM_044617478.1
5	Chalcone-flavanone isomerase (CHI)	*Hevea brasiliensis*	XM_021806745.1
		Pistacia vera	XM_031394262.1
		Triadica sebifera	MN906941.1
		Rhus chinensis	KC287083.1
6	Dihydroflavonol 4-reductase (DFR)	*Solanum lycopersicum*	NM_001247479.2
		Solanum pennellii	NM_001374410.1
		Lycium ruthenicum	KY287797.1
		Nicotiana sylvestris	XM_009761654.1

Amplification of selected genes

Target genes were amplified by running the gradient PCR using cDNA samples and gene specific primers. These amplified products of each gene were eluted out, diluted into different concentrations and used for primer efficiency analysis. PCR reaction conditions were optimized based on primer and target sequences. Trials were done with different annealing temperatures ranging from 52-64 °C. Finally optimised the annealing temperature for all the targets as 60 °C (Riet et al., 2017; Sambrook and Russell, 2001).

The PCR reaction mixture (25 µL) was prepared by combining 1.5 µL dNTPs, 3 µL 10× reaction buffer, 0.5 µL Taq polymerase enzyme, 2.5 µL cDNA, 1.5 µL forward primer (1 pmol) and 1.5 µL reverse primer (1 pmol) and the volume was adjusted to 25 µL with nuclease-free water. The mixture was then subjected to amplification cycles in a Gradient Master Cycler of HIMEDIA Wee 32 using the programme (Table 6.3).

Table 6.3: PCR programme outlining the step-wise temperature conditions, duration and cycle number used for amplification of the selected genes.

Step	Temperature	Time
Initial denaturation	94 °C	4 min
Denaturation (cycle)	94 °C	1 min
Annealing	58 °C	40 Sec
Extension	72 °C	1 min
Final extension	72 °C	10 min
Cycles	35 cycles	

Elution of target gene

To obtain each gene in purified form, the amplified gene products were separated on agarose gel. Agarose gel (1.5%) was prepared by dissolving 1.5 g agarose in 100 mL of 1× Tris acetate (TAE) buffer, followed by heating in a microwave oven until completely dissolved. The solution was cooled to approximately 50 °C, after which 2 µL of ethidium bromide (EtBr) (10 mg/mL) was added and mixed thoroughly. The gel was poured into a

casting tray and combs were placed to create wells. After solidification, the combs were carefully removed and the gel was placed in an electrophoresis tank containing the 1× TAE buffer.

The amplified gene product was mixed with the 6× loading dye and the mixture was added to each well. About 4 μL of 100 base pair DNA ladder was added to a separate well to determine the amplicon size after running the gel. The electrophoresis was run at 80 volts until the dye front reached 3/4 of the gel. After the gel had run, it was transferred to an open UV box. The gel was separated from the gel tray, as the plastic would absorb much of the UV light. Then, using a sterile stainless steel scalpel blade, the necessary portion of the gel containing the amplified DNA product was cut. Then elution was further performed by using XcelGen DNA Gel/ PCR Purification Kit (Xcelris Genomics, Ahmedabad) following manufacturer's protocol.

Procedure of elution of DNA

The agarose gel slice containing the DNA fragment was excised with a sterile scalpel and transferred to a pre-weighed 1.5 mL microcentrifuge tube. The weight of the gel was determined. Three volumes of Buffer (GC-optimised buffers) were added to one volume of gel in the microcentrifuge tube and the mixture was incubated at 55-60 °C for 8 min. The tube was mixed by tapping the bottom every 2 min until the gel had melted completely. The tube was then cooled to room temperature. One volume of isopropanol was added to the mixture. Up to 700 μL of the Buffer GC mixture was transferred to a spin column with a collection tube. The column was centrifuged at 13,000 g for 1 min at room temperature. The flow-through was discarded and the column was placed back into the collection tube.

Then 500 μL of Buffer GC was added to the DNA Mini Column. The column was centrifuged at 13,000 g for 1 min at room temperature to wash the DNA Mini Column. 650 μL of DNA wash buffer was added to the column and the column was centrifuged at 13,000 g for 1 min at room temperature. The flow-through was discarded and the column, with the lid open, was inserted back into the collection tube. This step was repeated. The empty DNA column, with the lid open, was centrifuged at 13,000 g for 2 min to dry any ethanol residue in the matrix.

The column was placed into a clean 1.5 mL microcentrifuge tube and 30-50 µL of pre-warmed (60 °C) Elution Buffer was added to the centre of the column. The column was incubated at room temperature for 1 min. The column was centrifuged at 13,000 g for 1 min to elute the DNA. The eluted DNA solution was reloaded onto the column for a second elution. The first elution typically yielded 60-70% of the DNA bound to the column. Reloading the eluted DNA solution onto the column for a second elution yielded an additional 20% of the DNA, making the total yield approximately 90%. The eluted PCR product was checked in 1.0% agarose gel and stored at -20 °C for future use.

Evaluation of primer efficiency

Primers were designed and synthesised for the amplification of target genes. The efficiency of each primer was validated using the standard curve method. For this, qRT-PCR reactions were set up to amplify serial 10-fold dilutions of the same target gene elution product. The Ct values were plotted on the Y-axis against the amount of DNA copies (ng) on the X-axis to generate the standard curve. The slope of the standard curve was calculated for each gene to determine primer efficiency, with efficient primers expected to produce a proportional dose-response relationship (Balcells et al., 2011; Lekanne et al., 2002). Then the efficiency of the primer is calculated by the following formula;

$$E = 10\left(-\frac{1}{Slope}\right) - 1$$

Selection of reference gene

Reference genes were expressed at a relatively constant level across different cell types, developmental stages and experimental factors. The housekeeping gene 18S rRNA was selected as a reference gene for normalisation of qRT-PCR data in this study (Chen et al., 2021; Kozera and Rapacz, 2013).

6.2.5. Quantitative real-time PCR for expression analysis of selected genes

qRT-PCR reactions were done for relative quantification of genes using the SYBR® Premix Ex Taq™ (TAKARA) polymerase enzyme following the manufacturer's protocol.

The qRT-PCR reaction mixture, with a total volume of 40 µL, was prepared by combining 2 µL of cDNA diluted 1:10, 2 µL of forward primer (10 pmol), 2 µL of reverse primer (10 pmol) and 10 µL of SYBR Green dye. The final volume of reaction mixture was made up by adding 24 µL nuclease-free water. This mixture was used to amplify and quantify the target genes during the real-time PCR assay. qRT-PCR was performed in thermocycler (Eppendorf Master cycler Ep Gradient Realplex 2) using the following scheme (Table 6.4).

Table 6.4: Optimized thermal cycling conditions used for the quantitative real-time PCR (qRT-PCR) analysis of the selected genes.

Temperature	Time
95 °C	2 min
95 °C	0.15 min
60 °C	0.15 min
72 °C	0.30 min
95 °C	0.15 min
Cycles	40 cycles

The fluorescence monitoring occurred at the end of each cycle. After thermal cycling, a melt curve analysis was performed to confirm that the fluorescence detected was due to target amplification. It aids in detecting non-specific amplification. It also helps to determine whether primer dimers produced during the qRT-PCR experiment.

The scheme of the PCR program is shown here. The relative fold gene expression was measured by comparative $2^{-\Delta\Delta CT}$ method. In this approach, the threshold cycle (Ct) values of the target genes were normalized to that of the reference (housekeeping) gene, 18S rRNA, to obtain the ΔCT value. The ΔCT of the experimental sample was then compared with that of the control sample to determine the ΔΔCT value. The fold change in gene expression was calculated as $2^{-\Delta\Delta CT}$, where the result represents the relative expression level of the target gene in the experimental sample compared to the control.

Statistical analysis

All experiments were conducted in triplicate for each sample. The mean values and corresponding standard deviations were calculated and used for comparative gene expression analysis. Data were log-transformed to satisfy the assumptions of normality and homogeneity of variance. Statistical analyses were performed using Python 3.10 software and GraphPad Prism 10.4.1 softwares. Analysis of Variance (ANOVA) was applied to assess overall differences among sample groups, followed by Tukey's post-hoc test to identify pairwise differences between normal leaves, gall leaves and galls. A threshold of $p < 0.05$ was adopted as significant for each gene expression analysis.

6.3. RESULTS AND DISCUSSION

6.3.1. RNA quality evaluation for qRT-PCR analysis

High-quality RNA was essential for accurate qRT-PCR analysis of genes in normal leaves, galled leaves and galls of *A. scholaris* and *F. racemosa*, to quantify gall-induced gene upregulation. RNA quality was assessed for 12 samples per plant species (4 each from normal leaf, gall leaf and gall) using UV-visible spectrophotometry (NanoDrop, Thermo Fisher) to measure the 260:280 ratio. All samples exhibited ratios between 1.8 and 2.1 (mean ± SD: 1.95 ± 0.08 for samples of *A. scholaris*, 1.97 ± 0.07 for tissues of *F. racemosa*), indicating minimal protein or phenolic contamination. RNA integrity was further verified by 1.2% agarose gel electrophoresis, with samples showing intact 28S and 18S rRNA bands (28S:18S ratio ~2:1), confirming no degradation.

All samples that met quality criteria were selected for further analysis ensuring reliable cDNA synthesis and qRT-PCR results. These rigorous checks validated the suitability of RNA for downstream analysis. The gel image of RNA of normal leaves, gall leaves and galls of *A. scholaris* is shown in Plate 6.3: Figure A. RNA from normal leaves, gall leaves and galls of *F. racemosa* is shown in Figure B, Figure C and Figure D respectively.

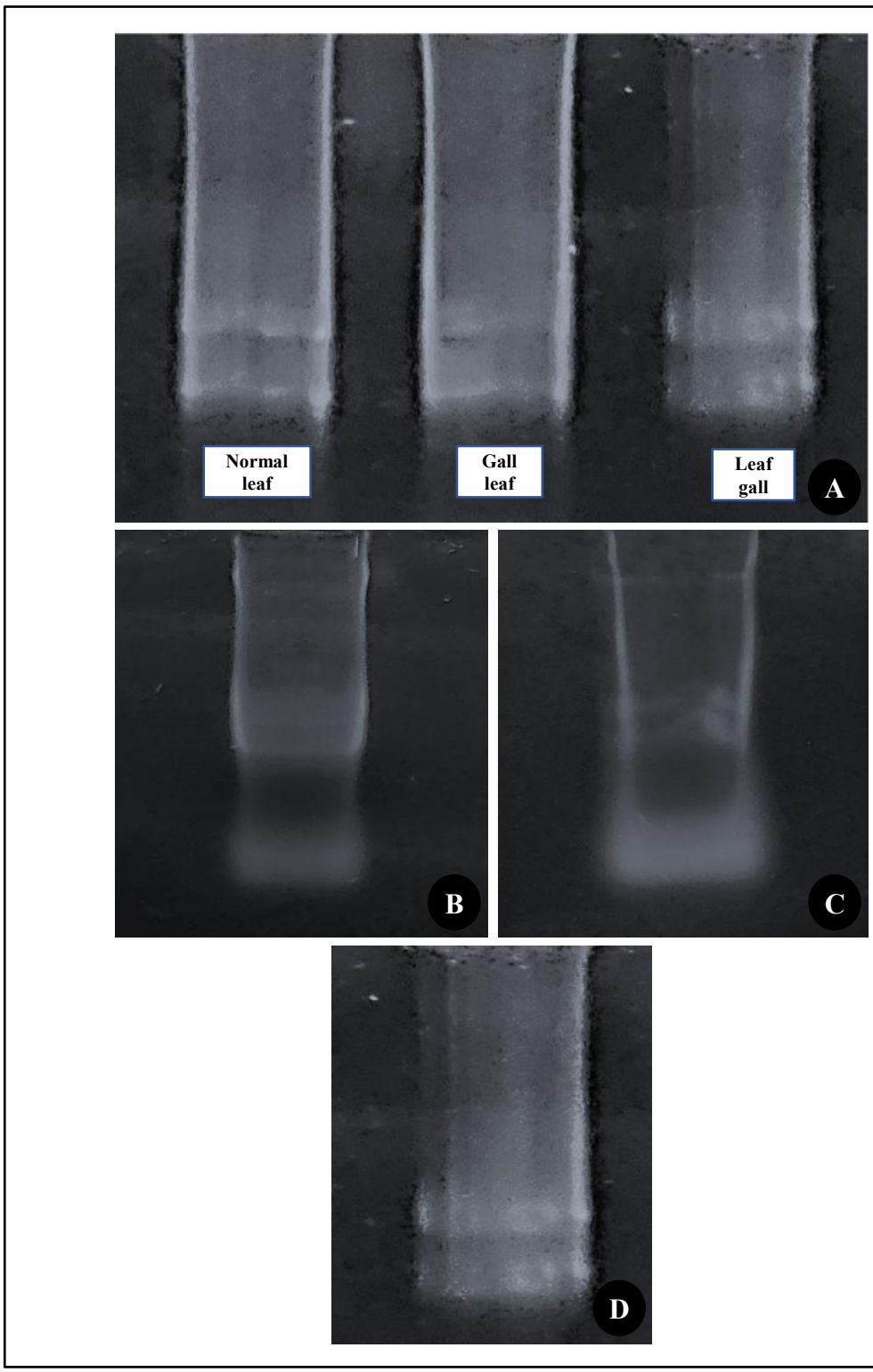


Plate 6.3: Extraction of RNA from different samples. Figure A: RNA from AS-NL, AS-GL and AS-GA, Figure B: RNA isolated from FR-NL, Figure C: RNA isolated from FR-GL and Figure D: RNA isolated from FR-GA.

6.3.2. Primer design and synthesis

Primers for qRT-PCR amplification of DAHPS, EPSPS, CS, PAL, CHI and DFR were designed to ensure specific and efficient gene expression analysis in gall-induced tissues. The sequences of primers used for synthesis of qRT-PCR primers were given in Table 6.5.

Table 6.5: List of forward and reverse primer sequences of selected genes used in this study with their respective product sizes (bp).

Sl. No.	Name of gene	Product size (bp)	Forward sequence (5′ to 3′)	Reverse sequence (5′ to 3′)
1	DAHPS	268	GCCGTCCATTCCGCC GATCC	AGCTCCTAGCCTCACCGGC A
2	EPSPS	104	AAAAAACCCACCTTC CTTCC	TCCCTTCTCCTGCTTTCTC
3	CS	136	CCATCTCCACCTGTCA AAATC	TACAACCAACACCTCCTCC
4	PAL	105	CCGGTGGTGAAGCTC GGTGG	GGCCCGGGCTGACTCAGA GA
5	CHI	104	CGGTGGCGCAGGTGT GAGAG	TGCCGGCGAGTGAAGACA CG
6	DFR	182	GCAGACAATGGACAA GACAAAG	CAGGGAACCAAGAAGCAA AC

6.3.3. Primer efficiency curve

The standard curve for primer efficiency analysis of each gene is attached in Plate 6.4 and 6.5. From the standard curves primer efficiency was calculated. All the six qRT-PCR primers synthesised have been found to have efficiency between 90-100%.

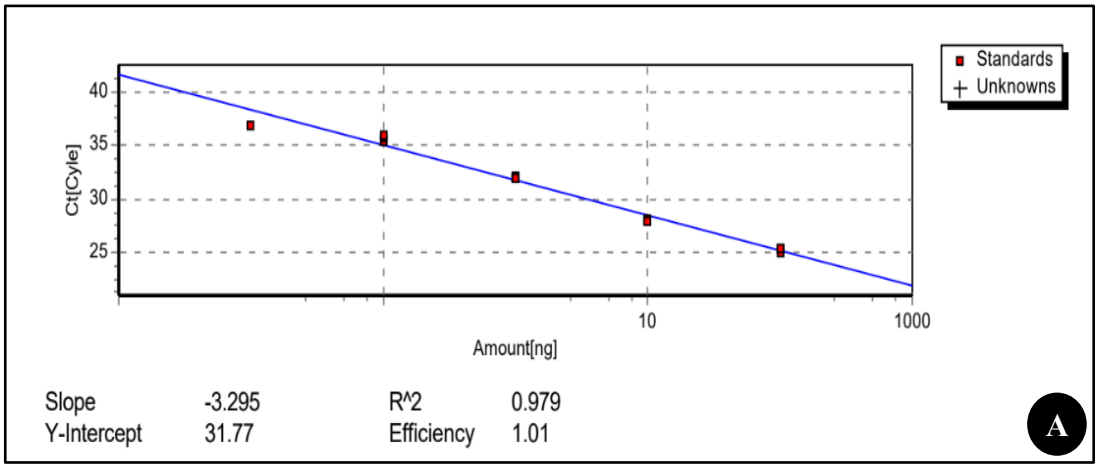
Gene expression analysis using qRT-PCR

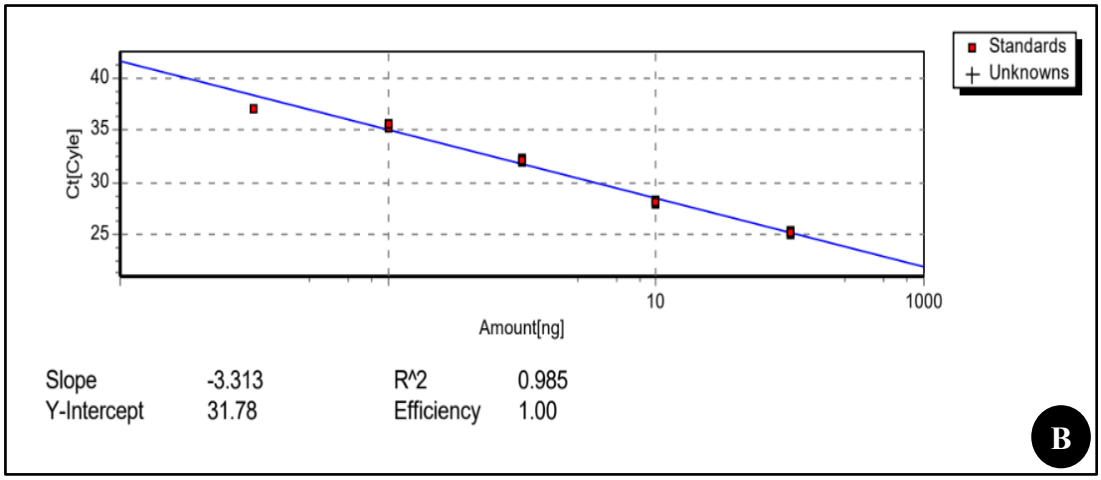
Plants have different systems of defence to resist the attacks of insects and pathogens, which are triggered by a diverse range of biotic stimuli. One of the defensive reactions triggered by the gall inducer attack is the production of different secondary metabolites with disease resistance properties. Phenolic compounds have the capacity to defend against the

pathogen in various ways; it may act as phytoalexins or as ROS scavengers, physical barriers or pathogenesis-related (PR) proteins or signal molecules that induce other defence related pathways. Due to the antioxidative characteristics of these compounds, which reduce cell membrane peroxidation and protect plant cells from the harmful impacts of oxidative stress, plants growing in stressful environments are able to biosynthesize more phenolic compounds than plants growing in normal conditions. It has been discovered through OHR-LC-MS that phenolics in gall leaves and galls of *A. scholaris* are 16 and 11 respectively. *A. scholaris* normal leaves have only 8 different phenolic compounds. In the normal leaves and gall leaves of *F. racemosa* found to contain 12 and 13 phenolic compounds respectively. 66 different phenolic compounds having various significant biological activities are identified in galls of *F. racemosa*.

Gall induction by *Pauropsylla* species triggers robust defense responses in *A. scholaris* and *F. racemosa,* marked by increased phenolic biosynthesis, a key mechanism for resisting insect attack. GC-MS and OHR-LC-MS analysis quantified phenolic compounds in normal leaves, galled leaves and galls, revealing distinct profiles. These findings, consistent with phytochemical profiles explained in the Chapter 3, underscore the role of phenolics as phytoalexins, antioxidants and structural barriers, contributing to the antimicrobial and antioxidant activities reported in Chapter 4. The marked phenolic increase in *F. racemosa* galls suggests a stronger metabolic reprogramming, potentially driven by differential gene expression.

Altered biosynthesis of phenolics is regulated by the increased activities of various key enzymes of phenolic biosynthetic pathways. This study focuses on 6 genes (DAHPS, EPSPS, CS, PAL, CHI and DFR) from shikimic acid pathway and phenylpropanoid pathway that are involved in the production of different classes of phenolic compounds; through qRT-PCR. Plant defence mechanisms involve alterations in gene expression mediated by several signalling pathways in response to herbivores, pathogens and mechanical injuries. Plant defensive genes are triggered by signalling molecules, which in turn causes the plant to become resistant to biotic and abiotic stressors. The shikimic acid pathway converts PEP and E4P to chorismate, the precursor of the aromatic amino acid tryptophan, phenylalanine and tyrosine, via seven metabolic steps. The majority of secondary phenolic compounds are





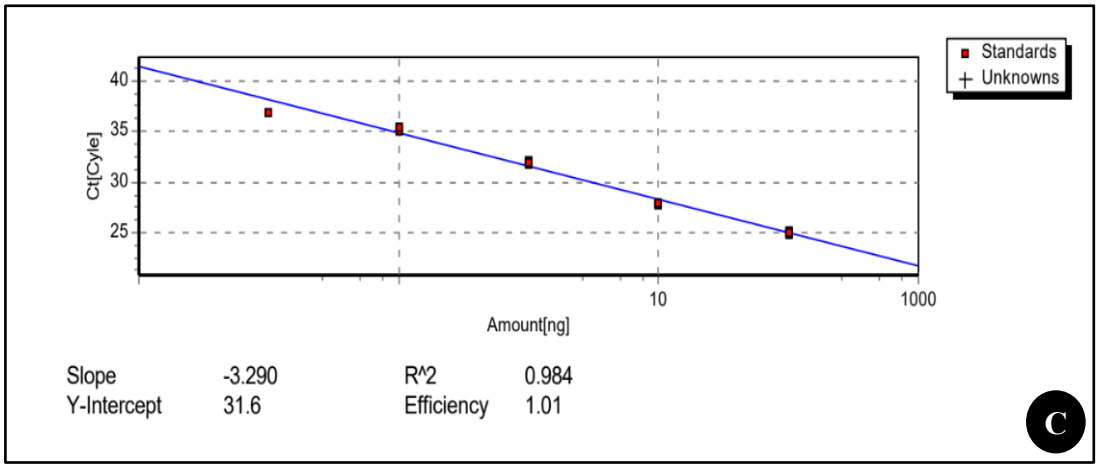


Plate 6.4: Primer efficiency analysis. Figure A: Standard curve for primer efficiency analysis of DAHPS gene primer, Figure B: Standard curve of EPSPS gene primer and Figure C: Standard curve of CS gene primer.

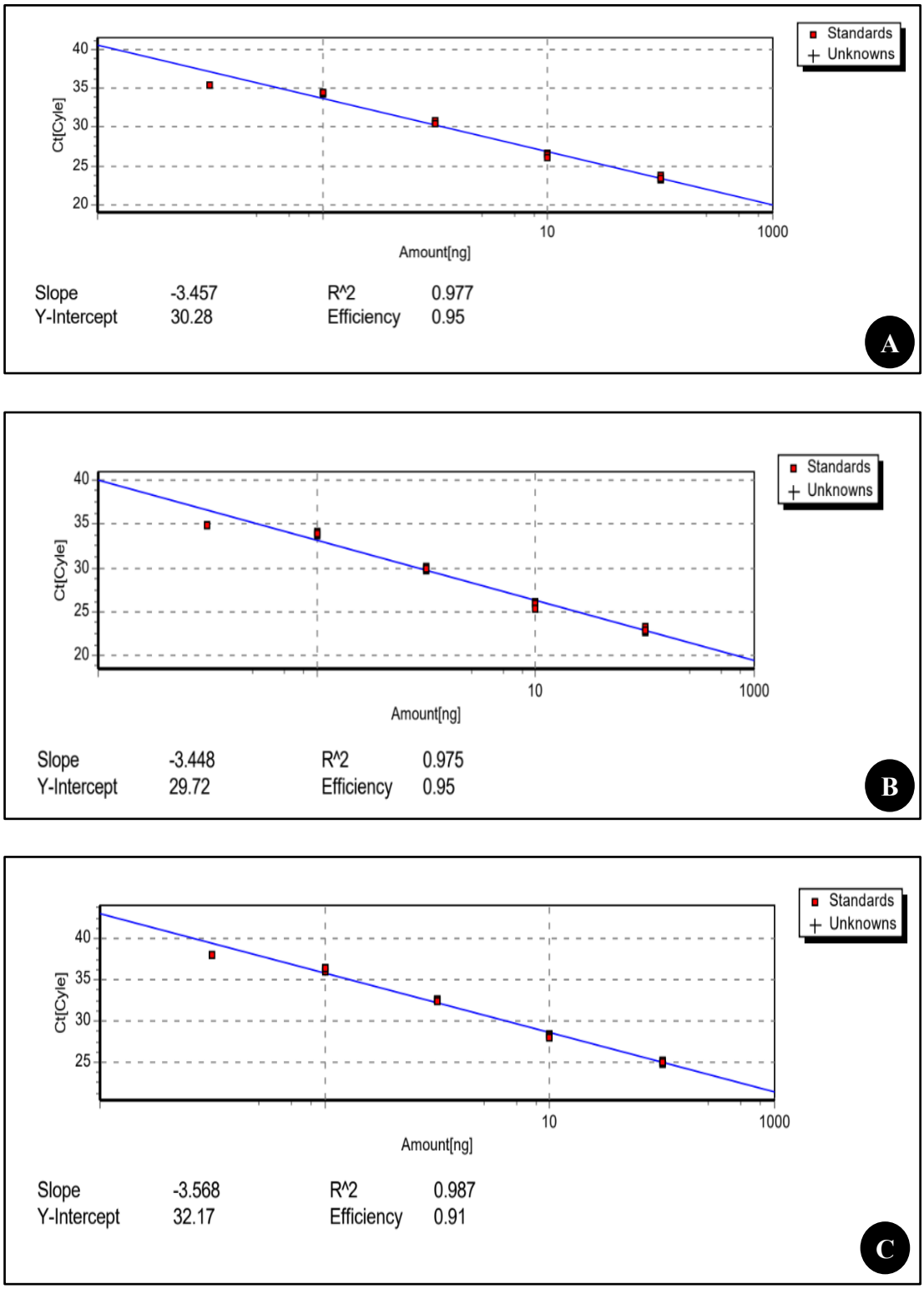


Plate 6.5: Primer efficiency analysis. Figure A: Standard curve for primer efficiency analysis of PAL gene primer, Figure B; Primer efficiency curve of CHI gene and Figure C: Primer efficiency analysis of DFR gene primer.

produced from phenylalanine through the action of several enzymes. The deamination of phenylalanine to cinnamic acid controls the formation of phenolic compounds such as lignin, flavonoids, pigments, phytoalexins and benzoic acid derivatives like salicylic acid. The pathway leads to the generation of flavonoids, flavanones, anthocyanins, catechins and procyanidins.

6.3.4. Gene expression profile of *A. scholaris*

qRT-PCR analysis quantified the expression of 3-Deoxy- D-arabino- heptulosonate-7-phosphate synthase (DAHPS), EPSP synthase (EPSPS), Chorismate synthase (CS), Phenylalanine ammonia-lyase (PAL), Chalcone-flavanone isomerase (CHI) and Dihydroflavonol 4-reductase (DFR) in galled leaves and galls of *A. scholaris*. Normal leaves served as the control, and their fold gene expression was normalized to 1. These genes, integral to the shikimic acid and phenylpropanoid pathways, exhibited differential expression in response to gall induction. Table 6.6 represents the data of fold gene expression in different samples of *A. scholaris.* A representation of the qRT-PCR analysis outcome file showing fluorescent profile of gene expression of *A. scholaris* is presented in Plate 6.6: Figure A.

DAHP synthase gene expression

In *A. scholaris,* the expression of genes involved in the shikimic acid and phenylpropanoid pathways exhibits distinct responses to gall-inducing biotic stress caused by psyllids, such as *P. tuberculata*. The present study provides novel insights into DAHPS gene expression (Plate 6.7: Figure A). The DAHPS gene, known to be upregulated under biotic and abiotic stresses, showed higher expression in the gall leaves with a fold gene expression (FGE) of 3.01 ± 0.39 and exhibited higher expression, approximately a 2-fold increase relative to the control. In the galls, the FGE is 6.14 ± 1.40. Notably, the DAHPS expression in gall leaves of *A. scholaris* was higher than that of all other genes (EPSPS, CS, PAL, CHI, DFR), underscoring its significant induction under biotic stress. These findings highlight the tissue-specific and species-specific regulation of DAHPS in response to biotic stress produced by gall inducer.

DAHPS gene expression is often upregulated in response to biotic and abiotic stresses. In Sitka spruce (*Picea sitchensis*), the DAHPS gene was induced by mechanical wounding and herbivory from spruce budworms (*Choristoneura occidentalis*) and white pine weevils (*Pissodes strobi*) in bark and shoot tips (Ralph et al., 2006b). Similarly, wounded carrots (*Daucus carota*) subjected to water stress exhibited a 20-fold increase in DAHPS gene expression (Becerra-Moreno et al., 2015). This study also supports these findings of stronger activation of DAHPS as observed in other plants like Sitka spruce (*P. sitchensis*) under herbivory stress (Ralph et al., 2006b), potentially supporting the production of defense-related secondary metabolites via the shikimate pathway. This increase implies greater carbon allocation to aromatic amino acids, which are precursors for phenolic biosynthesis, though the low FGE suggests a potential rate-limiting step in the pathway.

EPSP synthase gene expression

The present study investigated the effects of insect-induced gall formation on gene expression of EPSP synthase in the leaves of *A. scholaris.* EPSPS facilitating chorismate production in the pathway. The galls of *A. scholaris* exhibited a striking 21.52 ± 1.98-fold increase in galls, but only 2.53 ± 0.21 in gall leaves, reflecting gall-specific activation (Plate 6.7: Figure B).

Wounding stress influences phytochemical production in strawberry (*Fragaria* × *ananassa*) fruits, leading to differential phytochemistry and altered gene expression. Wounding significantly increased the levels of phenolic compounds, total soluble sugars and certain phenylpropanoids. The EPSPS gene, involved in the shikimate pathway, showed increased expression in fruits from plants wounded 14 days before harvest, with 6.25 ±1.03 fold increase in expression (Ibanez et al., 2019). Ralph and colleagues in 2006 also reported the upregulation of EPSPS gene in poplar (*Populus* spp.) leaves subjected to herbivory by forest tent caterpillars (*Malacosoma disstria*) (Ralph et al., 2006a). The EPSPS gene was significantly upregulated in the galls with an FGE of 21.52 ± 1.98, aligning with findings in wounded strawberry (*Fragaria* × *ananassa*) fruits where EPSPS expression increased (Ibanez et al., 2019).

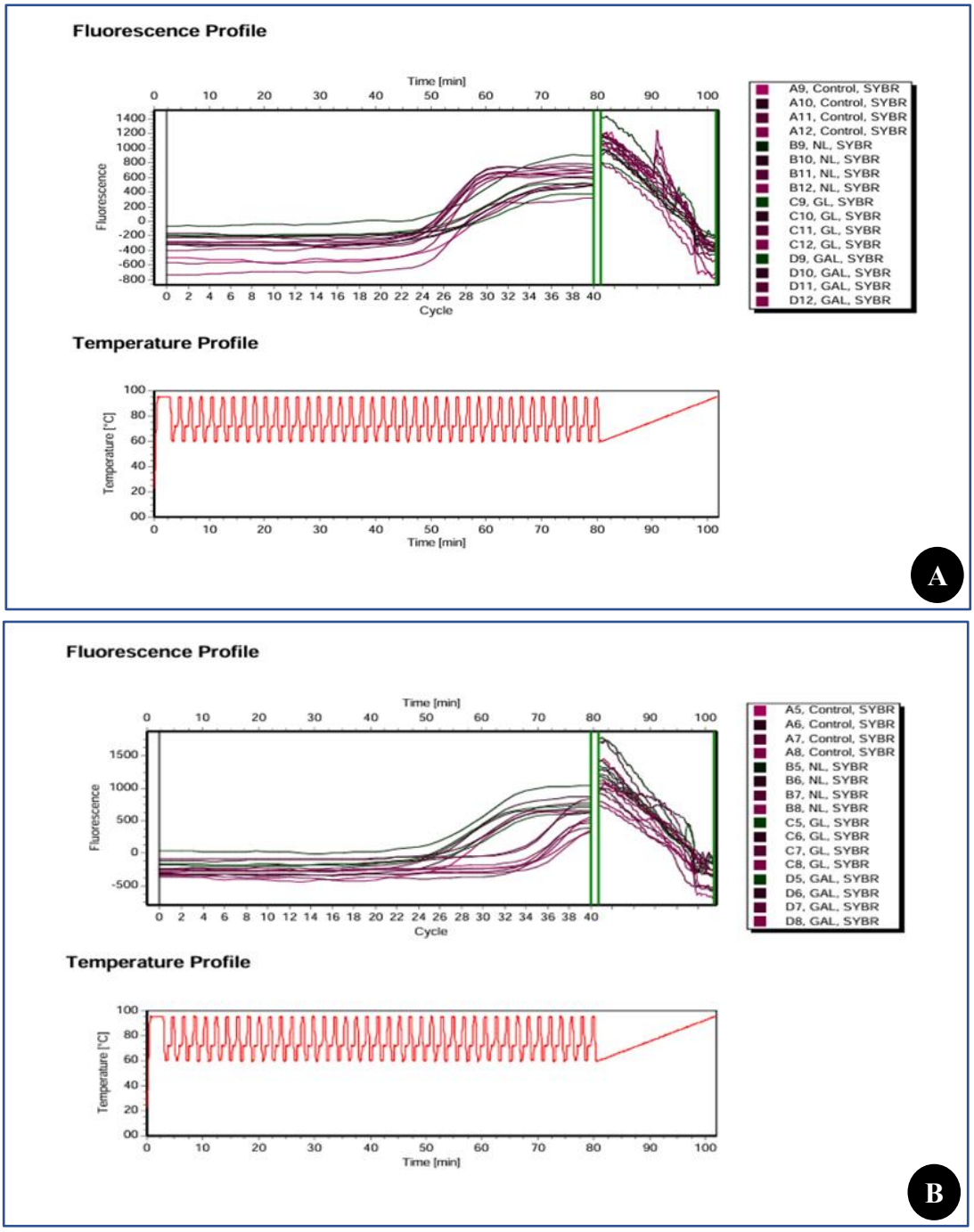


Plate 6.6: Figure A: Representation of qRT-PCR analysis outcome file of *A. scholaris*, showing fluorescent profile of gene expression and Figure B: Representation of qRT-PCR analysis outcome file of *F. racemosa*, showing fluorescent profile of gene expression.

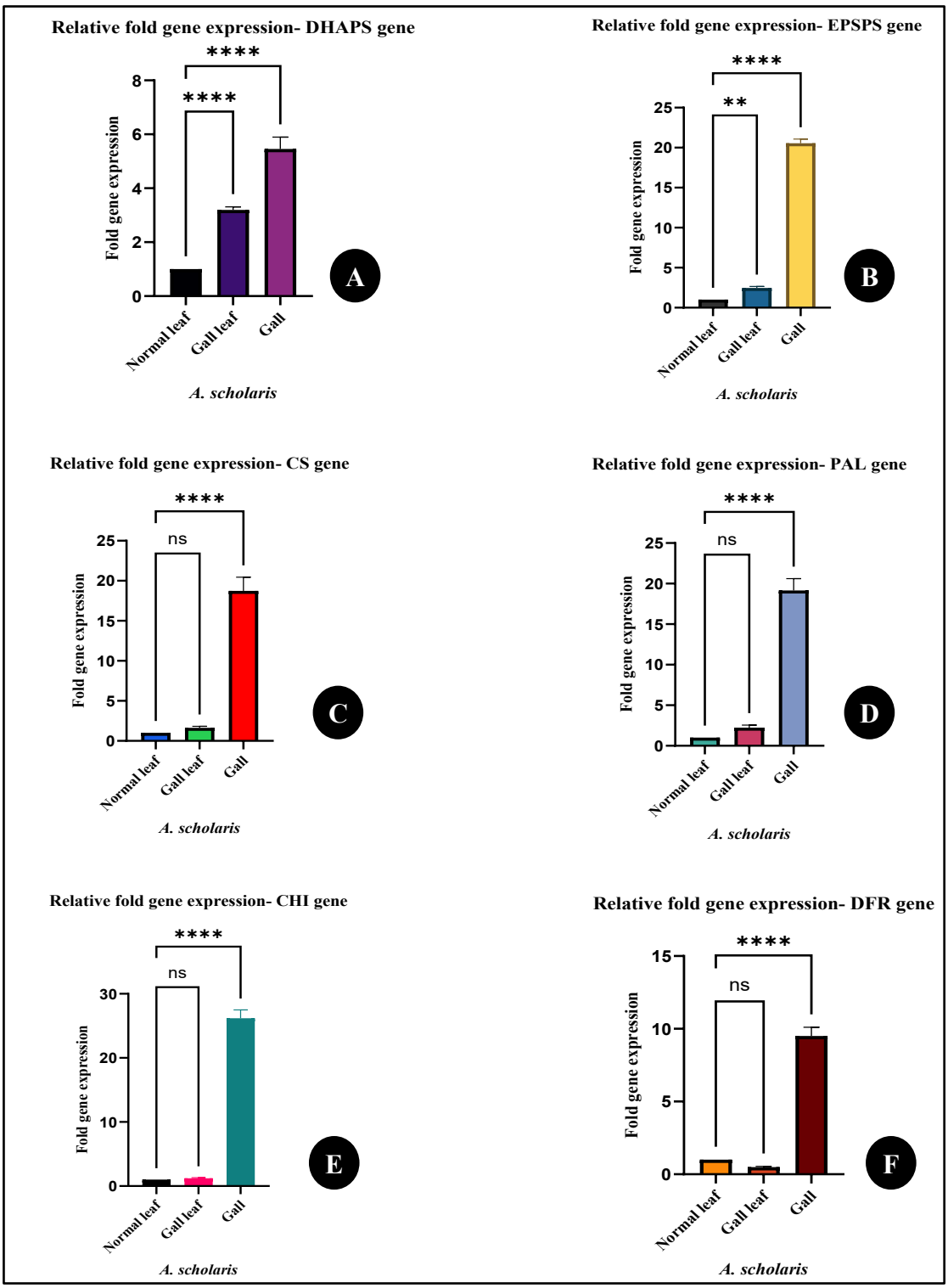


Plate 6.7: qRT-PCR expression analysis- Figure A-F: Relative fold gene expression (FGE) of DAHPS, EPSPS, CS, PAL, CHI and DFR genes in gall leaves and galls compared to control (normal leaves) in *A. scholaris*. Data representes mean ± standard deviation. **** indicated significance in gene expression at the level $p < 0.0001$ and ns denotes no significant difference.

Chorismate synthase (CS) gene expression

The CS gene expression in response to gall formation in *A. scholaris* was also investigated in this study. CS catalyzing the final step in the shikimic acid pathway. The CS gene was significantly more upregulated in galls, than gall leaves. In the present analysis it was found upregulated in galls (18.19 ± 1.78) with minimal gall leaves expression (1.81 ± 0.37), aligning with precursor accumulation (Plate 6.7: Figure C).

In broccoli (*Brassica oleracea*), differential gene expression was analyzed in heads subjected to two wounding intensities using qRT-PCR: floret cuts and chopping. The CS gene was significantly upregulated in chopped samples, exhibiting both early and late responses, indicating enhanced chorismate production to support defense pathways (Torres-Contreras et al., 2018). Similarly, in poplar (*Populus* spp.), herbivory by forest tent caterpillars (*Malacosoma disstria*) induced the expression of the CS gene, alongside DAHPS and EPSPS, reflecting activation of the shikimate pathway (Ralph et al., 2006a). Similarly, the CS gene exhibited an 18.19 ± 1.78-fold increase in galls, indicating enhanced chorismate production, a critical step in secondary metabolite biosynthesis, as seen in broccoli under wounding stress (Torres-Contreras et al., 2018) and poplar, due to herbivory by forest tent caterpillars (Ralph et al., 2006a). According to these results, gall induction triggers the defensive mechanism of the plant, which probably increases the production of chorismate. These findings, which show tissue-specific and species-specific expression patterns in response to gall formation, emphasize the crucial role of CS in the shikimate pathway under biotic stress.

Phenylalanine ammonia-lyase (PAL) gene expression

PAL is a key phenylpropanoid enzyme converting phenylalanine to trans-cinnamic acid, it is essential for phenylpropanoid biosynthesis. The gene showed similar upregulation in galls (18.74 ± 1.46) and gall leaves expression of FGE 2.02 ± 0.50, supporting increased phenolic synthesis. The expressions were matching the expression level of the CS gene in the same tissue. These findings suggest that gall-inducing biotic stress significantly enhances PAL-mediated phenolic biosynthesis in *A. scholaris* (Plate 6.7: Figure D).

In wounded carrots *(Daucus carota*), a 14-fold increase in PAL gene expression was observed, resulting in elevated phenolic compound levels during storage (Becerra-Moreno et al., 2015). In the resistant citrus species *Poncirus trifoliata,* PAL gene expression was activated during the early stages of infection by *Phytophthora parasitica,* suggesting a role in limiting pathogen invasion (Boava et al., 2011). Similarly, in the resistant rice cultivar 'Kasalath', PAL enzyme activity and transcript levels increased significantly in response to infestation by the small brown plant hopper, correlating with elevated levels of defensive phenolic compounds, such as tannins and quinones (Duan et al., 2014). In grapevine (*Vitis vinifera*), PAL gene expression differed between susceptible and resistant leaves infected with the host pathogen *Plasmopara viticola*, but not in non-host interactions with *Pseudoperonospora cubensis* (Kortekamp, 2006). This robust induction mirrors observations in wounded carrots and resistant citrus, where PAL activation led to elevated phenolic compound levels (Becerra-Moreno et al., 2015; Boava et al., 2011). According to these findings, herbivory-induced responses and wounds are frequently linked to upregulated PAL gene expression. Its function in gall-induced defense mechanisms is highlighted by the notable induction of PAL in *A. scholaris,* which most likely promotes the synthesis of phenolic compounds to fight insect induced stress.

Chalcone-flavanone isomerase (CHI) gene expression

qRT-PCR analysis found that *A. scholaris* gall formation activates the expression of the CHI gene. CHI is a critical gene for flavonoid production. It displayed the highest expression in gall (25.22 ± 2.21), with modest gall leaves induction (1.48 ± 0.53), indicating a strong gall-driven flavonoid response (Plate 6.7: Figure E).

Microarray gene expression profiling in Sitka spruce (*Picea sitchensis)* revealed differential expression of genes across functional categories, including plant defense, octadecanoid and ethylene signaling, transport, secondary metabolism and transcriptional regulation, in response to feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*). Real-time PCR analysis confirmed that flavonoid biosynthesis genes, such as chalcone synthase (CHS) and CHI, were upregulated following herbivory, highlighting the role of phenolic secondary metabolites in conifer defense against insects and

pathogens (Ralph et al., 2006b). In transgenic rice (*Oryza sativa*), stress-inducible CHI expression was significantly higher than in wild-type plants, with 1.63 to 3.83-fold increases under drought stress, 2.72 to 3.95-fold under salt stress and 1.81 to 3.46-fold under cold stress. These transgenic lines exhibited elevated total phenolic and flavonoid content and outperformed control plants under drought, salt and cold stress, demonstrating role of CHI in abiotic stress tolerance (Jayaraman et al., 2021). The CHI gene, involved in flavonoid biosynthesis, was upregulated in both galls (FGE: 25.22 ± 2.21) and gall leaves (FGE: 1.48 ± 0.53), consistent with its role in stress-induced flavonoid production in transgenic rice under abiotic stress (Jayaraman et al., 2021) and also with biotic stress tolerance by increased phenolic production in Sitka spruce (Ralph et al., 2006b). The substantial elevation of CHI in *A. scholaris* during gall development confirms its role in phenolic compound production as a defense against biotic stress caused by insects.

Dihydroflavonol 4-reductase (DFR) gene expression

The DFR gene is a significant part of the flavonoid pathway, involved in anthocyanin and flavanol synthesis. The present qRT-PCR analysis demonstrated the gene was significantly upregulated in galls (8.97 ± 1.18) but downregulated in gall leaves (0.45 ± 0.12), suggesting metabolite channeling to gall tissues. *A. scholaris* showed a lower DFR expression in galls than the results seen in *F. racemosa* in the current investigation, indicating species-specific responses to gall-inducing biotic stress (Plate 6.7: Figure F).

In transgenic tobacco (*Nicotiana tabacum*) overexpressing DFR and anthocyanidin reductase (ANR), elevated levels of alkaloids and flavonols conferred resistance against the tobacco leaf cutworm (*Spodoptera litura*) (Kumar et al., 2013). Abiotic stresses also influence DFR expression. Drought stress induced DFR gene expression in potato (*Solanum tuberosum*) tubers (André et al., 2009) and wheat (*Triticum aestivum*) leaves (Ni et al., 2020), suggesting a role in mitigating environmental stress through flavonoid accumulation. The lower expression in *A. scholaris* suggests species-specific metabolic priorities. DFR showed an FGE of 8.97 ± 1.18 in galls, contributing to defense against insect-induced gall formation, as seen in resistant *Camellia oleifera* under anthracnose stress (Yang et al., 2022).

ANOVA revealed significant differences in the relative fold gene expression levels across the three tissues ($p < 0.001$), indicating that gall induction markedly influences the transcriptional activity of key genes associated with secondary metabolite biosynthesis. The subsequent Tukey's HSD test confirmed a consistent expression trend of GA > GL > NL for all the analyzed genes, signifying that gene expression was highest in galls, moderate in gall leaves and lowest in normal leaves. Among the six genes analyzed, DAHPS and EPSPS showed significant upregulation in gall leaves compared to normal leaves, whereas CS, PAL, CHI and DFR exhibited non-significant differences. However, all genes were significantly upregulated in galls relative to the control at level of $p < 0.001$. This pronounced upregulation of secondary metabolism-related genes in gall tissues demonstrates that gall formation triggers enhanced biosynthetic activity, likely as part of a complex plant insect interaction. The elevated expression of these genes reflects a metabolic shift toward increased synthesis of phenolic and flavonoid compounds, which may serve defensive, structural or signaling roles during gall development. Graphical representation comparative analysis of the relative expression of all the selected genes in *A. scholaris* is provided as a bar diagram in Plate 6.8: Figure A.

Table 6.6: Relative fold gene expression levels of selected genes in *A. scholaris* normal leaves (control), gall leaves and galls, with values expressed as mean ± SD.

Sl. No.	Name of gene	Relative fold gene expression in *A. scholaris*		
		Normal leaves (Control)	Gall leaves	Galls
1	DAHPS	1	$3.01 \pm 0.39^{**}$	$6.14 \pm 1.40^{**}$
2	EPSPS	1	$2.53 \pm 0.21^{*}$	$21.52 \pm 1.98^{**}$
3	CS	1	1.81 ± 0.37	$18.19 \pm 1.78^{**}$
4	PAL	1	2.02 ± 0.5	$18.74 \pm 1.46^{**}$
5	CHI	1	1.48 ± 0.53	$25.22 \pm 2.21^{**}$
6	DFR	1	0.45 ± 0.12	$8.97 \pm 1.18^{**}$

Each value of relative fold gene expression represents mean ± standard deviation (n = 3). **indicates values significantly different from normal leaves (control) at $p < 0.001$. *indicates values significantly different from normal leaves at $p < 0.05$

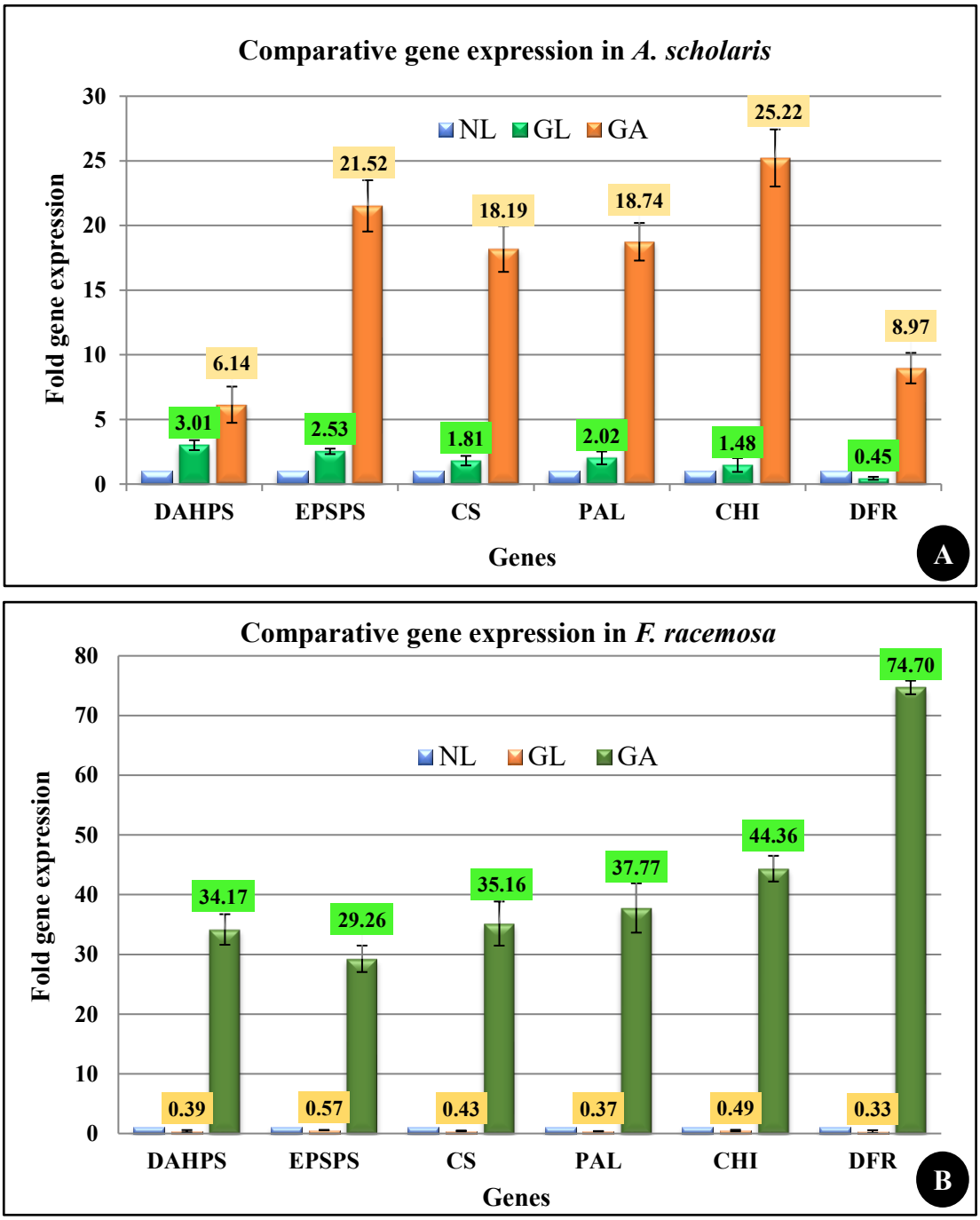


Plate 6.8: Comparative analysis of gene expression: Figure A: Fold gene expression of 6 selected genes in *A. scholaris* normal leaves (control), gall leaves and leaf galls and Figure B: Fold gene expression in *F. racemosa* normal leaf (control), gall leaves and leaf galls.

6.3.5. Gene expression profile of *F. racemosa*

qRT-PCR analysis of *F. racemosa* revealed pronounced upregulation of DAHPS, EPSPS, CS, PAL, CHI and DFR in galls induced by *P. depressa*, with normal leaves as the control (FGE = 1). The mean FGE of three different experiments calculated with standard deviation (Table 6.7). A representative output file of the analysis performed using the Eppendorf Mastercycler ep Gradient Realplex 2, with cDNA of *F. racemosa* as the template, is shown in Plate 6.6: Figure B.

DAHP synthase gene expression

The current study sheds new light on DAHPS gene expression in response to biotic stress generated by gall-inducing insects in *F. racemosa*. The expression study revealed that galls had significantly higher expression than normal leaves and gall leaves. In contrast, gall leaves expression has been found to be lower than the control. In galls, DAHPS expression was significantly higher in *F. racemosa* (29.84 ± 0.52), indicating stronger activation in *F. racemosa*. Conversely, in gall leaves the FGE < 1. The elevated expression in *F. racemosa* gall is linked to the higher production of defense-related secondary metabolites through shikimic acid pathway (Plate 6.9: Figure A).

In broccoli Torres-Contreras and colleagues found the differential expression of DAHPS also. Like other genes, wounding stress upregulated DAHPS expression also (Torres-Contreras et al., 2018). Additionally, poplar (*Populus* spp.) leaves showed increased DAHPS expression in response to herbivory by forest tent caterpillars, *Malacosoma disstria* (Ralph et al., 2006a). In contrast, strawberry fruit did not show altered DAHPS expression when subjected to wounding 7 or 14 days before harvest (Ibanez et al., 2019). As aromatic amino acids are required for protein synthesis and normal functioning of plants, DAHPS genes are constitutively transcribed and translated to produce enzymes. However, stress generated by gall inducers activates DAHPS and boosts their transcript level, enhancing the secondary metabolic pathway and producing a significant amount of phytochemicals. When compared to all other genes investigated, the DAHPS showed a lesser surge in gene expression in galls. This study also indicates a stronger activation of the shikimate pathway

in the *F. racemosa,* potentially linked to defense-related secondary metabolite production, as observed in poplar (*Populus* spp.) under herbivory by *M. disstria* (Ralph et al., 2006a).

EPSP synthase gene expression

The current study shows that the EPSPS gene was significantly upregulated in response to gall-inducing biotic stress, with a FGE greater than 30 in the galls. The results showed strong upregulation in gall with FGE of EPSPS 30.26 ± 1.88 and low in gall leaves expression (0.57 ± 0.04) (Plate 6.9: Figure B).

These findings align with previous observations of herbivory-induced activation of secondary metabolism genes in poplar. Microarray transcriptome profiling of poplar (*Populus* spp.) leaves subjected to herbivory by forest tent caterpillars, *M. disstria* revealed differential expression of genes across multiple functional classes, including stress response, secondary metabolism, photosynthesis, transport, octadecanoid and ethylene signaling, transcriptional regulation and signaling pathways. These genes were either upregulated or downregulated, reflecting a complex metabolic reprogramming (Ralph et al., 2006a). These results highlight the role of wounding and herbivory in modulating secondary metabolism, with EPSPS playing a key role in the shikimate pathway to support the biosynthesis of defense-related compounds. The upregulation of EPSPS in *F. racemosa* underscores the conserved response to biotic stress across plant species. The EPSPS gene was highly upregulated in galls with an FGE of 30.26 ± 1.88, reflecting its critical role in the shikimic acid pathway, similar to findings in strawberry fruits wounded 14 days before harvest (Ibanez et al., 2019).

Chorismate synthase (CS) gene expression

This study examined the expression of the CS gene in response to gall formation in *F. racemosa.* Compared to gall leaves, galls showed a much higher upregulation of the CS gene facilitating chorismate synthesis, showed strong upregulation (35.16 ± 3.70), suggesting localized precursor production (Plate 6.9: Figure C).

Torres-Contreras and colleagues reported that differential CS gene expression was observed in *Brassica oleracea* in response to chopping injuries and floret cuts, which elevated the synthesis of chorismate to support defense pathways as same as seen in DAHPS

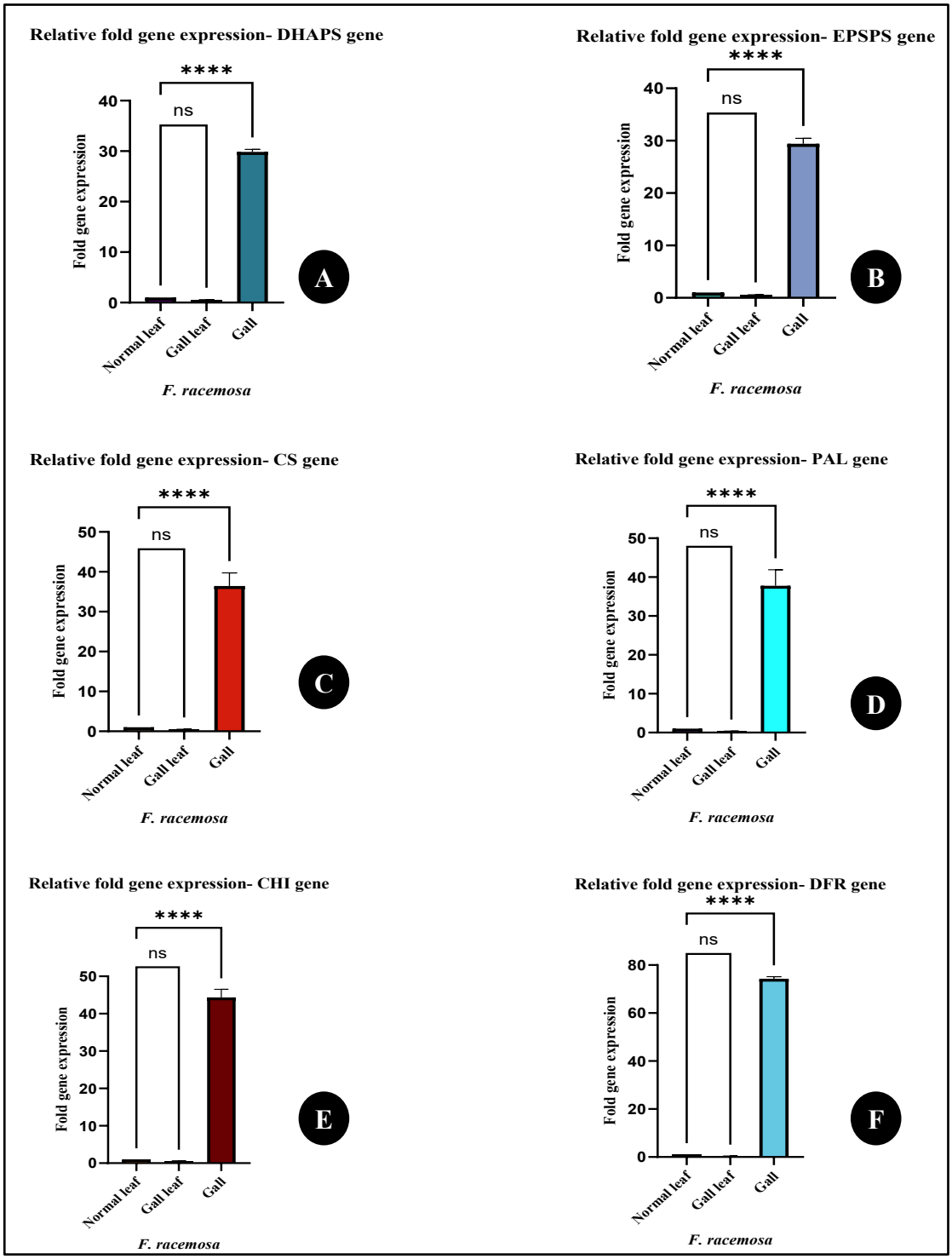


Plate 6.9: qRT-PCR analysis. Figure A-F: Relative expression levels of DAHPS, EPSPS, CS, PAL, CHI and DFR genes in gall leaves and galls of *F. racemosa* expressed as fold change compared to normal leaves (control). Data representes mean ± standard deviation. **** indicated significance in gene expression at the level $p < 0.0001$ and ns denotes no significant difference.

(Torres-Contreras et al., 2018). Likewise, the herbivory of *M. disstria* on poplar (*Populus* spp.) resulted in the expression of the CS gene, which indicates the activation of the shikimate pathway (Ralph et al., 2006a). The CS gene showed a 35.16 ± 3.70-fold increase in galls, supporting enhanced chorismate production for secondary metabolism, as reported in broccoli under wounding stress (Torres-Contreras et al., 2018). These findings suggest that gall-inducing biotic stress activates the plant defense system, likely enhancing chorismate production to support secondary metabolite biosynthesis. These results highlight the critical role of CS in the shikimic acid pathway under biotic and abiotic stress, with tissue-specific and species-specific expression patterns in response to gall formation.

Phenylalanine ammonia-lyase (PAL) gene expression

The expression of the PAL gene in response to gall development in *F. racemosa* was examined in the current study. The expression in gall leaves was 0.37 ± 0.04-fold, which is much less than normal (control) leaf. In *F. racemosa*, about 37.77 ± 4.13-fold increase in PAL expression was observed in galls, reflecting insect-induced activation of defense-related pathways (Plate 6.9: Figure D).

Microarray gene expression profiling in Sitka spruce (*Picea sitchensis*) revealed upregulation of PAL genes in response to insect feeding or mechanical wounding, contributing to large-scale transcriptomic changes (Ralph et al., 2006b). In broccoli, wounding stress induced the upregulation of five PAL-encoding genes (Torres-Contreras et al., 2018). In hybrid poplar (*Populus* spp.), PAL gene expression was also activated following infection by forest tent caterpillars, *M. disstria* and in Strawberry, a 1.85-fold increase in PAL expression was observed in response to wounding (Ibanez et al., 2019; Ralph et al., 2006a). The current study results also indicate a strong defense response through phenolic compound production, consistent with observations in resistant rice cultivars under plant hopper infestation (Duan et al., 2014).

However, not all biotic stresses induce PAL expression. In soybean (*Glycine max*) infected with *Rhizoctonia solani*, PAL gene expression remained unaffected (Chen et al., 2009). Similarly, cDNA array analysis of rice cultivars (resistant B5 and susceptible MH63) infested by the piercing-sucking insect *Nilaparvata lugens* showed downregulation of the

PAL gene, contradicting typical patterns of PAL induction under biotic stress (Zhang et al., 2004). These results underscore the context-specific regulation of PAL gene expression, with upregulation commonly associated with wounding and herbivory-induced defense responses, but not universal across all plant-pathogen or plant-insect interactions. The significant induction of PAL in *F. racemosa* highlights its role in gall-induced defense mechanisms, likely contributing to the production of phenolic compounds to counter insect stress.

Chalcone-flavanone isomerase (CHI) gene expression

This study demonstrates that CHI gene expression is activated during gall development in *F. racemosa*. The CHI gene, involved in flavonoid synthesis exhibited the second-highest FGE in galls among the studied genes, with an FGE of 44.36 ± 2.16, indicating significant induction due to gall-forming biotic stress by *P. depressa*. In gall leaves, CHI expression was also less than the control like other genes (Plate 6.9: Figure E).

In *Gossypium barbadense* (sea island cotton), which is highly susceptible to Fusarium wilt, seedlings overexpressing CHI genes showed enhanced resistance to the disease, underscoring protective role of CHI against pathogens (Zu et al., 2023). Additionally, in the lawn grass *Eremochloa ophiuroides,* wounding and methyl jasmonate treatments increased CHI transcript levels and total anthocyanin content in shoots, further linking CHI to stress-induced flavonoid production (Chung et al., 2019). The CHI gene expression was the second-highest among the studied genes, aligning with its stress-induced expression in *E. ophiuroides* under wounding (Chung et al., 2019). These findings highlight the critical role of CHI in flavonoid-mediated defense and stress responses across diverse plant species. The significant upregulation of CHI in *F. racemosa* during gall formation suggests its involvement in producing phenolic compounds to counter insect-induced biotic stress, consistent with its stress-inducible expression in other systems.

Dihydroflavonol 4-reductase (DFR) gene expression

The current study investigated DFR gene expression in response to gall formation and found that the expression is significantly higher in galls, with a 74.70-fold increase compared to all other genes studied, showing a markedly greater level of expression. The DFR expression observed in gall is the highest among studied genes (Plate 6.9: Figure F).

In Sitka spruce (*Picea sitchensis*), feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) upregulated DFR gene expression, highlighting its importance in flavonoid metabolism for conifer defense (Ralph et al., 2006b). In a resistant variety of *Camellia oleifera*, increased flavonoid accumulation was associated with anthracnose caused by *Colletotrichum gloeosporioides*, driven by the overexpression of flavonoid biosynthetic genes, including DFR. This overexpression likely enhances resistance to anthracnose (Yang et al., 2022).

However, DFR expression is not universally induced by biotic stress. In hybrid poplar (*Populus* spp.), cDNA microarray analysis showed no significant change in DFR gene expression in response to poplar leaf rust (*Melampsora medusae*) infection (Miranda et al., 2007). Similarly, cDNA array analysis of rice cultivars (resistant B5 and susceptible MH63) infested by the brown plant hopper (*Nilaparvata lugens*) revealed downregulation of DFR genes. Among 17 flavonoid pathway genes analyzed, two in B5 and five in MH63 were downregulated following insect damage, indicating a context-specific response (Zhang et al., 2004). Notably, in this study, the DFR gene displayed the highest upregulation in galls with an FGE of 74.70 ± 1.14, underscoring its critical role in flavonoid-mediated defense against gall-inducing insects, as seen in transgenic tobacco resistant to leaf cutworms (Kumar et al., 2013).

These findings suggest that DFR plays a significant role in flavonoid-mediated defense against insect-induced gall formation, particularly in *F. racemosa.* These findings highlight the context-specific regulation of DFR gene expression, with significant upregulation in response to certain biotic and abiotic stresses, but not others (e.g., leaf rust, plant hopper infestation). The pronounced induction of DFR in *F. racemosa* galls underscores its critical role in flavonoid-mediated defense against gall-inducing insects.

The expression profiles of all the studied genes across the different samples were statistically analyzed to determine the level of significance in their differential expression. ANOVA revealed that the observed differences in relative fold expression in galls were statistically significant ($p < 0.001$) in all cases, indicating that the variations in transcript abundance among the tissues were not due to random variation but reflected genuine

biological responses. The subsequent Tukey's HSD test confirmed that the gall induction and associated physiological changes leads to a measurable effect on gene expression patterns. Among the six genes analyzed in *F. racemosa*, none of the genes showed significant changes in expression in gall leaves compared to normal leaves $p > 0.05$, as indicated by the absence of significant differences in their relative fold expression values. However, all six genes (DAHPS, EPSPS, CS, PAL, CHI and DFR) exhibited strong and statistically significant upregulation in galls relative to normal leaves $p < 0.001$, suggesting that gall induction triggers a pronounced transcriptional activation of the shikimic acid and phenylpropanoid pathway genes. The activation of these genes is likely a part of the biochemical response of the plant to the infection caused by the gall-inducing insect *P. depressa*. Such differential gene expression indicates that *F. racemosa* enhances the synthesis of key secondary metabolites as a defense strategy to counter the stress induced by insect infestation. Graphical representation comparative expression of all selected genes in *F. racemosa* is provided as a bar diagram in Plate 6.8: Figure B.

Table 6.7: Relative fold gene expression levels of six selected genes in *F. racemosa* normal leaves, gall leaves and galls, with values expressed as mean ± SD.

Sl. No.	Name of gene	Relative fold gene expression in *F. racemosa*		
		Normal leaves (Control)	Gall leaves	Galls
1	DAHPS	1	0.39 ± 0.19	29.84 ± 0.52**
2	EPSPS	1	0.57 ± 0.04	30.26 ± 1.88**
3	CS	1	0.43 ± 0.08	35.16 ± 3.70**
4	PAL	1	0.37 ± 0.04	37.77 ± 4.13**
5	CHI	1	0.49 ± 0.14	44.36 ± 2.16**
6	DFR	1	0.35 ± 0.09	74.70 ± 1.14**

Each value of relative fold gene expression represents mean ± standard deviation (n = 3). **indicates values significantly different from normal leaves (control) at $p < 0.001$.

6.3.6. Comparison of gene expression

Compared to *F. racemosa*, the *A. scholaris* exhibits lower FGE in galls, particularly for DFR, reflecting species-specific responses to gall inducers. The moderate upregulation in *A. scholaris* possibly due to less aggressive induction of *P. tuberculata* compared to *P. depressa.* The downregulation of DFR in gall leaves suggests resource allocation to galls, a strategy to localize defense compounds. Higher FGE in galls of *F. racemosa* (DFR: 74.70) compared to *A. scholaris* (DFR: 8.97) reflects a more intense response to *P. depressa*. This may stem from greater phenolic diversity of *F. racemosa*, driven by DFR and CHI. The consistent downregulation of gall leaves in *F. racemosa* contrasts with moderate upregulation in gall leaves of *A. scholaris*, suggesting species-specific differences in defense strategies. The large range of phenolics in *F. racemosa* leaf galls compared to healthy leaf tissue also reflect the stress tolerance established due to the activity of the insect *P. depressa.*

This study showed that gall induction consistently increased the expression of all of the selected genes in leaf galls of both plants. Genes with low transcript abundance can be considered as a limited step in the pathway, whereas genes with high expression can be regarded as highly active enzymes in the system. Plants growing in stressed environments could biosynthesize more phenolic chemicals than plants living under normal conditions. As a result, the concentration of phenols in plant tissue serves as an excellent indicator of plant stress resistance.

Plants undergo significant metabolic reprogramming in response to herbivore interactions, activating both direct and indirect defense mechanisms that place substantial demands on primary metabolism. This reprogramming often involves a trade-off between growth-related processes and defense responses. Specifically, transcripts associated with photosynthesis are significantly downregulated, while those involved in stress responses, wound repair, pathogen defense and the transport of carbon and nitrogen for defense are markedly upregulated. For instance, in *Nicotiana attenuata,* herbivory by *Manduca sexta,* a moth induces an inverse relationship between photosynthesis and defense-related gene expression, prioritizing defense over photosynthetic activity (Hermsmeier et al., 2001). A similar pattern was observed in *Sorghum bicolor* during infestation by the phloem-feeding greenbug aphid (*Schizaphis graminum*), where defense gene upregulation coincided with

reduced expression of photosynthetic genes (Zhu-Salzman et al., 2004). Likewise, in *Picea sitchensis* (Sitka spruce), mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reduced the expression of photosynthesis-related genes in shoot tips and bark (Ralph et al., 2006b). These studies collectively demonstrate that plants reallocate metabolic resources from primary functions, such as photosynthesis to defense mechanisms under herbivore stress. This metabolic shift underscores the adaptive plasticity of plants, enabling them to prioritize survival and defense over the growth when faced with stressors. Collectively, such transcriptional reprogramming highlights the dynamic molecular adaptability of plants, where gene expression patterns shift rapidly upon herbivore or pathogen attack. This molecular shift promotes the enhanced synthesis of defensive phytochemicals, ensuring effective protection against biotic stress.

Comparative gene expression in gall leaves of *A. scholars* and *F. racemosa* (Plate 6.10)

In *A. scholaris*, all genes except DFR showed a fold gene expression (FGE) of greater than 1, indicating upregulation. The genes CS and CHI exhibited an FGE between 1 and 2, suggesting a moderate increase in expression. The genes EPSPS and PAL showed an FGE between 1 and 3, indicating a relatively higher upregulation compared to CS and CHI. The gene DAHPS displayed an FGE greater than 3, representing the highest level of upregulation observed among all the genes studied. In contrast, the gene DFR had an FGE of 1 or less, indicating no significant upregulation. In *A. scholaris*, all genes except DFR showed FGE greater than 1, indicating upregulation. In *F. racemosa*, all six genes exhibited FGE less than 1, indicating downregulation in gall leaf tissue.

Comparative gene expression in gall of *A. scholars* and *F. racemosa* (Plate 6.11)

In gall tissue, *F. racemosa* displayed higher FGE for all genes compared to *A. scholaris.* In *A. scholaris*, the genes DAHPS and DFR showed a FGE of less than 10. The genes CS and PAL exhibited an FGE of less than 20. The genes EPSPS and CHI displayed an FGE of less than 30. In *F. racemosa*, the gene DAHPS had an FGE of less than 30, representing the lowest among all the genes studied in *F. racemosa*. The genes EPSPS, CS The gene DFR showed the highest expression with an FGE of 74.70 ± 1.14.

Fold gene expression in gall leaves of *A. scholaris* and *F. racemosa*

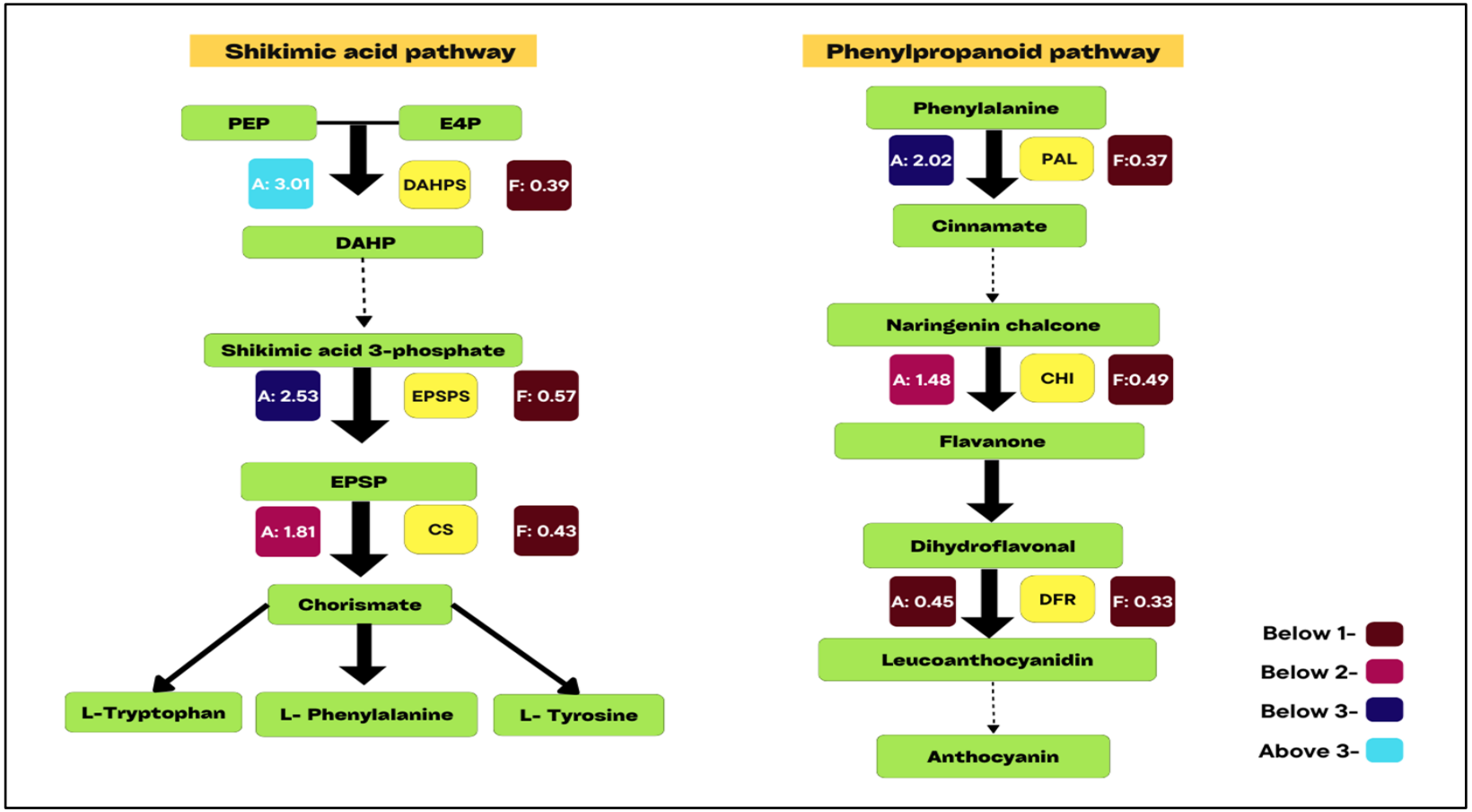


Plate 6.10: Gene expression in gall leaves: Fold gene expression (FGE) of selected genes in gall leaves of *A. scholaris* denoted with the letter 'A' and FGE of selected genes in gall leaves of *F. racemosa* represented with letter 'F'. Different colored boxes indicated with the range of FGE. Dotted lines indicate multiple steps.

Fold gene expression in galls of *A. scholaris* and *F. racemosa*

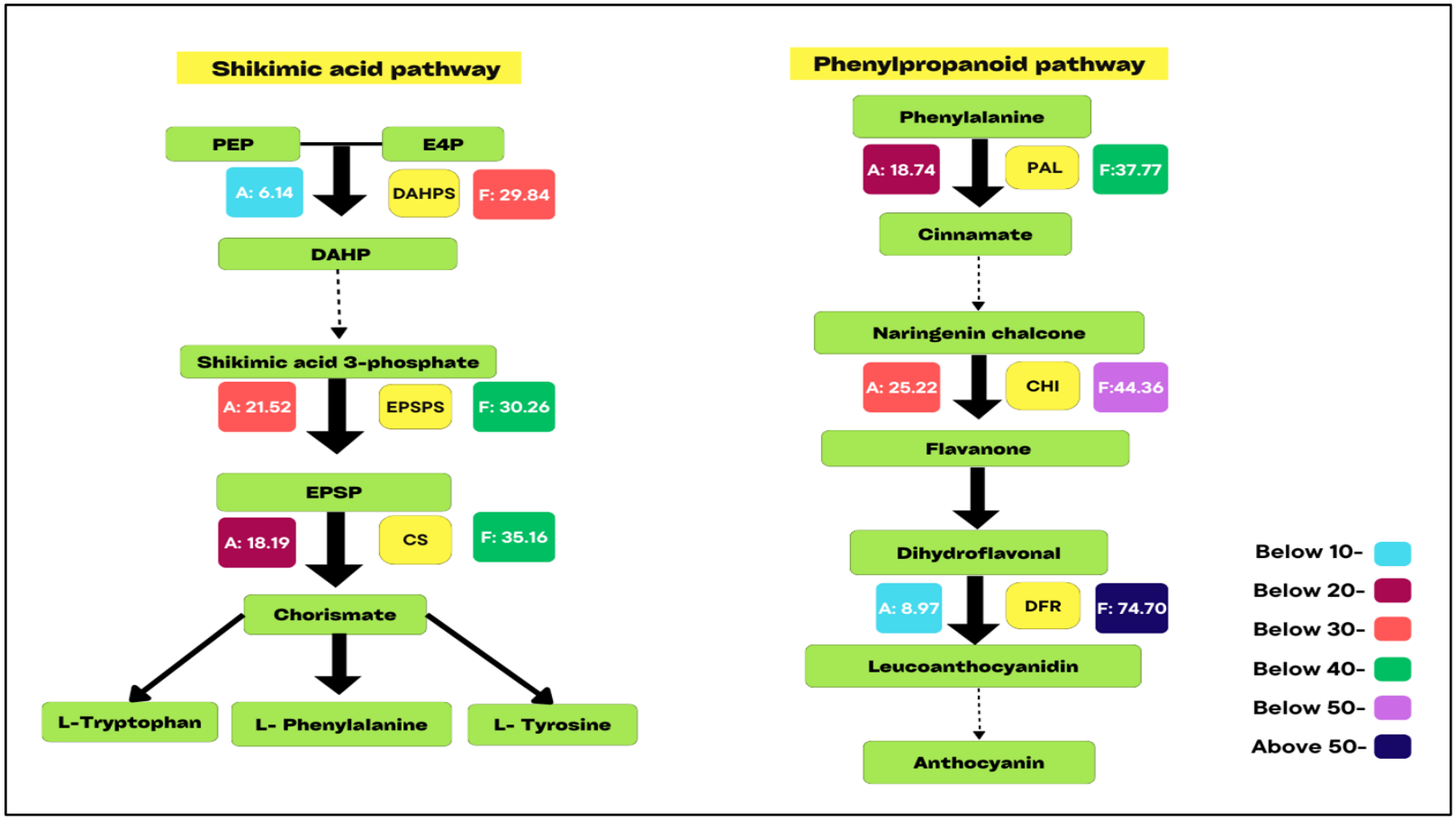


Plate 6.11: Gene expression in galls: FGE of selected genes in galls of *A. scholaris* denoted with the letter 'A' and FGE of selected genes in galls of *F. racemosa* represented with 'F'. Different colored boxes indicated with the range of FGE. Dotted lines indicate multiple steps.

This study elucidates the tissue-specific and species-specific regulation of shikimic acid and phenylpropanoid pathway genes (DAHPS, EPSPS, CS, PAL, CHI and DFR) in *F. racemosa* and *A. scholaris.* In the galls of *F. racemosa* exhibited significantly higher FGE for all genes compared to *A. scholaris.* This marked upregulation in *F. racemosa* galls, driven by *P. depressa* infestation, reflects enhanced transcription of secondary metabolic pathway genes, leading to elevated phenolic and flavonoid levels that strengthen defense against insect stress. In contrast, *A. scholaris* showed higher FGE in gall leaves (higher than control) compared to *F. racemosa* (all genes FGE < 1), indicating differential metabolic priorities between species.

The low FGE (<1) for all genes in *F. racemosa* gall leaves indicates no significant upregulation of defense-related pathways in the leaf tissue without gall. In gall leaves, unaffected by galling, *F. racemosa* maintains baseline metabolism, prioritizing growth and photosynthesis over defense gene expression, as seen in other systems under non-stressed conditions (Mithöfer and Boland, 2012; Taiz and Zeiger, 2010). This tissue-specific response aligns with studies showing herbivore-induced gene expression confined to attacked tissues, with unattacked tissues exhibiting minimal defense gene induction (War et al., 2012). Additionally, *F. racemosa* may allocate resources to gall tissues to support insect-derived structures, suppressing systemic defense gene expression in gall leaves to minimize metabolic costs (Mithöfer and Boland, 2012; Nabity et al., 2009).

However, earlier chapters revealed higher levels of bioactive chemicals and bioactivity in *F. racemosa* gall leaves compared to galls, despite the lack of gene upregulation. This paradox may be attributed to translocation of these compounds from other tissues (galls), maintaining defense response without requiring active gene expression during galling stress (Mithöfer and Boland, 2012; Schwachtje et al., 2006). Alternatively, post-transcriptional regulation or enzyme activity in gall leaves could sustain bioactive compound levels, bypassing the need for transcriptional upregulation (Ng et al., 2018; Staiger et al., 2013). These findings highlight the adaptive strategies of *F. racemosa* and *A. scholaris* in balancing growth and defense, with *F. racemosa* employing localized gall-specific responses. The study provides valuable insights into plant-insect interactions and secondary metabolite regulation, with implications for understanding defense mechanisms in gall-forming systems.

6.4. CONCLUSIONS

The molecular processes that take place within the cell or tissue will determine its physiological and biochemical properties. Normal leaves, gall leaves and galls of *A. scholaris* and *F. racemosa* were analysed using qRT-PCR, which demonstrated that the gall induction influenced the expression of genes. It will generate a distinct phytochemical profile in tissues of gall leaves and galls compared to normal or healthy leaf tissues. The infection by *P. tuberculata* on leaves of *A. scholaris* leads to the induction of genes in the pathway to a higher level up to 25-fold in galls. All the genes except DFR showed activation to a lesser extent in gall leaves also.

The insect *P. depressa* infected *F. racemosa* leaves also alter the transcript levels of genes of secondary metabolic pathway. The induction of gene expression leads to higher phenolic levels in *F. racemosa* galls also. There was no evidence of upregulation of gene expression in the gall leaves of *F. racemosa.* However, in the earlier chapters, it was discovered that the bioactive chemicals and bioactivity of the gall leaves was higher. Therefore, it can be explained by the possibility that phytoconstituents get synthesized in greater quantities in gall tissues and may be translocated or diffused to the surrounding leaf tissues. It may be a defensive mechanism shown by the plant. A different possibility might be post-transcriptional or post-translational regulation, in which changes in enzyme activity occur without variations in mRNA levels. The increased production of transcript in gall can be considered as a defensive action against gall inducers. The presence of a large number of phenolic compounds in galls and the rich transcript profile of important genes involved in phenolic biosynthesis will shed light on phenolic-mediated stress tolerance in these plants.

Thus gall and gall associated tissues have higher phytoconstituents and greater bioactivity. A comprehensive study of the phenylpropanoid and shikimic acid pathway, which is triggered by external stimuli, at the genic level can be used to enhance the therapeutic industrial production of plant-based formulations. It can also be used in plant breeding to improve the useful traits in crops like disease resistance and quality improvements. The results can be useful for further studies in the field of development of plants based drugs with much higher medicinal properties and bioactivities.

CHAPTER 7 – SUMMARY

This study elucidates the complex plant responses to the infection by gall-forming insects, emphasizing the integration of molecular and biochemical data to understand secondary metabolism dynamics in insect-induced galls. Comparative analysis of biomolecules in healthy leaves, galled leaves and galls provides insights into gall development processes. Insect-induced galls are recognized as sites of enhanced secondary metabolism, prompting the investigation of two gall-forming systems: *Pauropsylla tuberculata*-induced leaf galls on *Alstonia scholaris* and *Pauropsylla depressa*-induced leaf galls on *Ficus racemosa*. The study initially compared phytochemical profiles between normal (healthy) leaf tissues and insect-induced galls. Standard phytochemical assays revealed significantly higher levels of phenolics, flavonoids and other bioactive compounds in galls and galled leaves compared to normal leaves, indicating that gall formation triggers defense signaling and elevates bioactive defense compound production through upregulated secondary metabolic pathways.

To explore the relationship between biochemical changes and molecular pathway activation during gall development, key genes in the shikimate and phenylpropanoid pathways were analyzed using quantitative real-time PCR (qRT-PCR). The genes included 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), chorismate synthase (CS), phenylalanine ammonia-lyase (PAL), chalcone-flavanone isomerase (CHI) and dihydroflavonol 4-reductase (DFR). Results showed significant upregulation of these genes in galls compared to normal leaves, with *F. racemosa* galls exhibiting higher fold gene expression (FGE) than *A. scholaris* galls. This transcriptional activation correlates with the observed phytochemical accumulation, confirming that gall induction enhances secondary metabolism. In contrast, gall leaves showed significant biochemical differences but minimal gene expression changes compared to normal leaves, with *F. racemosa* gall leaves exhibiting FGE < 1 for all genes and *A. scholaris* gall leaves showing low FGE. This suggests that phytochemical accumulation in gall leaves may result from systemic translocation of metabolites from galls rather than local

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gene expression, as insects manipulate host tissues to act as nutrient sinks while plants redistribute defense compounds to adjacent uninfected tissues.

The medicinal potential of plants is closely tied to their bioactive phytochemical content. Using standard bioactivity assays, this study compared the antibacterial, antifungal, antioxidant and cytotoxic properties of normal leaves, galled leaves and galls in both species. Galls and galled leaves exhibited significantly higher bioactivity than healthy leaves, attributed to elevated secondary metabolite concentrations. These findings align with reports of galls being used in traditional medicine and highlight their potential in phytotherapy fields such as pharmacology, ethnomedicine and drug discovery. The results also support the validation of traditional medicinal practices, the development of sustainable natural products and applications in ecological and agricultural contexts.

This study enhances understanding of the complex reprogramming of plant defense and metabolic pathways triggered by gall-inducing insects. It elucidates the molecular basis for physiological, morphological and biochemical changes during gall formation, demonstrating how insects manipulate plant tissues for their benefit while plants adapt by enhancing defense compound production and distribution. The results underscore the plasticity of plant tissues and the role of galls as unique microenvironments that reflect intricate ecological interactions. Galls serve as valuable models for studying inducible secondary metabolism, offering insights into plant-insect interactions and the potential for novel therapeutic agent discovery.

CHAPTER 8 - RECOMMENDATIONS

Based on the study of structural, phytochemical and molecular characteristics of leaf galls of *Alstonia scholaris* and *Ficus racemosa*, the following recommendations can be suggested for further research and practical applications in the field of phytochemistry and plant biotechnology. The recommendations aim to deepen the understanding of gall-induced metabolic changes and explore their ecological and therapeutic implications.

1. More research into the phytochemical substances responsible for increased bioactivity in galls and galled leaves is required. This can be accomplished with modern techniques like high-resolution mass spectrometry. These techniques will make it possible to identify new chemicals. Such findings could have important phytopharmacological applications.
2. Transcriptomic and proteomic analyses can be used to identify all differentially expressed genes. These approaches help to understand the biochemical and molecular mechanisms behind the increased production of secondary metabolites during gall formation. They also provide insights into how gall-inducing insects change plant metabolism. This knowledge can support the development of strategies for metabolic engineering.
3. To explore the ecological role of gall formation in plant defense and its evolutionary significance, field studies should be conducted to assess how gall formation affects plant fitness, herbivory rates and interactions with other organisms. Comparative studies across different populations or species could reveal geographic or species-specific variations. These investigations will help clarify the adaptive significance of gall formation and its role in plant-insect co-evolution.
4. Metabolomics and genomics should be used to compare the phytochemical profiles and bioactivities of galled and non-galled tissues across different species and populations of *Alstonia* and *Ficus*. This approach will help identify species-specific or population-specific traits that influence gall-induced metabolic changes. Additionally, studies on other gall-inducing species should be conducted to determine whether these effects are specific to *Pauropsylla* species.
5. To advance the application of gall-induced bioactive compounds from *A. scholaris* and *F. racemosa* in drug discovery and phytopharmacology, screen the identified compounds for

therapeutic potential. Conduct *in vitro* studies using cell culture models to assess efficacy and safety. If promising, proceed with additional *in vivo* validations or preclinical trials to confirm effects. This could develop novel natural products or drugs, backing traditional uses of these plants.

6. To understand the impact of environmental factors on gall formation and secondary metabolism in *A. scholaris* and *F. racemosa*, research should assess how climate change influences these processes. Ecological studies suggest that climate variations could alter gall frequency and plant responses, affecting their medicinal properties. Long-term field studies under diverse environmental conditions should be conducted to evaluate these effects.
7. Future research should focus on exploiting upregulated secondary metabolite genes found in gall tissues for targeted metabolic engineering. These genes, critical for the medicinal properties of plants, offer opportunities to enhance phytochemical production in whole plants or cultured cells. Genetic engineering techniques should be used to upregulate these genes in target or related species. Plant cell cultures can enable scalable, consistent production of high-value metabolites *in vitro*.
8. To promote the conservation and sustainable use of *A. scholaris* and *F. racemosa*, efforts should focus on raising awareness. These plants, vital for their ecological and medicinal value, hold significant potential for sustainable utilization. Collaboration with local communities and organizations is recommended to develop effective management plans for their responsible use.

In conclusion, these recommendations provide a strategic framework for advancing research on *A. scholaris* and *F. racemosa*, harnessing their gall-induced bioactive potential for pharmaceutical innovation and ensuring their continued ecological and medicinal value for future generations.

RESHMA K RAMESH - Revised non core

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CHAPTER 1 - GENERAL INTRODUCTION

India is one among the most biodiverse countries globally and is home to approximately 8,000 plant species used in both traditional and modern medicine (Kala et al., 2006; Sharma et al., 2014). India has varied climatic zones including Himalayan heights, tropical rainforests and arid desert, which supports the growth of wide range of medicinal plants. Plants have been an essential part of Indian traditional medicinal system for centuries. Plants have been used in systems like Ayurveda, Unani and Siddha, as well as being recognized in modern pharmacology. The Ayurvedia documented over 2,000 plants have therapeutic value and used as medicine (Chandra, 2016; Mehta et al., 2021).

Plants produce different secondary metabolites including alkaloids, flavonoids, terpenoids, tannins and polyphenols, which are essential for defense, adaptation and ecological interactions. These phytochemical compounds exhibit diverse pharmacological properties such as antibacterial, anti-inflammatory, antioxidant and anticancer activities. Phytochemistry is the study of plant-derived compounds especially their structure, biosynthesis and beneficial functions. It played a significant role in the development of many modern drugs from traditional herbal medicines. These modern drugs derived from bioactive phytochemical constituents, highlights the importance of scientific validation for the development of new herbal medications (Kala et al., 2006; Marrelli, 2021).

1.1. Plant galls

Plants continually interact with biotic and abiotic environmental stresses throughout their growth and development. These stresses trigger the modification of physiological, biochemical and genetic processes of plants. Plant galls, known as cecidia, are abnormal growths induced by insects, mites, fungi, bacteria or viruses, representing a plant-parasite relationship. Due to the activities of these foreign organisms, the normal plant developmental patterns will change and these modifications will result in the development of galls. The significant changes due to plant-parasite interaction will appear in the morphology, biochemistry and molecular composition of affected plant tissues. In this relationship, the host plant will provide nutrition and shelter to the inducing organisms.

Cecidology is the branch of science that deals with the scientific study of plant galls, explores these complex interactions between plant and inducer organism (Albert et al., 2011; Mani, 2000; Raman, 2011; Shorthouse and Rohfritsch, 1992).

Galls show a varied pattern from normal plant development as the inducing organisms use the gall for meet their own needs. Therefore plant galls are often considered an extension of the phenotype of an inducer organism (Bailey et al., 2015; Lu et al., 2021). Galls are formed entirely of plant tissue; but they exhibit reprogrammed transcriptional pathways. So, galls are unique structures that example for how biotic stimuli like insects or other organisms can redirect plant developmental processes. After gall induction, the affected plant tissues produce much higher amounts and often different types of secondary metabolites. The research indicating higher concentrations of phytochemicals in gall tissues than unaffected plant tissue may potentially enhancing plant defense systems against herbivores (Hartley, 1998; Lu et al., 2021).

Approximately 13,000 species from various insect groups produce galls. The insect induced galls are the most complex and diverse among plant galls (Stone & Schönrogge, 2003). These galls may be develop on different plant parts like leaves, flowers, stems and roots, comprising highly organized structures with specialized tissue types (Raman, 2011; Stone & Schönrogge, 2003). Gall-inducing insects significantly modify the host plants for producing specialized tissues that are capable of provide nourishment, shelter and protection from natural enemies. Plants undergo morphological, anatomical, physiological, biochemical and molecular alterations during gall formation due to their dynamic environmental interactions (Rawat et al., 2012; Shorthouse & Rohfritsch, 1992).

1.1.1. Gall induction

Although elicitors or effectors responsible for gall formation have been reported, their precise mechanisms remain largely unknown and vary across insect classes. Plant growth regulators, such as auxins, cytokinins, indole-3-acetic acid (IAA), certain amino acids and proteins may act as potential inducing stimuli generated by the gall inducer (Gätjens-Boniche, 2019; Raman, 2011; Uma & Verghese, 2008).

Gall induction may begin when insects feed on immature leaves, then their salivary secretions acting as stimuli. Alternatively, oviposition can initiate gall formation, with female venom gland secretions triggering the process. In mites, stylets puncture epidermal cells, injecting saliva, while in Diptera and Lepidoptera, wounds formed by larval feeding release induction signals. Post-induction, insect excrement or other signals may contribute to gall development (Albert et al., 2011; Desnitskiy et al., 2023; Gätjens-Boniche, 2019; Raman, 2011; Tooker & Helms, 2014). Two fundamental events occurring during gall formation are cellular hypertrophy (cell enlargement) and hyperplasia (cell proliferation), leading to the development of galls. Reactive oxygen species (ROS) produce some metabolic reactions leading to the enhanced production of phenolic and hormones (auxins and cytokinins), that will leads to the development of gall (Dos et al., 2014).

1.1.2. Structure of gall

Galls are formed on any parts of plants and leaf galls are the most common in occurrence. Leaf galls produced on different flowering plants differ in their appearance, shape and color. Based on their complexity, they can be classified from simple thickenings to complex pouch galls enclosing the insect within differentiated organs. Complex insect-induced galls differ markedly from the host organ, forming unique tissues like nutritive tissue that nourishes the developing insect (Schultz et al., 2019). Galls mainly consist of three regions; they are (1) lignified zone; protecting the nymphs from environmental damage or enemies, (2) larval chamber; where nymphs develop and (3) nutritive area; surrounding the chamber. The cells of nutritive region are rich in cytoplasm, organelles, lipids, carbohydrates, nitrogen, proteins and other nutrients, with fragmented vacuoles and hypertrophied nuclei (Gätjens-Boniche, 2019; Raman, 2011; Shorthouse & Rohfritsch, 1992). The outer tissues of the gall are rich in defensive chemicals such as phenolics and tannins, which protect the insect from pathogens and predators. In contrast, the inner tissues function as nutrient sinks, providing the food necessary for the growth and development of the inducer insect (Hall et al., 2017).

1.1.3. Biochemical alterations due to gall development

Plants produce diverse bioactive secondary metabolites that have antibacterial and antioxidant properties. The phenolic compounds are critical for plant defense are often more abundant in gall tissues than in normal plant tissues. The flavonoid profiles in galled leaves are modified by stress responses produced by the gall-inducer organisms. Tannins are frequently occurring in higher amounts in galled tissues, which have astringency and therapeutic properties. Alkaloids exhibit difference in their composition during gall formation. Many of these exhibited neuroprotective and antibacterial activities. A similar observation was made for terpenoids, which showed different composition in galled tissues and are widely used in medicine for their diverse therapeutic effects (Biswas et al., 2014; Eshwarappa et al., 2015; Khurshid et al., 2021; Nyman and Julkunen-Tiitto, 2000; Shorthouse & Rohfritsch, 1992; Ushir et al., 2015). These modifications in phytochemical composition are the result from altered gene expression in biosynthetic pathways of secondary metabolites. In galls, stress-induced metabolic reprogramming increasing phytochemical production to strengthen plant defenses against the inducer (Chen et al., 2018; Oates et al., 2021; Wang et al., 2017a).

These modifications in secondary metabolite composition due to gall induction may serve as defensive mechanism of plants and potentially enhance therapeutic efficacy by altering biosynthesis of phytochemical compounds (Rawat et al., 2012; Shorthouse & Rohfritsch, 1992). Gall induction also affects phytohormone metabolism, stress-response pathways, photosynthesis, organ development and nutrient mobilization. Gall inducers alter source-sink interactions by making galls as nutrient sinks vital for nymph growth in insects like *Pauropsylla* (Hirano et al., 2020; Raman, 2011; Rawat et al., 2012). Phytohormones such as auxins, cytokinins and jasmonic acid regulate plant growth and defense, with elevated levels in galled tissues causing abnormal development and altered secondary metabolite synthesis (Roy & Das, 2023; Tooker & Helms, 2014; Yamaguchi et al., 2012).

1.1.4. Molecular alterations due to gall development

Gall development results from molecular reprogramming within the host plant, with inducers altering gene expression to favor their survival. Differential expression of genes

linked to secondary metabolic pathways drives these changes, enabling inducers to reorganize host metabolic pathways (Chen et al., 2018; Oates et al., 2021; Shorthouse & Rohfritsch, 1992; Wang et al., 2017a). Transcriptomic studies of gall tissues reveal the enhanced expression of genes for alkaloid, phenylpropanoid, flavonoid and shikimic acid biosynthesis. This will leads to increased production of bioactive compounds during gall induction and thus that may enhance medicinal value of plants (Mizuki et al., 2024; Shorthouse & Rohfritsch, 1992).

The shikimic acid pathway is takes place in plastids and it links primary and secondary metabolism. It is a seven step pathway producing chorismate, a precursor to aromatic amino acids (L-phenylalanine, L-tyrosine, L-tryptophan) and secondary metabolites like phenolics and flavonoids (Herrmann & Weaver, 1999; Maeda & Dudareva, 2012; Santos-Sánchez et al., 2019). The phenylpropanoid pathway in plants produces flavonoids, tannins and lignins. During gall development key genes of this pathway get upregulated and leads to increased phenolic synthesis, potentially enhancing medicinal properties of plants (Chung et al., 2019; Hartley, 1998; Yang et al., 2022).

1.2. Significance of phytochemical and molecular study of plant galls

In plants, gall development results from complex interactions between biotic stresses produced by inducer and plant metabolism. This interaction will drive significant biochemical and molecular changes in metabolism. So, the study of these types of interactions in medicinal plants is crucial for understanding plant responses to biotic stress and their potential to enhance therapeutic compounds. Galled plant tissues show enhanced synthesis of flavonoids, tannins, alkaloids and terpenoids, suggests that gall induction modifies secondary metabolism. Increased phenolics compunds protects inducers from pathogens and herbivores and also helps to improve therapeutic properties of plants (Boava et al., 2011; Duan et al., 2014; Ferreira et al., 2017; Hall et al., 2017).

The accumulation of particular compounds in gall tissue highlights the co-evolutionary interaction between gall inducers and host plants. Increased synthesis of bioactive compounds may enhance the effectiveness of herbal formulations; while reduction in significant medicinal metabolites reflects the need of strict quality assurance of traditional medicine formulations (Nacoulma et al., 2013). Gall-induced biochemical

and molecular alterations could enhance therapeutic substance concentrations, supporting improved herbal formulations. By studying the differentially expressed genes in gall tissues may reveal targets for metabolic engineering to produce phytochemicals under controlled conditions (Albert et al., 2011; Dsouza & Ravishankar, 2014; Ferreira et al., 2017; Kariñho-Betancourt et al., 2019; Raman, 2011). Understanding the gall induced changes is essential for finding how the therapeutic value of affected plants gets modified. It also helps in development of new plant-based medication approaches.

Morphological, phytochemical and molecular investigations are necessary to find the impacts of gall formation on medicinal plants. Morphological studies reveal structural alterations of normal plant tissues, phytochemical analyses identify difference in phytochemical composition and molecular investigations provide insights into regulatory mechanisms of biosynthetic pathways (Nyman & Julkunen-Tiitto, 2000; Schultz et al., 2019).

This study directly compares the gall-induced changes in two medicinally significant plants. This comparative approach is unique because it examines how distinct biochemical and molecular responses to gall induction occurs in these plants. By identifying the bioactive compounds and gene expression changes due to gall induction, the present study aims to elucidate whether gall formation enhances or diminishes medicinal properties, offering insights for phytopharmacology and plant defense strategies.

1.3. Aim of the study

The study focuses on gene expression profiling and phytochemical analysis of leaf galls of *Alstonia scholaris* (L.) R. Br. and *Ficus racemosa* L.

1.4. Objectives of the study

1. Extraction and identification of the phytochemicals in galls and regular (normal) leaves of selected plants both qualitatively and quantitatively.
2. Comparison of the gall and regular leaves for phytochemical profile.
3. Bioactivity analysis of the phytochemicals for its anti-microbial, cytotoxic and anti-inflammatory activity.

4. Bioactivity guided fractionation of the active phytochemicals.
5. Genetic profiling of the regular leaves and galls for the expression of selected genes in different phytochemical pathways involved in secondary metabolites production through quantitative RT-PCR.

1.5. Plants selected for the study

Through a thorough literature search, lists of plants that produce galls on their leaves were prepared. In order to guarantee the scientific rigor, relevance and potential for novel discoveries of the research, a number of important variables were thoroughly evaluated before the plants were chosen. The criteria adopted for selection of plants were, the therapeutic value of the plants, having medicinally important plant parts, taxonomic diversity and ecological considerations, availability and prevalence of gall formation, taxonomic position and life cycle of the inducer, phytochemical potential of their galls, practical considerations including accessibility and ensuring sufficient material for phytochemical and molecular analyses. Also based on the gall size and structures, that are helpful for precise sampling of galled and non-galled tissues, enabling accurate comparisons. The details of the selected plants are given below;

1. ***Alstonia scholaris* (L.) R. Br.**

Systematic position

Kingdom	: Plantae
Clade	: Tracheophytes
Clade	: Angiosperms
Clade	: Eudicots
Clade	: Asterids
Subclade	: Lamiids
Order	: Gentianales
Family	: Apocynaceae
Genus	: *Alstonia*
Species	: *Alstonia scholaris*

A. scholaris is a medium to large sized tree, grows up to 30 m height. It is commonly known as ‘Devil Tree’, ‘Milk wood pine’ or ‘Saptaparna’. In Kerala, the plant is known as *Ezhilamppala* or *Yekshippala*. It has corky, grey to grey-white, rough bark, which is lenticellate. Produce latex when injured, which is milky white. Branches whorled; branchlets terete, glabrous. Leaves are in a whorl of 4-7, coriaceous, glabrous, elliptic to oblanceolate, entire margin, acute base and rounded apex. The inflorescence is an umbellate cyme. Flowers bisexual, light green, pubescent, strongly perfumed. Fruits are follicles, linear with more than 20 cm long; pendulous, cylindrical and green. The Seeds are many, cylindrical, glabrous, and smooth with a tuft of hairs on both ends. Plate 1.1 represents the habit of the plant *A. scholaris.*



Plate 1.1: Habit of *Alstonia scholaris* (L.) R. Br.

2. *Ficus racemosa* L.

Systematic position

Kingdom	: Plantae
Clade	: Tracheophytes
Clade	: Angiosperms
Clade	: Eudicots
Clade	: Rosids
Order	: Rosales
Family	: Moraceae
Genus	: *Ficus*
Species	: *Ficus racemosa*

The *F. racemosa* is a large deciduous evergreen tree, which grows up to a height of 15-25 m (Plate 1.2). It is commonly known as 'Cluster Fig' or 'Indian fig'. In *Malayalam*, the plant is known as *'Atthi*'. It is one of the four species of *Ficus* that make up the well-known Ayurvedic preparation known as *'Nalpamara'*. Young shoots are glabrous, pubescent. Bark rusty brown with a fairly smooth and soft surface. Leaves are simple, dark green colored and rough when mature. Flowers are unisexual. Male and female flowers within the same fig. Male flowers found near apical pore, sessile, calyx lobes 3 or 4, stamens 2. Female flowers are pedicellate, calyx lobes linear, style lateral, stigma clavate. Fruits, 1-2 cm in diameter, subglobose or pyriform, found in large clusters on the main trunk or large branches (cauliflorous). When ripe, they are orange, reddish or dark crimson.



Plate 1.2: Habit of *Ficus racemosa* L.

CHAPTER 2 - LITERATURE REVIEW

Plant galls are abnormal growths induced by organisms like insects, mites, fungi, bacteria and viruses and are a striking example of plant-parasite interactions. The leaf galls are not only morphologically unique but also exhibit distinct biochemical and molecular profiles, serving as nutrient-rich shelters for inducers (Gätjens-Boniche, 2019; Mani, 2000; Raman, 2011). In medicinal plants, such as *Alstonia scholaris* and *Ficus racemosa*, galls may alter the production of bioactive compounds, impacting their therapeutic potential (Biswas et al., 2014; Chauhan & Chauhan, 2020; Eshwarappa et al., 2015; Muhammad et al., 2023; Ushir et al., 2015). There are inconsistencies in the findings about gall physiology and phytochemistry particularly in medicinal species, so this chapter critically evaluates the literature on plant galls, focusing on their formation and biochemical changes, focusing on *A. scholaris* and *F. racemosa*, to identify gaps that the current study addresses.

2.1. Medicinal plants and importance of secondary metabolites

In India, for more than thousands of years, medicinal plants are the essential part of traditional medicine, where about 7,500 species are used in traditional health care systems like Ayurveda, Siddha and Unani (Kala et al., 2006). The World Health Organization (WHO) estimates that 80% of people in developing countries rely on plant-based drugs, with over 21,000 plant species being utilized for medicinal purposes globally (Mehta et al., 2021). The secondary metabolites such as alkaloids, flavonoids, phenolics and terpenoids, that are produced from plants possess different pharmacological properties, including antibacterial, antioxidant and anti-inflammatory effects (Jain et al., 2019; Pagare et al., 2015).

The oldest form of medicine is the direct use of medicinal plants, which have been employed as traditional medicine for thousands of years in different countries worldwide (Marrelli, 2021). The pharmaceutical research has improved its reliability by identifying

the function of the active phytochemical constituents found in medicinal plants and identifying their action in both human and animal systems (Jain et al., 2019). The phytochemical constitution and possible health advantages of numerous plants have not yet been thoroughly examined and require further research. Future herbal medications will thus be made possible by the essential scientific validation of these plant-based medical benefits (Kala et al., 2006; Marrelli, 2021).

Chemical defense is one of the most important mechanisms in plant resistance to diseases and herbivores. The secondary metabolites present in plants can shield them from a wide range of herbivores (such as arthropods and vertebrates) and microorganisms (such as bacteria, fungus and viruses). Additionally, these compounds play a significant role in the adaptation of plants to their environment. Thus these phytochemicals are important in defending plants against both biotic and abiotic stresses (Pagare et al., 2015; Zaynab et al., 2018). They also serve a variety of purposes, such as providing vitality, structure, signaling, stimulating and inhibiting enzymes and enhancing their own catalytic activity. Their range within the plant kingdom is relatively constrained and unique. For a given plant species growing in different environmental conditions, the phytochemicals may vary in both the quantity and quality. They are usually generated by particular cell types at different developmental stages in lower quantities (Jain et al., 2019; Pagare et al., 2015).

2.2. Introduction to plant galls

Plant galls are induced by a diverse array of organisms, including bacteria, nematodes, insects, mites and fungi, with insects and mites being the most common inducers (Bailey et al., 2015; Raman, 2011; Uma & Verghese, 2008; Ushir et al., 2015). Galls can be developed on various plant organs, among them leaf galls are predominant (Schultz et al., 2019; Ushir et al., 2015). Globally, approximately 13,000 insect species induce galls, with 2,000 reported in India and 87 gall inducing insects found from Northern Kerala (Saleem & Nasser, 2015; Stone & Schönrogge, 2003). Galls vary in shape, size and color, influenced by the host plant, inducer species and plant developmental stage (Ananthakrishnan, 1998; Mani, 2000). Uma and Abraham reported that gall making habit was found in 6 different Orders such as Diptera (flies), Hemiptera (aphids), Coleoptera

(beetles), Hymenoptera (sawflies and wasps), Lepidoptera (moths and butterflies) and Thysanoptera (thrips), 90% of these insects are host specific (Uma & Verghese, 2008). The development of galls proceeds through distinct stages, including initiation, growth and differentiation, maturation and finally dehiscence (Gätjens-Boniche, 2019; Ushir et al., 2015). According to Takeda and colleagues, gall development can be divided into 4 steps: secretion of signaling molecules from insects, perception of the signals by plants, plant cell regeneration and differentiation and organization of gall tissue (Takeda et al., 2019).

Martinson and colleagues (2021) demonstrated that gall is transcriptionally as well as anatomically complex structures (Martinson et al., 2022). Galls arise as an extension of the host-plant phenotype (Datta & Tomar, 2022) and some researchers report galls as an extension of inducers phenotype (Bailey et al., 2015; Lu et al., 2021). Galls provides food and protection for the larvae of the inducer and also protecting them from abiotic stresses and other predators (Davidovich-Rikanati et al., 2022; Gätjens-Boniche, 2019; Ushir et al., 2015). A rich cytoplasm with numerous cell organelles, broken vacuoles, a hypertrophied nucleus and nucleolus and dedifferentiated plastids grouped around the nucleus are the characteristics of a typical nutritive tissue (Schultz et al., 2019; Ushir et al., 2015). According to the ‘nutrition hypothesis’ the gall interiors are rich in nutrients like sugars, lipids and proteins, while exteriors are enriched with defensive compounds like phenolics and tannins to deter herbivores (Hall et al., 2017; Price et al., 1987). Nyman and Julkunen-Tiitto in 2000 support this, by reporting higher phenolic concentrations in gall exteriors to deter herbivores (Nyman & Julkunen-Tiitto, 2000). However, there exist some conflicting findings, that uniform distribution of secondary metabolites, such as terpenoids, throughout galls (Davidovich-Rikanati et al., 2022).

2.2.1. Gall induction and development

The exact mechanism of gall induction is still unknown, so that there exist different hypotheses on the induction process of galls. A physiological fluid injected during oviposition by insects would mediate gall induction. Another hypothesis proposes that gall formation is the result of mechanical irritation produced by an external organism. The third hypothesis proposes that the saliva of the inducing insect contains some active proteins

along with lytic enzymes that acts as a chemical shock and leads to induction of galls. Some research suggested that rather than adult feeding, larval feeding damages the host epidermal cells, which act as a stimulus for gall induction. Finally, it has also been hypothesized that the excretion of metabolic products from the insect may act as inductive stimuli for gall initiation (Desnitskiy et al., 2023; Gätjens-Boniche, 2019; Raman, 2011).

Researchers found that hormones like auxin and cytokinins are found to accumulate during gall induction. The interaction between these plant hormones and phenolic compounds will helps in the growth of the galls (Gätjens-Boniche, 2019; Hall et al., 2017; Hartley, 1998; Isaias et al., 2014). In combination with auxin, the cytokinins promote cell division and thus help in gall development. There are reports that, the cytokinins have been found to upregulate genes controlling the phenylpropanoid pathway, which is responsible for the synthesis of various phenolic compounds. So, they helps in the production of significantly higher amounts of phenolics and tannins in insect induced galls (Hall et al., 2017). There is no details available in literature about the working of these chemicals and the general processes by which the insect could regulate and alter the physiology and development of plants (Gätjens-Boniche, 2019; Raman, 2011).

2.2.2. Effects of gall inducers on host plants

The relationship between gall development and photosynthesis continues to be a controversial and actively researched topic. There exist several studies suggesting the reduced photosynthetic activity of galls. In this case, galls will functioning as nutrient sinks that take nutrients from surrounding tissues (Desnitskiy et al., 2023; Martinson et al., 2022). Martinson and colleagues found that the photosynthetic genes in gall tissues are downregulated, supporting the ‘sink hypothesis’ (Martinson et al., 2022). Similarly, Florentine and colleagues reported decreased transpiration and stomatal conductance in galled tissues (Florentine et al., 2005). These results suggest that the galls function as sinks, taking nutrients from surrounding non-gall tissues (Hall et al., 2017). In contrast, Fay and colleagues found enhanced photosynthesis in *Silphium integrifolium* galls and they suggest it as a compensatory response to overcome stress induced by the inducer. The reaerschers found that, this discrepancy may arise from differences in plant species, gall inducers or

experimental conditions (Fay et al., 1993). Retuerto and colleagues in 2004 demonstrated that gall tissue itself increases photosynthesis relative to uninjured tissue (Retuerto et al., 2004). For instance, Takeda and colleagues in 2019 found consistent downregulation of photosynthetic genes that is in contradictory to the compensatory hypothesis (Takeda et al., 2019). These conflicting findings point to the important gap in the effect of gall induction on photosynthetic efficiency in medicinal plants, where energy allocation could influence secondary metabolite production. Pathways for amino acid synthesis and degradation were shown to be altered in aphid galls in a way that is advantageous for the inducer. The production of glucose was found increased after gall induction (Lu et al., 2021). There are many research studies showing that gall-inducing insects influence their host plants in various ways, including biochemically, physiologically, and genetically. These alterations will be performed with the aim to promote inducers growth (Biswas et al., 2014; Saini & Sarin, 2012).

2.2.3. Use of plant galls

In medicinal plants, gall induction can significantly impact the concentration of bioactive compounds, such as phenolics, flavonoids, tannins, alkaloids and terpenoids. These compounds contribute to the pharmacological activities of plants, including antioxidant, antimicrobial, anti-inflammatory and anticancer properties (Ahmed & Urooj, 2010; Yamaguchi et al., 2012). Various plant galls have been reported to possess medicinal uses. Uddin in 2011 reported that galls of *Pistacia chinensis* were used in treatment of asthma, respiratory tract infection, dysentery, bronchitis, cough, vomiting skin diseases, fever and also to increase hunger (Uddin et al., 2011). In 2008 a work by Ganesh and colleagues proved the use of leaf galls of *Pongamia glabra* in the treatment of bronchitis and cough (Ganesh et al., 2008). *F. racemosa* plant is used in different systems of medicines including Ayurveda. Bark, fruits, leaf, roots, latex and seeds were found to be used along with other herbs. But no use of leaf galls of *F. racemosa* reported (Ushir et al., 2015).

2.3. *Alstonia scholaris*

The plant belongs to the Family Apocynaceae. It is a large evergreen tree growing up to 200 m height. Almost all parts of the plant are used for treatment of different ailments in various systems of medicines like Ayurvedic, Unani and Siddha. It possesses different pharmacological activities and is potentially used as an antimalarial drug. Various extracts of different plant parts have possessed anticancer, antibacterial, antifertility, antitussive, antitumour, antidiabetic, anthelmintic and antiepileptic properties (Bonvicini et al., 2014; Kanase & Mane, 2018; Khan et al., 2003; Saini & Sarin, 2012). The parts were used as febrifuge, also used as tonic for digestion and stimulation of appetite. Used in treatment of liver and intestinal troubles, malaria, diarrhea, dysentery, gonorrhea, hypertension, fever, leprosy, skin diseases, bronchitis, asthma and lung cancer. The bark is anthelmintic, astringent and antiperiodic. It is used against chronic diarrhoea, dysentery and bowel movements. Alkaloids present in it can cause a rapid fall in the blood pressures and are hypotensives. Leaves are used against liver congestion, dropsy and ulcers. The latex is useful as an application for ulcers, sores, tumours and in rheumatoid pain (Bonvicini et al., 2014; Khan et al., 2003). The stem-bark has been used in the antimalarial drug formulation ‘Ayush64’, developed by the Central Council for Research in Ayurvedic Sciences, Calcutta (Das et al., 2020).

Different classes of secondary metabolic compounds have been isolated from the plant, including alkaloids, flavonoids and terpenoids. Alkaloids such as alstonidine, alstonine, chlorogenic acid, chlorogenine, echitine and echitamine. Triterpenoids include linoleate and lupeol (Bonvicini et al., 2014; Das et al., 2020). Echitamine, Echitamine chloride, Scholarine, Scholaricine, monoterpenoid indole alkaloids, iridoids, coumarins, phenolics, steroids, saponins and tannins were also reported to be present in the leaves and bark of the plant (Saini & Sarin, 2012).

Leaf galls of *A. scholaris*

Leaf galls of *A. scholaris* are pouch-like and globular formed on the upper surface of the leaf as isolated or as agglomerate structures (Tripathy et al., 2019). Young galls are green or light green in colour and when they mature change to brownish or yellowish with a hard woody texture (Bhatnagar et al., 2022). The galls are produced due to infection by

the insect, *Pauropsylla tuberculata*. The life cycle of this insect passes through the egg stage, nymphal instars and then the adult stage. *Pauropsylla* is a hemimetabolous insect, which has no larval stage (Albert et al., 2011; Pasayat & Tripathy, 2020). Biochemical analysis shows that phenolic compounds were found to be higher in galls compared to leaves (Biswas et al., 2014; Muhammad et al., 2023). High proline and phenol contents and low chlorophyll level was observed in gall by Rasool and colleagues in 2019 (Rasool et al., 2019).

2.4. *Ficus racemosa*

F. racemosa is an important medicinal tree in India. The plant is used for treatment of diabetes, liver disorders, respiratory, urinary diseases and inflammatory conditions. Different parts of the plant such as root, bark, latex, leaves, fruit, seeds and galls were medicinally important (Eshwarappa et al., 2015; Kumari et al., 2020; Singh et al., 2023). The various extracts of plant parts have diverse biological activities such as anti-inflammatory, hepatoprotective, chemopreventive, antidiabetic, antipyretic, cardio-protective, mosquito larvicidal, gastroprotective, antidiuretic and hypolipidemic activity. The roots of the plants are used for the treatment of diarrhea and hydrophobia. The milky juice produced from the bark is used to treat piles and diarrhea. In combination with sesamum oil, there is report of use of it to treat cancer. The leaves are also useful for bronchitis and are astringent to the bowels. The bark and fruit are astringent and used in the treatment of haematuria, menorrhagia and hemoptysis (Bagyalakshmi et al., 2019; Singh et al., 2023; Ushir et al., 2015) The flavonoids and polyphenols present in the plants help in prevention and cure of many diseases (Bagyalakshmi et al., 2019; Chaware et al., 2020).

Leaf galls of *F. racemosa*

Leaf galls found in *F. racemosa* are induced by the insect, *Pauropsylla depressa*. The pouch galls developed on the leaves are simple, fleshy, globose, sessile, unilocular, sometimes agglomerate or multilocular. Colour varies from yellow to reddish brown. The galls measure about 15- 30 mm in diameter (Savitha et al., 2013; Singh et al., 2023). Galls were initially green during development, turned brown as they matured and then turned reddish brown during the senescence stage (Ushir et al., 2015). The galls contained different active phytochemicals such as flavonoids, triterpenoids, alkaloids and phenolics.

They also reported that the leaf galls are pharmacologically significant as the TLC analysis of ethanolic extract also proved the presence of active phytoconstituents (Ushir et al., 2015). The use of leaf galls of *F. racemosa*, reported by Kumari and colleagues in the preparation of 'Chvyanprash avaleha' and 'Kumari Asava' (Eshwarappa et al., 2015; Kumari et al., 2020).

2.5. Phytochemical composition of the selected plants

Notable differences between the phytochemical composition of healthy leaves and galls were reported in previous studies on selected plants, *A. scholaris* and *F. racemosa*. During the insects attack, the plants produce more defensive chemicals and thus the galls have higher levels of certain compounds compared to normal leaves. Advanced techniques like Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS) help identify these compounds, revealing the phytochemical profiles in galls.

2.5.1. Phytochemical composition of normal leaves

There are reports on the chemical constituents of *A. scholaris* and *F. racemosa* leaves. For *A. scholaris*, Labaran and colleagues (2024) found that methanolic extracts contain phenolics, flavonoids and alkaloids, but lack proteins, anthraquinone or reducing sugar. In contrast, aqueous extracts include reducing sugar, amino acids, phenolic compounds, anthraquinone and saponins (Labaran et al., 2024). For *F. racemosa*, both aqueous and methanolic leaf extracts revealed saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids (Mpala et al., 2019). Additional studies identified lanosterol, tetracyclic triterpene-glauanol acetate alkaloids, tannins and significant amounts of total phenols and flavonoids in *F. racemosa* leaves (Pahari et al., 2022; Yadav et al., 2019).

2.5.2. The effect of insect attack on phytochemical composition of plants

Plants produce defensive secondary metabolites, including nitrogenous alkaloids, terpenes, phenols and flavonoids, in response to insect attacks. This is supported by

multiple studies done in 2019-2022 (Davidovich-Rikanati et al., 2022; Qi et al., 2020; Takeda et al., 2019). For instance, tobacco plants infected with *Rhodococcus fascians* exhibit significant metabolic changes, yielding new compounds with therapeutic potential (Nacoulma et al., 2013). Qi and colleagues found that, the insect attacks can trigger the production of over 200 distinct volatile compounds, which helps in enhancing plant defense (Qi et al., 2020).

2.5.3. Phytochemical profile of plant galls

Galls exhibit unique phytochemical profiles compared to normal leaf tissues. High-performance thin layer chromatography (HPTLC) fingerprinting revealed clear variations in phytoconstituents between leaves with and without galls (Das et al., 2020). Mizuki and co-workers identified that, galls typically contain higher concentrations of total phenolics, terpenes and tannins across all growth stages (Mizuki et al., 2024). However, some studies report no significant difference in volatile compounds between galled and normal tissues (Hall et al., 2017). The 'nutrition hypothesis' suggests that galls accumulate higher nutrient concentrations internally with reduced defensive compounds, while their exterior has elevated phenolics and tannins to deter herbivory (Hall et al., 2017; Price et al., 1987). This is supported by the findings of (Nyman & Julkunen-Tiitto in 2000 that bioactive phytochemicals are rich in gall exteriors to reduce predation risk (Nyman & Julkunen-Tiitto, 2000).

Each type of gall exhibits a distinct metabolomic profile. In some cases there exist similarities in metabolomic alterations following gall induction across different plant species; it may indicate the presence of common induction mechanisms. But the differences in metabolomics profile could reflect species-specific gall structures or causal agents (Markel et al., 2024).

Specific findings of phytochemical constituents in galls of *A. scholaris*

In galled leaves of *A. scholaris* reported the presence of an alkaloid Alstorisine A by Chen and colleagues in 2016 (Chen et al., 2016). Other identified compounds include ursolic acid, lupeol acetate, 17-nor-excelsinidine, 19, 20 E-vallesamine and an unidentified monoterpene indole alkaloid, 2-hydroxy deacetyl akuammiline (Pandey et al., 2023). Galls

are considered a promising source of bioactive phytochemicals (Pandey & Kate, 2024). Proline content in galled tissue is significantly elevated; indicating stress, with mature galls containing approximately twice as much as young galls (Albert et al., 2011). A negative correlation exists between phenolic compound production and chlorophyll content, with perforated galls showing higher phenolic content than mature or young galls (Biswas et al., 2014; Muhammad et al., 2023).

Specific findings of phytochemical constituents in galls of *F. racemosa*

F. racemosa galls are rich in pharmacologically important phytochemicals, including alkaloids, phenolic compounds and terpenoids, with high levels of phenolics, lignins and tannins (Bagyalakshmi et al., 2019; Ushir et al., 2015). Phenolic content in galls exceeds that in healthy or galled leaves, with elevated orthodihydric and total phenols. Polyphenols and dihydroxyphenols act as IAA-oxidase inhibitors, contributing to gall development (Dsouza & Ravishankar, 2014). Methanolic gall extracts exhibit higher phenolic and flavonoid content, resulting in greater antioxidant activity (Eshwarappa et al., 2015).

2.5.4. Analytical techniques used to find metabolomic profiles

Advanced analytical methods, such as Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS), are effective for identifying bioactive phytochemicals in plants (Goyal & Shinde, 2020). For *A. scholaris*, GC-MS analysis of stem bark identified compounds like hexadecanoic acid methyl ester, 1,4-benzenedicarboxylic acid dimethyl ester and dodecanoic acid methyl ester (Bagheri et al., 2020). Carranza and colleagues found 18 volatile compounds from dichloromethane bark extracts (Carranza et al., 2020). Analysis of leaf extract confirmed alkaloids such as Akuammidine, Voacristine, Strictamine and Nicotine (Hamdiani et al., 2018), with metabolic extracts identifying seven phytoconstituents (Chakraborthy et al., 2016). In case of *F. racemosa*, GC-MS revealed bioactive secondary metabolites in leaves, with isoamyl nitrite showing the highest peak area (Sharma & Kumar, 2021). Phytochemical profiling identified 48 phytoconstituents in fruit extract and 80 in leaf extract (Rani et al., 2024),

with other studies reporting six to ten compounds in fruits (Kannan et al., 2024; Swargiary et al., 2020).

LC-MS/MS analysis of *A. scholaris* latex detected quercetin, kaempferol and ursolic acid (Banik et al., 2023), while bark analysis yielded different active phytochemicals (Carranza et al., 2020). For *F. racemosa*, LC-MS of aqueous extract confirmed 11 phytochemical compounds, with phloretin (64.165%) and choline (11.933%) showing the highest area percentages (Dhas et al., 2021). Chloroform extract analysis identified 10 active compounds in *F. racemosa* leaves (Shaikh et al., 2020).

2.6. Biological properties of different parts of selected plants

2.6.1 Antioxidant activity

Research indicates that *A. scholaris* exhibit significant antioxidant activity. Methanol extracts of stem bark and leaves demonstrate strong DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging, with ethyl acetate extracts following closely. The phenolic compounds are key contributors of antioxidant activity (Itam et al., 2018). The bark shows mild to moderate DPPH scavenging activity (Bellah et al., 2017), while ethanolic leaf extracts are highly effective across various assays (Arulmozhi et al., 2010). In leaf galls, antioxidant enzymes (catalase and peroxidase) correlate positively with phenolic content, enhancing the antioxidant capacity of plants (Biswas et al., 2014).

For *F. racemosa*, DPPH assays reveal that fruit extracts have greater antioxidant activity than leaf extracts (Bagyalakshmi et al., 2019). Seeds outperform leaves in antioxidant potential and leaf extracts exhibit dose-dependent reducing power (Hosain et al., 2011). Yadav conducted different methods of antioxidant assays in leaves of the plant and found that it has dose dependent reducing power activity (Yadav et al., 2019). *F. racemosa* stem bark and leaves found to exhibit significant radical scavenging activity (Sultana et al., 2014). DPPH radical scavenging activity, reducing power assay and nitric oxide scavenging activity analysis were done by Londhe and colleagues 2012 to prove the antioxidant activity of ethanolic extracts of leaves of *F. racemosa* and found that the leaves were potential source of plant antioxidants (Londhe et al., 2012). Sumi and colleagues also investigated reducing capacity of the leaves of the plant by using different approaches and

proved the same (Sumi et al., 2016). A work done by Eshwarappa and colleagues proves the significance of *F. racemosa* leaf gall extracts as a natural antioxidant and justify its ethnobotanical use (Eshwarappa et al., 2015). With an 80% radical scavenging potential, young galls seem to be a strong source of antioxidants (Savitha et al., 2013)**.**

2.6.2. Antibacterial activity

Different parts of *A. scholaris* and *F. racemosa* were checked for its antibacterial property. The stem bark extracts of *A. scholaris* were determined active against reference strains (Bonvicini et al., 2014; Lalchhandama et al., 2024) and clinical isolates (Bonvicini et al., 2014). Methanolic extracts of leaf, stem, root and bark extracts of *A. scholaris* have been reported to have potent antimicrobial activity (Khan et al., 2003). Flower extracts (benzene, methanol, water) are more effective against Gram-positive bacteria, while hexane and water extracts lack activity against *Pseudomonas aeruginosa* and *Salmonella typhi* (Thankamani, 2011). Hexane, water, benzene, isopropanol and methanol fractions of *A. scholaris* bark and leaf were tested against different bacteria and except isopropanol and methanol extract none of the other extracts showed no activity (Bagheri et al., 2020). Researchers found that metabolic extracts of the leaves of *A. scholaris* have higher activity than other extracts (Labaran et al., 2024).

The disc diffusion assay showed no antibacterial activity from methanolic and aqueous extracts of *F. racemosa* leaves against a variety of human pathogenic bacteria, such as *Staphylococcus aureus* and *Escherichia coli* (Mpala et al., 2019). But there is a report that leaves of *F. racemosa* were found active against the growth of *Actinomyces viscosus* (Shaikh et al., 2010). Antibacterial activity of leaf and fruit of *F. racemosa* proved that the extracts possess good inhibition activity against wound isolates (Bagyalakshmi et al., 2019). The seeds and leaves of *F. racemosa* were tested against Gram-positive and Gram-negative bacteria and found that both showed moderate activity (Hosain et al., 2011). Kumari and colleagues checked the antibacterial activity of leaf gall extracts of *F. racemosa* and reported that gall extracts are more inhibitory to Gram-negative bacteria in comparison to Gram-positive bacteria. The gall extracts have more inhibitory effects than leaf extracts against the fifteen bacterial isolates tested (Kumari et al., 2020). It was found that silver nanoparticles made from *F. racemosa* gall extracts had strong antibacterial action against *S. aureus* and *E. coli*. Significant anticancer activities have also been demonstrated (Sabira et al., 2024).

2.6.3. Antifungal activity

Previous studies on different parts of both plants proved its antifungal activity. Bonvicini and colleagues proved the antifungal activity of stem bark extracts of *A. scholaris* against *Candida albicans* (Bonvicini et al., 2014). Isopropanol, methanol and water extract of leaf found to inhibit the growth of *Candida* spp and *Cryptococcus* spp (Bagheri et al., 2020). The bark carbon tetrachloride and chloroform extracts showed growth inhibition to *Aspergillus niger* and *C. albicans* (Bellah et al., 2017). Crude ethanolic extract of leaves showed antifungal activity against *C. albicans* (Ara et al., 2020).

Methanolic extracts of leaves of *F. racemosa* showed better antifungal action against both *C. albicans* and *A. niger* (Mishra et al., 2023). The ethanolic and ethyl acetate extract of fruits also showed antimicrobial activity against *C. albicans* and *A. niger*, these properties support the traditional use of *F. racemosa* for treating fungal infections (Pingale et al., 2019).

2.6.4. Cytotoxic activity

A. scholaris found to have strong cytotoxic effects against cancer cells. Studies show that the n-hexane fraction of *A. scholaris* is highly toxic to breast cancer (MCF-7) cells, while the ethanol fraction is less harmful to vero cells (Zuraida & Mariya, 2020). Bark extracts (methanol) also showed significant cytotoxicity in brine shrimp lethality tests (Bellah et al., 2017). Bagheri and colleagues identified that, hexane extracts of stem bark and leaves has promising anticancer activity against various cancer cell lines using different assays such as trypan blue exclusion, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and apoptotic assays (Bagheri et al., 2016). Leaf extracts (ethyl acetate fraction), show high cytotoxicity against brine shrimp (*Artemia salina*) and leaves are noted for significant bioactivity (Ara et al., 2020; Sarkar et al., 2021).

Evidence indicates that *F. racemosa* has notable cytotoxic properties. Methanol extracts of seeds show good cytotoxicity in brine shrimp lethality tests (Hosain et al., 2011). Hexane extracts exhibit strong anticancer activity against human lung cancer cell lines in XTT cell proliferation assays (Gorla & Shankar, 2016). *In-vivo* studies of methanolic extracts from bark, fruits and leaves demonstrate anticancer effects in Swiss

albino mice with Dalton lymphoma ascites tumors, using trypan blue and MTT assays (Poongothai & Annapoorani, 2021).

2.6.5. Anti-inflammatory activity

Inflammation is the natural way of the body for protecting itself and is important for survival. But if it continues for too long, due to constant release of certain chemicals and ongoing harmful signals, it can turn into a long-lasting problem. So, finding safe and effective ways to stop inflammation is important. Medicinal plants are an important source for developing new and effective medicines (Nunes et al., 2020). There are so many *in vitro* assays for finding anti-inflammatory activity; they are inhibition of protein denaturation method, human red blood cell membrane stabilization test, proteinase inhibitory assay etc. The water extracts of *F. racemosa* bark demonstrated a concentration-dependent increase in inhibition of egg albumin denaturation. Among the extracts, hot water preparations exhibited significantly higher inhibition compared to cold water extracts. The IC_{50} values were 3691.97 µg/mL for the cold water extract and 4207.1 µg/mL for the hot water extract (Dharmadeva et al., 2018). Arulmozhi and colleagues in 2007 evaluated the anti-inflammatory activity of ethanolic extracts of *A. scholaris* leaves using carrageenan-induced rat paw oedema in male albino Wistar rats and reported significant activity (Arulmozhi et al., 2007). Banik and Das demonstrated that the latex of *A. scholaris* exhibited significant anti-inflammatory activity when assessed using the egg albumin denaturation method (Banik & Das, 2023). Mandal and colleagues reported that *F. racemosa* leaf extract exhibited significant anti-inflammatory activity in carrageenan, serotonin, histamine and dextran-induced rat hind paw oedema models at different doses (Mandal et al., 2000a). Aqueous extracts of *F. racemosa* bark demonstrated significant anti-inflammatory activity in rat models, with intraperitoneal administration at 20 mg/100 g inhibiting carrageenan-induced acute inflammation and at 30 mg/100 g suppressing formalin-induced subacute inflammation (Mehta et al., 2012).

2.7. Genes involved in regulation of secondary metabolic pathways

Biotic and abiotic stresses, such as insect attacks, mechanical wounding, drought and fungal infections, induce significant metabolic shifts in host plants by altering gene

expression in secondary metabolite pathways. These changes enhance the synthesis and accumulation of phytochemicals, including phenolics, terpenoids and flavonoids, which serve as defense compounds or structural components (Boava et al., 2011; Løvdal et al., 2010; Ralph et al., 2006b). The shikimic acid and phenylpropanoid pathways are critical for producing these compounds and genes regulating key steps in these pathways were selected for the current study.

2.7.1. Gall induction and gene expression

Gall-inducing insects, such as aphids manipulate host plants to enhance their ecological fitness by upregulating genes in secondary metabolic pathways. For example, aphids increase the expression of terpenoid pathway genes, leading to high monoterpene concentrations in galls (Davidovich-Rikanati et al., 2022). Gene expression in galls shows similarities with adjacent host tissues, but galls experience the greatest stress from the inducer, resulting in more pronounced gene expression changes (Lu et al., 2021). Gall inducers control host plant metabolism, upregulating genes involved in DNA synthesis, secondary metabolism, cell division, tissue development and formation of structural proteins (Lu et al., 2021). Research by Wang and colleagues (2017) and Martinson and co-workers (2022) identified numerous genes associated with secondary metabolism that are more active in galls than in normal leaf tissues (Martinson et al., 2022; Wang et al., 2017a). *De novo* transcriptome analysis of *Quercus rubra* galls, using RNA extracted from inner and outer gall tissues and adjacent leaves, revealed increased expression of genes related to morphogenesis and secondary metabolite production (Martinson et al., 2022). A study by Lu and colleagues (2021) found significant differences in gene expression between galls and normal leaves, with 18 genes involved in phenylpropanoid metabolism (e.g., flavonols, isoflavonoids, anthocyanins) upregulated and only 9 downregulated (Lu et al., 2021). Genes related to carotenoid and terpenoid backbone biosynthesis also showed altered expression in galls. Phenylpropanoids contribute to defense against herbivores, regulate hormone transport and support new cell wall formation in growing galls (Martinson et al., 2022).

2.7.3. Genes selected for the study

3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS) is an important gene in shikimic acid pathway which catalyzes the condensation of phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P). cDNA microarray and RNA-seq analyses show elevated transcript levels in response to mechanical wounding or insect feeding (Ralph et al., 2006a; Ralph et al., 2006b). 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene produces carboxyvinyl transferase enzyme, which involved in the shikimic acid pathway. The gene expression found to vary in tissue, time and abiotic stresses like drought, salt and cold (Garg et al., 2014). According to RNA-seq and transcriptome profiling, the chorismate synthase (CS) gene, which is involved in the production of lignin, showed elevated expression during wounding. There are evidences for the accumulation of phenolic compounds linked to this gene in broccoli (Torres-Contreras et al., 2018). Boava and colleagues reported that mechanical wounding enhanced the expression of phenylalanine ammonia-lyase (PAL) gene (Boava et al., 2011). Investigations on upregulation of PAL due to fungal infections (Chen et al., 2009; Rukmini et al., 2017; Sayari et al., 2014), herbivores (Duan et al., 2014) and abiotic stresses (Løvdal et al., 2010) are reported. RNA-seq has confirmed its role in phenylpropanoid biosynthesis. Chalcone-flavanone isomerase (CHI) gene involved in flavonoid production found to be modified by insect attacks, microbes or mechanical wounding. Dao and co-workers done RNA-seq and qPCR studies to highlight its expression in stressed condition (Dao et al., 2011). Finally RNA-seq investigations of the Dihydroflavonol 4-reductase (DFR) gene demonstrate upregulation in response to fungal infections (Yang et al., 2022), drought stress (André et al., 2009; Ma et al., 2014) and biotic stresses (Kumar et al., 2013), leading to flavonoid accumulation.

The review of literature found several unresolved aspects related to plant galls and their development, such as photosynthetic activity, secondary metabolite distribution, and differential gene expression. There are conflicting reports on photosynthetic activity whether galls reduce, maintain or increase photosynthesis (Fay et al., 1993; Martinson et al., 2022). It requires clarification, especially in medicinal plants where energy allocation affects metabolite production. Secondary metabolite distribution in normal leaves and galls

need more detailed analysis to resolve the discrepancies of whether metabolites are concentrated in gall exteriors or interiors and to study the impact in their therapeutic potential. Also need to reveal the molecular basis of structural, biochemical and physiological alteration due to gall formation (Davidovich-Rikanati et al., 2022; Hall et al., 2017).

CHAPTER 3 - STRUCTURE AND PHYTOCHEMISTRY OF LEAF GALLS ON SELECTED MEDICINAL PLANTS

3.1. INTRODUCTION

The present chapter deals with the qualitative and quantitative phytochemical analysis of healthy leaves (normal leaves), galled leaves and galls of selected medicinal plants. Two plants were selected for the study: *Alstonia scholaris* (L.) R. Br. and *Ficus racemosa* L. This chapter provides insights into the differential phytochemical profiles resulting from gall formation in comparison with healthy leaves. The chapter also sheds light on the anatomical differentiations produced in healthy leaves due to gall induction. This provides a complete comprehension of anatomical and biochemical differentiation in plants due to gall formation.

3.1.1. Plant galls

Plant galls are abnormal growths on formed on different plant parts resulting from hypertrophy and hyperplasia. The galls are produced by the activity of foreign organism, which may range from microorganisms to macroorganisms such as insects, bacteria, fungi, nematodes or mites. These structures represent complex interaction between plants and gall-inducing organisms (Mani, 2000; Uma & Verghese, 2008). The galls are classified into leaf, bud, stem, flower or fruit galls according to the part of the plant in which they originate. They exhibit diverse shapes and textures (round, oblong, globular, discoid, egg-shaped, spindle-shaped or bottle-shaped). Some galls are hairy or spiny, while others have rough or glabrous surfaces. Based on the number of cavities, galls are categorized as unilocular or multilocular. Gall showed three distinct phases of growth: the developmental stage, the maturation stage and finally senescence stage (Raman, 2011; Ushir et al., 2015).

Among gall-inducing organisms, insects and midges are the most common. In India, over 2,000 species of gall-inducing insects have been identified, with 87 morphotypes of insect-induced galls reported in Northern Kerala (Saleem & Nasser, 2015; Uma & Verghese, 2008). Most gall-inducing insects are specific to particular host plants. Insect-mediated gall induction often starts with the activity of salivary secretions, which act as a chemical stimulus. Inducer insects create a small wound, allowing salivary amino acids

and proteins to enter the host plant, change the osmotic balance and create stressed condition. Then stress driven excessive growth and cell division occur. This may be the result of altered enzymatic reactions and modified chemical constituents in the affected tissues. Gall inducers modify host plant biochemistry and physiology through altered metabolic pathways to support their development, resulting in a chemical composition of gall distinct from surrounding normal plant tissue. These differences arise from altered metabolic pathways of host plants that arise through differential molecular processes in response to stress (Hartley, 1998; Raman, 2011).

Gall induction causes tissue redifferentiation, transcriptional changes, increased protein content, decreased chlorophyll content, transition of leaves from source to sink, accumulation of phytochemicals such as phenolic compounds and elevated proline levels in the affected tissues. These changes are produced due to altered gene expression patterns induced by the gall inducer. The secondary metabolites like phenolics, determines therapeutic potential of plants as they have antioxidant, anti-inflammatory, anticancer and antimicrobial properties. The gall induction enhances the medicinal value of plants for applications such as antioxidant and anti-inflammatory treatments (Albert et al., 2011; Dsouza & Ravishankar, 2014; Ferreira et al., 2017). The therapeutic potential of plant galls is attributed to their unique phytochemical composition, which is distinct from that of normal plant tissues. The accumulation of secondary metabolites like phenolics, flavonoids and tannins in galls provides them with enhanced biological activities, making them valuable resources for developing natural antioxidants and other medicinal agents (Saleem & Nasser, 2015).

3.1.2. Overview of plant secondary metabolites and their functions

Secondary metabolites are diverse group of compounds produced by plants and are not directly involved in growth or development of plant, but play critical roles in plant interactions. These compounds have significant role in defense against pathogens and herbivores, facilitation of symbiosis, signaling and stress response etc. (Croteau et al., 2000; Taiz & Zeiger, 2010). Recent advancements in biochemical and sequencing technologies have significantly enhanced our understanding of these compounds, revealing

their biosynthetic pathways, genetic regulation and ecological significance (Dixon & Dickinson, 2024; Wang et al., 2024).

Plant secondary metabolites can be divide into three distict chemical catogaries; they are terpenoids, phenolics and nitrogen containing compounds. Terpenoids are the largest class of secondary metabolites (30,000 compounds). They are derived from isoprene units having five carbons. They are synthesized by two separate mechanisms; methylerythritol phosphate (MEP) pathway (plastids) and mevalonate pathway (cytosol). Terpenoids perform a variety of functions in plants, including functioning as hormones (e.g., gibberellins, abscisic acid), pigments (e.g., carotenoids) and mediators of plant-plant or plant-insect interactions. They also participate in the defense mechanisms against herbivores and diseases. Some volatile terpenoid molecules repel herbivores or attract herbivore predators, while others are feeding deterrents (Taiz & Zeiger, 2010).

Phenolic compounds have a structure consisting of one or more hydroxyl groups attached to aromatic rings. These chemicals are mostly synthesized through the shikimate pathway which results in the formation of phenylalanine, the precursor of the majority of phenolic compounds. There are different types of phenolic compounds; they are flavonoids, tannins, lignins etc. Flavonoids play important roles in UV protection, flower colouration and disease defense. Tannins deter herbivores by making plant tissues less palatable. Lignins give plants structural support by reinforcing cell walls, especially in vascular tissues. Phenolics have antioxidant properties, which helps plants to survive oxidative stress produced by environmental conditions such as high light intensity, drought and disease infections. Some of the compounds are phytoalexins, useful against micro-organisms (Johnson et al., 2021; Mamari, 2021; Taiz & Zeiger, 2010).

Nitrogen-containing secondary metabolites are a broad collection that includes alkaloids, cyanogenic glycosides and glucosinolates. These chemicals frequently play important roles in plant defense. Alkaloids are nitrogen-containing molecules produced from amino acids like tryptophan, tyrosine and ornithine. They are known for their significant biological activities like antiviral, antibacterial, anti-inflammatory and anticancer properties. Because of their bitter taste and toxicity, alkaloids are frequently used to deter herbivores, thus helping in plant defense. Cyanogenic glycosides are

produced from amino acids and can generate hydrogen cyanide (HCN) when plant tissue is injured. They are found in a number of plant species and serve as a defense mechanism against herbivores. When an animal bites on the plant, the glycosides are hydrolysed, producing cyanide, which inhibits the cellular respiration of herbivores and thus inhibits feeding (Adamski et al., 2020; Taiz & Zeiger, 2010).

Glucosinolates are sulfur containing chemicals generated from amino acids. When plant tissue is damaged, it produces isothiocyanates that are harmful to many insects and microorganisms. Saponins are glycosides from both triterpenes and steroids. This reduces serum cholesterol by preventing its reabsorption. Research suggests that saponins may be able to cure hypercholesterolaemia by preventing the uptake of cholesterol. It possesses cardiac depressant and hypotensive actions. Tannins are complex phenolic polymers. It has the ability to bind to proteins and carbohydrates, decreasing their digestibility and so inhibiting microbial development. Tannins possess astringent properties (Soetan & Aiyelaagbe, 2009).

Plant galls can be viewed as extensions of the host plant phenotype as they have the same genetic makeup as normal tissues, but exhibit distinct phytochemistry and this may due to differential gene expression (Raman, 2011). The accumulation of secondary metabolites like phenolics and flavonoids in galls not only aids in plant defense but also provides valuable bioactive compounds for medicinal applications, particularly as natural antioxidants and anti-inflammatory agents (Dsouza & Ravishankar, 2014; Ferreira et al., 2017). In India, plant galls have been used for their therapeutic potential. Galls from genera such as *Rhus, Pistacia, Quercus* and *Terminalia* are utilized in Indian folk remedies, including Ayurveda, Unani and Siddha, for treating various ailments (Patel et al., 2018). This chapter compares the structure and phytochemistry of normal leaf tissues and gall tissues in selected plants to elucidate the biochemical differences, particularly the enhanced phenolic content in galls, which may increase their therapeutic potential.

3.2. METHODOLOGY

3.2.1. Structure of normal leaves and leaf galls

Collection of plants

Mature plants of *Alstonia scholaris* (L.) R. Br. and *Ficus racemosa* L. grown in different regions of Palakkad district were chosen to collect normal leaves and leaves with galls. Normal leaves are those that were fully grown, healthy and showed no symptoms of infection or abnormal growth. Leaf galls are abnormal outgrowths present on leaves, varying in size, shape and position. In order to prevent damage, some normal and galled leaves were carefully collected by hand using sterile scissors. The collection was done during the active growing season, when both kinds of leaves were abundant.

Every sample collected, identified and authenticated using Flora of the Presidency of Madras (Gamble and Fischer, 1936) and Flowering Plants of Kerala (Sasidharan, 2013). During collection, the sample was tagged with the date, plant part collected and species name. For reference, field photography was also done. To preserve tissue integrity, fresh samples were brought to the lab in insulated containers and wrapped in moist paper or towels. Voucher specimens of all collections prepared as herbarium following standard procedures and were deposited in Herbarium of Government Victoria College (GVCH), Palakkad, Kerala, with accession numbers GVCHP-SV 768 and GVCHP-SV 769 for *A. scholaris* and *F. racemosa* respectively. From the leaf galls of *A. scholaris* and *F. racemosa,* the inducer organism is isolated. The identification and authentication of the organism was done by Dr. K Abdul Rasheed, Department of Zoology, Government Victoria College, Palakkad.

Morphological study

The healthy leaves (normal leaves) and leaf galls were collected from the field and maintained their freshness for morphological and anatomical studies. Galls were separated from galled leaves and analysed under a dissection microscope in the laboratory. For morphological documentation, the Leica Sapo Stereo Microscope was used. Photographs of different developmental stages of leaf galls were taken from the field as well.

Anatomical study

Anatomical study was conducted by taking free hand sections. Thin sections were selected, stained with safranin, mounted in glycerin, observed through microscope and images were transferred to the computer. Leica DM 1000 Stereo Microscope with Leica DFC 295 digital camera connected to the computer and Leica Application Software LAS Version 3.6.1 was used for anatomical study. The transverse sections taken were observed and compared to find out the difference induced by the gall inducing insects in the normal leaves (Johansen, 1940).

3.2.2. Phytochemical analysis of plant extracts

Collection of plants

Healthy plants of *Alstonia scholaris* (L.) R. Br. and *Ficus racemosa* L. were selected and their leaves were collected. *A. scholaris* and *F. racemosa* plants with galls were located in different regions of Palakkad district and gall containing leaves were collected from areas of its abundance. The gall tissues were separated from the leaves immediately after collection. The normal leaves, gall leaves and the galls were separately dried in shade for pulverization and for further extraction.

Extraction of plant samples with selected solvents

The collected tissues, including normal leaves, galled leaves and galls of *A. scholaris* and *F. racemosa*, were thoroughly cleaned and air-dried. The dried samples were pulverized into a fine powder using a mechanical blender. The powdered samples were transferred into airtight containers, properly labeled and stored at room temperature for subsequent use. For extraction, 25 g of each pulverized sample was weighed and extracted using a Soxhlet apparatus with solvents of varying polarity to capture a broad spectrum of secondary metabolites.

The choice of solvents was guided by their polarity, which determines their ability to extract specific secondary metabolites, such as phenolics, flavonoids, tannins, alkaloids, terpenoids and saponins, known to be present in *A. scholaris* and *F. racemosa*. The solvents cover a polarity gradient, ensuring comprehensive extraction of both polar and

nonpolar compounds, which is critical for comparing phytochemical profiles across normal leaves, gall leaves and galls. The solvents selected for our study are listed below;

1. **Water** is a highly polar solvent, it is ideal for extracting highly polar compounds such as phenolic glycosides, hydrolyzable tannins, saponins and cardiac glycosides.
2. **Ethanol** is a moderately polar solvent having amphiphilic nature, with a polar hydroxyl group and a non-polar ethyl group, allowing it to extract both polar and moderately non-polar compounds. Ethanol is highly effective for extracting flavonoids, phenolics, tannins and some alkaloids.
3. **Hexane** is a highly non-polar solvent, suitable for extracting lipophilic compounds. Targeting terpenoids, phytosteroids and fatty acids.

The extracts were concentrated by evaporating the solvents using a rotary evaporator or hot air oven, yielding dry extracts free of solvents. These extracts were stored in glass vials for further phytochemical analysis.

Qualitative phytochemical analysis

The various extracts of *A. scholaris* and *F. racemosa* were subjected to qualitative phytochemical analysis to detect the presence of various phytoconstituents. The tests were conducted using standard procedure on normal leaves, gall leaves and gall of both plant extracts and the results were compared to get the phytochemical profile of the plants (Edeoga et al., 2005; Harborne, 1998).

1. **Test for alkaloids:**
   a) Dragendorff's test: To 1 mL of the crude extract, a few drops of Dragendorff's reagent were added. The presence of alkaloids was indicated by the formation of an orange-red precipitate.
   b) Hager's test: To 1 mL of each sample solution taken in a test tube, a few drops of Hager's reagent (saturated aqueous picric acid solution) were added. The formation of a yellow precipitate confirmed the presence of alkaloids.

c) Wagner's test: To a few drops of each sample solution taken in a test tube, a few drops of Wagner's reagent (iodine in potassium iodide) were added. The appearance of a brown flocculent precipitate indicated the presence of alkaloids.

2. Test for phenolics:

Ferric chloride test: To 2 mL of extract 0.5 mLof 5% aqueous ferric chloride solution was added and observed for the colour change. Formation of deep blue or black colour was taken as positive for the presence of phenols.

3. Test for flavonoids:

a) Lead acetate test: A few drops of each sample solution were taken in a test tube, and aqueous basic lead acetate was added. The formation of a white turbid precipitate indicated the presence of flavonoids.

b) Alkaline reagent test: To about 3 mL of the extract, 2 mL of 20% sodium hydroxide solution was added and the mixture was observed for the formation of an intense yellow colour, which became colourless upon the further addition of dilute hydrochloric acid, confirming the presence of flavonoids.

4. Test for saponins:

Froth test: To 1 mL of each sample solution taken in a test tube 2 mL of distilled water was added and shaken well. Appearance of froth on shaking of the mixture indicated presence of saponins.

5. Test for quinones:

HCl Test: A small amount of extract was treated with 2 mL of concentrated hydrochloric acid and observed for the formation of yellow precipitate which indicated the presence of quinones.

6. Test for coumarins:

Ferric chloride test: To 1 mL of each sample solution taken in a test tube, one drop of ferric chloride solution was added, resulting in the formation of a dark green colour. The appearance of a dark green colour that turned yellow upon the addition of five drops of concentrated nitric acid indicated the presence of coumarins.

7. Test for cardiac glycosides:

Keller killani test: To 5 mL of each sample taken in a test tube added 2 mL of acetic acid along with 1 drop of ferric chloride solution and 1 mL of concentrated sulphuric acid. The appearance of the brown ring indicates the presence of cardiac glycosides.

8. Test for triterpenoids:

a) Libberman's test: To 1 mL of each sample taken in a test tube added 3 drops of acetic anhydride and boiled. Then the sample was cooled and added concentrated sulphuric acid along the side of the test tube. A brown ring formed at the junction and deep red colour in the lower region indicated the presence of triterpenoids.

b) Salkowski test: To 1 mL of each of the samples taken in a test tube added a few drops of chloroform on the side of the test tube. Then carefully added a few drops of concentrated sulphuric acid. Reddish brown colour at the interphase indicated the presence of triterpenoids and steroids.

9. Test for tannin:

Potassium dichromate test: To 1 mL of each sample added a few drops of strong potassium dichromate solution. Yellow colour precipitate indicated the presence of tannin.

Quantitative phytochemical analysis

1) Quantitative phytochemical analysis by GC-MS

The normal leaf, gall leaf and gall extract of *A. scholaris* and *F. racemosa* were subjected to Gas Chromatography analysis along with Mass Spectrum analysis using the equipment by Shimadzu QP-2010 Plus with Thermal Desorption System TD 20. About 1µL of ethanolic extracts of *A. scholaris* and *F. racemosa* were injected into the machine using the micro syringe and the scanning was done for 45 min. The injector and detector temperatures were maintained at 260 ºC, and the operation temperature at 50-280 ºC. The column temperature was programmed at 50-120 ºC, with a 4 ºC increase per min which

was maintained for 1 min. Then it was programmed at 120-300 ºC, with 6 ºC increases per min and held on for 3 min, with retention time (Rt) 60 min. Column oven temperature was 50.0 ºC, injection temperature was 260.00 ºC and injection in split mode. Pressure was kept at 69.0 k Pa. Total flow was 16.3 mL/min and column flow was maintained at 1.21 mL/min. The identification of the compounds was done using the Software named GC-MS solution. The determination of structure of compounds was done based on the comparison of mass spectra and their fragmentation profiles using published data, Wiley, NIST library search. The analysis was done at Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University, New Delhi.

2) Identification of phytochemicals by OHR-LC-MS analysis

To obtain the detailed phytochemical profile of the normal leaves and galls, Orbitrap High Resolution Liquid Chromatography Mass Spectroscopy (OHR-LC-MS) analysis was performed in all the 3 samples of both plants. It is done using the equipment Q Exactive Plus Biopharma, Thermo Scientific Xcalibur, version 4.2.28.14. The analytic instrument is composed of two outer electrodes and a central electrode, which act as an analyzer and detector. It performs both negative and positive ionisation modes with mass (m/z) ranges of 50 to 8000 amu. The mobile phase contains 0.1% formic acid in Milli-Q water, methanol and acetonitrile. The running time is kept at 35 min. Metabolite identification was performed using Thermo Compound Discoverer 3.2 and BioPharma Finder Software 2.0. Resulted data were analysed with Compound Discoverer 3.2. The experiment was conducted at Sophisticated Analytical Instrument Facility (SAIF), IIT, Bombay. The biological properties of identified chemical compounds in normal leaves, gall leaves and galls were identified using Pub-Chem and Chem-spider.

3.2.3. Other preliminary quantitative analysis

Estimation of chlorophyll content

Photosynthetic pigments are essential biomolecules that enable plants to absorb and utilize light energy for photosynthesis. Carotenoids and chlorophyll are important among them. Chlorophylls are green pigments that absorb light energy and transform it into

chemical energy. They are mostly composed of chlorophyll a and chlorophyll b. The proportion and amount of these pigments can change depending on the environment and frequently indicate the physiological state of the plant. In plant physiological investigations, the estimation of chlorophylls and carotenoids is crucial because they work together to provide the photosynthetic system with the maximum possible light absorption and protection. Each of the pigments has a characteristic absorption spectrum which can distinguish one pigment from another.

Protocol:

Fresh leaf samples (50 mg each) from normal leaves, gall-infected leaves and isolated galls were weighed and thoroughly crushed using a mortar and pestle in 10 mL of acetone. The homogenized mixture was then centrifuged at 5000 rpm for 10 min, and the resulting supernatant was carefully collected. The remaining residue was re-extracted using 80% acetone and centrifuged under the same conditions. This extraction process was repeated several times until the pellet turned colourless, indicating complete extraction of pigments. All supernatants from successive extractions were pooled together and the final volume was noted. The absorbance of the pooled extract was measured at 663 nm, 646 nm, 470 nm and 750 nm using 80% acetone as the solvent blank.

The quantities of different pigments were calculated using the following formula; (Arnon, 1949; Prabhakaran & Neravathu, 2019).

$$\text{Chlorophyll a} = 12.69\,(A_{663} - A_{750}) - 2.69\,(A_{646} - A_{750}) \times V \div W$$

$$\text{Chlorophyll b} = 22.9\,(A_{646} - A_{750}) - 4.68\,(A_{663} - A_{750}) \times V \div W$$

$$\text{Total chlorophyll} = 20.12\,(A_{646} - A_{750}) + 8.02\,(A_{663} - A_{750}) \times V \div W$$

$$\text{Carotenoid} = 1000\,(A_{470}) + 3.72\,(\text{Chl a} - \text{Chl b}) \times V \div W \times 229$$

Where A is the absorbance, V is volume and W is fresh weight of the sample

Estimation of Phenylalanine ammonia-lyase (PAL) enzyme activity

Phenylalanine ammonia-lyase (PAL) is an important enzyme in the phenylpropanoid pathway, which converts phenylalanine into trans cinnamic acid. The amount of PAL that catalyzes the conversion of 1 µmol of phenylalanine into NH3 and trans-cinnamic acid at pH 8.5 and 30 ºC per min was regarded as the unit of activity. PAL enzyme activity was calculated based on the following protocol (Babich et al., 2020; Safari et al., 2021; Yasmin et al., 2023).

Protocol

About 10 mg each of the fresh plant samples of both *A. scholaris* and *F. racemosa* - gall, gall leaf and normal leaf was taken. Each sample was ground with 0.5 mL of 0.1 M trisodium borate buffer (pH 8.8) and mercaptoethanol was added to make up the final volume to 3 mL. The homogenates were centrifuged at 8000 × g for 20 min at 4 °C using a refrigerated centrifuge. The resulting supernatant was collected and used as the enzyme source for determining PAL activity. For the assay, 1 mL of the enzyme extract was mixed with 1 mL of borate buffer and 1 mL of 0.05 M, L-phenylalanine. The reaction mixture was incubated at 37 °C for 1 hour, after which 30% trichloroacetic acid (TCA) was added to terminate the reaction. The absorbance of the resulting solution was measured at 270 nm using a UV-Visible spectrophotometer. PAL activity was calculated based on the rate of conversion of L-phenylalanine to trans-cinnamic acid at 270 nm.

The amount of trans-cinnamic acid synthesized was calculated, by using its extinction coefficient and enzyme activity of sample was calculated by using the following equation;

$$Units/mL\ enzyme\ = \frac{(\Delta A270nm/\ min\ Test - \Delta A270nm/\ min\ Blank)\ (3)(df)}{(19.73)(0.1)}$$

Where ΔA is the change in absorbance per min, 3 is the total volume (in mm) of assay, df is the dilution factor (1), 19.73 is the mM extinction coefficient of trans-cinnamate at 270 nm and 0.1 is the enzyme volume.

CHAPTER 4 - BIOACTIVITY ANALYSIS OF NORMAL LEAVES, GALL LEAVES AND LEAF GALL EXTRACTS

4.1. INTRODUCTION

This chapter deals with the bioactivities of normal leaves, gall leaves and galls of *Alstonia scholaris* and *Ficus racemosa*. The previous chapter provided the details of phytochemicals present in the different samples of both plants. It is well established that the secondary metabolites present in plants are the basis of their medicinal properties and thus biological activities. The leaf galls induced by insects are rich in phenolic compounds, as demonstrated in Chapter 3, so here in this chapter the bioactivities such as antimicrobial, antioxidant and cytotoxic activities of normal leaves, gall leaves and gall of *A. scholaris* and *F. racemosa* were analyzed to see if gall induction has any positive effect on the bioactive properties of the tissues being infected.

Plants have been used for therapeutic purposes for thousands of years. Initially known as herbalism, the use of plants to treat diseases has evolved into modern phytotherapy, defined as the application of plants or their extracts for therapeutic and medical purposes to treat illnesses and enhance human health. According to the World Health Organization (WHO), approximately 80% of the global population relies on herbal medicines for primary healthcare, utilizing either plant extracts or their active principles (Farnsworth et al., 1987; Houghton, 1995). Indian Vedas outline the use of plants in medicine to treat a variety of ailments. The traditional medicinal systems of India, including Ayurveda, Siddha, Unani and Naturopathy, employ over 7,500 plant species, establishing the country as a global leader in phytopharmacology (Shakya, 2016).

Plants can be used in medicine in various forms, including crude preparations, refined extracts, which are partially purified or rationalized extracts having a few defined components or as single phytochemical species. There are three ways for use of plants in therapeutic fields, they are direct, semi-synthetic and total synthetic systems. In a direct system, the parts of plants that are applied straight in treatments. Semi-synthetic is the application of fundamental chemical components that are plant-derived but have been modified to have better qualities including increased efficacy and reduced toxicity. The plants with elements that are not being

therapeutically beneficial, but can be converted into medicinal agents through different chemical or microbiological means. In the total synthetic system, natural molecules from plants can also be used as templates to create new compounds with enhanced activity, a higher therapeutic index or fewer side effects (Fowler, 2006; Houghton, 1995).

Many significant pharmaceuticals used in modern healthcare contain 'active elements' from traditional medicines. They still remain the recommended medications for specific ailments. So some of the bioactive compounds from plants can be directly used in medication (Houghton, 1995). Plant metabolites are divided into primary metabolites (e.g., glucose, proteins and lipids) essential for growth and development and secondary metabolites (e.g., phenolics, terpenes, alkaloids) that mediate ecological interactions like defense against herbivores and pathogens. Secondary metabolites are the primary source of therapeutic properties of plants, exhibiting bioactivities such as antioxidant, antimicrobial and cytotoxic effects. Plants synthesize secondary metabolites from primary metabolites through different metabolic pathways. They play no direct role in growth and reproduction, although they do participate in ecological processes such as defense mechanisms and pigment production (Shakya, 2016).

Phytochemicals and bioactivity

A medicinal plant is defined as a plant having chemicals with therapeutic value or containing precursors for chemo-pharmaceutical production. Phytochemical compounds present in medicinal plants possess immense therapeutic properties. The mixtures of secondary products found in plants usually produce the beneficial therapeutic effects of plants (Jain et al., 2019). These therapeutic effects typically arise from combinations of secondary products that exhibit a wide range of bioactivities including antioxidant, antidiabetic, antiviral, antibacterial, anticancer, neuroprotective, antimutagenic, antiallergic and anti-inflammatory properties (Soetan & Aiyelaagbe, 2009). Bioactivity possessed by different phytochemical compounds present in normal leaf, gall leaf and galls of both selected plants were detailed in chapter 3. In this chapter, the experimental approaches used to analyze the antioxidant, antibacterial, antifungal and cytotoxic activities of these extracts are presented, based on the hypothesis that elevated concentrations of secondary metabolites in gall tissues enhance their bioactivity.

4.1.1. Antioxidant activity

Antioxidants are bioactive substances that inhibit oxidation by neutralizing free radicals, thereby mitigating oxidative damage associated with diseases such as cancer, cardiovascular disorders and neurodegenerative conditions. Medicinal plant-derived bioactive chemicals, particularly polyphenols like flavonoids and phenolic acids, exhibit potent antioxidant properties by scavenging peroxyl radicals, alkyl peroxyl radicals, superoxide, hydroxyl radicals and nitric oxide across various chemical oxidation systems, often by transferring a hydrogen atom from an aromatic hydroxyl group to the free radical. Phenolic substances, including phenols, phenolic acids, flavonoids, anthocyanins and tannins, are well-known for their strong antioxidant capabilities (Altemimi et al., 2017). Antioxidant activity can be assessed using methods such as DPPH radical scavenging assay, ABTS (2,2'-azino-bis (3-ethyl benzothiazoline -6- sulphonic acid) assay, superoxide scavenging, lipid peroxidation, β-carotene bleaching, nitric oxide scavenging, reducing power, iron chelation, hydroxyl radical scavenging and FRAP (ferric reducing antioxidant power) assay (Alam et al., 2013; Altemimi et al., 2017; Shen et al., 2010).

4.1.2. Antimicrobial activity

Medicinal plants possess notable antibacterial and antifungal properties attributed to their diverse secondary metabolites. Phenolic compounds such as flavonoids and tannins, along with alkaloids and saponins, demonstrate potent antibacterial effects against both Gram-positive and Gram-negative bacteria by disrupting bacterial cell membranes or inhibiting critical enzymes essential for growth and metabolism. Certain bioactive substances also enhance the defense mechanisms of the body by modulating immune responses to combat infections. Similar to antibacterial substances, they may work by enhancing the immune system, disrupting fungal cells and inhibiting essential enzymes etc. (Dar et al., 2023).

4.1.3. Cytotoxic activity

Cytotoxicity refers to the potency of a chemical to cause changes in cellular functioning, negatively impact structures or essential processes and ultimately lead to cell death, often through DNA damage or inhibition of the cell cycle (Kmail et al., 2015). The use of herbal remedies for cancer treatment spans centuries and plant-derived bioactive secondary metabolites, such as vincristine and paclitaxel, are extensively investigated for their cytotoxic potential with

fewer adverse effects compared to synthetic drugs (Mansour et al., 2022). The MTT assay, utilizing 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl tetrazolium Bromide, is a common colorimetric technique for assessing cell viability by measuring the activity of mitochondrial dehydrogenase, which converts the yellow MTT dye into insoluble purple formazan crystals in viable cells (Kmail et al., 2015; Mansour et al., 2022).

4.1.4. Anti-inflammatory activity

When tissues in the body get injured by an infection, physical trauma, hazardous substances or microbes like bacteria and viruses, they naturally react with inflammation to protect them. Redness, heat, swelling (edema), discomfort and loss of function are its main characteristic symptoms. Inflammation is a complicated bodily response that frequently results in pain and involves modifications to cellular membranes, protein denaturation and vascular permeability. The egg albumin denaturation assay is one of the techniques used to evaluate the anti-inflammatory properties of plant extracts. This *in vitro* technique is widely utilized, fast and reliable. It functions on the principle that denaturation of proteins can cause inflammation; hence, substances that prevent denaturation are thought to have anti-inflammatory properties. In this study selected extracts were used for protein denaturation assay for assessing its anti-inflammatory property (Chandra et al., 2012; Mirke et al., 2020; Vinchurkar et al., 2014).

The current application of plant-derived medications merely touches the surface of their vast potential. The biological and chemical diversity in plants, coupled with novel developments in plant biotechnology, molecular biology and genetic engineering, can provide innovative medicinal molecules for disease treatment in the future (Fowler, 2006). In this context, the present study aims to evaluate the bioactivity of insect-induced plant tissues to elucidate their significance and therapeutic potential.

4.2. METHODOLOGY

4.2.1. Antioxidant activity analysis

Reducing power method

Different concentrations (20, 40, 60, 80 and 100 µg/mL of water, hexane and ethanol) of extracts were prepared. To 1 mL of extracts added 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% Potassium ferricyanide. The resulting mixture was incubated at 50 °C for 20 min, followed by the addition of 2.5 mL of 10% Trichloro acetic acid. The mixture was centrifuged at 5000 rpm for 10 min to collect the upper layer of the solution (2.5 mL), mixed with distilled water (2.5 mL) and 0.5 mL of 0.1% ferric chloride. The absorbance was then measured at 700 nm against a blank. Each reaction was done in triplicate. The increase in absorbance indicates the reducing power of the extracts (Alam et al., 2013; Jayaprakasha et al., 2001).

Reducing power method based on the principle of increases in the absorbance of the reaction mixtures. Increase in the absorbance indicates an increase in antioxidant activity. Antioxidant compounds cause the reduction of ferric form to the ferrous form because of their reductive capabilities. The Prussian blue coloured complex is formed by adding ferric chloride. Therefore, the reduction can be determined by measuring the formation of Prussian blue at 700 nm absorbance (Alam et al., 2013; Jayaprakasha et al., 2001).

DPPH radical scavenging activity

The free radical scavenging activity of the extracts was measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH). 0.1 mM solution of DPPH in ethanol was prepared. 2.96 mL of DPPH solution was added to various concentrations of different samples. The sample solution was made up to 40 µL by adding Dimethyl sulfoxide (DMSO) to 2.5, 5, 7.5 and 10 µL of sample solutions taken from the stock solution of concentration 100 mg/mL to make different dilutions of the sample (0.25, 0.5, 0.75 and 1 mg/mL). The mixture was shaken vigorously and allowed to stand for 30 min at room temperature, absorbance was measured at 517 nm using a UV-Visible spectrophotometer (Alam et al., 2013; Shen et al., 2010). Ascorbic acid was used as reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. All

the tests were performed in triplicate mean value was calculated and the percentage radical scavenging activity was calculated by using the following equation;

$$\% RSA = Abs\ Control - Abs\ Sample / Abs\ Control \times 100$$

'RSA' is radical scavenging activity, 'Abs control' is the absorbance of the control reaction (containing all reagents except the test sample) and 'Abs sample' is the absorbance of the plant extracts.

4.2.2. Antibacterial activity analysis

Bacterial strains and growth conditions

Various cultures of human pathogenic Gram-positive and Gram-negative bacteria were selected for antibacterial assay. They are *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (MCMB 310), *Escherichia coli* (ATCC 35218) and *Pseudomonas aeruginosa* (MCMB 407). The cultures were obtained from Agharkar Research Institute, Pune, Maharashtra.

Nutrient broth preparation

Nutrient broth was prepared by dissolving 13 g of nutrient broth powder (HiMedia) in 1000 mL of distilled water. The mixture was stirred thoroughly until completely dissolved. The solution was then transferred to a clean conical flask and sterilized by autoclaving at 121 °C for 15 min. A loop full test organism was inoculated on nutrient broth and incubated for 24 h at 37 ± 1ºC and maintained in sterile condition.

Screening for antibacterial properties by agar well diffusion method

Nutrient agar was prepared by dissolving 28 g of nutrient agar powder (HiMedia) in 1000 mL of distilled water. The mixture was stirred thoroughly until fully dissolved. The medium was then sterilized by autoclaving at 121 °C for 15 min. After sterilization, the molten medium was poured into sterile petri dishes and allowed to solidify at room temperature.

The culture plates were prepared by pouring 20 mL of sterile nutrient agar. After the solidification of the medium, the inoculum was swabbed uniformly over the entire agar surface

using a sterile cotton swab and allowed to dry for 5 min. A sterile cork borer (8.5 mm) was used to make wells in each plate. These plates were labeled and 10 µL, 20 µL and 30 µL of each plant extracts were added aseptically into the well using a micropipette and the plates were then maintained at room temperature for 2 h to allow the diffusion of the solution into the medium. Then the plates were incubated for 24 h at 37 ºC for microbial growth to occur. For each bacterial strain, pure solvent was used as negative control. Streptomycin was used as positive control. Each test was repeated three times and the antibacterial activity was expressed as the mean diameter of the inhibition zones (mm) produced by each plant extracts when compared to the control (Mandal et al., 2000b; Misra et al., 2011).

4.2.3. Antifungal activity analysis

Fungal strains and growth conditions

Candida albicans (NFCCI 5123) and *Aspergillus niger* (MTCC1344) were standard fungal strains collected from Biovent Innovations, Department of Biotechnology, University of Kerala, Trivandrum. The fungal strains were cultured in sterile potato dextrose agar (PDA) at 37 °C for 48 h and the strains were also maintained as glycerol stocks.

PDA medium-preparation

Fresh potatoes (200 g) were washed thoroughly to remove surface dirt, peeled, and cut into small slices. The sliced potatoes were boiled in 1000 mL of distilled water for 20-25 min using a hot plate. The extract was filtered through muslin cloth to obtain the potato infusion.

To the potato infusion, 20 g of dextrose and 15 g of agar were added. The mixture was stirred using a magnetic stirrer and heated until all components were completely dissolved. The final volume was adjusted to 1000 mL with distilled water. The medium was transferred to a conical flask, closed with a cotton plug and sterilized by autoclaving at 121 °C (15 psi) for 20 min.

Antifungal assay of *C. albicans* by agar-well diffusion method

Agar-well diffusion method was conducted to evaluate the antifungal activities of healthy leaves, gall leaves and galls of both plants against *C. albicans*. All the three extracts (water,

hexane and ethanol) of plant samples were used for the analysis. *C. albicans* colonies that were stored on PDA agar slant were transferred to PDA broth and incubated at 35 °C for 3 days for complete growth. Prior to inoculation, 20 mL of PDA medium was poured into petri dishes and kept for solidification. The organisms were inoculated uniformly with sterile swabs on the surface of the solidified PDA plate. After inoculation, 8 mm diameter wells were made by using a sterile cork borer.

For primary screening of activity, 100 mg/mL stock solution was made for aqueous, hexane and ethanol extracts. From that stock solution 20 µL and 40 µL dilutions taken for screening (2 mg and 4 mg per well respectively) was added into the respective wells using a micropipette. After inoculation, the plates were incubated for 48 h at 37 °C. The results were measured as zone of inhibition in mm and the results were photographically recorded. The extracts that gave positive results were only selected for further analysis. These extracts were tested with different concentrations and each test was done in triplicates. Zone of inhibition was measured in triplicates and SD was calculated. A standard antifungal drug, Itraconazole 20 µL, 40 µL and 60 µL from 50 mg/mL stock, was used as a positive control (Gizaw et al., 2022; Raj et al., 2021; Sales et al., 2016).

Antifungal assay of *A. niger* by food poisoning method

The crude extracts of ethanol were used for antifungal analysis against *A. niger*. A standard antifungal drug, Itraconazole 60 µL from 50 mg/mL stock (3 mg), was used as a positive control. Here preliminary screening was conducted with different dilutions of ethanolic extracts.

The food poisoning approach was used to demonstrate antifungal activity against *A. niger*. Different dilutions of plant extracts were mixed with PDA media and added to sterile petri plates. A mycelial disc measuring 6 mm was then positioned in the middle of the plate after solidification of medium. Every treatment was carried out three times. The plates were incubated at 30 ± 2 °C for seven days. The growth of the fungal colony was measured on alternate days and percentage inhibition was calculated by using the following formula;

25

$$PGI = (AC - AT)/AC \times 100$$

Where 'AC' is the average mycelial growth of the control, 'AT' is the average mycelial growth of the treatment with plant extract and 'PGI' is the percentage growth inhibition. Values were given as the mean ± standard deviation (SD) of tests performed in triplicates (Chakrapani et al., 2020; Rafiq et al., 2022; Schoss et al., 2022).

4.2.4. Cytotoxic activity analysis

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay is performed to evaluate the cytotoxicity of different extracts of *A. scholaris* and *F. racemosa*. MTT in the living cells generate purple formazan crystals. The amount of formazan produced is determined by measuring the absorbance. The cytotoxicity studies were done at Stem cell and Regenerative Biology lab, School of Biotechnology, Amrita Vishwavidyapeetham, Vallikavu, Kollam, Kerala.

Cell culture

The extract was tested for cytotoxicity on 3T3 cells. The 3T3 cell line is derived from mouse embryonic fibroblasts. Freshly harvested cells were used for assay. Seeded an appropriate quantity of the cells in a cell culture plate for the assay and incubated at 37 °C with 5% CO_2. Allow the cells to grow for up to 24 h, or until confluence occurs. Harvested the cells and used them for the experiment.

Assay procedure

The harvested cells were cultured using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were grown in 96-well plates at a density of 5×10^3 cells and maintained for 24 h at 37 °C with 5% CO_2. After 24 h the medium is replaced by DMEM containing 10% FBS and different amounts of plant extracts. Test concentrations of extracts of *A. scholaris* and *F. racemosa* were used to screen cytotoxic activity initially. The extracts that showed lower cell viability percentages were selected for further evaluation of cytotoxic effects at different dilutions (50 mg/mL - 500 mg/mL).

Cells treated with dimethyl sulfoxide (DMSO) and phosphate buffered saline (PBS) served as negative controls and cells treated with Triton X-100 served as positive control. After

an additional 24 h incubation at 37 °C with 5% CO_2, the medium was replaced with 90 µL of DMEM and 10 µL of MTT reagent (3-(4,5- dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide) per well. The plates were incubated for 4 h at 37 °C with 5% CO_2 to allow the reduction of MTT to formazan crystals by metabolically active cells. Then, 100 µL of solubilisation solution was added to each well to dissolve the formazan crystals and the plate was placed on a shaker for 30-45 min.

Absorbance was measured at 570 nm with a reference wavelength of 630 nm using a microplate reader. The average values of 570 nm absorbance values of the control wells was subtracted from the average values of 570 nm absorbance values of corresponding experimental wells to calculate relative cell viability (Abdoli et al., 2021; Kmail et al., 2015). All the experiments were done in triplicates and mean OD values were taken for calculation. The cell viability was calculated using the following formula;

$$Percentage\ cell\ viability = (AS/AC) \times 100$$

Where, AS = absorbance of extract treated cells and AC = absorbance of control cells

4.2.5. Anti-inflammatory activity analysis

The anti-inflammatory activity of the plant extract was determined *in vitro* by assessing its inhibition of egg albumin (protein) denaturation.

Assay procedure

A reaction mixture was prepared by mixing 0.2 mL of a 1-2% egg albumin solution (obtained from fresh hens eggs), 2 mL of the sample extract and 2.8 mL of phosphate-buffered saline (pH 7.4), resulting in a total volume of 5 ml. Samples were prepared using five dilutions of 1, 2, 3, 4 and 5 µg/µL. A total volume of 5 mL of the control was created by combining 2 mL of distilled water, 0.2 mL of 1-2% egg albumin solution and 2.8 mL of phosphate-buffered saline. Diclofenac sodium was used as a standard drug at a final concentration of 50 mg/ml. The reaction mixtures were incubated at 37 ± 2 °C for 30 min and subsequently heated in a water bath at 70 ± 2 °C for 15 min. After cooling, their absorbance was measured at 660 nm. The assay was performed in triplicate. The percentage inhibition of protein denaturation was calculated using the following formula;

$$Percentage\ inhibition = (Abs\ Control - Abs\ sample) \times 100 / Abs\ control$$

Statistical analysis

For each treatment or sample type, three independent replicates (n = 3) were used to generate all experimental data in order to validate the reliability and repeatability of the results obtained. The mean ± standard deviation (SD) was used to express the data. One-way analysis of variance (ANOVA) was used to compare the overall differences among group means using Python 3.10 software. Significant variations in the measured parameters between the tissue types might be identified with this approach. Tukey's honestly significant difference (HSD) post-hoc test was used to identify the particular group pairs that differed significantly from one another where the ANOVA displayed significant variation ($p < 0.05$). Prior to analysis, all statistical presumptions, such as homogeneity of variances and normality were verified. Throughout the study, $p < 0.05$ was deemed statistically significant.

CHAPTER 5 - ISOLATION OF BIOACTIVE FRACTIONS RESPONSIBLE FOR POTENT BIOLOGICAL ACTIVITY

5.1. INTRODUCTION

Chromatography is used for separation of a mixture into its components and allows more accurate study of each compound instead of the whole mixture. It is used in industries like pharmaceuticals, biotechnology and clinical research (Kaale et al., 2011). Thin-layer chromatography (TLC) is a widely used chromatographic technique for the analysis of plant materials, separation and identification of medicinal plants and their extracts. TLC offers advantages such as low cost, ease of operation, rapid analysis and minimal sample preparation (Bele & Khale, 2011; Cimpoiu, 2006).

TLC operates on the principle of differential distribution of compounds between a stationary phase and mobile phase. The stationary phase consists of a thin layer of adsorbent material, made up from silica gel, cellulose or aluminum oxide, coated onto glass, plastic or aluminum sheet plate. The mobile phase is selected based on the properties of the compounds to be separated. When the mobile phase moves upward through capillary action, compounds partition between the stationary and mobile phases based on their relative affinities. This will result in separation of components (Ahamed et al., 2017; Bele & Khale, 2011). The retention factor (Rf) value is calculated as the ratio of the distance traveled by a compound to the distance traveled by the solvent front. The polar compounds interact more strongly with a polar stationary phase like silica gel, resulting in lower Rf values, while less polar compounds move higher up the plate, yielding higher Rf values. Factors influencing Rf values include nature of adsorbent, nature of mobile phase, temperature conditions, layer thickness, nature of developing tank, sample mass and also based on the type of chromatographic technique (Bele & Khale, 2011; Stoddard et al., 2007).

TLC allows multiple samples to be evaluated on a single plate, supports flexible selection of mobile and stationary phases and enables multiple detection methods and specific derivatization. TLC fingerprints, based on Rf values and spot colors, facilitate compound identification. These fingerprints can be captured as images, aiding in result communication and

documentation (Cimpoiu, 2006). TLC can be used to monitor the reaction progression, identification of the components of the mixture and the determination of the purity of the mixture. It is used for checking the identity and purity of drugs. It also helps find any adulterations or substitutions in a simple and accurate way (Ahamed et al., 2017; Asante et al., 2016; Porika et al., 2014).

Preparative thin-layer chromatography (PTLC) is a method used to purify and isolate larger amounts of compounds for synthesis, testing or analysis. It processes larger sample sizes (10-1000 mg) to obtain sufficient purified material. It uses stationary phase layers of 0.5-2 mm or more thickness to handle increased sample volumes. It also uses larger plates (20 x 20 cm or bigger) to accommodate more sample volume and simplify collection after separation (Rabel & Sherma, 2017; Sherma & Fried, 1987). This technique is commonly applied in pharmaceutical and organic chemistry labs to purify compounds to get large amounts of pure material. PTLC is used to isolate pure compounds for further analysis such as gas chromatography, analytical TLC, high-performance liquid chromatography (HPLC), visible spectrometry, fluorescence spectrometry, infrared (IR) spectrometry, ultraviolet (UV) spectrometry, nuclear magnetic resonance (NMR) spectrometry (^{1}H and ^{13}C) etc. Multiple rounds of PTLC may be needed to achieve sufficient compound purity for tasks like structure elucidation (Rabel & Sherma, 2017; Sherma & Fried, 1987).

Nuclear Magnetic Resonance (NMR) spectroscopy is a fundamental analytical technique widely used for the structural elucidation of natural products. It provides detailed insights into molecular structure, stereochemistry and dynamics through the interaction of atomic nuclei with an external magnetic field. The measurement of metabolites can be effectively done using NMR spectroscopy. In plant metabolomics, NMR aids in identifying and measuring different plant metabolites present in mixtures. NMR-based metabolomics is developing rapidly and is becoming a common method because it is reliable, fast and needs very little sample preparation. It can also be used for studying samples directly in living systems (*in vivo*). One-dimensional proton (^{1}H) NMR is the most commonly used NMR because it gives highly reproducible results, making it suitable for creating shared databases. However, in the case of complex mixtures, additional two-dimensional NMR techniques are often employed to resolve overlapping signals and obtain clearer spectral data. These advanced methods enable accurate identification of

individual metabolites within complex mixtures. The resulting chemical shift values and signal patterns are then compared with reference databases such as Biological Magnetic Resonance Data Bank for metabolite identification. Using advanced two-dimensional NMR techniques, it can identify both known and unknown compounds in complex mixtures. It also offers the advantages of quick sample preparation and less time-consuming. Another major benefit of NMR is that it is non-destructive, meaning the same sample can be used for further studies after the analysis (Brennan, 2014; Cvetkovic et al., 2020; Halabalaki et al., 2014). NMR-based metabolomics mainly uses proton detection because it provides high sensitivity. Although ^{13}C NMR offers valuable structural information with broader spectral separation and clear signals, it is less commonly used due to its low natural abundance and sensitivity. NMR spectroscopy plays a central role in natural products chemistry, serving as a basic technique for structural elucidation, dynamic studies and metabolite identification (Brennan, 2014; Clendinen et al., 2014).

Here in this chapter, various chromatographic and spectroscopic techniques such as TLC, PTLC and NMR were employed to isolate, purify and characterize the bioactive fractions from the selected plant extracts. These methods helped in identifying the specific compounds responsible for the potent biological activities observed in the study.

5.2. METHODOLOGY

5.2.1. Selection of most bioactive plant sample

Bioactivity analyses (antioxidant, antibacterial, antifungal and cytotoxicity assays), were performed on samples AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA. The data obtained from these analyses were subjected to statistical evaluation. Based on the results of bioactivity assays and statistical analyses, the sample exhibiting the highest bioactivity was selected for further fractionation and characterization. The selection criteria for the potent bioactive sample included: (i) the lowest IC_{50} value in antioxidant assays, indicating higher radical scavenging efficiency; (ii) maximum zone of inhibition in antibacterial and antifungal assays; (iii) highest percentage inhibition in cytotoxicity assays and (iv) finally statistically significant differences compared to control and other samples.

5.2.2. Optimization of chromatographic conditions of the targeted plant extracts

Preliminary TLC analysis was done for selected plant sample using pre-coated silica plates. To determine suitable solvents for the separation of compounds in the selected sample, trial TLC analysis was performed using pre-coated silica gel plates 60 F_{254} (Merck). TLC silica plates on aluminum (20 × 20 cm) were cut into smaller strips (2 × 10 cm) and employed for the TLC separation. The selected plant extracts were applied onto pre-coated TLC plates using capillary tubes and developed in a TLC chamber using various solvent systems (mobile phases). The developed TLC plates were air-dried and examined under visible light and ultraviolet (UV) light at both 254 nm and 366 nm. The solvent system that achieved optimal separation was selected for preparative TLC analysis (Bele & Khale, 2011; Jesionek et al., 2015).

5.2.3. Preparative TLC analysis for separation of compounds

Preparative TLC analysis was done for separation of compounds from the selected sample. The following are the different steps for performing preparative TLC (Hostettmann et al., 1998; Iborra et al., 1992; Kagan & Flythe, 2014; Sherma & Fried, 1987).

(i) Plate preparation

Clean the TLC plates by developing it in one or more polar, neutral solvents, in order to move adsorbed contaminants away from the zone of development. A slurry of 25 g of silica gel G and 75 mL distilled water is stirred in a beaker with a glass rod for 5 min to assure complete mixing and poured on to a pre cleaned 20 × 20 cm glass plate. While holding the plate in one hand, it is tilted to spread the layer evenly over the whole plate. The plate is then placed on a level surface at ~25 °C for 2 h before placing it overnight in a storage rack to dry slowly by spontaneous evaporation. The plate is activated at 130 °C for 2 h and cooled slowly to ~25 °C. The edges are scraped straight with a spatula and the plate is ready for sample application. The adsorbent thickness is made to 2 mm (Hostettmann et al., 1998; Iborra et al., 1992; Kagan & Flythe, 2014; Sherma & Fried, 1987).

(ii) Sample application

Fine tipped capillaries were used to apply the concentrated solutions of the extracts to be resolved in a band. The sample is applied 1 cm above the application line.

(iii) Solvent selection

Solvent selection for preparative TLC was done on the basis of separation obtained in TLC plate 60 F_{254}. Different solvent systems were tried and the best resolution was obtained for Hexane: Acetone (8:2).

(iv) Plate development

The plates were developed by an ascending technique inside the glass TLC developing chambers. Inserted the plates into the chamber in a way that the tops lean against the chamber walls. The chamber was covered and let the solvent move up the plate by capillary action. When solvent reached the top of the plates, removed plates from the chamber and arranged in a standing position within the fume hood until solvent had evaporated (Kagan & Flythe, 2014).

(v) Component detection and isolation

The components separated were detected by using a UV chamber in visible, long and short UV range. The movement of the sample was monitored to find its retention factor (Rf). Each band was measured and expressed as Rf using the following formula;

$$Retension\ factor\ (Rf) = \frac{Distance\ travel\ by\ solute}{Distance\ travel\ by\ solvent}$$

The bands located were removed by scraping off with a spatula. Solvents were added and the adsorbent was separated by filtration and centrifugation (Hostettmann et al., 1998; Iborra et al., 1992; Kagan & Flythe, 2014; Sherma & Fried, 1987).

5.2.4. Antibacterial assay of separated fractions using disc diffusion method

To examine the antibacterial activity of components of the most bioactive crude extract, disc diffusion method was used. TLC separated and purified fractions were evaluated for antibacterial activity against Gram-positive and Gram-negative bacteria using two concentrations of sample (20 µL and 80 µL). Filter paper discs infused with each fraction were placed on agar plates inoculated with the bacteria. After incubation, the zone of inhibition was measured to determine the antibacterial activity of each fraction. Ethyl acetate (solvent used in the extraction), was used as negative control. Discs with ethyl acetate showed no inhibition, verifying that the antibacterial activity originated from the fractions, not from the solvent (Sbhatu & Abraha, 2020; Subramaniam et al., 2020).

5.2.6. Characterization of most active fractions using NMR technique

Proton (^{1}H) NMR were done for identification of the compound that showed the highest antibacterial activity. The structure, dynamics, reaction state and chemical environment of molecules will all be clearly demonstrated through this method. The intra-molecular magnetic field surrounding an atom in a molecule alters the resonance frequency. This gives data on the electronic structure and individual functional groups of the compound. NMR analysis was done using the equipment Bruker Avance 400 MHz FT-NMR Spectrometer at Central Laboratory for Instrumentation and Facilitation, University of Kerala, Karivattom Campus Trivandrum.

CHAPTER 6 - EXPRESSION PROFILING OF GENES INVOLVED IN THE SECONDARY METABOLIC PATHWAY FROM DIFFERENT SAMPLES

6.1. INTRODUCTION

Secondary metabolic pathway in plants helps to adapt to environmental stresses by producing bioactive compounds, critical for stress response and defense. This chapter examines gene expression changes in *Alstonia scholaris* and *Ficus racemosa* due to gall induction by *Pauropsylla* species, focusing on selected genes. Using quantitative real-time PCR (qRT-PCR), the gene expression is compared between normal leaves, galled leaves and galls to test the hypothesis that gall induction changes the expression of genes, enhancing phenolic biosynthesis. This study is to elucidating the molecular mechanisms, to addresses gaps in understanding gall-induced metabolism in medicinal plants, offering insights for phytopharmacological applications (Mir et al., 2015).

Secondary metabolites are chemical compounds that are not involved directly in the primary metabolic processes of plants, but they have function in defense systems, pollinator attraction and interactions with other species. Chapter 3 explains the use of these phytochemical compounds for mankind as they possess various biologically significant properties. The major classes of secondary metabolites are terpenoids, phenolics and nitrogen-containing compounds, which include alkaloids, cyanogenic glycosides and glucosinolates. Each category of secondary metabolites is synthesised through distinct biosynthetic pathways. They also function in the interaction of the plant with its environment. Here in this chapter, we will be looking deep into the pathways involved in this secondary metabolic production. The present work mainly focused on the production of phenolic compounds as these are found to be expressed as major compounds after gall induction by insects. It means phenolic compounds may be the important defensive compound produced by the plants as defence against the action of the gall inducers (Abrahamson et al., 1991; Hartley, 1998; Taiz & Zeiger, 2010).

6.1.1. Secondary metabolites and plant defence

Secondary metabolites are pivotal in enabling plants to withstand biotic and abiotic stresses, with phenolic compounds playing a central role in defense against insect-induced gall

formation in *A. scholaris* and *F. racemosa.* Biotic stresses, such as infection or attack by other organism and abiotic stresses, including UV radiation and drought will trigger the biosynthesis of secondary metabolites, regulated by intricate phytohormone-mediated signaling pathways (Akula & Ravishankar, 2011; Ashraf et al., 2018; Saddique et al., 2018). Phenolics are synthesized primarily through the shikimic acid and phenylpropanoid pathways, encompass flavonoids, lignin, phenolic acids, stilbenes and tannins, which confer antioxidant, antibacterial and insect-repellent properties (Sharma et al., 2019; Taiz & Zeiger, 2010). This study hypothesizes that gall induction enhances phenolic production, fortifying plant resistance and yielding bioactive compounds with therapeutic potential, as evidenced in phytochemical analysis in Chapter 3.

Plants employ both constitutive and induced defense mechanisms to counter biotic stressors like insects and pathogens. Induced defenses include systemic acquired resistance (SAR), induced systemic resistance (ISR) and hypersensitive responses (HR). Induced defese involve cellular changes such as cell wall lignification, reactive oxygen species (ROS) generation and accumulation of pathogenesis-related (PR) proteins (Kortekamp, 2006). These responses rely on transcriptional activation of defense genes, encoding enzymes, transcription factors and proteinase inhibitors that enhance resistance (Boava et al., 2011; Duan et al., 2014). In *A. scholaris* and *F. racemosa,* gall induction by *Pauropsylla* likely upregulates phenolic biosynthesis, with flavonoids acting as phytoalexins to deter insect feeding and lignin reinforcing gall tissues against further invasion. The upregulation of phenolic pathways is critical, as these compounds not only limit insect damage but also contribute to plant-microbe interactions, facilitating communication and symbiosis under stress (Mohammadi et al., 2012).

Beyond their role in defense, the significance of phenolics extends to phytopharmacology, as gall tissues rich in flavonoids and phenolic acids found to exhibit antimicrobial and antioxidant activities (Chapter 4). By focusing on gall-induced phenolic accumulation, this study addresses a gap in understanding how medicinal plants respond to specific biotic stressors, offering insights into metabolic engineering for enhanced bioactive compound production. This chapter reveals the molecular mechanisms behind the defense response and their relation to secondary metabolites that enhance plant resilience and therapeutic potential.

6.1.2. Secondary metabolic pathways and important genes

The shikimic acid and phenylpropanoid pathways are two essential pathways for phenolic biosynthesis which is critical for plant defense against biotic stresses like attack by gall inducer. The shikimic acid pathway, a seven-step process, produces chorismate, a precursor to aromatic amino acids (phenylalanine, tyrosine and tryptophan) that feed into secondary metabolism (Mir et al., 2015).

The pathway begins with 3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS), which catalyzes the condensation of phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate to form 3-Deoxy-D-arabino-heptulosonate-7-phosphate (DAHP). In the next step, 3-dehydroquinic acid (DHQ) is produced by cycling DAHP after eliminating its phosphate. This cyclization is catalysed by 3-dehydroquinate synthase (DHQS). DHQ is converted to 3-dehydroshikimic acid (DHS) by the enzyme 3-dehydroquinate dehydratase. A reduction reaction involving DHS and nicotinamide adenine dinucleotide phosphate (NADPH) produces shikimic acid. In the next step, shikimic acid is activated with adenosine triphosphate (ATP) to form shikimic acid 3-phosphate (S3P). The enzyme catalyzes this reaction is shikimate kinase (SK). PEP is then added to S3P to produce 5-enolpyruvyl shikimic acid 3-phosphate (EPSP). The conversion is catalysed by the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Chorismate synthase (CS) catalyses the seventh and final step in the shikimic acid pathway, *i.e.*, conversion of EPSP to chorismate. Chorismate mutase catalyses the reaction of chorismate to prephenate, the first committed step in phenylalanine and tyrosine biosynthesis. The pathway products are the precursors of an array of natural products (Mir et al., 2015; Ralph et al., 2006b).

The phenylpropanoid pathway transforms phenylalanine into diverse phenolics, including flavonoids and lignins, vital for gall-induced defense. Phenylalanine ammonia-lyase (PAL) initiates the pathway by converting phenylalanine to trans-cinnamic acid, a critical step regulating carbon flux from primary to secondary metabolism (Dae & Hwang, 2014). The trans-cinnamic acid is hydroxylated to 4-coumarate by the action of cinnamic acid 4-hydroxylase (C4H) activity. Then 4-coumarate is converted to 4-coumaroyl-CoA by 4-coumarate-CoA ligase (4CL). The synthesis of flavonoids, which begins with the generation of chalcones, is initiated by the incorporation of 4-coumaroyl-CoA into the flavonoid biosynthesis pathway. Chalcone synthase (CHS) combines one molecule of 4-coumaroyl-CoA with three molecules of malonyl-

CoA to form naringenin chalcone. The chalcone is converted into a flavanone by the enzyme chalcone-flavanone isomerase (CHI) (Keykha et al., 2016).

The pathway split into various branches from these centre intermediates, each of which produces a distinct class of flavonoids. Flavanones are then subsequently transformed by flavanone 3-hydroxylase (FHT) and by dihydroflavonol 4-reductase (DFR) to leucoanthocyanidins (flavanols), which are the precursors of both anthocyanidins and flavan-3-ols (Hua et al., 2013; Lillo et al., 2008). DAHPS, EPSPS, CS, PAL, CHI and DFR were selected for this study due to their regulatory roles in channeling carbon toward phenolics, hypothesized to be upregulated in galls induced by *Pauropsylla* species. The shikimic acid and phenylpropanoid pathway is shown in Plate 6.1 and 6.2 respectively.

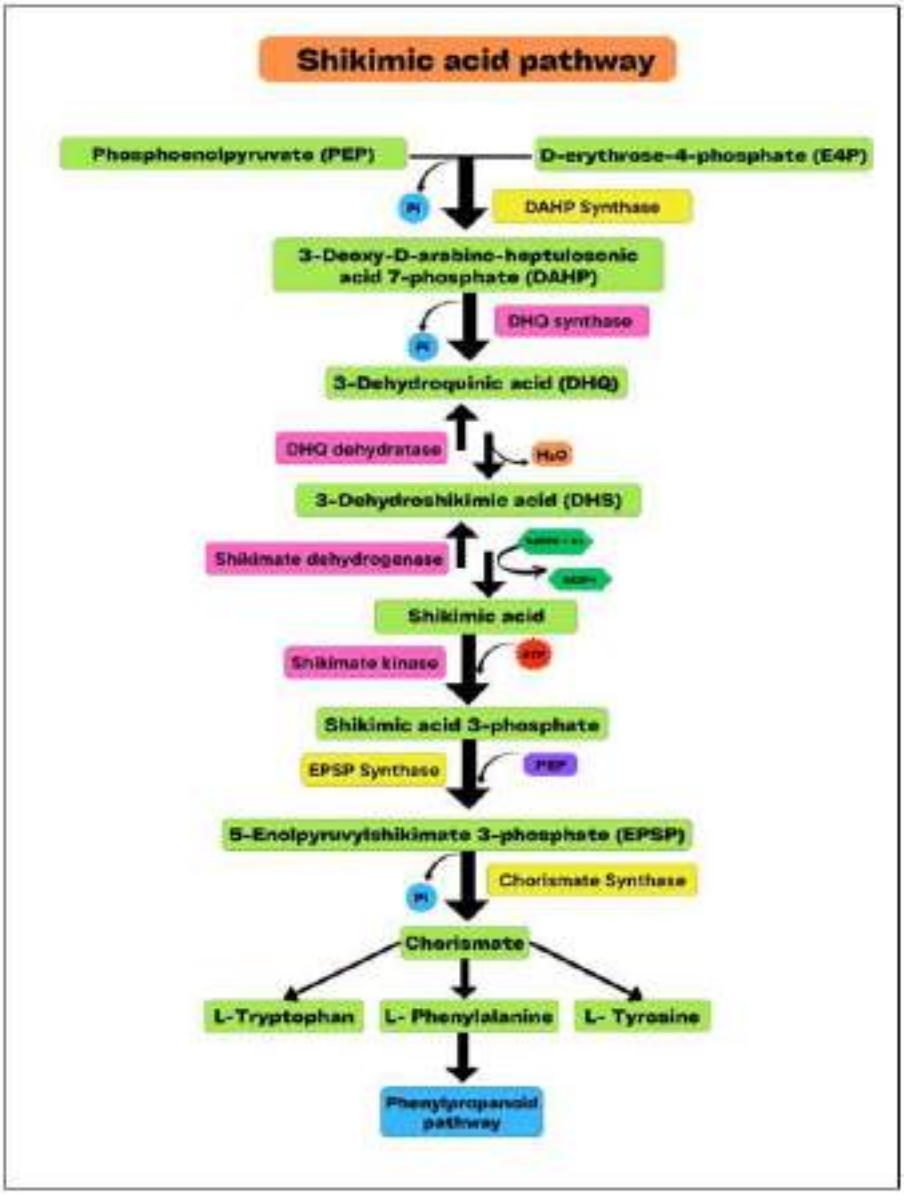


Plate 6.1: Diagrammatic representation of shikimic acid pathway with important genes and pathway products. Genes selected for the present expression analysis are shown in yellow boxes (Liu et al., 2014).

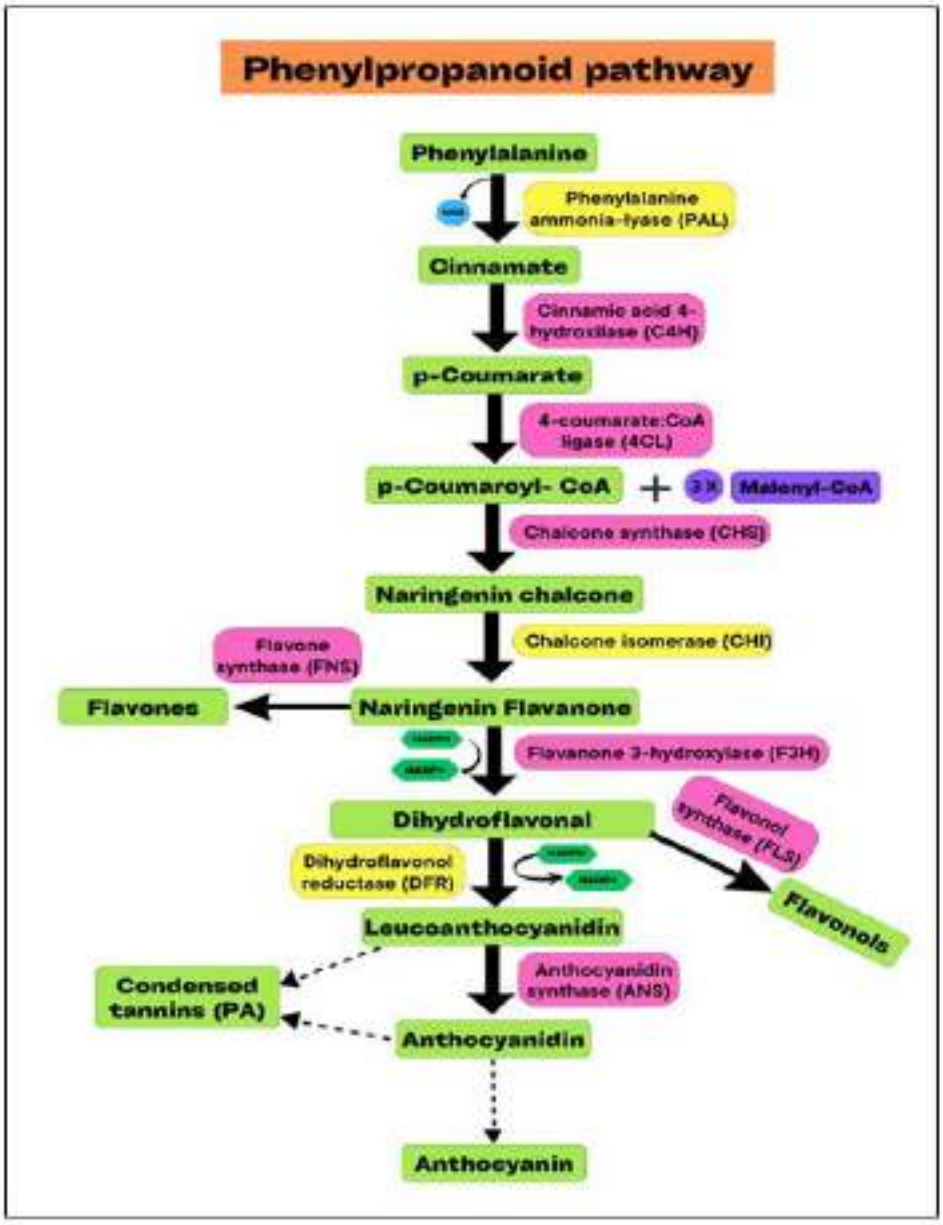


Plate 6.2: Diagrammatic representation of phenylpropanoid pathway. Genes selected for the present expression analysis are shown in yellow boxes. Dotted line arrow represents multiple steps (Ali et al., 2011; Choi et al., 2021).

6.1.3. Gene expression profiling

Gene expression is the process through which DNA is transcribed into RNA and subsequently translated into proteins, it forms the basis of all biological functions and represents the central dogma of molecular biology. Differential gene expression enables cells with identical genetic information to perform specialized roles, shaping tissue-specific phenotypes and organismal responses to environmental stimuli, such as gall induction in *A. scholaris* and *F. racemosa* (Busch & Lohmann, 2007). In this study, gene expression profiling is employed to investigate how *Pauropsylla*-induced galls alter the expression of six genes (DAHPS, EPSPS, CS, PAL, CHI and DFR) in the shikimic acid and phenylpropanoid pathways, hypothesizing that gall formation upregulates these genes to enhance phenolic biosynthesis, as detailed in the Chapter 3.

Different techniques can be used for gene expression profiling. Northern Blotting, Reverse Transcription PCR, *In Situ* Hybridization are conventional methods for expression profiling. The foundation for our current knowledge of gene regulation and expression was established based on these gene expression profiling techniques (Bachman, 2013; Dozois et al., 1997; Drea et al., 2005; Kumlauf, 1991). Depending on their underlying technology, expression profiling techniques fall into three categories: PCR based, sequencing based and hybridization-based approaches. Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) is a PCR based method, which allows simultaneous profiling of gene expression for a limited number of genes. Serial analysis of gene expression (SAGE) is one of the sequencing-based expression analysis methods. Nowadays RNA sequencing technologies using next generation sequencing (NGS) methods are widely employed for profiling. It analyses transcriptomes with high speed and accuracy. It is a powerful tool for measurement which is practical and affordable (Matsumura et al., 2010; Yamamoto et al., 2001).

Microarrays gene expression uses hybridization as a basic principle. Microarrays consist of DNA probes that are attached to a solid support that hybridise with complementary RNA or cDNA samples. The hybridisation of nucleic acid leads to the emission of signals as fluorescence. The signal intensity is directly proportional to the level of expression. Microarray helps in the measurement of a large number of genes simultaneously. It can be used for analysis

of known genes and transcripts. Signal saturation at the high end and background at the low-end limit signal measurement (Galbraith, 2006; Jain, 2012; Seki et al., 2010).

The choice of qRT-PCR reflects its ability to quantify subtle expression changes in response to *Pauropsylla*-induced stress, offering insights into how gall formation drives phenolic accumulation. By profiling DAHPS, EPSPS, CS, PAL, CHI and DFR, this study addresses gaps in understanding molecular mechanisms underlying gall-induced metabolism in medicinal plants for elucidating plant defense strategies and informing phytopharmacological applications, such as developing gall-derived therapeutic compounds.

Quantitative real-timePCR

Quantitative real-time PCR (qRT-PCR) is a molecular technique for analyzing gene expression. It works on the polymerase chain reaction (PCR), which amplifies DNA using primers and a thermostable Taq polymerase from *Thermus aquaticus*. qRT-PCR enables precise, real-time quantification of mRNA levels (Erlich, 1989; Zhu et al., 2020). Unlike traditional PCR, qRT-PCR monitors amplification through fluorescent reporters. It uses threshold cycle (Ct), the point where fluorescence exceeds background-indicating mRNA abundance for expression analysis. Lower Ct values reflect higher gene expression, making qRT-PCR ideal for detecting small changes in response to biotic stress. Three steps make up the qRT-PCR process: first, RNA is converted to cDNA using reverse transcriptase enzyme; second, the cDNA is amplified using PCR and third, is the quantification of amplified products (Derveaux et al., 2010; Jozefczuk & Adjaye, 2011).

It has high sensitivity, speed and accuracy, make it ideal method for the present study. It targets a small gene set (less than 10) to investigate gall-induced upregulation in normal leaves, galled leaves and galls. qRT-PCR has the ability to compare expression across tissues under experimental conditions, such as biotic stress and has established it as a cornerstone for plant molecular studies (Chen et al., 2009). For instance, qRT-PCR has revealed gene expression changes in plants under insect herbivory, supporting its application to use in the expression analysis of gall induction systems.

By elucidating how gall induction alters secondary metabolism, qRT-PCR provides insights into plant defense mechanisms and their pharmacological potential. This targeted

approach not only advances our understanding of plant-insect interactions but also informs strategies for metabolic engineering and stress-resistant crop breeding (Chen et al., 2009). Detailed study of differential gene expression might lead to a better understanding of plant physiology. It will also help to uncover the physiological and molecular processes associated with plant interactions.

6.2. METHODOLOGY

Plant material collection and storage

Healthy leaves and gall possessing leaves were collected from both *A. scholaris* and *F. racemosa* plants with care to avoid contamination with RNases. The galls were separated from gall leaves immediately after collection. All the collected samples were stored in liquid nitrogen soon after collection. The samples were stored at -80 °C in a deep freezer until RNA extraction (Bilgin et al., 2009).

Extraction of total RNA

To extract RNA from the selected plant samples, various RNA extraction techniques were done. They include the Guanidine thiocyanate (GTC) method, Lithium chloride precipitation, Trizol method, modified Trizol method and modified method from Chan and colleagues (Chan et al., 2007). However, due to the increased concentrations of secondary compounds in the selected plant tissues, none of these approaches were able to isolate pure RNA from the plant tissues. Phenolic chemicals prevent pure RNA from being isolated from the tissues. Latex content in the tissue is also intercepted in the isolation of pure RNA. Using various doses of polyvinylpyrrolidone (PVP), we modified the methods in order to reduce the interference of phenolics in RNA extraction. There observed a little impact (Johnson, 2023; Shu et al., 2014; Vennapusa et al., 2020).

Due to the inability of above-mentioned methods to isolate RNA, we used Phytolyze Reagent (Origin) following the manufacturer protocol for RNA extraction and it worked in the tissues selected. The total RNA was isolated from normal leaves, gall leaves and gall of both plants using Phytolyze Reagent. It is a quick and convenient reagent for use in the simultaneous

isolation of RNA from plants. Phytolyze Reagent performs well with large or small amounts of plant tissues. Prior to isolation of RNA, mortars and pestles were autoclaved at a temperature of 121 °C and 15 psi for 30-45 min. Mortar and pestle were stored at -20 °C overnight before use. Eppendorf tubes, tips, forceps etc. were also double autoclaved to remove any RNAse contamination.

RNA extraction procedure

Total RNA was extracted from plant tissues using Phytolyze Reagent following a standard protocol. Fresh plant samples were first frozen in liquid nitrogen and finely ground using a pre-cooled mortar and pestle. Approximately 100 mg of the powdered tissue was transferred into a 1.5 mL microcentrifuge tube. To this, 700 µL of Phytolyze Reagent and 20 µL of β-mercaptoethanol were added, followed by vortexing for 10-20 seconds to ensure complete homogenization. The mixture was then incubated at 65 °C for 30 min, with intermittent vortexing.

After incubation, 700 µL of chloroform was added to the lysate, vortexed and centrifuged at 12,000 rpm (~13,400 ×g) for 10 min. The upper aqueous phase was carefully transferred to a new RNase-free tube, avoiding the interphase. For RNA precipitation, 700 µL of ice-cold isopropanol was added to the supernatant, mixed by gentle inversion and incubated at room temperature for 5-10 min. The mixture was then centrifuged at 12,000 rpm for 10 min at 2-8 °C to pellet the RNA.

The resulting RNA pellet was washed with 75% ethanol (1 mL per 700 µL of Phytolyze Reagent used), vortexed and centrifuged again at 12,000 rpm for 5 min at 2-8 °C. The pellet was then briefly air-dried for 5-10 min to remove residual ethanol, taking care not to overdry. The RNA was resuspended in an appropriate volume of RNase-free water by gentle pipetting, followed by incubation at 55-60 °C for 10-15 min to enhance solubilization. To eliminate any contaminating genomic DNA, the resuspended RNA was subsequently treated with DNase I.

Evaluation of RNA quality

For each sample, RNA concentration was determined using a UV-visible spectrophotometer (Eppendorf, Thermomixer Comfort) by measuring absorbance at 260 nm. The

260:280 optical density (OD) ratio of RNA was checked to find the quality of RNA (Vennapusa et al., 2020).

Agarose gel electrophoresis to check purity and integrity of RNA

1.2% agarose gel was prepared by dissolving 1.2 g agarose in 100 mL of 1× Tris acetate EDTA (TAE) buffer. The mixture was heated in a microwave oven until the agarose was completely dissolved. After cooling down to 50 °C, 2 µL of ethidium bromide (EtBr) (10 mg/mL) was added to the melted agarose, mixed well. The gel was added into a casting tray and a comb was placed in order to form wells. After the gel was solidified, it was submerged in a 1× TAE buffer within the electrophoresis tank. An aliquot of 8 µL RNA was mixed with the 2 µL 10× RNA loading dye and placed into each well. 10× RNA loading dye was prepared by mixing 3.9 mL Glycerol, 0.5 mL 10% SDS, 0.2 mL 0.5 M EDTA, 25 mg Bromophenol Blue and 25 mg Xylene Cyanol. The mixture was made up to 10 mL with RNase-free water.

The electrophoresis was run at 60 volts for 45 min or till dye front reached to 3/4th part of the gel (Armstrong & Schulz, 2015; Sambrook & Russell, 2001; Vennapusa et al., 2020). After that, a UV transilluminator was used to view the RNA bands and a gel image was taken as record.

Complementary DNA (cDNA) synthesis:

Isolated RNA, which is treated with DNase I and then subjected to reverse transcription PCR reaction using a commercial kit (PrimeScript™ 1st Strand cDNA Synthesis Kit, TAKARA) according to the manufacturer's instructions.

Procedure of cDNA synthesis

The reaction was set up by mixing 5 µL RNA, 0.5 µL oligo dT primer, and 12 µL nuclease-free water, followed by incubation at 65 °C for 5 min and immediate chilling on ice for 2 min. Subsequently, 5 µL 5× reaction buffer, 1 µL 10 mM dNTPs, 0.5 µL RNase inhibitor (RNase I) and 1 µL reverse transcriptase enzyme were added to the vial. The mixture was incubated at 42 °C for 60 min in a Gradient Master Cycler (HIMEDIA Wee 32) and then heated at 70 °C for 10 min, after which the cDNA was collected. Then it was diluted into 1:10 ratio with sterile double distilled water and stored at -80 °C to use for PCR reactions (Sambrook & Russell, 2001).

Genes selected for the study

Initially, a literature search was done and identified 10 genes from both the phenylpropanoid and shikimic acid pathways for gene expression analysis. Following an initial screening, six genes were selected for further investigation. Table 6.1 presents the genes selected for the current expression profiling analysis.

Table 6.1: List of selected genes with their reaction substrates and products

Sl. No	Name of gene	Substrates for the reaction	Product of the reaction
1	3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS)	Phosphoenolpyruvate (PEP) and Erythrose-4-phosphate (E4P)	3-Deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP) and Phosphate
2	5-Enolpyruvylshikimate 3-phosphate synthase (EPSPS)	Shikimic acid 3-phosphate (S3P) and Phosphoenolpyruvate (PEP)	5-Enolpyruvylshikimate 3-phosphate (EPSP) and phosphate
3	Chorismate synthase (CS)	5-Enolpyruvylshikimate 3-phosphate (EPSP)	Chorismate and phosphate
4	Phenylalanine ammonia lyase (PAL)	Phenylalanine	Trans-cinnamic acid and ammonia
5	Chalcone-flavanone isomerase (CHI)	Chalcone	Flavanone
6	Dihydroflavonol 4-reductase (DFR)	Dihydroflavanone	Leucoanthocyanidins

Primer design and synthesis

Due to the lack of genomic sequences for *A. scholaris* and *F. racemosa* in the NCBI GenBank, the gene specific primers for real time amplification were designed by using blast results of similar accessions selected from NCBI. The accessions selected and used were listed in Table 6.2. Conserved regions of each gene were identified from blast results and the primers were designed using conserved sequences. The best set of primers was identified based on the region to be amplified. FAST PCR primer design software (version 6.7) was used for this purpose. The parameters considered for primer design were primer size of 19-22 bp, Tm of 55-62 ºC, GC% of 50-60%, product size of 100-300 bp and rest of the parameters were set to default (Li et al., 2011). The designed primer sequences were given to manufacture for synthesis (Sigma, Bangalore, India).

Table 6.2: Gene accessions used for primer design, with their accession numbers and plant species.

Sl. No	Selected genes	Plant species	Accession no.
1	DAHP synthase (DAHPS)	*Hevea brasiliensis*	XM_021786494.1
		Mangifera indica	XM_044652266.1
		Citrus sinensis	XM_006472513.3
		Pistacia vera	XM_031395831.1
2	EPSP synthase (EPSPS)	*Solanum tuberosum*	XM_006347816.2
		Capsicum annuum	XM_016719734.2
		Nicotiana tabacum	NM_001325913.1
		Coffea arabica	XM_027250652.1
3	Chorismate synthase (CS)	*Manihot esculenta*	XM_021770219.2
		Ricinus communis	XM_015725629.2
		Mangifera indica	XM_044651584.1
4	Phenylalanine ammonia-lyase (PAL)	*Manihot esculenta*	XM_021766727.2
		Hevea brasiliensis	XM_021804780.1
		Ricinus communis	XM_002519475.3
		Mangifera indica	XM_044617478.1
5	Chalcone-flavanone isomerase (CHI)	*Hevea brasiliensis*	XM_021806745.1
		Pistacia vera	XM_031394262.1
		Triadica sebifera	MN906941.1
		Rhus chinensis	KC287083.1
6	Dihydroflavonol 4-reductase (DFR)	*Solanum lycopersicum*	NM_001247479.2
		Solanum pennellii	NM_001374410.1
		Lycium ruthenicum	KY287797.1
		Nicotiana sylvestris	XM_009761654.1

Amplification of selected genes

Target genes were amplified by running the gradient PCR using cDNA samples and gene specific primers. These amplified products of each gene were eluted out, diluted into different

concentrations and used for primer efficiency analysis. PCR reaction conditions were optimized based on primer and target sequences. Trials were done with different annealing temperatures ranging from 52-64 °C. Finally optimised the annealing temperature for all the targets as 60 °C (Riet et al., 2017; Sambrook & Russell, 2001).

The PCR reaction mixture (25 µL) was prepared by combining 1.5 µL dNTPs, 3 µL 10× reaction buffer, 0.5 µL Taq polymerase enzyme, 2.5 µL cDNA, 1.5 µL forward primer (1 pmol) and 1.5 µL reverse primer (1 pmol) and the volume was adjusted to 25 µL with nuclease-free water. The mixture was then subjected to amplification cycles in a Gradient Master Cycler of HIMEDIA Wee 32 using the programme (Table 6.3).

Table 6.3: PCR programme outlining the step-wise temperature conditions, duration and cycle number used for amplification of the selected genes.

Step	**Temperature**	**Time**
Initial denaturation	94 °C	4 min
Denaturation (cycle)	94 °C	1 min
Annealing	58 °C	40 Sec
Extension	72 °C	1 min
Final extension	72 °C	10 min
Cycles	35 cycles	

Elution of target gene

To obtain each gene in purified form, the amplified gene products were separated on agarose gel. Agarose gel (1.5%) was prepared by dissolving 1.5 g agarose in 100 mL of 1× Tris acetate (TAE) buffer, followed by heating in a microwave oven until completely dissolved. The solution was cooled to approximately 50 °C, after which 2 µL of ethidium bromide (EtBr) (10 mg/mL) was added and mixed thoroughly. The gel was poured into a casting tray and combs

were placed to create wells. After solidification, the combs were carefully removed and the gel was placed in an electrophoresis tank containing the 1× TAE buffer.

The amplified gene product was mixed with the 6× loading dye and the mixture was added to each well. About 4 µL of 100 base pair DNA ladder was added to a separate well to determine the amplicon size after running the gel. The electrophoresis was run at 80 volts until the dye front reached 3/4 of the gel. After the gel had run, it was transferred to an open UV box. The gel was separated from the gel tray, as the plastic would absorb much of the UV light. Then, using a sterile stainless steel scalpel blade, the necessary portion of the gel containing the amplified DNA product was cut. Then elution was further performed by using XcelGen DNA Gel/ PCR Purification Kit (Xcelris Genomics, Ahmedabad) following manufacturer's protocol.

Procedure of elution of DNA

The agarose gel slice containing the DNA fragment was excised with a sterile scalpel and transferred to a pre-weighed 1.5 mL microcentrifuge tube. The weight of the gel was determined. Three volumes of Buffer (GC-optimised buffers) were added to one volume of gel in the microcentrifuge tube and the mixture was incubated at 55-60 °C for 8 min. The tube was mixed by tapping the bottom every 2 min until the gel had melted completely. The tube was then cooled to room temperature. One volume of isopropanol was added to the mixture. Up to 700 µL of the Buffer GC mixture was transferred to a spin column with a collection tube. The column was centrifuged at 13,000 g for 1 min at room temperature. The flow-through was discarded and the column was placed back into the collection tube.

Then 500 µL of Buffer GC was added to the DNA Mini Column. The column was centrifuged at 13,000 g for 1 min at room temperature to wash the DNA Mini Column. 650 µL of DNA wash buffer was added to the column and the column was centrifuged at 13,000 g for 1 min at room temperature. The flow-through was discarded and the column, with the lid open, was inserted back into the collection tube. This step was repeated. The empty DNA column, with the lid open, was centrifuged at 13,000 g for 2 min to dry any ethanol residue in the matrix.

The column was placed into a clean 1.5 mL microcentrifuge tube and 30-50 µL of pre-warmed (60 °C) Elution Buffer was added to the centre of the column. The column was incubated at room temperature for 1 min. The column was centrifuged at 13,000 g for 1 min to elute the DNA. The eluted DNA solution was reloaded onto the column for a second elution. The

first elution typically yielded 60-70% of the DNA bound to the column. Reloading the eluted DNA solution onto the column for a second elution yielded an additional 20% of the DNA, making the total yield approximately 90%. The eluted PCR product was checked in 1.0% agarose gel and stored at -20 °C for future use.

Testing of primer efficiency

Primers were designed and synthesised for the amplification of target genes. The efficiency of each primer was validated using the standard curve method. For this, qRT-PCR reactions were set up to amplify serial 10-fold dilutions of the same target gene elution product. The Ct values were plotted on the Y-axis against the amount of DNA copies (ng) on the X-axis to generate the standard curve. The slope of the standard curve was calculated for each gene to determine primer efficiency, with efficient primers expected to produce a proportional dose-response relationship (Balcells et al., 2011; Lekanne et al., 2002). Then the efficiency of the primer is calculated by the following formula;

$$E = 10\left(-\frac{1}{Slope}\right) - 1$$

Selection of reference gene

Reference genes were expressed at a relatively constant level across different cell types, developmental stages and experimental factors. The housekeeping gene 18S rRNA was selected as a reference gene for normalisation of qRT-PCR data in this study (Chen et al., 2021; Kozera & Rapacz, 2013).

Quantitative real-time PCR for expression analysis of selected genes

qRT-PCR reactions were done for relative quantification of genes using the SYBR® Premix Ex Taq™ (TAKARA) polymerase enzyme following the manufacturer's protocol.

The qRT-PCR reaction mixture, with a total volume of 40 µL, was prepared by combining 2 µL of cDNA diluted 1:10, 2 µL of forward primer (10 pmol), 2 µL of reverse primer (10 pmol) and 10 µL of SYBR Green dye. The final volume of reaction mixture was made up by adding 24 µL nuclease-free water. This mixture was used to amplify and quantify the target genes during the real-time PCR assay. qRT-PCR was performed in thermocycler (Eppendorf Master cycler Ep Gradient Realplex 2) using the following scheme (Table 6.4).

Table 6.4: Optimized thermal cycling conditions used for the quantitative real-time PCR (qRT-PCR) analysis of the selected genes.

Temperature	Time
95 °C	2 min
95 °C	0.15 min
60 °C	0.15 min
72 °C	0.30 min
95 °C	0.15 min
Cycles	40 cycles

The fluorescence monitoring occurred at the end of each cycle. After thermal cycling, a melt curve analysis was performed to confirm that the fluorescence detected was due to target amplification. It aids in detecting non-specific amplification. It also helps to determine whether primer dimers produced during the qRT-PCR experiment.

The scheme of the PCR program is shown here. The relative fold gene expression was measured by comparative $2^{-\Delta\Delta CT}$ method. In this approach, the threshold cycle (Ct) values of the target genes were normalized to that of the reference (housekeeping) gene, 18S rRNA, to obtain the ΔCT value. The ΔCT of the experimental sample was then compared with that of the control sample to determine the ΔΔCT value. The fold change in gene expression was calculated as $2^{-\Delta\Delta CT}$, where the result represents the relative expression level of the target gene in the experimental sample compared to the control.

Statistical analysis

All experiments were conducted in triplicate for each sample. The mean values and corresponding standard deviations were calculated and used for comparative gene expression analysis. Data were log-transformed to satisfy the assumptions of normality and homogeneity of variance. Statistical analyses were performed using Python 3.10 software and GraphPad Prism 10.4.1 softwares. Analysis of Variance (ANOVA) was applied to assess overall differences among sample groups, followed by Tukey's post-hoc test to identify pairwise differences between normal leaves, gall leaves and galls. A threshold of $p < 0.05$ was adopted as significant for each gene expression analysis.

RESHMA K RAMESH - Core

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CHAPTER 3 - STRUCTURE AND PHYTOCHEMISTRY OF LEAF GALLS ON SELECTED MEDICINAL PLANTS

3.3. RESULTS AND DISCUSSION

Gall formation is a complex phenomenon resulting from interactions between plants and various organisms, including insects, which induce pathological growths through feeding or oviposition. These galls exhibit a wide range of morphological complexities, from simple to highly elaborate structures. Key cellular processes involved include hypertrophy (cell enlargement) and hyperplasia (increased cell division), leading to the development of specialized tissues that support the growth of the inducing organism (Mani, 2000; Oliveira & Isaias, 2009). In India, over 2000 different gall forms have been documented, highlighting the richness of gall-inducing flora (Raman, 2007). This study focuses on two medicinal plants, *A. scholaris* and *F. racemosa*, which develop leaf galls induced by psyllids from the Order Hemiptera and Class Psyllidae. With approximately 350 species of psyllids known to induce galls on dicotyledonous leaves, these insects play a significant role in shaping plant morphology and phytochemistry (Raman, 2011).

3.3.1. Structure of normal leaves and leaf galls

Morphology of leaves of *A. scholaris*

The leaves are smooth and glossy, with a dark green upper surface and a paler, grayish underside. They are elliptic to broadly obovate in shape and exhibit a subcoriaceous to coriaceous (leathery) texture. The leaves measure approximately 20-25 cm in length and 5.5-6.5 cm in width. The apex is either rounded or emarginate, while the base is cuneate to decurrent. The petiole is short, typically less than 1 cm in length. Leaves are arranged in whorls of 6-7 leaflets and each leaflet displays numerous pairs of closely spaced lateral veins that are oriented nearly perpendicular to the midrib (Plate 3.1: Figure A and Figure B). *A.*

scholaris leaves with galls (Plate 3.1: Figure C) and single leaf with galls, adaxial surface (Plate 3.3: Figure A) and abaxial surface (Plate 3.3: Figure B) of leaf with galls were shown.

Morphology of leaf galls of *A. scholaris*

Both young and mature leaves of *A. scholaris* are susceptible to infection by leaf gall-inducing insects. The galls predominantly develop along the lateral veins and are fleshy, yellowish-green and spherical in shape, ranging from 5-10 mm in diameter. A single leaf may bear 1-190 galls, with an average of 20-30 galls per leaf. The galls are typically unilocular, containing a single chamber; however, in cases where galls are fused, they may become multilocular with 3-4 chambers. Severe infestations result in significant morphological deformities, with the leaf lamina often becoming entirely covered by dense clusters of galls. Mature galls possess an opening called ostiole, on the abaxial surface, which facilitates the emergence of the insect after development. After the adult insect exits, the gall becomes brownish-black, hard and woody in texture. Plate 3.3: Figure C represents the images of unilocular gall, bilocular gall and multilocular galls of *A. scholaris* and Figure D photograph showing the cross sections of galls.

The leaf galls of *A. scholaris* are induced by the insect *Pauropsylla tuberculata* Crawford (Psyllidae: Homoptera). The plate 3.5: Figure A showing the photograph of inducer insect *P. tuberculata* developing inside the leaf galls of *A. scholaris*, Figure B showing the photograph of the *P. tuberculata* and Figure C is the mature stage of *P. tuberculata*. The galls are epiphyllous, which can be unilocular or multilocular, with 3-4 chambers. The gall possesses a diameter between 0.1 and 1 cm. The galls can be single, dispersed or occasionally form agglomerated clusters. As the number of galls increases, the leaves get crumpled and distorted (Albert et al., 2011; Pasayat & Tripathy, 2020). Similar findings were made by the current study, which observed the occurrence of enormous galls that affected the natural shape and structure of *A. scholaris* leaves and led to change into totally distorted structures. The morphology of agglomerate galls of *A. scholaris* is shown in Plate 3.6: Figure A.



Plate 3.1: Figure A: *A. scholaris* plant with branches, leaves and leaf galls, Figure B: *A. scholaris* twig with healthy leaves and Figure C: *A. scholaris* twig possessing leaves with leaf galls.

Morphology of the leaves of *F. racemosa*

The leaves (Plate 3.2: Figure A and Figure B) are simple and arranged alternately along the stem, with a length ranging from 8-23 cm and a width of 3-7 cm. They are ovate, elliptical, lanceolate or sub-ovate in shape, with entire margins and a distinctly petiolated base. The leaf apex is acute and the margins are slightly wavy. The adaxial (upper) surface is dark green, while the abaxial (lower) surface is lighter green in colour. The petiole is angular, reddish brown in appearance and measures approximately 4.0-5.5 cm in length.

Morphology of leaf galls of *F. racemosa*

Galls are epiphyllous structures that develop on the adaxial surface of leaves, occurring singly or in multiple numbers. They may appear scattered across the leaf blade or grouped together, forming conspicuous spherical masses or convex bulges. The individual galls are simple, regular, globose, sessile, perfoliate and unilocular in structure, measuring approximately 5-10 mm in diameter. The number of galls per leaf ranges from 1 to 65, with an average of 15-20.

In cases of severe infestation, galls may coalesce, forming a single, large mass that can obscure the leaf lamina entirely. Such compound galls, resulting from the partial fusion of multiple galls, are typically irregular in shape, multilocular and measure between 15-30 mm in diameter (Plate 3.2: Figure C). Young galls are initially light green, gradually developing brownish or reddish-brown discoloration as they mature. In mature galls a small ostiole is found on the abaxial surface of the leaf, which is the way to release the adult insect out of gall after their complete development. Plate 3.4: Figure A and B represents the dorsal and ventral view of *F. racemosa* single leaf with leaf galls respectively, Figure C showing the leaf galls having single chamber to multiple chambers and Figure D represents the gall chambers of leaf galls of *F. racemosa*.

Galls induced on the leaves of *F. racemosa* by *Pauropsylla depressa* Crawf (Psyliidae, Hemiptera). Plate 3.5: Figure D shows the gall inducer *P. depressa* developing inside the leaf galls of *F. racemosa*, Figure E is the photograph of insect *P. depressa* and Figure F is the



Plate 3.2: Figure A: *F. racemosa* plant with branches, leaves and leaf galls, Figure B: *F. racemosa* twig with healthy leaves and Figure C: *F. racemosa* twig possessing leaves with leaf galls.

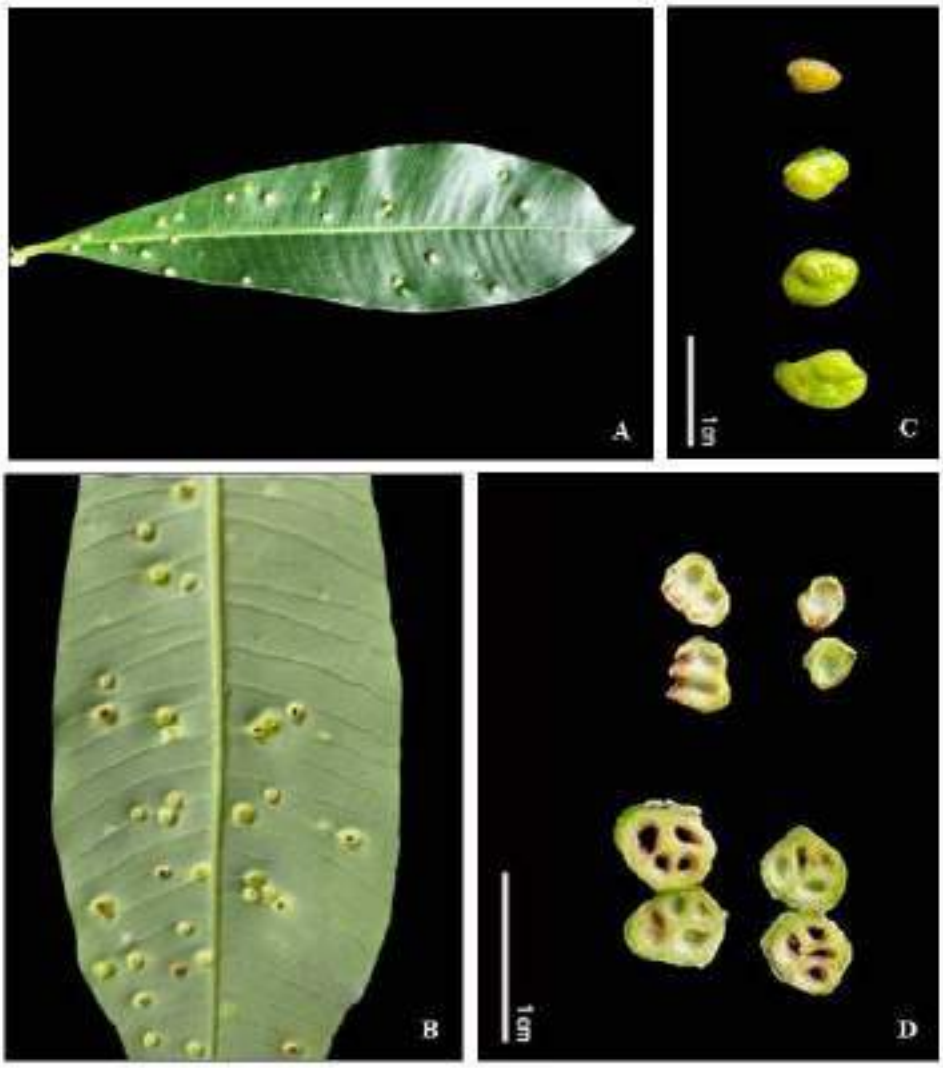


Plate 3.3: Figure A: *A. scholaris* single leaf with galls (Adaxial surface), Figure B: *A. scholaris* abaxial surface of leaf with galls, Figure C: Unilocular gall, bilocular gall and multilocular galls of *A. scholaris* and Figure D: Each gall is cut into two pieces to view the larval chamber.

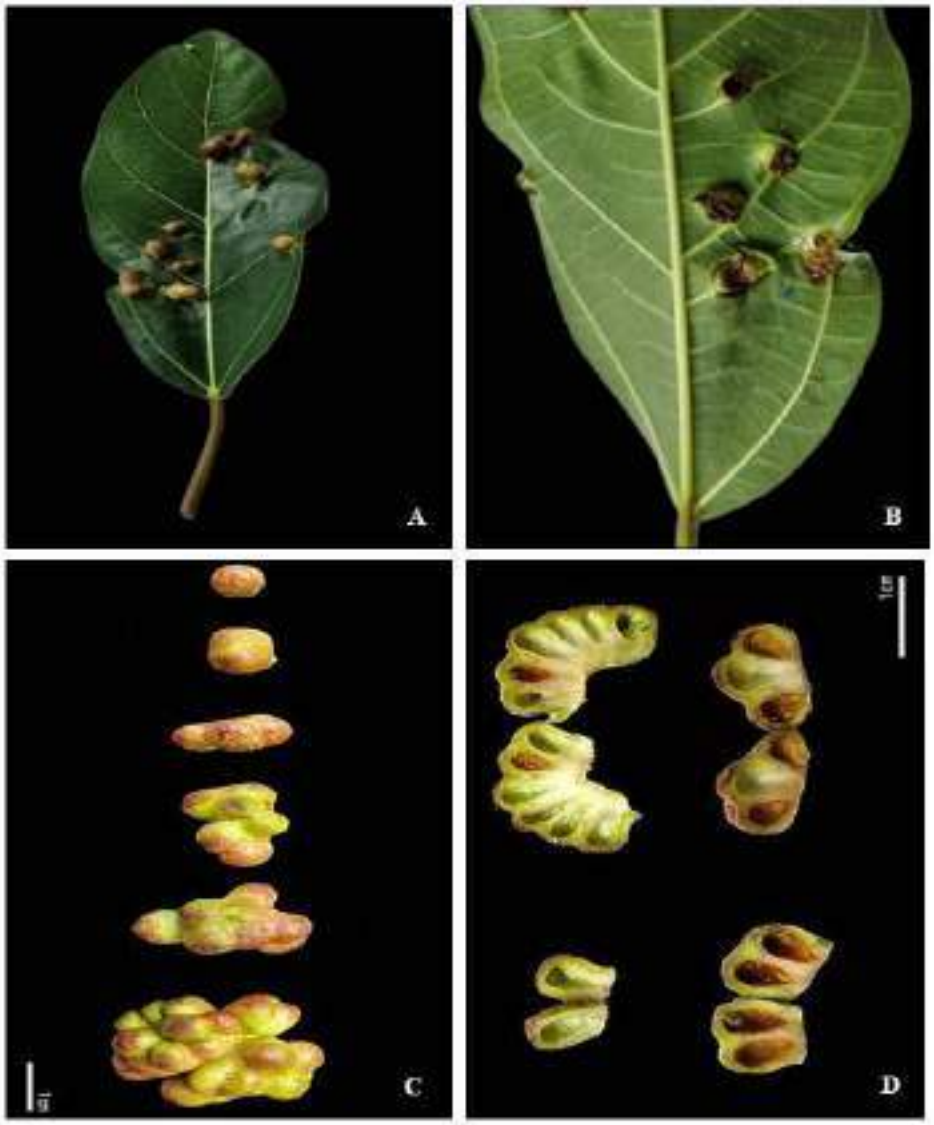


Plate 3.4: Figure A: *F. racemosa* single leaf with galls (Adaxial surface), Figure B: Abaxial surface of leaf with galls in *F. racemosa*, Figure C: *F. racemosa* galls having single to multiple chambers and Figure D: Each gall is cut into two pieces to view the larval chamber.



Plate 3.5 Figure A: The photograph of inducer insect *Pauropsylla tuberculata* developing inside the leaf galls of *A. scholaris*, Figure B: Photograph showing the insect *P. tuberculata*, Figure C: Mature stage of *P. tuberculata*, Figure D: The leaf gall inducer insect *Pauropsylla depressa* developing inside the leaf galls of *F. racemosa*, Figure E: Photograph of insect *P. depressa* and Figure F: Matured stage of *P. depressa* with wings.

picture of the mature stage of *P. depressa* with wings. The leaf galls are epiphyllous, simple, fleshy, globose, sessile, unilocular pouch galls or multilocular aggregates, clumps or masses. Our observations on *F. racemosa* galls found that the aggregate galls developed from individual galls are combined to generate complicated masses or clumps (Plate 3.6: Figure B). The gall typically lacks chlorophyll and is reddish or reddish-brown in color when mature. Its diameter ranges from 5 to 10 mm (Dsouza & Ravishankar, 2014; Ushir et al., 2015).

Anatomy of leaves of *A. scholaris*

Normal healthy leaves are dorsiventral in structure (Plate 3.7: Figure A and Figure B). The upper epidermis is covered by a thick cuticle, whereas the lower epidermis has a relatively thinner cuticle. The upper epidermal cells are tubular in shape and compactly arranged. Stomata are confined to the lower epidermis and are sunken in nature. The mesophyll is differentiated into palisade and spongy tissues. Palisade cells are cylindrical and tightly packed, while the spongy tissue is aerenchymatous, facilitating gaseous exchange. Resin canals are present within the mesophyll region. The vascular bundles are bicollateral and are surrounded by thick-walled supportive cells, which are sclerenchymatous. The vascular bundles are traversing the mesophyll, with the xylem strand flanked by phloem on both sides.

Anatomy of leaf galls of *A. scholaris*

The transverse section of leaf galls of *A. scholaris* is shown in Plate 3.8: Figure A and Figure B. The epidermis of galled leaves consists of rectangular cells covered by a thin cuticle. Beneath the epidermis lies the mesophyll, which appears homogeneous due to the absence of tissue differentiation. The typical palisade layer is replaced by thin-walled, compactly arranged parenchyma cells. These cells are large, round and closely packed. At the center of the gall is a prominent cavity known as the larval chamber, which serves as the site for insect development. This chamber is surrounded by nutritive parenchyma cells that contain conspicuously large starch grains, providing nourishment to the developing insect. Vascular bundles are seen around the larval chamber. They are scattered and embedded within the gall parenchymatous tissue irregularly.



Plate 3.6: Figure A: A twig of *A. scholaris* with clumped galls and Figure B: *F. racemosa* twig turned completely into galls.

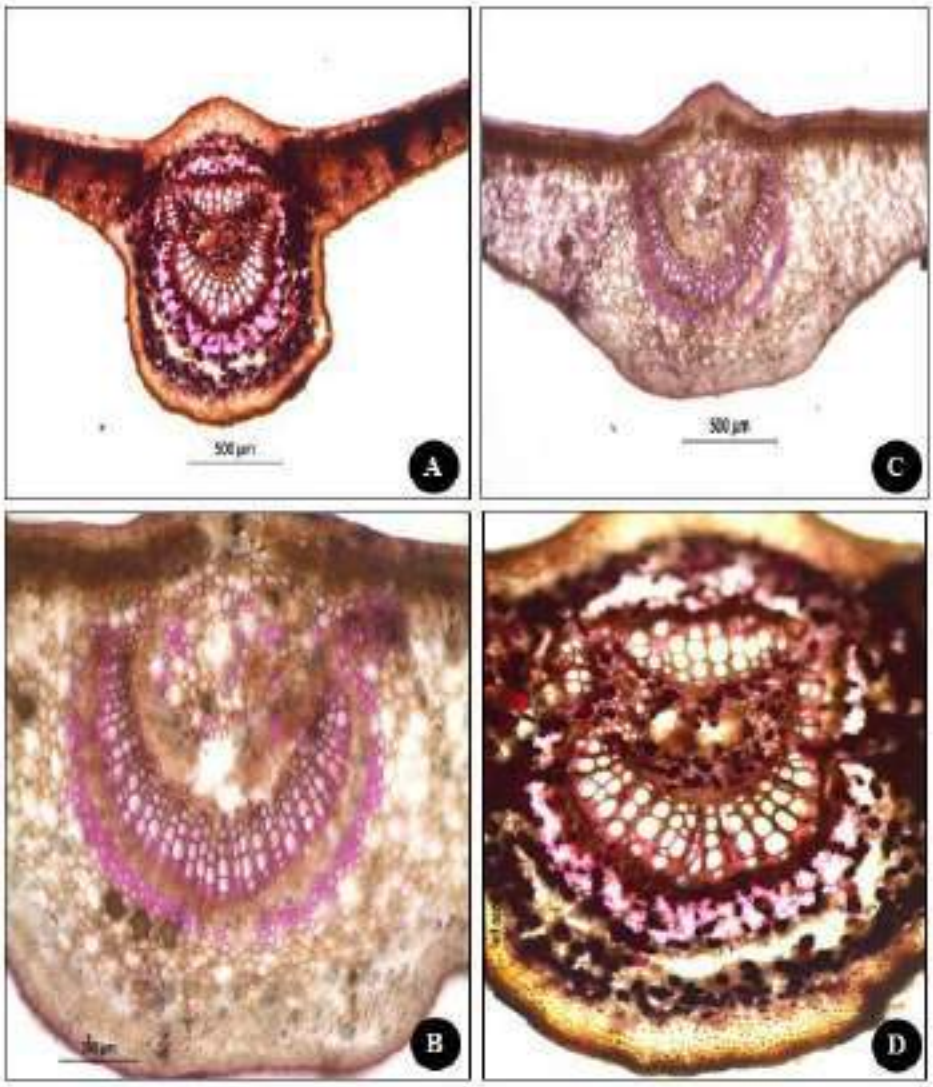


Plate 3.7: Anatomy of normal leaves. Figure A. Transverse section (T. S.) of normal (healthy) leaves of *A. scholaris*, Figure B: T. S. of mid rib portion of *A. scholaris* leaf, Figure C: T. S. of normal leaves of *F. racemosa* and Figure D: T. S. of mid rib portion of *F. racemosa* leaf.

The normal leaf anatomy of *A. scholaris* was investigated by Albert and colleagues and found a moderately thick layer of cuticle covers the tubular, densely packed upper epidermal cells. The lower epidermal cells are papillate. The thick, echinulate cuticle covering the lower epidermis is located near the papilla. Stomata are limited to the lower epidermis and are sunken. By analysing the transverse section of a normal leaf, there is no such difference observed from normal anatomy of a dicot leaf. Leaves were dorsiventral having a mesophyll tissue differentiated into palisade and spongy tissue. Albert and colleagues also analysed foliar galls of *A. scholaris* anatomically and discovered that there is a cavity at the center of the gall, surrounded by a closely packed mass of chlorophyll lacking cells, which are rich with starch grains. In this cavity, the insect development occurs and the surrounding tissue will supply nutrition for their development (Albert et al., 2011). Only a few works were reported on the anatomy of leaf galls of *A. scholaris.* A cavity in the middle of the leaf, encircled by starch-rich cells was visible in the transverse section. The former is commonly referred to as the larval chamber, while the latter is described as the nutritive tissue.

Anatomy of leaves of *F. racemosa*

The dorsiventral leaf consists of a central midrib and two lateral wings (Plate 3.7: Figure C and Figure D). The entire epidermis is covered by a thin cuticle and is composed of rectangular and cylindrical cells. Stomata are confined to the abaxial epidermis, which also bears simple trichomes. The adaxial epidermis is two-layered, while the abaxial epidermis is single-layered. Beneath the epidermis lies the mesophyll, which is clearly differentiated into palisade and spongy tissues. The adaxial side contains 2-3 layers of cylindrical, compactly arranged palisade cells. The spongy tissue comprises multiple layers of loosely arranged aerenchymatous cells with conspicuous intercellular spaces, facilitating gas exchange.

The midrib displays a convex outline on the ventral (upper) side and an arched profile on the dorsal (lower) side. Below the epidermis, the hypodermis consists of two layers. A central vascular bundle is present in the midrib region and is enclosed by a collenchymatous bundle sheath. The vascular bundle contains annular xylem vessels and 6-10 distinct phloem strands. Lithocysts are located in the adaxial region of the leaf, while crystals are observed

within the bundle sheath and in the midrib parenchyma. Tanniferous cells are distributed in both adaxial and abaxial regions.

Anatomy of leaf galls of *F. racemosa*

The T.S. of leaf galls shown in plate 3.8: Figure C and Figure D. The epidermis of the gall is a direct continuation of the normal leaf epidermis and consists of narrow, small-sized cells. It is externally covered by a thin cuticle. Beneath the epidermis lie 2-3 layers of lignified sclerenchyma cells, providing mechanical support to the gall structure. The cortex is differentiated into two distinct regions: an outer cortex and an inner cortex. The outer cortex is composed of spongy parenchyma interspersed with sclereids, while the inner cortical region contains parenchyma cells rich in starch grains. This inner cortical tissue serves as nutritive tissue essential for the development of the insect larvae.

The gall parenchyma contains a large number of superficial and deep vascular bundles that are irregularly scattered. The bundles have distinct xylem and phloem. The xylem is located on the adaxial side and the phloem is located on the inner side of the gall. At the center of the gall lies a prominent cavity known as the larval chamber, which serves as the site for the development of insects from larva to adult.

Ushir and colleagues reported that the mesophyll cells of the *F. racemosa* normal leaves are structured into layers. Adjacent to the adaxial epidermis are two layers of palisade cells. The cells of the palisade are closely spaced and cylindrical. The mesophyll tissues contain the vascular bundles buried within them. In the palisade region, cystoliths are often observed. In the case of leaf galls of *F. racemosa*, the gall tissue is made up of undifferentiated parenchyma that is several times thicker than the mesophyll of a normal leaf. These cells are large, thin-walled and are closely packed together. There are no stomata seen in galls. The parenchyma cells of mature galls are devoid of chlorophyll (Ushir et al., 2015). Our study also detected absence of chlorophyll in the gall tissue. Previous findings reported that the cells around the gall cavity show the accumulation of starch. This region is known as the nutritive region of the gall. Numerous vascular bundles are scattered across the gall parenchyma. The xylem is on the adaxial side of the gall, as seen in normal leaves and the phloem is on the inner side (Singh et al., 2023; Ushir et al., 2015). Transverse sections of galls

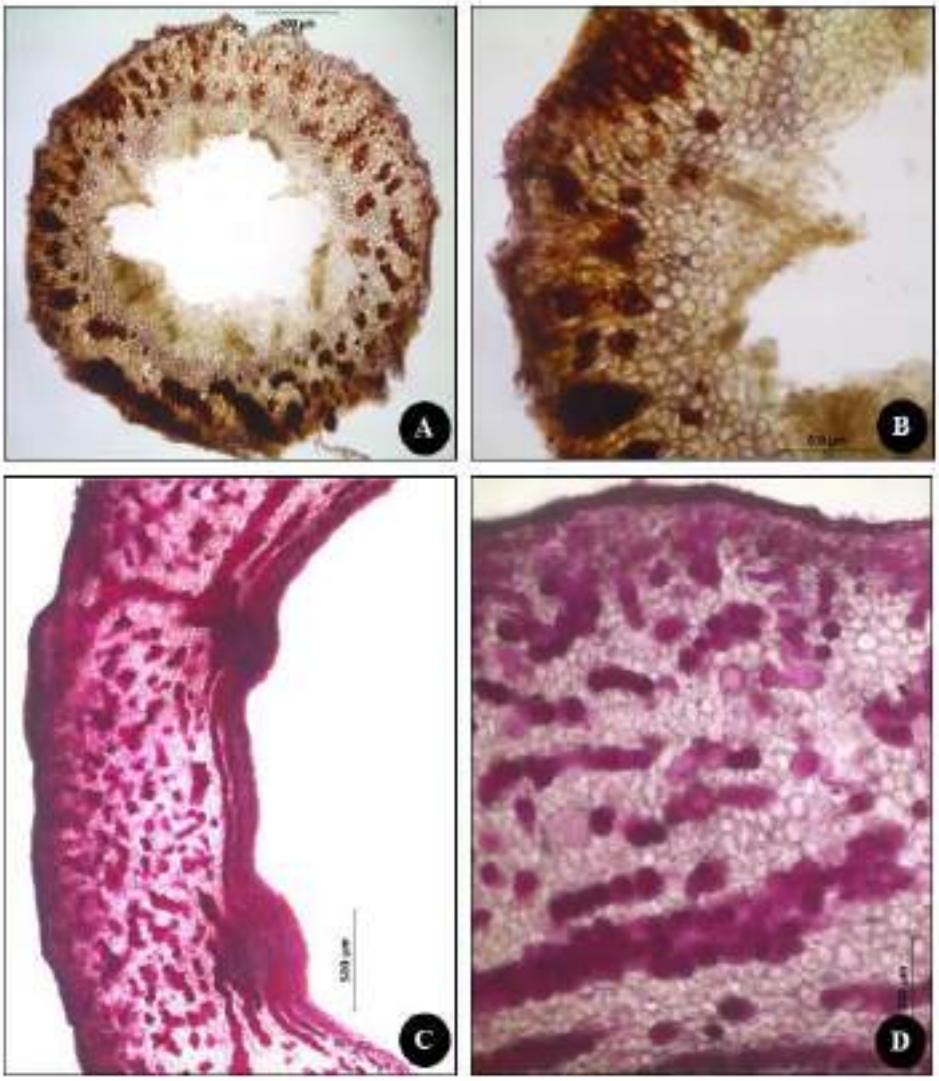


Plate 3.8. Anatomy of leaf galls. Figure A: T. S. of leaf galls of *A. scholaris*, Figure B: *A. scholaris* leaf galls; a portion enlarged, Figure C: T. S. of leaf galls of *F. racemosa* and Figure D: *F. racemosa* leaf galls; a portion enlarged.

have been examined which show the presence of a larval chamber and the presence of vascular bundles within the parenchyma underneath the chamber. The nutritive tissue around the chamber consisted of large, thin-walled cells with dense cytoplasm.

3.3.2. Results of phytochemical analysis of plant extracts

Preliminary phytochemical analysis of A. scholaris

Aqueous, hexane and ethanolic extracts of normal leaves, gall leaves and galls of *A. scholaris* plant were analysed for the presence of different phytochemicals. Analysis was done in triplicates to assess the consistency and significance of the observations. The frequency of positive results (presence of a phytoconstituent) was calculated for each phytoconstituent across the three replicates for each solvent extract (water, ethanol and hexane) and tissue type (normal leaves, gall leaves and galls) of both *A. scholaris* and *F. racemosa*. This provided a qualitative overview of the distribution of phytoconstituents.

Alkaloids were tested using Dragendorff's test, Hager's test and Wagner's test; found that Hanger's test gave positive result in aqueous extract of all samples and Wagner's test showed positive for ethanolic extracts. Phenolic compounds were detected in all samples of aqueous and ethanol extracts but only hexane extract of gall showed positive result. Lead acetate test of flavonoid were positive for all samples and extracts except ethanolic extracts of normal leaves and gall leaves. Saponins and coumarins gave positive results only in aqueous extracts and quinones were absent in all extracts and samples. Cardiac glycosides indicated their presence in all extracts except ethanolic extracts of normal leaf and gall leaf. Terpenoids were tested using Libberman's test and Salkowski test, found to be present in all extracts except hexane extract of gall leaves. Potassium dichromate test of tannins gave positive results only in ethanolic extracts. The results of qualitative phytochemical analysis of *A. scholaris* are summarized in Table 3.1. The photographs of the results of preliminary phytochemical analysis of *A. scholaris* normal leaf, gall leaf and gall aqueous extracts (Plate 3.9), hexane extracts (Plate 3.10) and ethanolic extracts (Plate 3.11) are shown.

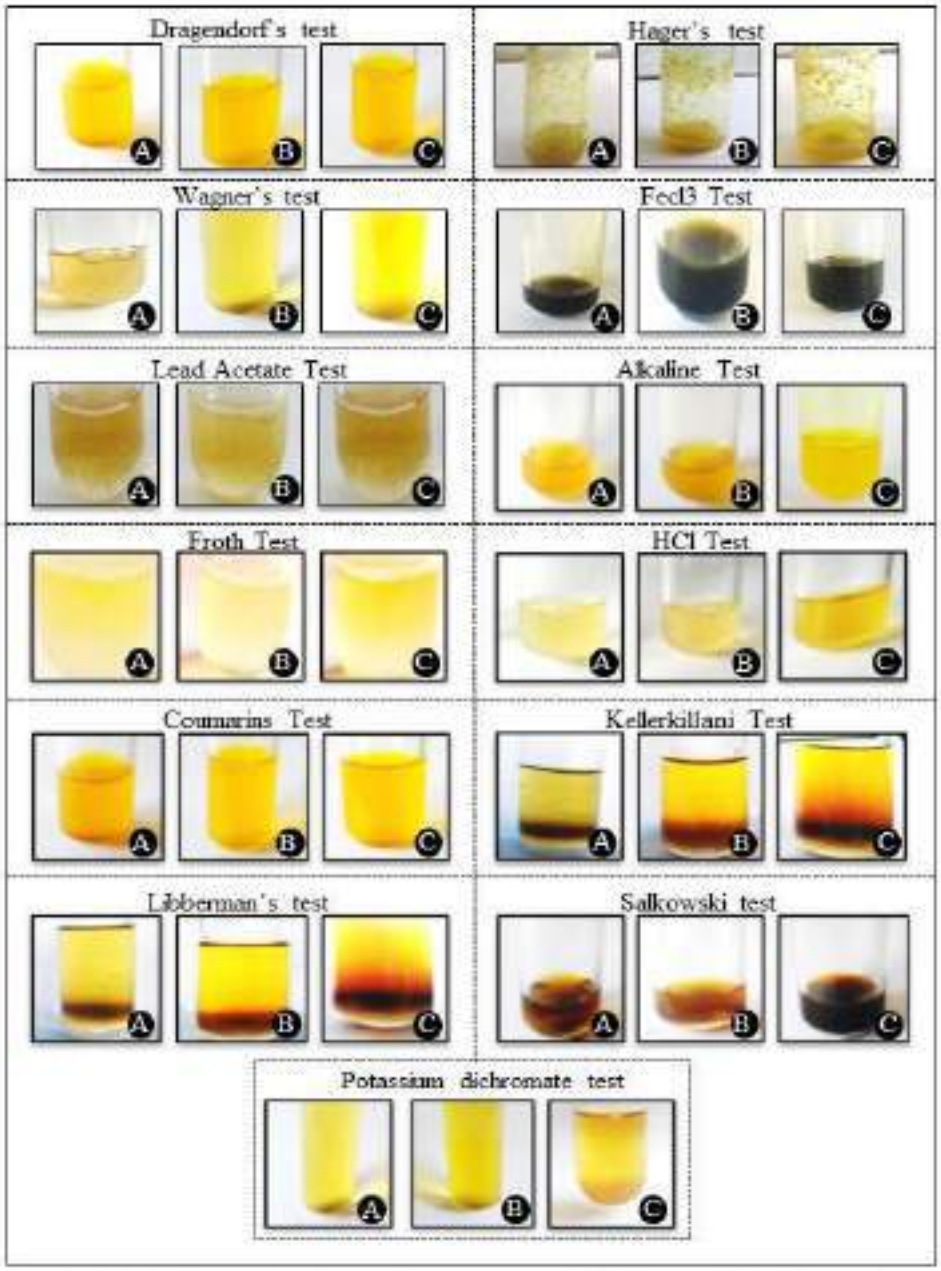


Plate 3.9: Preliminary phytochemical tests of normal leaf (A), gall leaf (B) and gall (C) aqueous extract of *A. scholaris*.

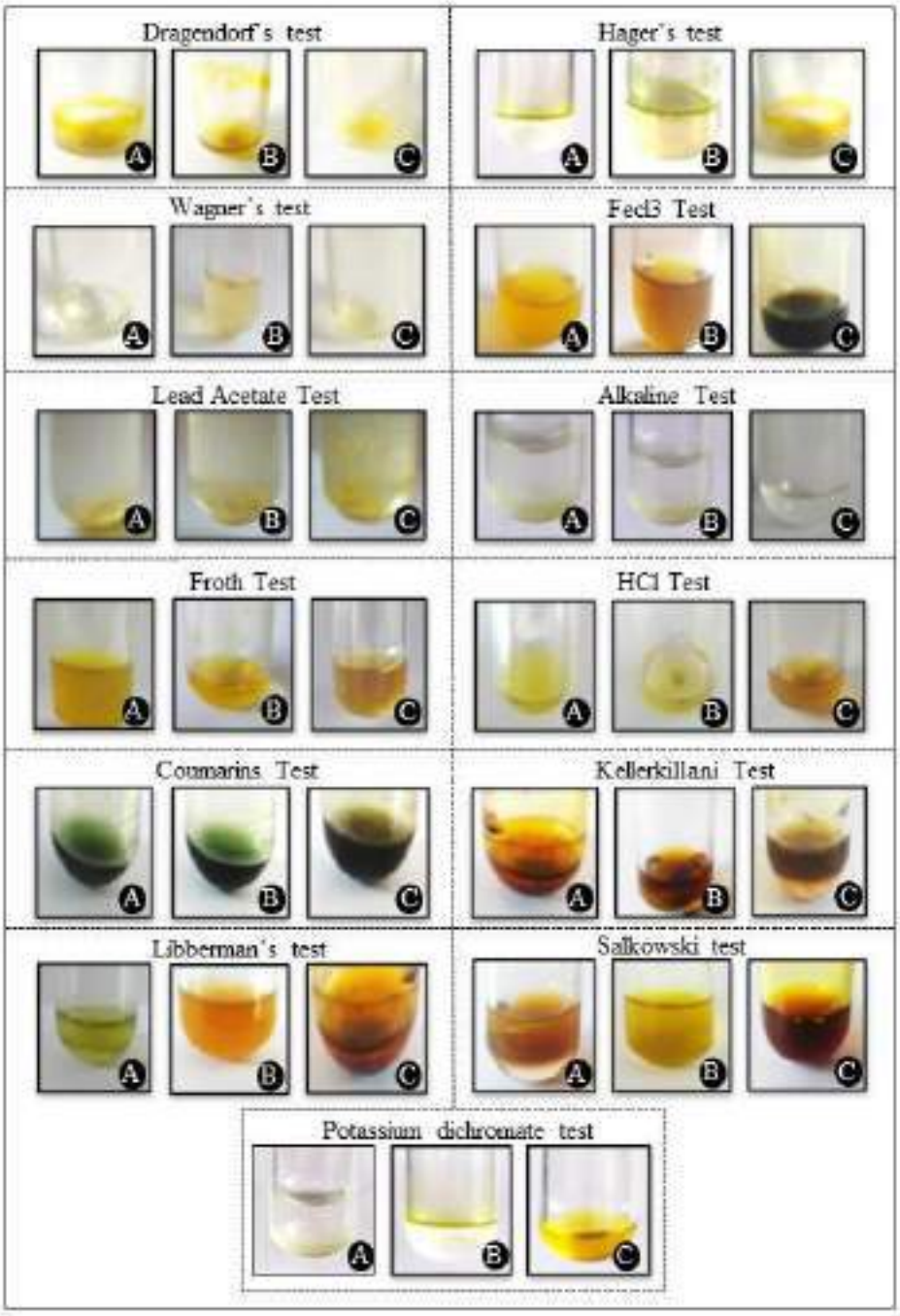


Plate 3.10: Preliminary phytochemical tests of normal leaf (A), gall leaf (B) and gall (C) hexane extract of *A. scholaris*.

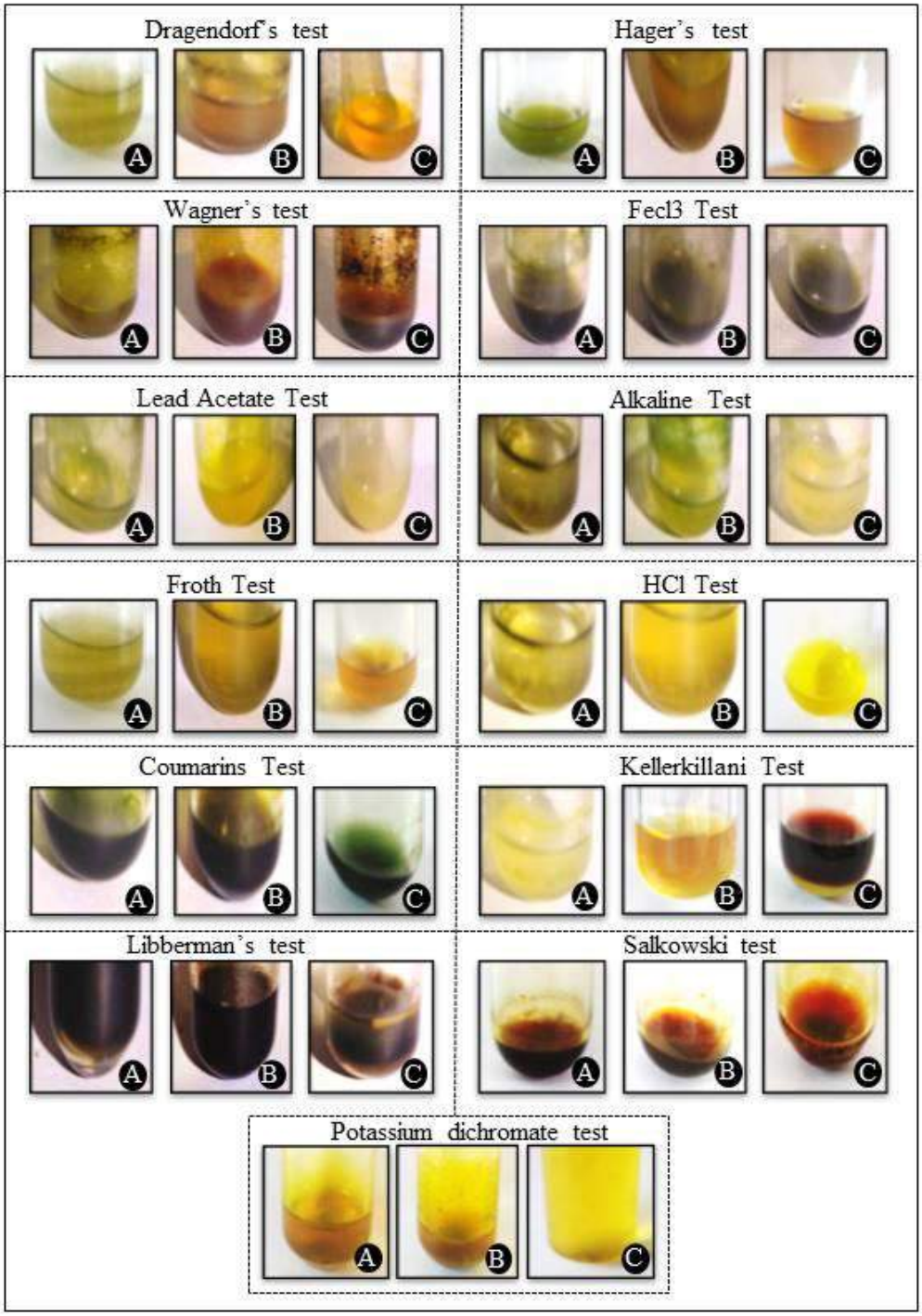


Plate 3.11: Preliminary phytochemical tests of normal leaf (A), gall leaf (B) and gall (C) ethanolic extract of *A. scholaris*.

Table 3.1: Results of preliminary phytochemical analysis of aqueous, hexane and ethanolic extracts of normal leaves, gall leaves and leaf galls of *A. scholaris*.

Compound	Name of test	*A. scholaris*								
		Water			Hexane			Ethanol		
		Normal leaf	Gall leaf	Gall	Normal leaf	Gall leaf	Gall	Normal leaf	Gall leaf	Gall
Alkaloids	Dragendorff's test	–	–	–	–	–	–	–	–	–
	Hager's test	+	+	+	–	–	+	–	–	–
	Wagner's test	–	–	–	–	–	–	+	+	+
Phenolics	Ferric chloride test	+	+	+	–	–	+	+	+	+
Flavonoid	Lead acetate test	+	+	+	+	+	+	–	–	+
	Alkaline reagent test	–	–	–	+	+	+	–	–	–
Saponin	Froth test	+	+	+	–	–	–	–	–	–
Quinones	HCl test	–	–	–	–	–	–	–	–	–
Coumarins	Ferric chloride test	+	+	+	–	–	–	–	–	–
Cardiac glycosides	Kellerkillani test	+	+	+	+	+	+	–	–	+
Terpenoids	Libberman's test	+	+	+	–	–	+	+	+	+
	Salkowski test	+	+	+	+	–	+	+	+	+
Tannin	Potassium dichromate	–	–	–	–	–	–	+	+	–

There were previously published reports on phytochemical compounds of *A. scholaris* leaves identified in various extracts. Alkaloids and steroids were the main constituents of the leaf hexane extracts, whereas alkaloids, terpenoids and saponins were found in the water extract of healthy leaves.

Phenols, alkaloids and saponins were detected in benzene extract (Misra et al., 2011). Methanol extracts showed the presence of alkaloids, coumarins, flavonoids, leucoanthocyanins, reducing sugars, simple phenolics, steroids, saponins and tannins (Khyade et al., 2009). Here in this study we analysed gall and gall leaf along with normal leaf of *A. scholaris.* Water extracts of all samples contain alkaloids, flavonoids, tannins, phenolics, coumarins, cardiac glycosides and steroids but no sample detected the presence of quinones. Gall hexane extracts indicated the presence of more compounds than other two samples. Alkaloids and phenols are only detected in gall hexane extracts. Ethanolic extracts were analyzed and detected the presence of flavonoids and cardiac glycosides in gall only.

Preliminary phytochemical analysis of *F. racemosa*

The photographs showing the results of preliminary phytochemical analysis of *F. racemosa* aqueous extracts, hexane extracts and ethanol extracts of normal leaf, gall leaf and gall are shown in Plate 3.12, Plate 3.13 and Plate 3.14 respectively. Hagers's test of alkaloids gave positive results for all samples in all three extracts but Wagner's test didn't show positive for all hexane extracts. Phenols and cardiac glycosides indicated their presence only in galls of *F. racemosa*. Flavonoids showed positive results for all samples of aqueous extracts and also in ethanolic extracts of galls. Aqueous extracts of galls indicated positive results for saponins and quinones. Terpenoids were present in all samples of aqueous extracts by Libberman's test and in the case of Salkowski test; results were positive for hexane extracts and only gall ethanolic extracts. Except for steroids and coumarins all the other phytochemical constituents showed positive results in gall aqueous and ethanolic extracts. The results of phytochemical analysis of *F. racemosa* are summarized in Table 3.2.



Plate 3.12: Preliminary phytochemical tests of normal leaf (A), gall leaf (B) and gall (C) aqueous extract of *F. racemosa*.



Plate 3.13: Preliminary phytochemical tests of normal leaf (A), gall leaf (B) and gall (C) hexane extract of *F. racemosa*.



Plate 3.14: Preliminary phytochemical tests of normal leaf (A), gall leaf (B) and gall (C) ethanol extract of *F. racemosa*.

Table 3.2: Result of preliminary phytochemical analysis of aqueous, hexane and ethanolic extracts of normal leaves, gall leaves and leaf galls of *Ficus racemosa.*

Compound	Name of test	*F. racemosa*								
		Water			Hexane			Ethanol		
		Normal leaf	Gall leaf	Gall	Normal leaf	Gall leaf	Gall	Normal leaf	Gall leaf	Gall
Alkaloids	Dragendorff's test	–	–	–	–	–	–	–	–	–
	Hager's test	+	+	+	+	+	+	+	+	+
	Wagner's test	+	+	+	–	–	–	+	+	+
Phenolics	Ferric chloride test	–	–	+	–	–	+	–	+	–
Flavonoids	Lead acetate test	+	+	+	–	–	–	–	+	–
	Alkaline reagent test	+	+	+	–	–	–	–	+	–
Saponins	Froth test	–	–	+	–	–	–	–	–	–
Quinones	HCl test	–	–	+	–	–	–	–	–	+
Coumarins	Ferric chloride test	–	–	–	+	+	+	–	–	–
Cardiac glycosides	Kellerkillani test	–	–	+	–	–	+	–	–	+
Terpenoids	Libberman's test	+	+	+	+	–	+	–	–	–
	Salkowski test	–	–	–	+	+	+	–	–	+
Tannin	Potassium dichromate	–	–	–	–	–	–	+	–	+

The previous report on the preliminary phytochemical analysis conducted in the leaves of *F. racemosa* found the presence of triterpenoids, tannins, kaempferol, rutin, arabinose, bergapten, psoralenes, flavonoids, ficusin, coumarin, phenolic glycosides and saponins (Ahmed & Urooj, 2010). Savitha and colleagues reported that saponins, flavonoids,

terpenoids, reducing sugars and tannins showed presence in both normal leaves and gall leaves. The presence of coumarins and quinones were found only in the galled leaves (Bagyalakshmi et al., 2019; Savitha et al., 2013). Here in the present study, phenolics and cardiac glycosides indicated their presence only in gall extracts. Quinones and saponis were found in water extracts of gall only. All aqueous extract samples contained steroids and terpenoids.

Quantitative phytochemical analysis

GC-MS analysis of *A. scholaris*

The GC-MS analysis report of methanolic extracts of normal leaves of *A. scholaris* found 54 peaks, gall leaves with 33 peaks and the gall having 63 peaks. Plate 3.15 displays the resulted chromatogram of GC-MS analysis conducted in normal leaf; Plate 3.17 represents the chromatogram of gall leaf whereas Plate 3.17 displays the chromatogram of gall. Compounds in *A. scholaris* with an area percentage of higher than 1 are listed in Table 3.3, along with their chemical formula and area percentage in different samples.

From the phytochemical compounds discovered in *A. scholaris*, 4 were unique to gall, 7 compounds were found unique to gall leaves and 6 were unique to normal leaves. 6 compounds were identified from all the analysed samples. There are 9 compounds detected as common to normal leaf and gall, but they were represented with different area percentages in different samples. Betulin is found in gall leaves and gall and 9, 19-cyclolanostan-3-ol, 24-methylene, (3.beta.) is the only compound common to normal leaf and gall leaf. By analyzing the data it has been found that from normal leaf to gall, some of the chemicals showed an increase in the area percentage.

Khyade and colleagues discovered more than 70 different types of alkaloids from different parts of *A. scholaris* such as root, stem bark, leaves, fruit and flower. The majority of alkaloids are found to occur in the leaves (Khyade et al., 2014). GC-MS analysis of *A. scholaris* was done by Goyal and Shinde in the year 2020 discovered that the methanol and hexane extracts possess 35 and 29 compounds respectively. The compounds like flavonoids, tannins, alkaloid, phenols, coumarins, ketones and terpenoids were extracted only in

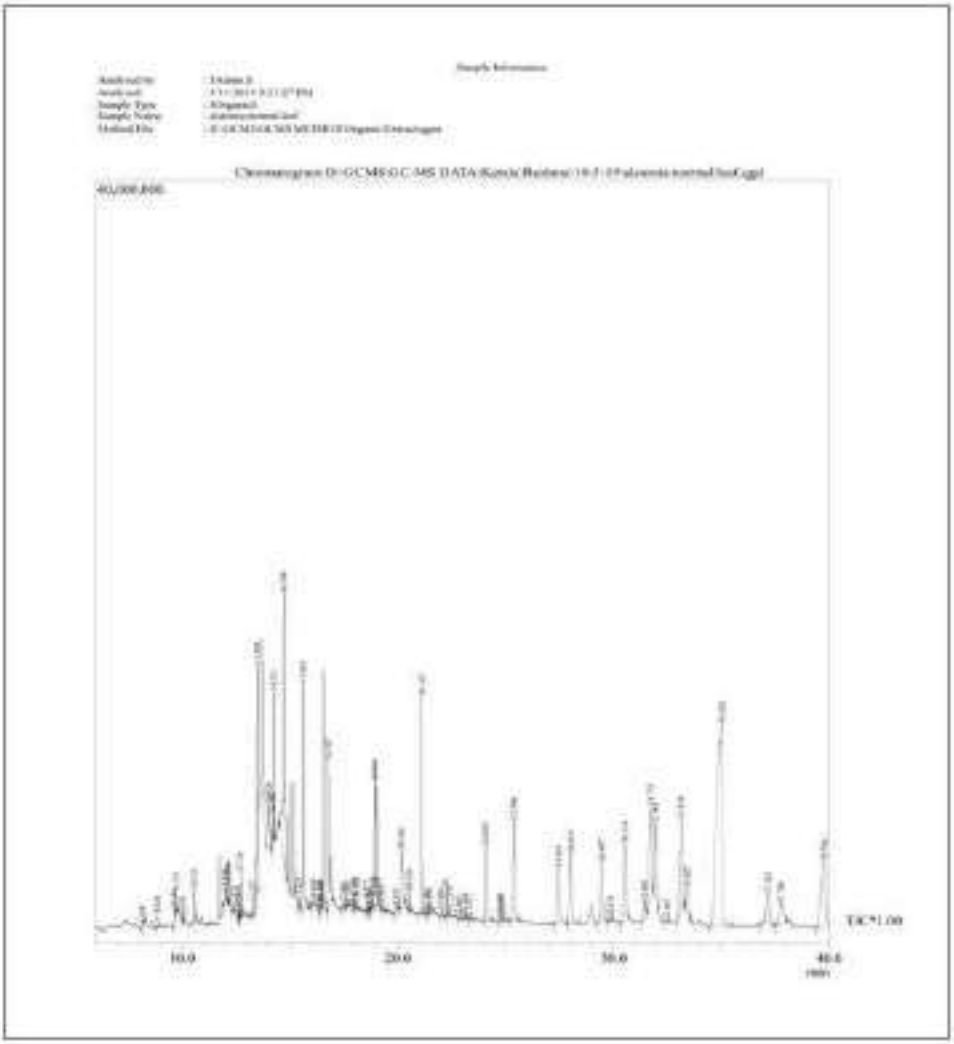


Plate 3.15: GC-MS chromatogram obtained through GC-MS analysis of the methanolic extract of *A. scholaris* normal leaves (AS-NL). Each peak represents a compound eluted at a specific retention time, indicating the presence of different phytoconstituents in the extract of *A. scholaris* normal leaf extracts, showing different peaks.

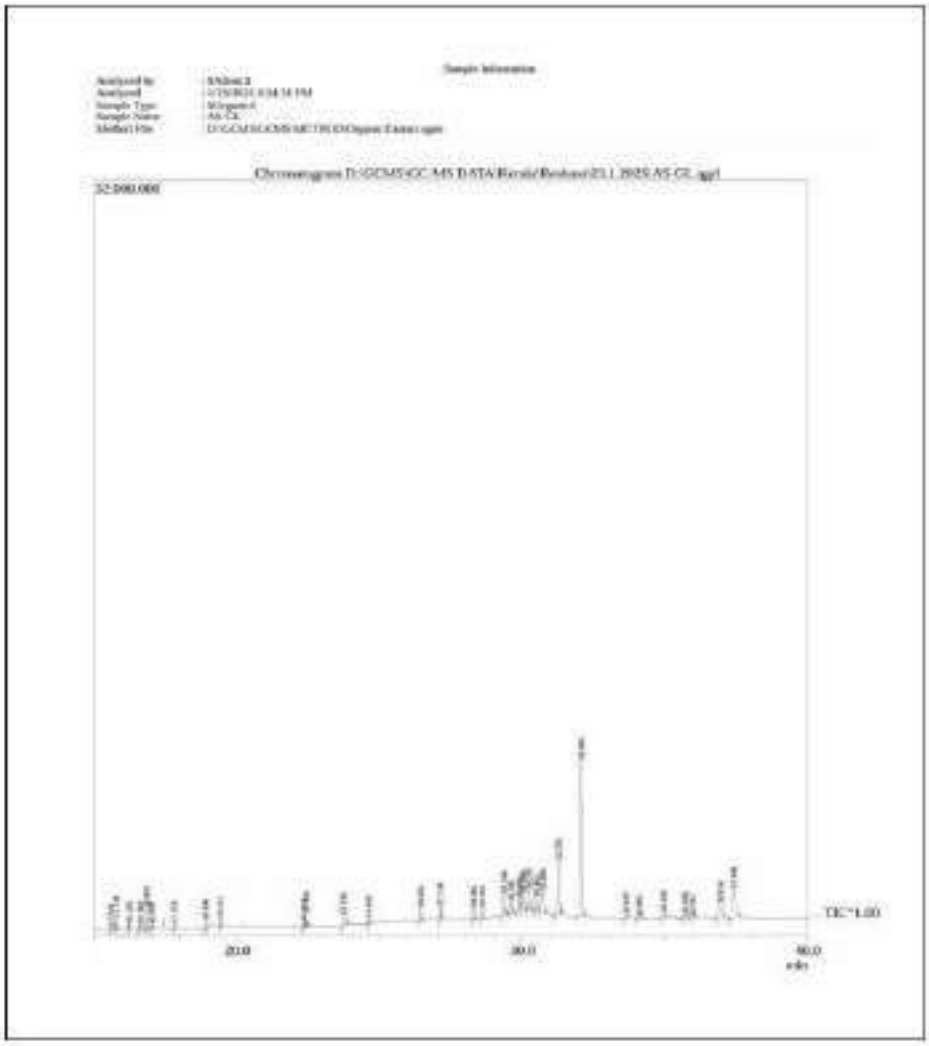


Plate 3.16: GC-MS profile of methanolic extract of gall leaf of *A. scholaris* (AS-GL). Each peak in this chromatogram corresponds to a bioactive compound separated based on its retention time, reflecting the metabolic diversity present in the sample.

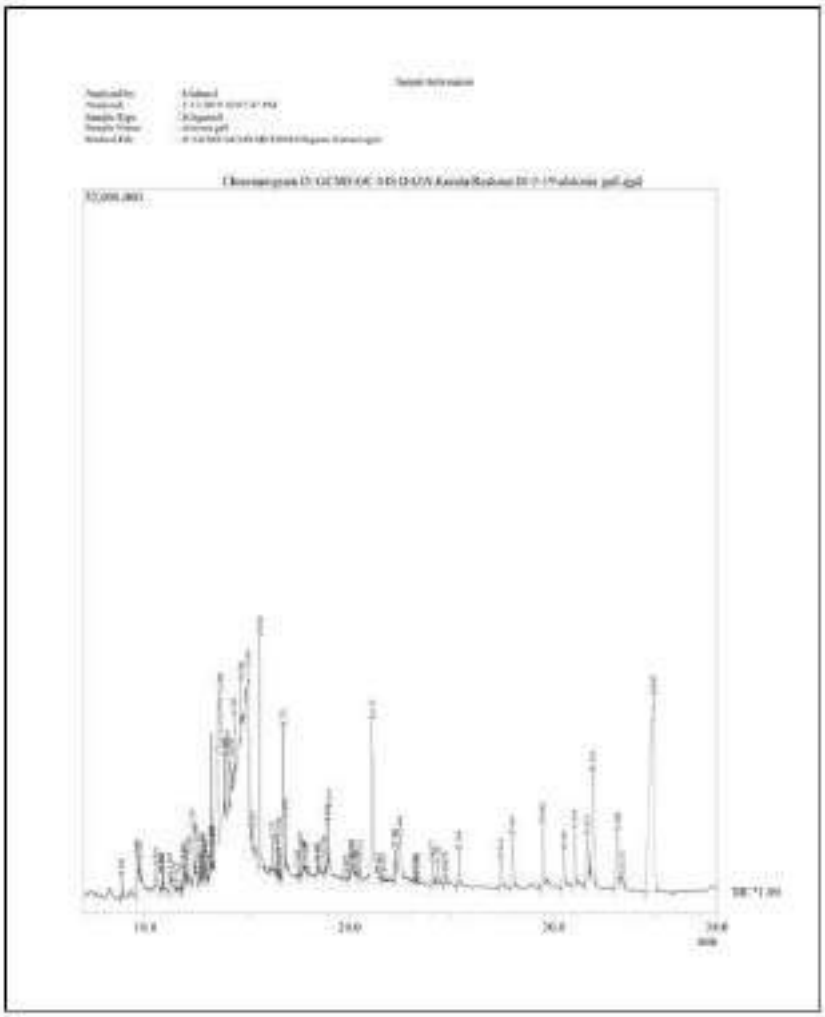


Plate 3.17: GC-MS chromatogram of *A. scholaris* gall (AS-GA) illustrates the separation of volatile constituents present in the methanolic extract of gall of *A. scholaris*. Each peak represents a distinct compound identified based on its retention time, highlighting the phytochemical complexity of the gall extract.

methanolic leaf extract whereas phenylethyl icosanoate, linoleic acid and unsaturated hydrocarbons from hexane extract (Goyal & Shinde, 2020).

Table 3.3: Results of quantitative phytochemical analysis on methanolic extracts of normal leaves, gall leaves and leaf galls of *A. scholaris* by GC-MS analysis.

Sl. No.	Name of compound	Molecular formula	Area percentage		
			Normal leaf	Gall leaf	Gall
1.	2-Furanmethanol,Tetrahydro-.Alpha.,.Alpha., 5 Trimethyl-5-(4-Methyl-3-Cyclohexen-1-Yl)	$C_{15}H_{26}O$	3.99	-	-
2.	2H-Pyran-3-Ol, Tetrahydro-2,2,6-Trimethyl-6-(4-Methyl-3-Cyclohexen-1-Yl)-, [3S-[3alpha,6alpha(R*)]]	$C_{15}H_{26}O$	1.19	-	-
3.	Mome Inositol	$C_7H_{14}O_6$	1.5	-	4.84
4.	Neophytadiene	$C_{20}H_{38}$	5.69	-	0.48
5.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	3.97	-	4.43
6.	9,12-Octadecadienoic Acid, Methyl Ester	$C_{19}H_{34}O_2$	2.49	-	-
7.	(Z,Z)-6,9-Cis-3,4-Epoxy-Nonadecadiene	$C_{19}H_{34}O$	3.81	-	-
8.	Hexadecanoic Acid, 2-Hydroxy- 1-(Hydroxymethyl)Ethyl Ester	$C_{19}H_{38}O_4$	2.79	-	0.71
9.	9-Octadecenoicacid (Z)-, 2,3-Dihydroxypropyl Ester	$C_{21}H_{40}O_4$	2.45	-	0.47
10.	Squalene	$C_{30}H_{50}$	3.24	1.49	4.61
11.	gamma-Tocopherol	$C_{28}H_{48}O_2$	1.97	1.35	0.7
12.	Vitamin E	$C_{29}H_{50}O_2$	3.27	1.39	1.11
13.	Ergost-5-En-3-Ol, (3.Beta.,24r)	$C_{28}H_{48}O$	2.51	1.8	1.46

14.	Stigmasterol	$C_{29}H_{48}O$	2.79	1.48	2.43
15.	Gamma-Sitosterol	$C_{29}H_{50}O$	2.73	-	3.16
16.	Cycloeucalenyl Acetate	$C_{32}H_{52}O_2$	3.46	-	-
17.	9,19-Cyclolanost-24-En-3-Ol, (3.Beta.)	$C_{30}H_{50}O$	4.53	-	2.22
18.	Lupeol	$C_{30}H_{50}O$	2.66	-	6.27
19.	Olean-12-En-3-Ol, Acetate, (3.Beta.)	$C_{32}H_{52}O_2$	4.51	1.76	2.95
20.	9,19-Cyclolanostan-3-ol,24-methylene-, (3.beta.)	$C_{31}H_{52}O$	1.12	2.54	-
21.	Lup-20(29)-En-3-Ol, Acetate, (3.Beta.)	$C_{32}H_{52}O_2$	23.13	-	23.51
22.	Phytyl Palmitate	$C_{36}H_{70}O_2$	1.57	-	-
23.	Catechol	$C_6H_6O_2$	-	-	1.13
24.	1,3,4,5-Tetrahydroxy-Cyclohexanecarboxylic Acid	$C_{15}H_{24}O$	-	-	11.12
25.	9-Octadecenoic Acid (Z)	$C_{18}H_{34}O_2$	-	-	1.05
26.	Betulin	$C_{30}H_{50}O_2$	-	10.03	3
27.	Lup-20(29)-en-3-one	$C_{30}H_{48}O$	-	-	2.91
28.	Oleoyl chloride	$C_{18}H_{33}ClO$	-	1.68	-
29.	Stigmast-5-en-3-ol,(3. beta.)	$C_{29}H_{50}O$	-	3.57	-
30.	.alpha.-Amyrin	$C_{30}H_{50}O$	-	2.57	-
31.	24-Norursa-3,12-diene	$C_{29}H_{46}$	-	4.14	-
32.	Ursolic aldehyde	$C_{30}H_{48}O_2$	-	1.28	-
33.	Uvaol	$C_{30}H_{50}O_2$	-	1.02	-
34.	Acetamide, 2-(diethylamino)-n-(2,6- dimethyl)	$C_{12}H_{18}N_2O$	-	1.55	-

GC-MS analysis of *F. racemosa*

When *F. racemosa* was analysed using GC-MS and found 53, 24 and 57 peaks in normal leaves, gall leaves and galls respectively. Plate 3.18 displays the resulting

chromatogram for normal leaf of *F. racemosa*, Plate 3.19 represents the chromatogram of gall leaf, while Plate 3.20 displays the chromatogram for leaf gall. A list of the phytochemicals in *F. racemosa* with an area percentage of above 1 listed with molecular formula and area percentage in different samples represented in Table 3.4.

The quantitative examination of phytochemicals from *F. racemosa* normal leaves, gall leaves and galls revealed variation in their composition across the tissues examined. There were 4 compounds unique to normal leaves, 8 compounds shared with galls and no compounds shared with gall leaves. Galls have 4 unique compounds and gall leaves have 10 unique compounds. No compounds found to be shared between gall leaf and gall. Despite being reported from all three samples, N-hexadecanoic acid, olean-12-En-3-Ol, acetate, (3.Beta.) and lup-20(29)-en-3-ol, acetate, (3.Beta.) were found to vary in their area percentage.

Ushir and colleagues reported the qualitative phytochemical analysis of *F. racemosa* leaf galls and revealed the presence of secondary metabolites such as alkaloids, flavonoids, steroids, tannins and terpenoids (Ushir et al., 2015). The gall portions extracted in methanol detected the presence of terpenoids. The chloroform extract of the galled portion indicated the presence of glycosides (Savitha et al., 2013).

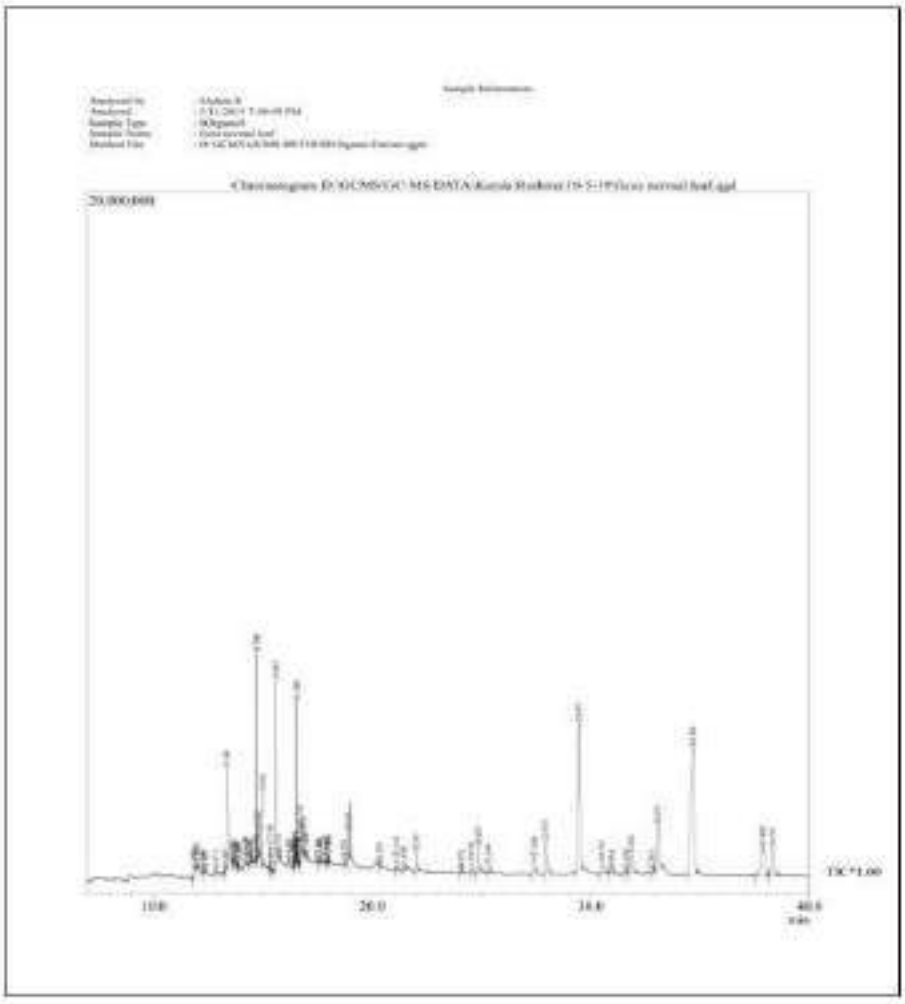


Plate 3.18: GC-MS chromatogram obtained through GC-MS analysis of the methanolic extract of normal leaf of *F. racemosa* (FR-NL). Each peak represents a compound eluted at a specific retention time, indicating the presence of different phytoconstituents in the extract.

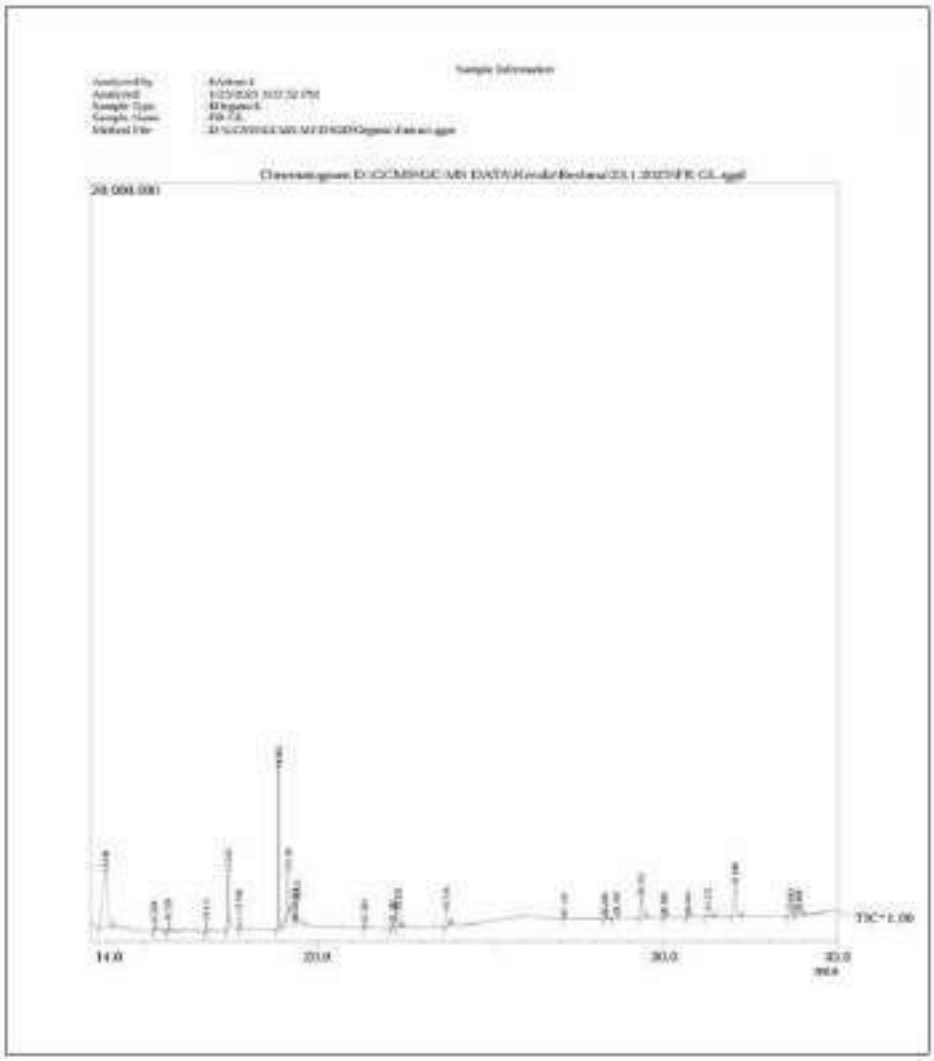


Plate 3.19: GC-MS profile of *F. racemosa* gall leaf (FR-GL) extract. Each peak in this chromatogram corresponds to a bioactive compound separated based on its retention time, reflecting the metabolic diversity present in the sample.

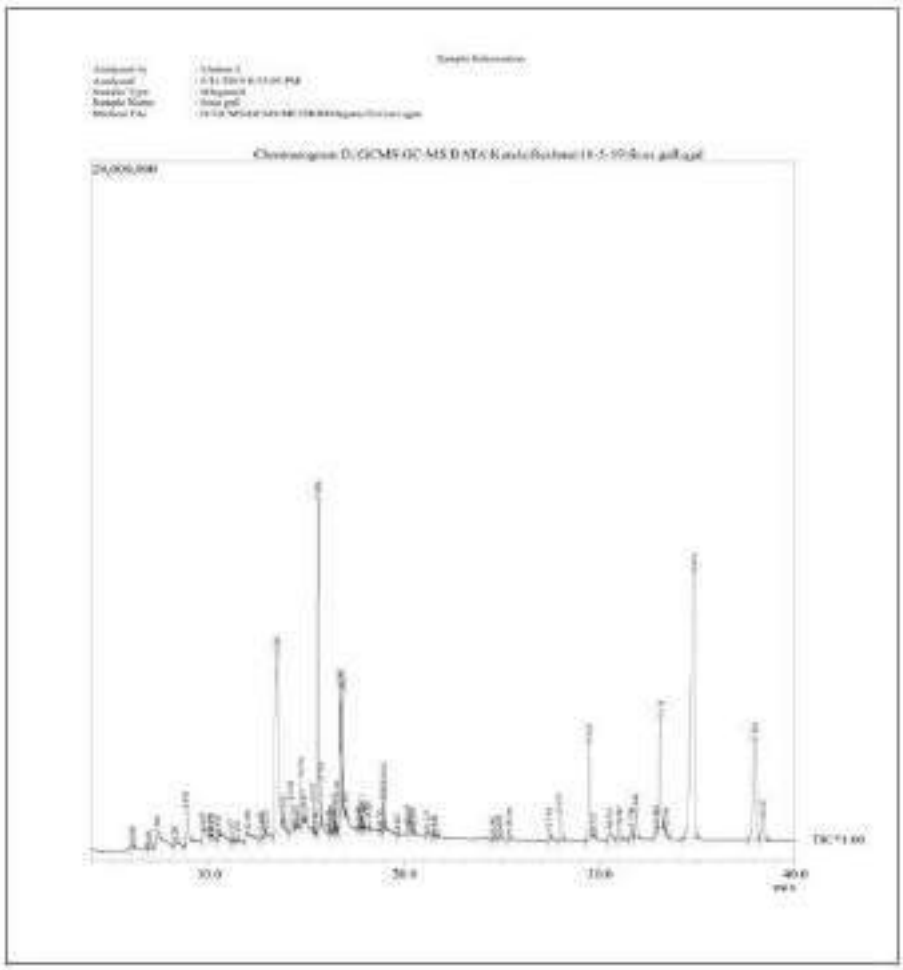


Plate 3.20: GC-MS chromatogram *F. racemosa* gall (FR-GA) extract illustrates the separation of volatile constituents present in the methanolic extract of *F. racemosa*. Each peak represents a distinct compound identified based on its retention time, highlighting the phytochemical complexity of the gall extract.

Table 3.4: Results of quantitative phytochemical analysis on methanolic extracts of normal leaves, gall leaves and leaf galls of *F. racemosa.*

Sl. No.	Name of compound	Molecular formula	Area percentage		
			Normal leaf	Gall leaf	Gall
1.	1,3,4,5-Tetrahydroxy-Cyclohexanecarboxylic Acid	$C_7H_{12}O_6$	10.45	-	15.5
2.	Neophytadiene	$C_{20}H_{38}$	4.02	-	0.61
3.	N-Hexadecanoic Acid	$C_{16}H_{32}O_2$	6.79	6.87	5.98
4.	2-Hexadecen-1-Ol, 3,7,11,15-Tetramethyl-, (R-(R*,R*-(E)))	$C_{20}H_{40}O$	3.27	-	-
5.	9,12-Octadecadienoic Acid (Z,Z)	$C_{18}H_{32}O_2$	1.77	-	-
6.	1-Hexacosanol	$C_{26}H_{54}O$	1.01	-	-
7.	1-Heptacosanol	$C_{27}H_{56}O$	1.97	-	-
8.	Ergost-5-En-3-Ol, (3.Beta.,24r)	$C_{28}H_{48}O$	1.64	-	0.76
9.	Stigmasterol	$C_{29}H_{48}O$	3.06	-	1.36
10.	Gamma.-Sitosterol	$C_{29}H_{50}O$	14.77	-	5.18
11.	Methyl Commate D	$C_{31}H_{50}O_4$	2.37	-	1.51
12.	Olean-12-En-3-Ol, Acetate, (3.Beta.)	$C_{32}H_{52}O_2$	4.79	1.87	7.02
13.	Methyl Commate B	$C_{31}H_{50}O_3$	21.62	-	0.89
14.	Lup-20(29)-En-3-Ol, Acetate, (3.Beta.)	$C_{32}H_{52}O_2$	4.99	8.49	8.33
15.	Betulin	$C_{30}H_{50}O_2$	4.04	-	1.8
16.	1,2,3-Propanetriol	$C_3H_8O_3$	-	-	1.09
17.	4H-Pyran-4-One, 2,3- Dihydro -3,5-Dihydroxy-6-Methyl	$C_6H_8O_4$	-	-	2.05

18.	(Z,Z)-6,9-Cis-3,4-Epoxy-Nonadecadiene	$C_{19}H_{34}O$	-	-	4.23
19.	Ethyl (9z,12z)-9,12- Octadecadienoate	$C_{20}H_{36}O_2$	-	-	1.92
20.	Methyl beta-D-glucopyranoside	$C_7H_{14}O_6$	-	34.21	-
21.	Puchidiol	$C_{13}H_{20}O_2$	-	1.65	-
22.	Phytol	$C_{20}H_{40}O$	-	17.06	-
23.	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	-	1.41	-
24.	cis-9-Hexadecenal	$C_{16}H_{30}O$	-	6.02	-
25.	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)	$C_{20}H_{34}O_2$	-	1.68	-
26.	Hexadecanoic acid, 2-hydroxy -1-(hydroxy methyl) ethyl ester (2-Palmitoylglycerol)	$C_{19}H_{38}O_4$	-	3.54	-
27.	Oleoyl chloride	$C_{18}H_{33}ClO$	-	4.78	-
28.	Stigmast-5-en-3-ol,(3. beta.)	$C_{29}H_{50}O$	-	4.54	-
29.	Faradiol	$C_{30}H_{50}O_2$	-	1.25	-

Both normal leaf and gall of *A. scholaris* showed a higher area percentage for the triterpenoid lup-20(29)-en-3-ol, acetate, (3. beta.), which has an unidentified biological action. Betulin showed the highest area percentage in gall leaf of *A. scholaris*, which has reported antiviral, analgesic, anti-flu, cytotoxic, anti-inflammatory and antineoplastic activities (Bębenek et al., 2019; Kim et al., 2023). The substance with the largest area percentage in *F. racemosa* normal leaf is methyl commate B, which is known to have antibacterial and anti-inflammatory properties (Arora et al., 2017; Motlhanka et al., 2010) and in gall- 1,3,4,5-tetrahydroxy- cyclohexanecarboxylic acid, which has antimicrobial, anti-inflammatory, antioxidant and analgesic activities (Mujeeb et al., 2014). This compound has the second-highest area percentage in the gall of *A. scholaris.* Both plant galls and leaves contain N-

hexadecanoic acid, which possesses cytotoxic, insecticidal and nematicide qualities (Abubakar & Majinda, 2016; Tyagi & Agarwal, 2017).

Olean-12-en-3-ol, acetate, (3.beta.) was reported from all the 3 samples of both plants, which have antidyslipidemic activity (Maurya et al., 2012). It was discovered that Stigmasterol (found in all samples except the gall leaves of *F. racemosa*), has cytotoxic activity (Padmashree et al., 2018; Singh & Chaturvedi, 2019; Sudha et al., 2013; Tyagi & Agarwal, 2017). The 9-octadecenoic acid (Z) found in *A. scholaris* gall has been shown to have insectifuge irritant effect (Kim et al., 2023; Selvan & Velavan, 2015). 1-heptacosanol, which exhibits nematicidal activity, is found in the normal leaves of *F. racemosa* (Begum et al., 2016; Raman et al., 2012). Most of the compounds were discovered to possess anti-inflammatory, antibacterial and antioxidative qualities. Table 3.5 shows the biological activity of compounds having an area greater than 1% in normal leaves, gall leaves and galls of both plants.

Table 3.5: List of compounds identified from *A. scholaris* and *F. racemosa* with their reported biological activities.

Sl. No.	Name of compounds	Reported bioactivities
1.	2-Furanmethanol, Tetrahydro-.Alpha., .Alpha., 5-Trimethyl -5- (4- Methyl -3-Cyclohexen -1-Yl)	Anti-hyperalgesic and anti-edematous activities (Tomić et al., 2014).
2.	Mome Inositol	Anti-cirrhotic, anti-neuropathic, cholesterolytic and lipotropic activities (Arora & Saini, 2017).
3.	2H-Pyran-3-Ol, Tetrahydro -2,2,6- Trimethyl-6-(4-Methyl-3-Cyclohexen-1-Yl) [3S-[3alpha, 6alpha (R*)]]	Antifungal activities (Tolouee et al., 2010).
4.	Neophytadiene	Analgesic, antipyretic, anti-inflammatory, antimicrobial and antioxidant activities (Kim et al., 2023; Singh & Chaturvedi,

		2019).
5.	N-Hexadecanoic Acid	Antioxidant, hypocholesterolemic, pesticide, nematicide, antiandrogenic, hemolytic, 5-Alpha reductase inhibitor, antipsychotic, antibacterial, antifungal and cytotoxic activities (Abubakar & Majinda, 2016; Tyagi & Agarwal, 2017).
6.	9,12-Octadecadienoic Acid, Methyl Ester	Antifungal and antioxidant activity (Pinto et al., 2017).
7.	Hexadecanoic Acid, 2-Hydroxy-1-(Hydroxymethyl) Ethyl Este	Hemolytic and antioxidant activity (Arora et al., 2017; Tyagi & Agarwal, 2017).
8.	9-Octadecenoic Acid (Z)-, 2,3- Dihydroxypropyl Ester	Antioxidant and anti- inflammatory activities (Shaheed et al., 2019).
9.	Squalene	Antibacterial, antioxidant, anti-tumour, immunostimulant, lipoxygenase-inhibitor and chemo-preventive activity (Padmashree et al., 2018; Singh & Chaturvedi, 2019; Sudha et al., 2013).
10.	Gamma.-Tocopherol	Antioxidant, cardioprotective, anticancer, anti-inflammatory and hypocholesterolemic activities (Padmashree et al., 2018; Rahim et al., 2017).
11.	Vitamin E	Antioxidant, cytoprotective, anti-aging, analgesic, antidiabetic, anti-dermatitis, antileukemia, anticancer, vasodilator, hepatoprotective, hypocholesterolemic, anti-bronchitis, anti-alzheimer's, immunostimulant, anti-inflammatory, antiulcerogenic and antispasmodic activities (Arora et al., 2017; Padmashree et al., 2018; Singh & Chaturvedi, 2019).
12.	Ergost-5-En-3-Ol, (3. Beta.,24r)	Antimicrobial, anticarcinogenic, antiangiogenic, hypocholesterolemic and antioxidant activities (Arora et al., 2017).
13.	Stigmasterol	Anti-angiogenic, anticancer, anti-inflammatory, antioxidant, antidiabetic, cytotoxic and antimicrobial activities (Padmashree et al., 2018; Singh & Chaturvedi, 2019; Sudha et al., 2013; Tyag & Agarwal, 2017).
14.	Gamma.-Sitosterol	Antioxidant, antimicrobial, antidiabetic, antiasthma, diuretic, hypolipidemic and prophylactic activities (Padmashree et al., 2018; Singh & Chaturvedi, 2019; Sudha et al., 2013).
15.	Cycloeucalenyl Acetate	Antibacterial and antifungal activities (Zubair et al., 2017).

16.	9,19-Cyclolanost-24-En-3-Ol, (3.Beta.)	Antibacterial, anticancer, antidiabetics and pesticidal activity (Naidoo et al., 2023).
17.	Lupeol	Cytotoxic and anti-inflammatory activities (Padmashree et al., 2018; Singh & Chaturvedi, 2019; Sudha et al., 2013).
18.	Olean-12-En-3-Ol, Acetate, (3. Beta.)	Antidyslipidemic activity (Maurya et al., 2012).
19.	9,19-Cyclolanostan-3-Ol, 24-Methylene-, (3. Beta.)-	Antibacterial activity and anti-HIV compound used to prevent the HIV virus (Arora et al., 2017).
20.	Lup-20(29)-En-3-Ol, Acetate, (3.Beta.)	Data deficient.
21.	Phytyl Palmitate	Antimicrobial and cytotoxic activity (Damasceno et al., 2019).
22.	Catechol	Antimicrobial, antioxidant, anti-inflammatory and analgesic activities (Juch & Rüedi, 1997; Kocaçalışkan et al., 2006).
23.	1,3,4,5-Tetrahydroxy-Cyclohexanecarboxylic acid	Antimicrobial, anti-inflammatory, antioxidant and analgesic activities (Mujeeb et al., 2014).
24.	9-Octadecenoic Acid (Z)	Antibacterial, antioxidant, allergenic, anemia genetic, antiandrogenic, anti-inflammatory, cancer preventive, choleretic, hypocholesterolemic and insectifuge irritant activities (Kim et al., 2023; Selvan & Velavan, 2015).
25.	Cis-9-Hexadecenal	Antimicrobial activity (Mujeeb et al., 2014).
26.	Betulin	Antiviral, analgesic, anti-flu, cytotoxic, anti-inflammatory and antineoplastic activities (Bębenek et al., 2019; Kim et al., 2023).
27.	Lup-20(29)-En-3-One	Trypanocidal activity (Polanco-Hernández et al., 2012).
28.	2-Hexadecen-1-Ol,3,7,11,15 Tetramethyl-,(R-(R*, R*-(E)))	Antioxidant, anti-inflammatory, anticancer neuroprotective, antinociceptive, antioxidant, antimicrobial and diuretic activities (Rahim et al., 2017).
29.	9,12-Octadecadienoic Acid (Z, Z)	Antioxidant, antiproliferative, anti-inflammatory and antiarthriti activities (Padmashree et al., 2018; Singh & Chaturvedi, 2019).
30.	1-Hexacosanol	Antileishmanial activity (Padmashree et al., 2018).
31.	1-Heptacosanol	Nematicidal, anticancer, antioxidant and antimicrobial activities

		(Begum et al., 2016; Raman et al., 2012).
32.	Methyl Commate D	Antimicrobial and anti-inflammatory activities (Motlhanka et al. 2010).
33.	Methyl Commate B	Antimicrobial and anti-inflammatory activities (Arora et al., 2017; Motlhanka et al., 2010).
34.	4H-Pyran-4-One, 2,3-Dihydro-3,5-Dihydroxy- 6-Methyl	Antimicrobial, anti-inflammatory, antiproliferative, anticancer and antioxidant activities (Mujeeb et al., 2014; Yu et al., 2013).
35.	(Z,Z)-6,9-Cis-3,4-Epoxy-Nonadecadiene	Antibacterial activity (Kumar & Sati, 2021).
36.	Ethyl (9z,12z)- 9,12-Octadecadienoate	Anti-inflammatory activity (Kim et al., 2023).
37.	Methyl beta-D-glucopyranoside	Unknown activity.
38.	Puchidiol	Antioxidants, antiviral and anti-inflammatory activity (Kavitha, 2021; Lina & Eman, 2022; Zacharia et al., 2024).
39.	Phytol	Antimicrobial, anticancer, antiinflammatory antioxidant, anti-diabetic, anti-diuretic, neuroprotective, antidepressant, anticonvulsant and antiviral activity (Kavitha, 2021; Zacharia et al., 2024).
40.	Hexadecanoic acid ethyl ester	Antibacterial, antioxidant, hemolytic, hypocholesterolemic, nematicide and anti-androgenic activities (Shaaban et al., 2021; Tyagi & Agarwal, 2017).
41.	cis-9-Hexadecenal	Antifungal, cytotoxic and antibacterial activity (Hoda et al., 2019; Lykholat et al., 2021).
42.	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)	Anti-melanogenesis, anti-inflammatory, antioxidant and antimicrobial properties (Huh et al., 2010).
43.	Hexadecanoicacid, 2-hydroxy -1-(hydroxymethyl)ethyl ester	Sugar-phosphatase inhibitor, lipid metabolism regulator, antiinfective, anti-inflammatory, histamine release inhibitor and antiprotozoal activity (Adnan et al., 2019).
44.	Oleoyl chloride	Unknown activity.
45.	Stigmast- 5-en-3-ol,(3. beta.)	Methyltransferase inhibitor, an anti cholesteremic drug and an antioxidant (Kim et al., 2023).
46.	Faradiol	Antiinflammatory activity (Reznicek & Zitterl-Eglseer, 2003).

47.	Alpha.-Amyrin	Anti-inflammatory and antioxidant agent (Melo et al., 2010).
48.	24-Norursa-3,12-diene	Antibacterial and antifungal activity (Ullah et al., 2022).
49.	Ursolic aldehyde	Unknown activity.
50.	Uvaol	Anti-proliferative and pro-apoptotic effect (Bonel-Pérez et al., 2020).
51.	Acetamide, 2-(diethylamino) -N-(2,6-dimethyl)	Unknown activity.

Quantitative phytochemical analysis using OHR-LC-MS

OHR-LC-MS analysis was performed on normal leaves, gall leaves and gall ethanolic extracts of plants, yielding different phytochemical compounds, fatty acids and essential amino acids from various tissues. The data from each sample was evaluated and a phytochemical profile was developed for each sample. The phytochemical makeup of normal leaf samples was compared to gall leaf and gall information in order to detect modifications in plant tissue associated with infection by gall causer. The composition of phytochemical compounds in different samples of both plants, they are: *A. scholaris*-normal leaf (AS-NL), *A. scholaris*-gall leaf (AS-GL), *A. scholaris*-gall (AS-GA), *F. racemosa*-normal leaves (FR-NL), *F. racemosa* -gall leaves (FR-GL) and *F. racemosa*- gall (FR-GA) were represented graphically in Plate 3.21.

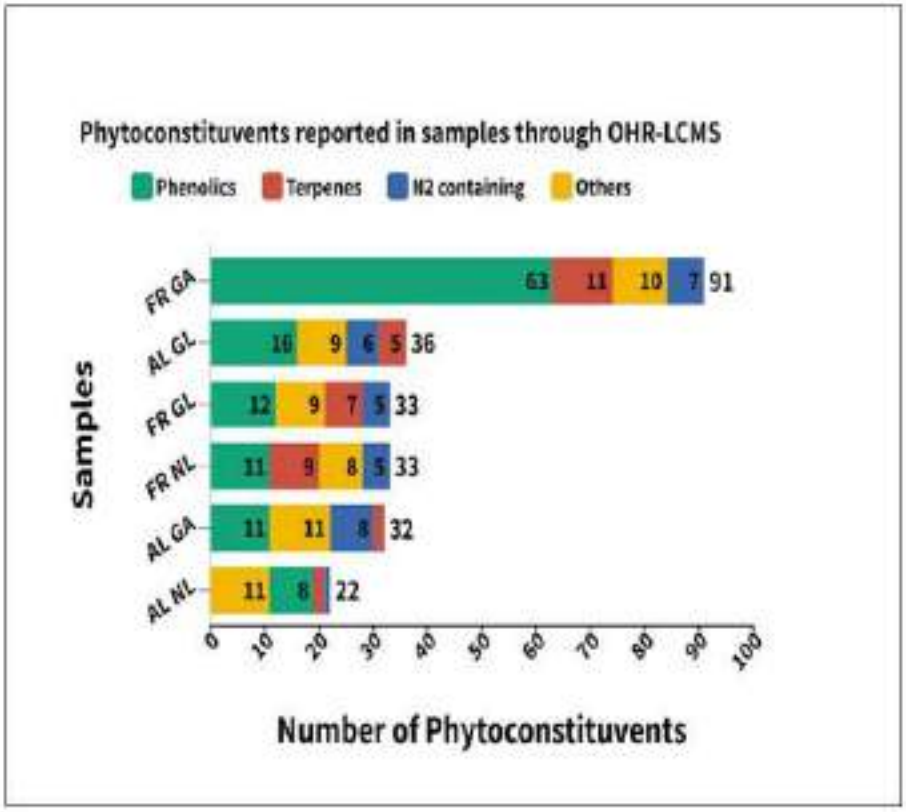


Plate 3.21: Bar diagram representing the number of different phytochemical compound classes identified in normal leaves, gall leaves and leaf galls of *A. scholaris* and *F. racemosa*.

Quantitative phytochemical analysis of *A. scholaris*-normal leaf (AS-NL)

The AS-NL ethanolic extracts were analysed using OHR-LC-MS analysis to find out the quantitative phytochemical profile. The analysis expressed the presence of 22 different chemical compounds. Among the compounds 8 were phenolic compounds of different categories, they are; 1- cardiac glycoside, 2 were terpenes, 10 were fatty acids and 1-amino acid. 9 (10)-epoxy-12Z-octadecenoic acids present in AS-NL has a role as chemical mediators of insects. Another compound, chlorogenic acid is an intermediate metabolite in the biosynthesis of lignin. Lignin helps in strengthening tissues. Another fatty acid, 16-hydroxyhexadecanoic acid is a component of the plant cuticle. Cuticles are important plant cell structures that act as barriers limiting water and nutrient loss and protecting plants from radiation and pathogens. The chromatogram of AS-NL is displayed in Plate 3.22: Figure A. Table 3.6 represents the list of compounds detected in AS-NL extracted through OHR-LC-MS with molecular formula, chemical class and reported bioactivities. Plate 3.23 represents a sunburst diagram showing the phenolic compounds present in the AS-NL with their reported bioactivity.

Quantitative phytochemical analysis of *A. scholaris*-gall leaf (AS-GL)

36 distinct chemical compounds have been identified in AS-GL through OHR-LC-MS analysis. Of these, 16 were phenolic compounds (including lignin-1, phenolics-7 and flavonoids-6), 5 were terpenes, 6 were nitrogen-containing compounds, 7 were fatty acids and 1-aminoacid. There were twice as many phenolic chemicals in AS-GL compared to AS-NL. Chlorogenic acid, an intermediate in the production of lignin, was found in gall leaves. Similar to normal leaves, 16-hydroxyhexadecanoic acid, a cuticle component, is also found. Another fatty acid that was found in AS-GL was 12-oxo phytodienoic acid, which is an intermediary in the production of jasmonic acid. The chromatogram of AS-GL is displayed in Plate 3.22: Figure B. Table 3.7 represents the list of compounds detected in AS-GL extracted through OHR-LC-MS with molecular formula, chemical class and reported bioactivities. Plate 3.24 represents a sunburst diagram showing the phenolics compounds present in the AS-GL with their reported bioactivity.

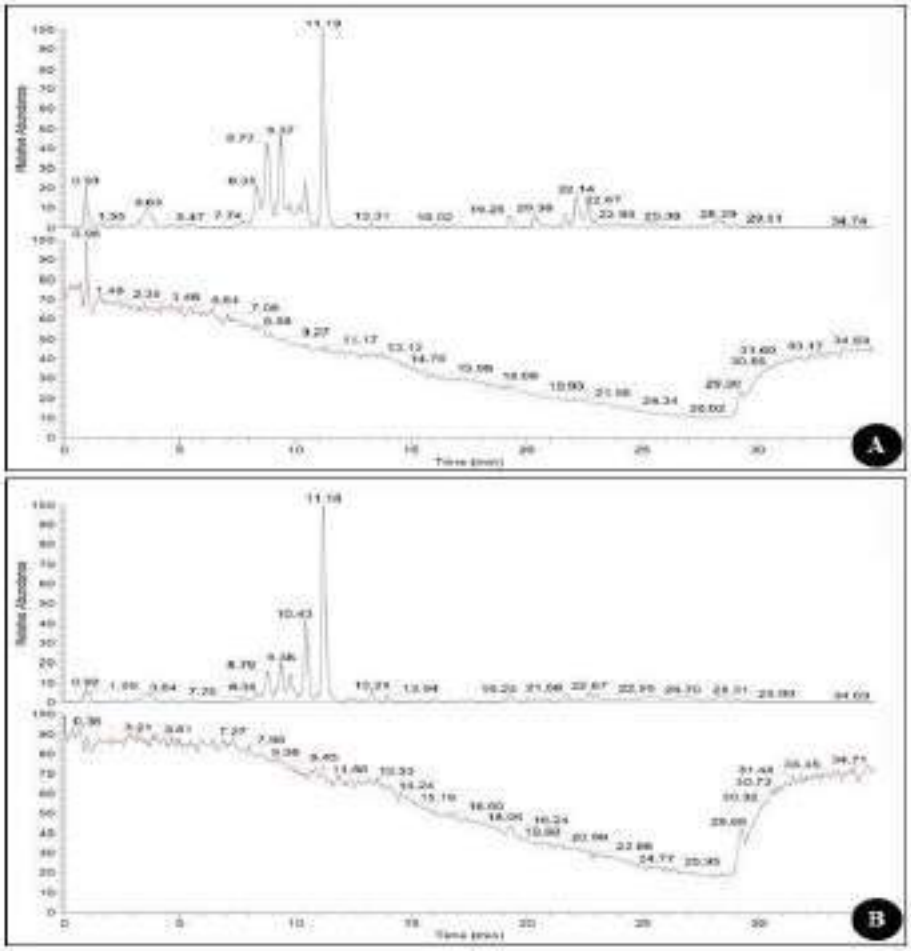


Plate 3.22: Figure A: OHR-LC-MS-Chromatogram of ethanolic extracts of AS-NL presents the metabolic profile of the AS-NL, revealing the presence of several bioactive compounds and Figure B: OHR-LC-MS Chromatogram of ethanolic extracts of AS-GL shows the chemical profile of the gall leaf, indicating the presence of a variety of secondary metabolites based on distinct retention time peaks.

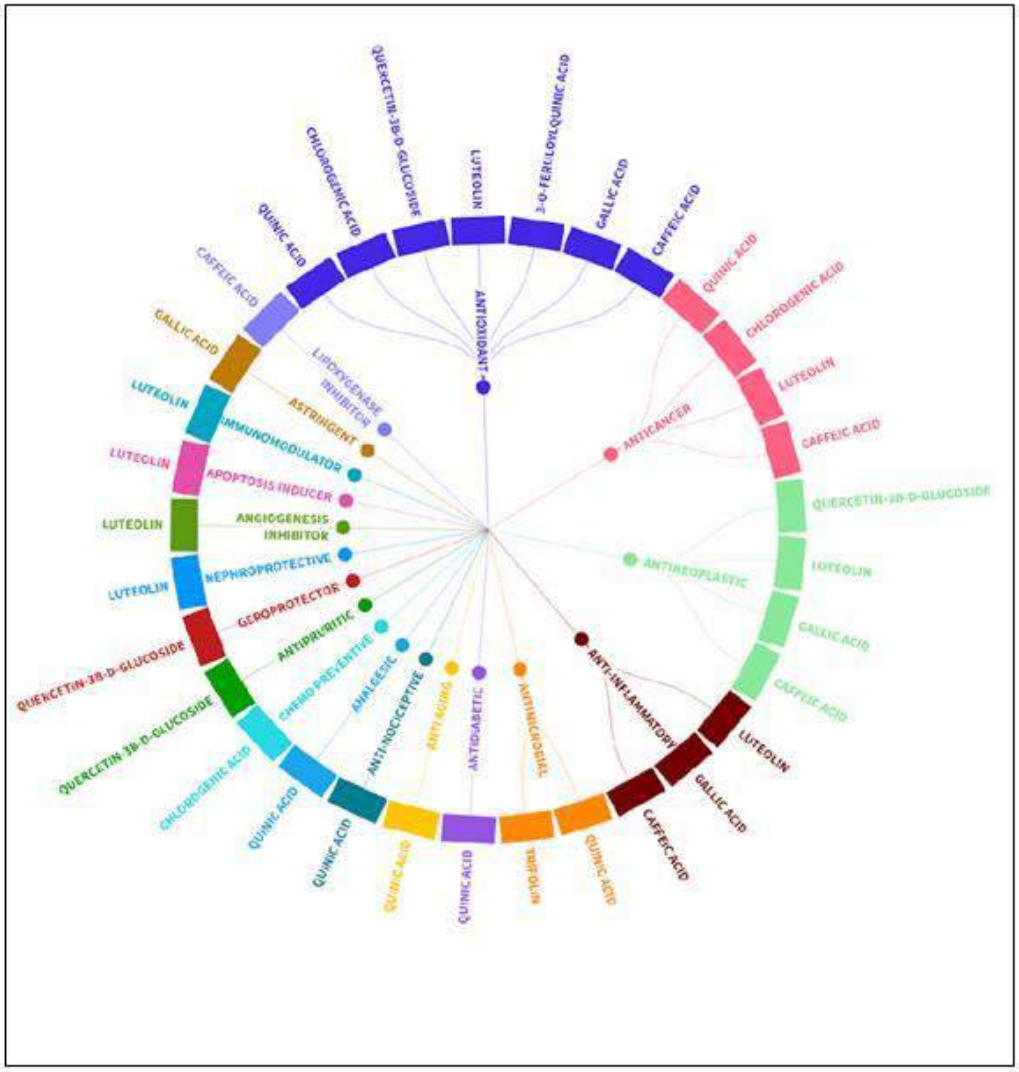


Plate 3.23: The diagram illustrates the diverse pharmacological properties of phenolic constituents present in AS-NL. These activities are represented with lines linking specific compounds to their known bioactivities, highlighting the potential therapeutic value of the identified phytochemicals.

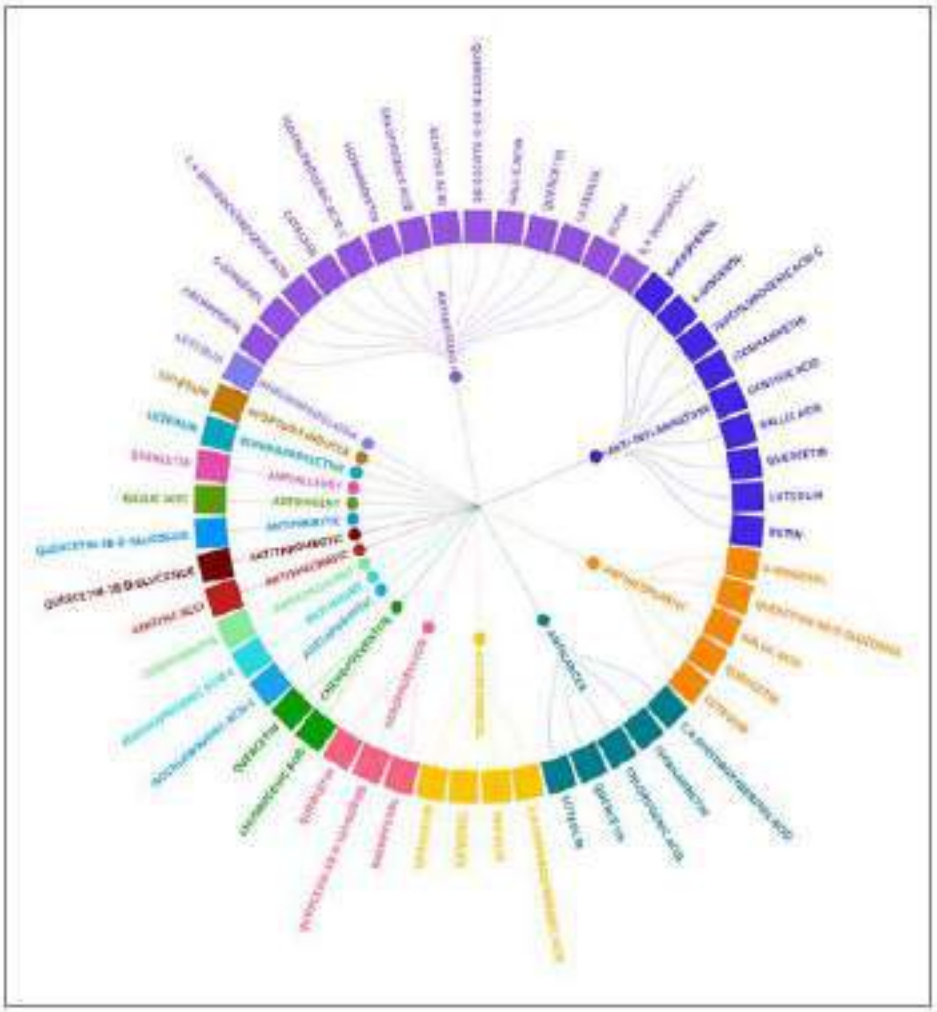


Plate 3.24: Schematic diagram illustrating the bioactivities of phenolic compounds identified in AS-GL. Each line links a specific phenolic compound to its corresponding biological activity.

Quantitative phytochemical analysis of *A. scholaris*-gall (AS-GA)

AS-GA yielded 32 peaks through OHR-LC-MS analysis. Of these chemical compounds 11 were different phenolic compounds, they include phenols, flavonoids and lignins. The gall also indicated the presence of terpenes, nitrogen containing compounds, fatty acids and amino acids. N-acetyldopamine, which is a reactive intermediate in sclerotization, was found in gall. It may act as sclerotizing agent of the insect cuticle (Karlson & Sekeris, 1962). Similar to AS-GL and AS-NL, gall also contained 12-oxo phytodienoic acid, a fatty acid. The chromatogram of gall is shown in Plate 3.25: Figure A. Table 3.8 represents the list of compounds detected in AS-GA through OHR-LC-MS, with molecular formula, chemical class and their bioactivities. Plate 3.26 represents the diagram showing the phenolics compounds present in the AS-GA with their reported bioactivity.

By analysing the results it has been found that 7 compounds were common to normal leaves, gall leaves and galls of *A. scholaris*, they are trifolin, ursolic acid, quercetin-3β-D-glucoside, 12,13- dihydroxy octadec -9-enoic acid, azelaic acid, gallic acid and 18-β-glycyrrhetinic acid. Out of reported chemical compounds 11 each were found exclusive to normal leaves and gall leaves and 9 were unique to galls. About 15 compounds were found to be shared between gall and associated tissues. Only 3 common compounds were present in normal leaves and gall leaves and only 1 compound shared between normal leaves and gall of *A. scholaris*.

Previous works reported lower carbohydrate levels in mature galls. Gall tissue showed no appreciable alterations in its protein and total amino acid composition. Compared to young ungalled leaves, mature galls exhibited higher lipid content. Among them, some lipids may serve as building blocks for the insect moulting hormone, ecdysteroid, while others may give structural role in cellular membranes of insects (Manna et al., 2018; Muhammad et al., 2023). The present work identified the presence of N-acetyldopamine from gall tissue, which is a reactive intermediate in sclerotization of insects (Karlson & Sekeris, 1962). As gall develops phenol production of gall tissues also increases (Biswas et al., 2014; Khurshid et al., 2021). In this study, OHR-LC-MS analysis of normal leaf of *A. scholaris* yielded 8 phenolic compounds belonging to different categories such as flavonoids, phenols, lignin etc. In the case of gall

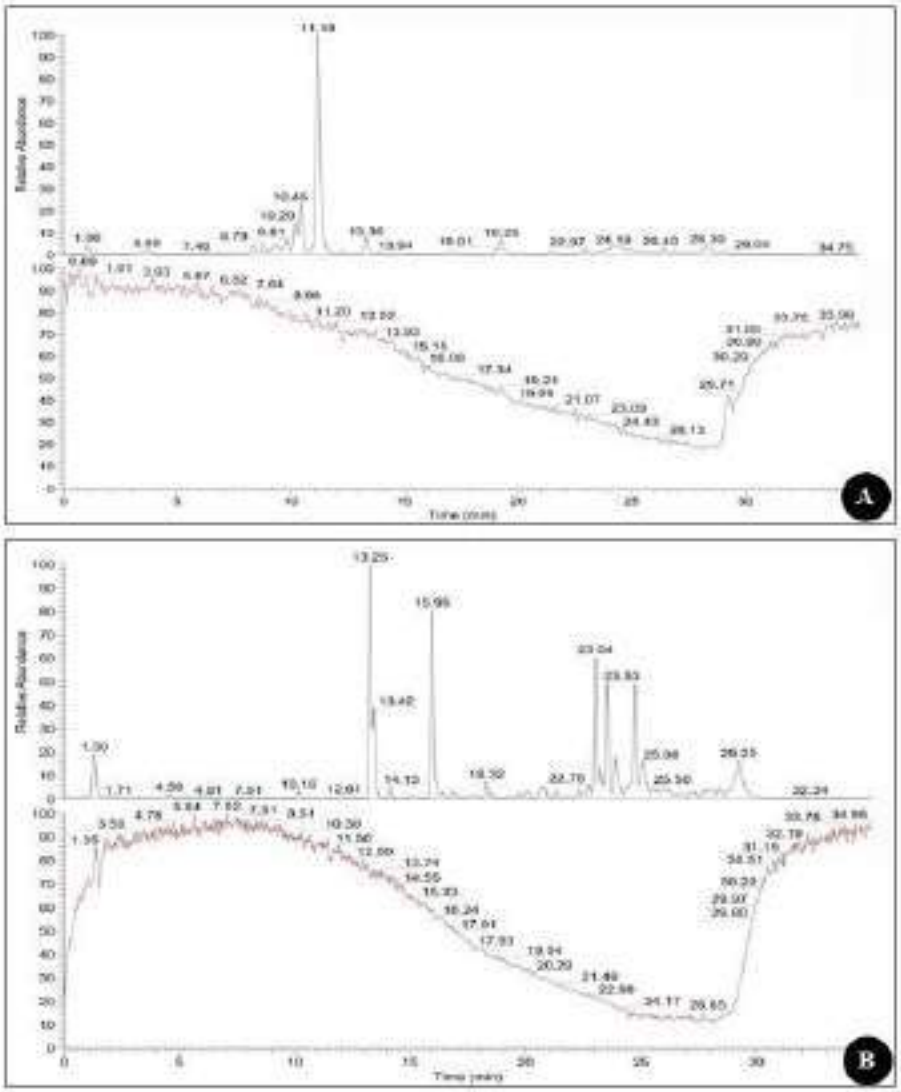


Plate 3.25: Figure A: Shows the chromatographic profile of AS-GA, characterized by several sharp and well-resolved peaks at different retention times (RT), indicating the presence of distinct secondary metabolites and Figure B: displays the OHR-LC-MS chromatogram of ethanolic extracts of FR-NL with distinct peaks corresponding to individual compounds.

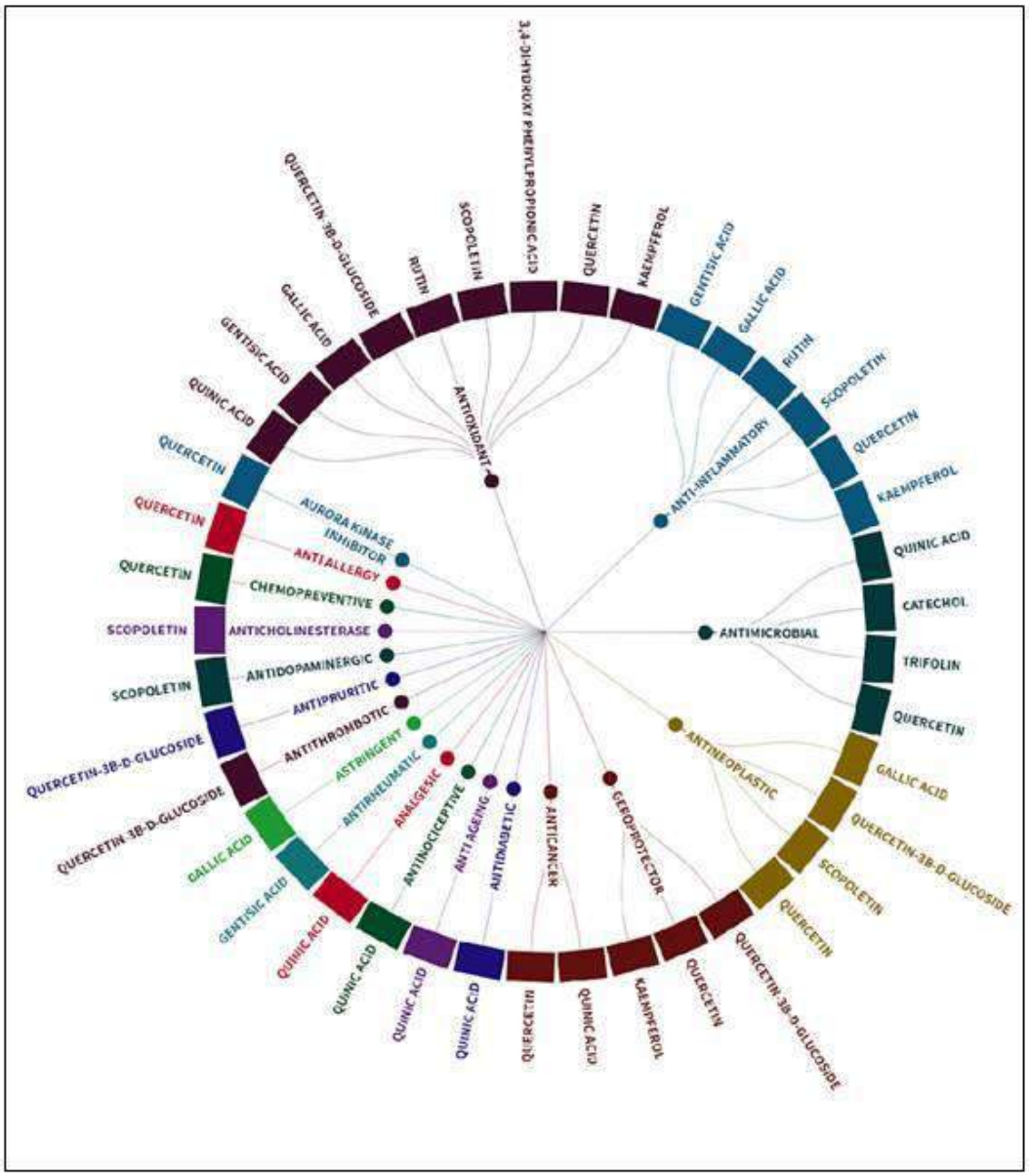


Plate 3.26: The diagram illustrates the pharmacological potential of phenolic constituents specifically present in the AS-GA. Compounds and their respective activities are connected by lines and many of the compounds have multiple bioactivity.

leaf and gall, the total number of compounds identified was 36 and 32 respectively. Among them 16 phenolic compounds were reported from gall leaves and 11 phenolics found from gall. The majority of them found to have different bioactivities. Our observation also supports the previous finding of increase in the production of phenolic compounds as a result of gall induction.

Biswas and colleagues in 2014 found that during gall development, ROS were produced, which increased the production of plant hormones and polyphenols in gall tissues. This may lead to the disruption of some biochemical pathways and ultimately resulting in gall formation. Elicitors produced by insects activate the synthesis of different enzymes and some secondary metabolites which are important for resistance in plants. Different antioxidant enzymes (catalase and peroxidase) act as biochemical markers of foliar gall resistance in *A. scholaris* (Biswas et al., 2014). A monoterpene indole alkaloid, 2-hydroxy deacetyl akuammiline and other compounds such as ursolicacid, lupeol acetate, 17-nor-excelsinidine and 19, 20 E-vallesamine were isolated from methanolic and extracts of leaf galls of *A. scholaris* (Pandey et al., 2023). Phytochemical study of the gall discovered the presence of a nor-monoterpenoid indole alkaloid, alstorisine A, in both gall and gall leaves and not found in normal healthy leaves (Chen et al., 2016). Our work also detected compounds like trifolin, ursolic acid, quercetin-3β-D-glucoside, 12, 13- dihydroxy octadec-9-enoic acid, azelaic acid, gallic acid and 18-β-glycyrrhetinic acid from all the samples analysed.

Quantitative phytochemical analysis of *F. racemosa*-normal leaves (FR-NL)

The chromatogram of FR-NL shown in Plate 3.25: Figure B. Table 3.9 represents the list of compounds discovered with its molecular formula and bioactivity. Plate 3.27 represents a sunburst diagram showing the phenolics compounds present in the FR-NL with their reported biological activity.

OHR-LC-MS analysis of FR-NL detected 33 different phytochemical compounds of important pharmacological activities. Among these compounds, the majority of them are important phenolic compounds of different categories like phenols, flavonoids, flavonol, flavone and coumarins. The other compounds present in the normal leaf include various classes of terpenes (monoterpene, diterpene and sesquiterpene), alkaloids and fatty acids. The majority

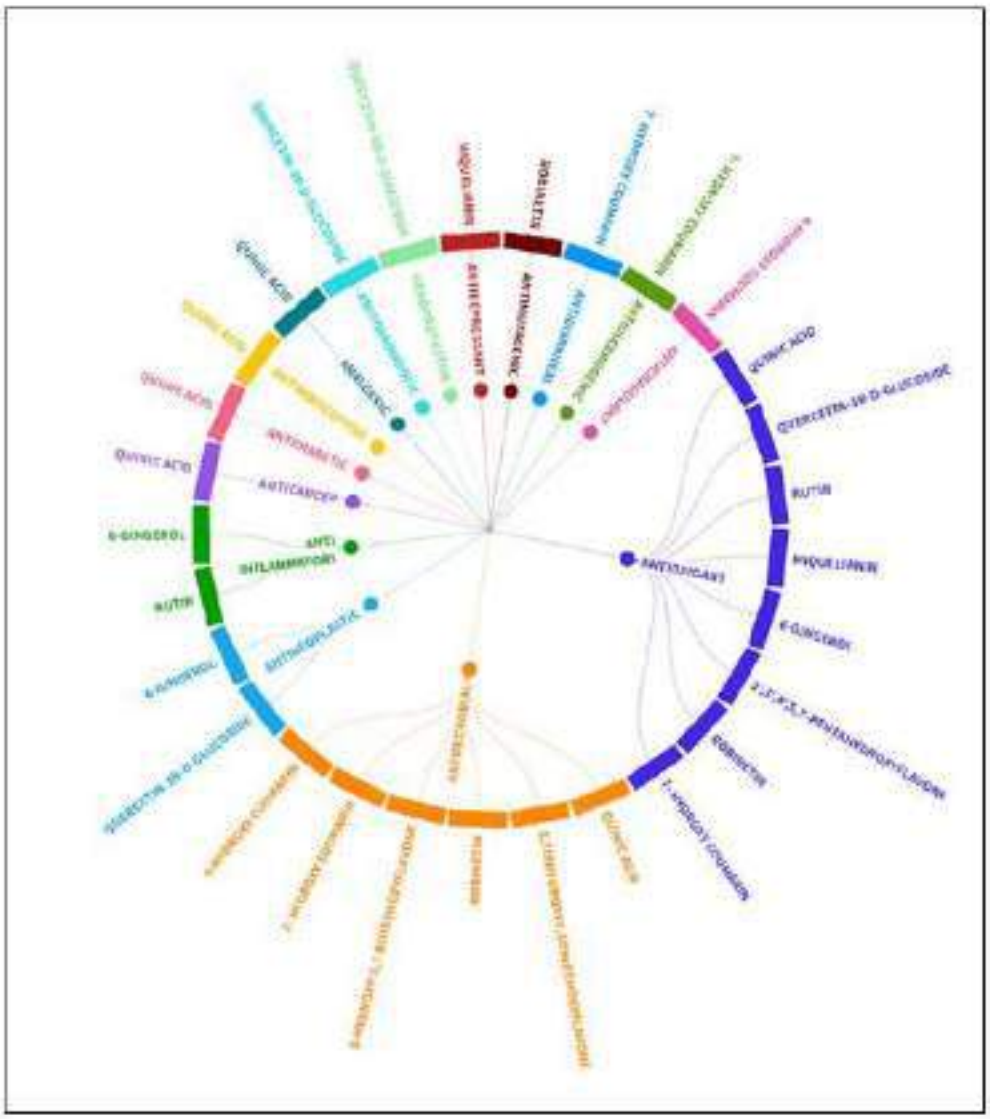


Plate 3.27: The diagram illustrates the diverse pharmacological properties of phenolic constituents present in FR-NL. These bioactivities are represented with lines linking specific compounds, highlighting the potential therapeutic value of the identified phytochemicals.

of the identified phenolic compounds have been found to possess antioxidant properties. 12-oxo phytodienoic acid, which was reported from AS-GL and AS-GA through our analysis, was also discovered in FR-NL and found to have roles in defense responses.

Quantitative phytochemical analysis of *F. racemosa*-gall leaves (FR-GL)

The chromatogram of FR-GL is displayed in Plate 3.28: Figure A. Table 3.10 represents the list of compounds discovered with its molecular formula and bioactivity. Plate 3.29 represents the diagram showing the phenolics compounds present in the FR-GL with their reported bioactivity.

In the case of FR-GL, 33 components were discovered, 12 of which were bioactive phenolic compounds of different categories like phenols, flavonoids, flavonols, flavone and coumarins. It is also found to possess terpenes, alkaloids and fatty acids. 7-hydroxycoumarin, 4-hydroxycoumarin, 2′,3′,4′,5,7-pentahydroxyflavone, 5,7-dihydroxy-3,8-dimethoxyflavone, 12-oxo-phytodienoic acid, and several other compounds were common to both FR-NL and FR-GL.

Quantitative phytochemical analysis of *F. racemosa*-gall (FR-GA)

The chromatogram of gall is shown in Plate 3.28: Figure B. Table 3.11 represents the list of compounds discovered with its molecular formula and bioactivity. Plate 3.30 and Plate 3.31 together represent the sunburst diagrams showing the phenolics compounds present in the FR-GA with their reported bioactivity. Analysis revealed 91 compounds, including 63 phenolic compounds, 11 terpenes, 7 alkaloids and various fatty acids. The number of compounds found from FR-GA is thrice compared to FR-NL and FR-GL. Phenolic compounds detected belonged to different categories (phenols- 22, flavonoid-12, flavonol-5, flavone-10, isoflavone-5, isoflavonoid-1, coumarin-7, lignin-2 and anthocyanin-2). Other compounds identified from the analysis include terpenes, alkaloids and fatty acids.

Dhas and colleagues identified active phytoconstituents from aqueous leaf extracts of the *F. racemosa* through LC-MS analysis, they include citronellol, delcosine and amitenone

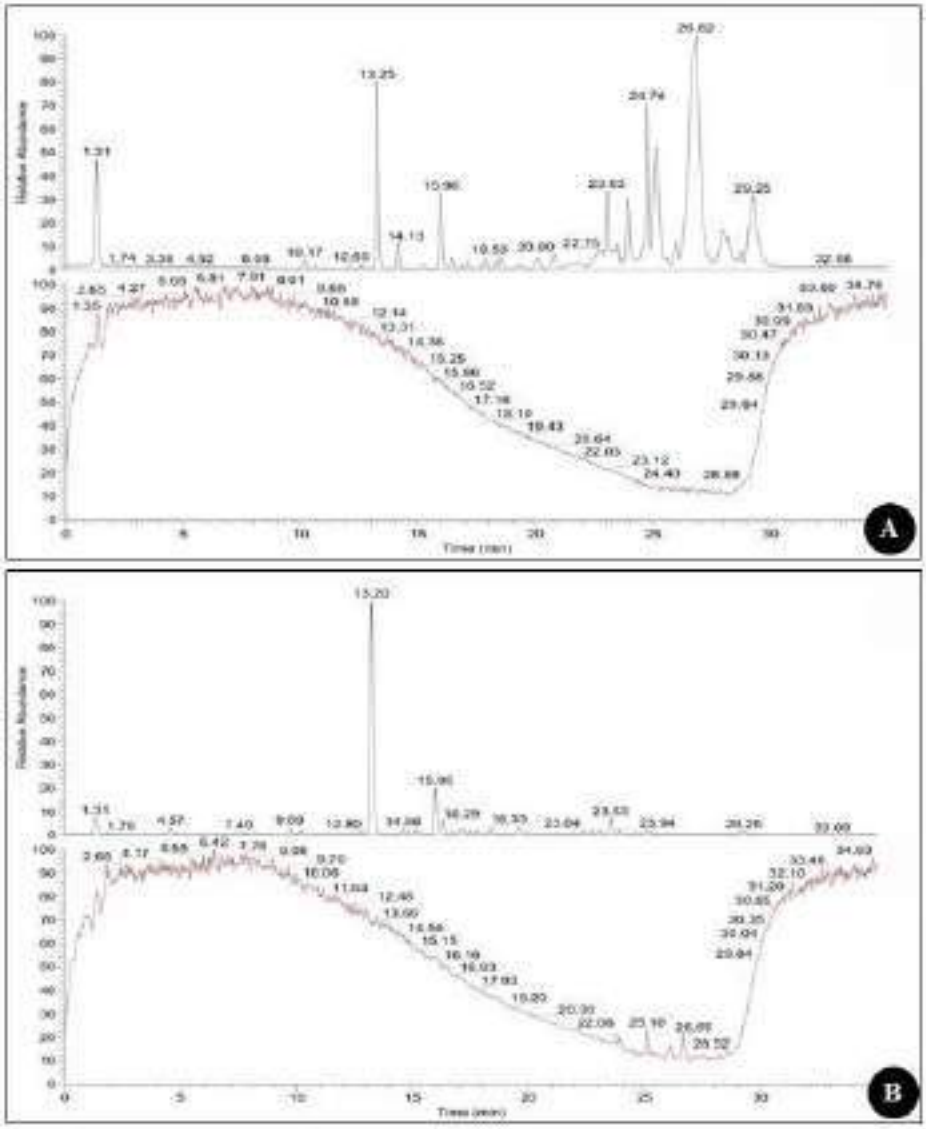


Plate 3.28: Figure A: OHR-LC-MS chromatogram of ethanolic extracts of FR-GL represents the metabolic profile of the FR-GL, revealing the presence of several bioactive compounds and Figure B: OHR-LC-MS chromatogram of ethanolic extracts of FR-GA shows the chemical profile of the gall, indicating the presence of a variety of secondary metabolites based on distinct retention time peaks.

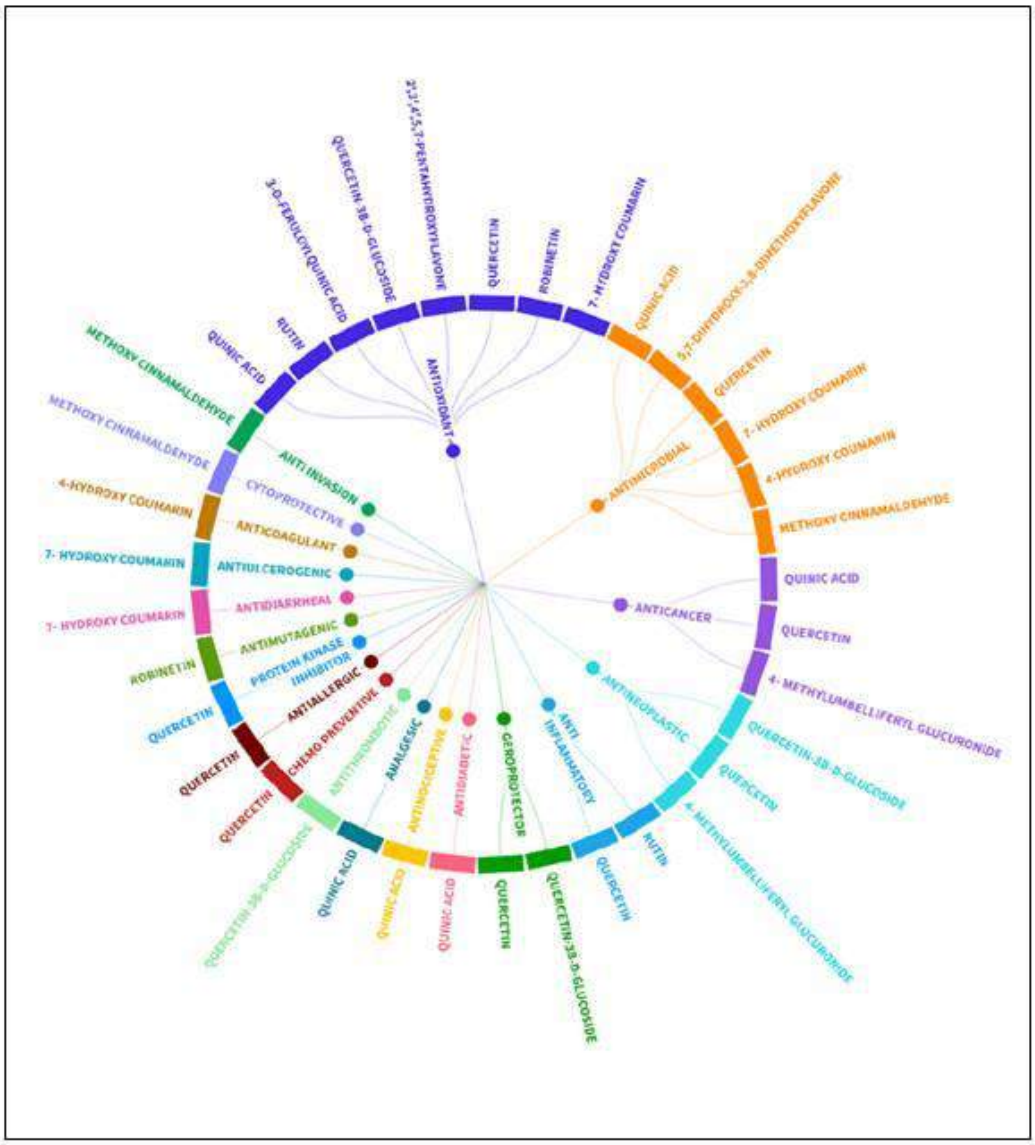


Plate 3.29: The schematic illustration represents the bioactivity of phenolic compounds found in the FR-GL. The lines in the diagram connect individual phenolic compounds to their respective bioactivities.

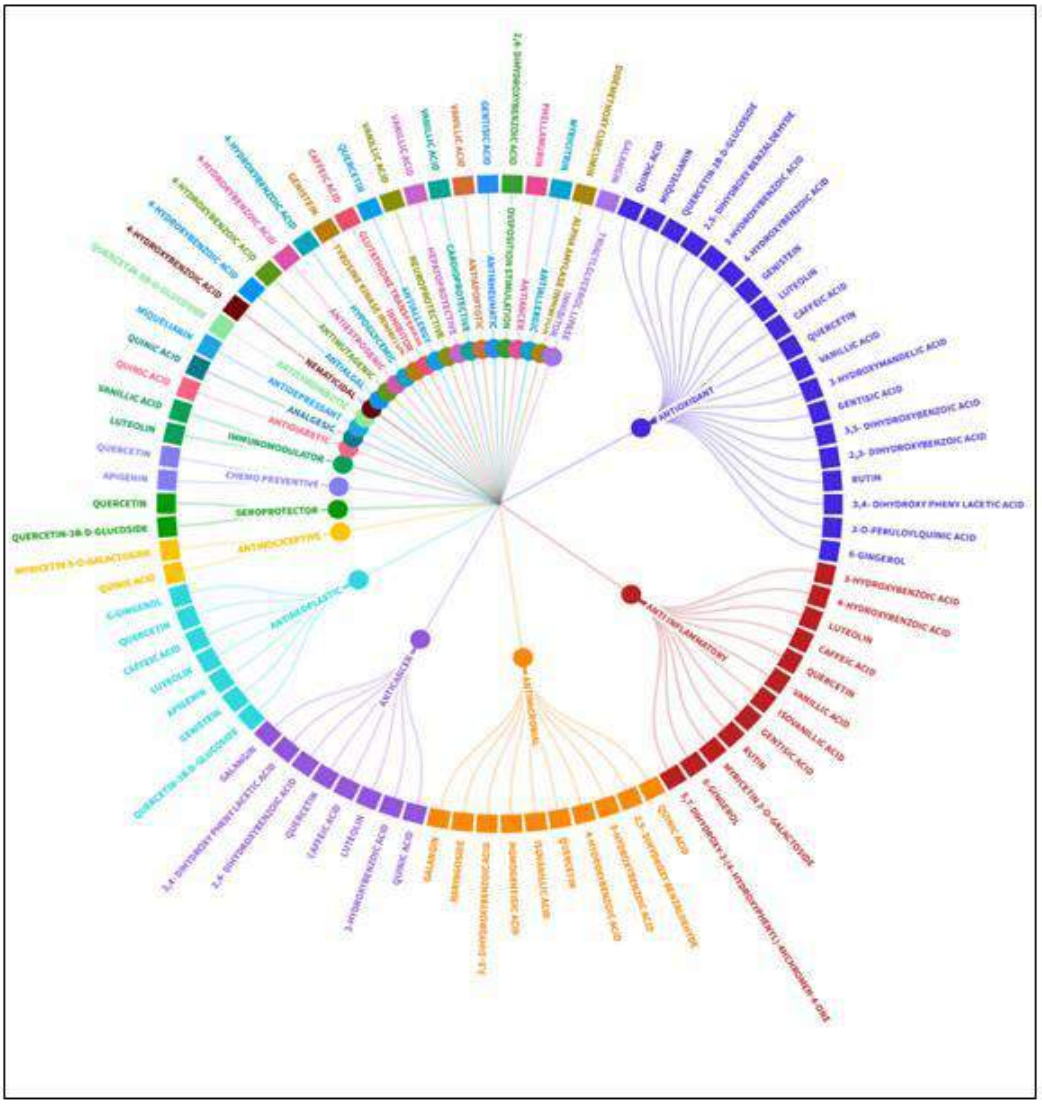


Plate 3.30: The diagram (part-1) illustrates the pharmacological relevance of phenolic constituents found in FR-GA. Each compound is connected to its corresponding biological activities through lines. This is part 1 of the complete diagram; the remaining phenolic compounds and their bioactivities are presented in Part 2.

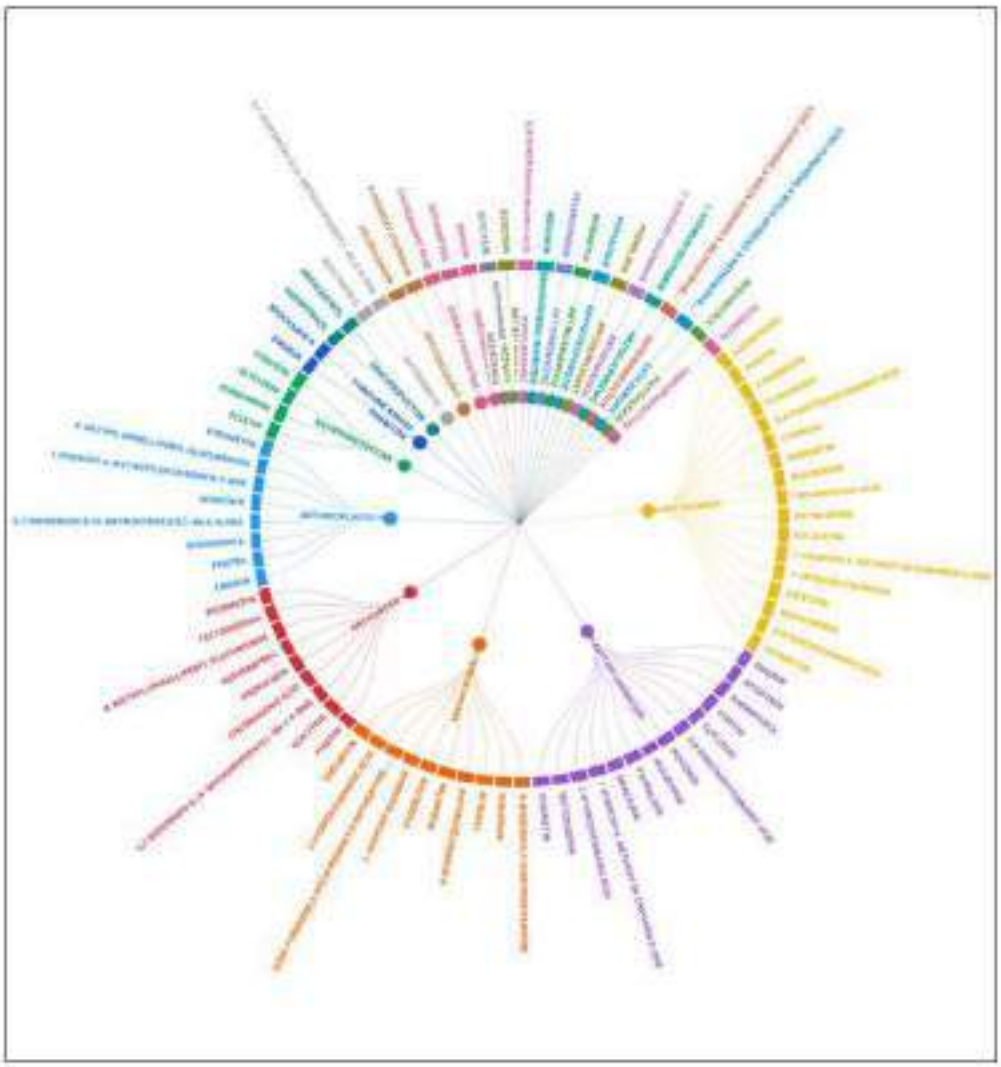


Plate: 3.31: The diagram (Part-2) is the continuation of bioactivities of phenolic compounds identified in FR-GA. This second part completes the illustration of bioactive phenolic compounds found in FR-GA. Together, both parts emphasize the complex and diverse bioactive profile of gall-derived phenolic compounds.

(terpenoids), gamma-nonalactone and gallocatechin-4 beta-ol (flavonoids), phloretin (phenolics) etc. (Dhas et al., 2021). Terpenes, alkaloids, phenolic and fatty acids were identified from normal leaves of the plant through OHR-LC-MS analysis done during this work. All the samples of *F. racemosa* examined were shared 11 components, including quinic acid, quercetin-3β-D-glucoside, azelaic acid, rutin, robinetin, 7-hydroxycoumarin, 4-hydroxycoumarin etc. Among the 33 phytochemicals found, 16 were unique to normal leaves. 75 of the 91 compounds were peculiar to gall. About 18 of the 33 were exclusive to gall leaves. Quercetin-3β-D-glucoside and azelaic acid were found to be present in all the 3 samples of both plants analysed.

Higher levels of total phenols and ortho-dihydric phenols were discovered in the galls, compared to the gall leaves (Dsouza & Ravishankar, 2014). The same observation was found by Ushir and colleagues in 2015 (Ushir et al., 2015). Savitha and colleagues discovered variations in phenolic content among different stages of gall development such as young, medium, and mature; and reported that higher levels of phenols were present in young galls compared to medium and mature galls (Savitha et al., 2013). Eshwarappa and colleagues in 2014 viewed the presence of phenolics, flavonoids, phytosterols, terpenoids and reducing sugars in both aqueous and methanol extracts of leaf galls of *F. racemosa* (Eshwarappa et al., 2015). The presence of phytochemical substances in plant extracts responsible for defense mechanisms of the host plant against pathogens. Following psylloid herbivory, *A. scholaris* and *F. racemosa* both showed notable alterations in their biochemistry. From our study, a total of 33 components from different categories were detected by OHR-LC-MS analysis in the FR-GL, 12 of which were bioactive phenolic compounds. However, FR-GA showed an enormous increase in the amount of phytochemicals, gall extracts showed 91 peaks, including 63 phenolic compounds, 11 terpenes, 7 alkaloids, and different fatty acids. Out of the 91 compounds detected in gall, 67 were unique to galls. This illustrates how significantly the phytochemistry of a plant is impacted by gall induction. Similar to earlier findings, this study supports the trend of increased levels of phenolic compounds in the gall and gall-associated tissues.

Table 3.6: List of compounds of AS-NL identified by OHR-LC-MS analysis with molecular formula, chemical class and reported bioactivities.

Sl. No	Name of compound	Molecular formula	Class of compound	Reported bioactivity	Reference
1	Quinic acid	$C_7H_{12}O_6$	Phenolic compound	Antioxidant, antidiabetic, anticancer, antimicrobial, antiviral, aging protective, anti-nociceptive and analgesic effects.	(Benali et al., 2024)
2	Chlorogenic acid	$C_{16}H_{18}O_9$	Phenolic acids. Intermediate in the biosynthesis of lignin.	Potential antioxidant, chemo preventive and carcinogenic inhibitor.	(Kim et al., 2023)
3	Trifolin	$C_{21}H_{20}O_{11}$	Flavonoid	Antifungal agent.	(Kim et al., 2023)
4	Ursolic acid	$C_{30}H_{48}O_3$	Pentacyclic triterpenoid	Anti-inflammatory, antioxidant, antiviral, serum lipid-lowering and antineoplastic activities. Promotes apoptosis and inhibits cancer cell proliferation.	(Kim et al., 2023)
5	Quercetin-3β-D-glucoside	$C_{21}H_{20}O_{12}$	Flavonoid	Antineoplastic, antioxidant activity. Bone density conservation agent, an osteogenesis regulator, histamine antagonist, an antipruritic drug and a geroprotector.	(Kim et al., 2023)
6	12,13-Dihydroxy octadec-9-enoicacid	$C_{18}H_{34}O_4$	Long-chain fatty acid.	Prevention of obesity and metabolic diseases.	(Macêdo et al., 2022)
7	Luteolin	$C_{15}H_{10}O_6$	Tetrahydroxy flavone	Antioxidant, a free radical scavenger, an anti-inflammatory agent, anticancers, antineoplastic agent, nephroprotective	(Kim et al., 2023)

				agent, an angiogenesis inhibitor, apoptosis inducer and immunomodulator.	
8	Azelaic acid	$C_9 H_{16} O_4$	Fatty acid-Saturated dicarboxylic acid	Antibacterial, antineoplastic, antioxidant activity. Dermatologic drug.	(Kim et al., 2023)
9	16-Hydroxy hexadecanoic acid	$C_{16} H_{32} O_3$	Omega-hydroxy-long-chain fatty acid	A component of the plant cuticle. These apoplastic structures are important plant-environment interfaces that act as barriers limiting water and nutrient loss and protecting plants from radiation and pathogens.	(Kim et al., 2023)
10	Oleoyl-L-α-lysophosphatidic acid	$C_{21} H_{41} O_7 P$	Lysophosphatidic acid (LPA) species	Role in breast cancer metastasis.	(Nam et al., 2018)
11	3-O-Feruloylquinic acid	$C_{17} H_{20} O_9$	Phenolic compound	Antioxidant activity.	(Liu et al., 2021)
12	(3beta,9xi)-3-(beta-D-Glucopyranosyloxy)-14-hydroxycard-20(22)-enolide	$C_{29} H_{44} O_9$	Cardenolide glycoside.	Cardioprotective agent.	(Degtyarenko et al., 2007)
13	Methylmalonic acid	$C_4 H_6 O_4$	Fatty acid	Vital intermediate in the metabolism of fat and protein.	(Kim et al., 2023)
14	L-Phenylalanine	$C_9 H_{11} N O_2$	Essential aromatic amino acid nutraceutical, a micronutrient	Anti-inflammatory activity.	(Davis et al., 1968)

15	Gallic acid	$C_7 H_6 O_5$	Phenolic acid	Astringent, a cyclooxygenase 2 inhibitor. Antioxidant, antineoplastic, anti-inflammatory activities.	(Kahkeshani et al., 2019)
16	9-hydroxyoctadeca-10,12 dienoic acid	$C_{18} H_{32} O_3$	Fatty acid	Markers of oxidative stress.	(Vangaveti et al., 2010)
17	Coriolic acid	$C_{18} H_{32} O_3$	Fatty acid	Antibacterial and anti-inflammatory activity.	(Hanh et al., 2011; Mundt et al., 2003)
18	9,10-dihydroxy- 12Z-octadecenoicacid	$C_{18} H_{34} O_4$	Polyunsaturated fatty acid	Antifungal activity.	(Christopher et al., 2023)
19	13S-hydroxy-9Z,11E, 15Z-octadecatrienoic acid	$C_{18} H_{30} O_3$	Fatty acid	Antiviral activity.	(More et al., 2022)
20	9(10)-Epoxy-12Z-octadecenoic acid	$C_{18} H_{32} O_3$	Medium-chain fatty acid	Chemical mediators in insects.	(Xu et al., 2014)
21	Caffeic acid	$C_9 H_8 O_4$	Caffeic acid is a hydroxycinnamic acid that is cinnamic acid & polyphenol	Glutathione transferase inhibitor, lipoxygenase inhibitor, histone deacetylase inhibitor, antioxidant, anti-inflammatory, anticancer and antineoplastic activities.	(Kim et al., 2023)
22	18-β-Glycyrrhetinic acid	$C_{30} H_{46} O_4$	Pentacyclic triterpenoid	Immunomodulator, antiviral, potential expectorant, anticancer, hepatoprotective, antioxidant and antiulcer activities.	(Hardy et al., 2012; Wang et al., 2018)

Table 3.7: List of compounds found in AS-GL through OHR-LC-MS analysis with molecular formula, chemical class and reported bioactivities.

Sl. No	Compound name	Molecular formula	Class of compound	Reported bioactivity	Reference
1.	Betaine	$C_5 H_{11} N O_2$	Amino acid derivative	Osmoprotectant, anti-inflammatory, antioxidant activities. Beneficial for treatment of obesity, diabetes, cancer and Alzheimer's disease.	(Guangfu et al., 2018)
2.	Palmitic Acid	$C_{16} H_{32} O_2$	Long chain fatty acid	Anti-inflammatory, antioxidant, hypocholesterolemic, pesticide, nematicide, antiandrogenic, hemolytic, antipsychotic, antibacterial, antifungal and cytotoxic activities. 5-Alpha reductase inhibitor.	(Ortega & Campos, 2021)
3.	Methyl 2-[4- ethenyl -2,6-dihydroxy-3-(3-hydroxy prop -1-en-2-yl) -4-methylcyclohexyl] prop-2-enoate	$C_{16} H_{24} O_5$	Sesquiterpenoid.	Unknown activity.	-
4.	9-Oxo-10(E),12(E)-octadecadienoic acid	$C_{18} H_{30} O_3$	Fatty acid	Anticancer agent and potent PPAR α agonist to decrease triglyceride accumulation.	(Kim et al., 2011; Zhao et al., 2015)
5.	Kaempferol	$C_{15} H_{10} O_6$	Flavanol	Anti-inflammatory, antioxidant activity and geroprotector.	(Kim et al., 2023)
6.	Monolaurin	$C_{15} H_{30} O_4$	Fatty acid-Monoglyceride	Antibacterial, antiviral, antifungal and immunomodulatory activity.	(Barker et al., 2019; Nitbani et al., 2022)

7.	12-Oxo phytodienoic acid	C_{18} H_{28} O_3	Fatty acid (Intermediate in biosynthesis of jasmonic acid)	Defence responses, suppresses neuroinflammation and anti-inflammatory activities.	(Stelmach et al., 1998; Taki-Nakano et al., 2016)
8.	DL-Stachydrine	C_7 H_{13} N O_2	Proline derivative	Anti-obesity, anti-fibrotic, anticancer, neuroprotective, anti-inflammatory property and cardiovascular system protection properties.	(Cheng et al., 2020)
9.	6-Gingerol	C_{17} H_{26} O_4	Phenolics	Antineoplastic, antioxidant and anti-inflammatory activity.	(Kim et al., 2023)
10.	Betulin	C_{30} H_{50} O_2	Triterpenoid	Antiviral, analgesic, anti-carcinomic, anti-flu, cytotoxic, anti-inflammatory and antineoplastic activities.	(Bębenek et al., 2019; Kim et al., 2023)
11.	2,4-Dihydroxy benzoic acid	C_7 H_6 O_4	Phenolic acid	Antioxidant, antimicrobial and antiproliferative activity.	(Popiołek et al., 2023)
12.	Nicotinic acid	C_6 H_5 N O_2	Alkaloid	Antidote and antilipemic drug. A vasodilator agent, nicotinamidase inhibitor and antihyperlipidemic activity.	(Kim et al., 2023)
13.	Catechin	C_{15} H_{14} O_6	Polyphenolic (Flavonoid)	Antioxidant.	(Kim et al., 2023)
14.	3,4-Dihydroxy phenylpropionic acid	C_9 H_{10} O_4	Phenolic compound	Antioxidant property.	(Degtyarenko et al., 2007)
15.	Cinchonidine	C_{19} H_{22} N_2	Alkaloid	Antimalarial, anti-obesity, anticancer, antioxidant,	(Gurung & De, 2017)

		O		anti-inflammatory and antimicrobial activities.	
16.	Gelsemine	$C_{20}H_{22}N_2O_2$	Indole alkaloid	Anti-inflammatory, anticancer and antioxidant activities.	(Lin et al., 2015)
17.	Isochlorogenic acid C	$C_{25}H_{24}O_{12}$	Phenolic compound	Anti-inflammatory, antioxidant, anti-apoptotic, anti-injury and anti-hepatitis B virus effects.	(Liu et al., 2023; Lodise et al., 2019; Yin et al., 2018)
18.	Nicotinamide	$C_6H_6N_2O$	Alkaloid	Antioxidant, neuroprotective, anti-inflammatory activities. Histone deacetylase inhibitor and geroprotector.	(Kim et al., 2023)
19.	Catharanthine	$C_{21}H_{24}N_2O_2$	Monoterpenoid indole alkaloid	Anti-parasitic, vasodilatory, antimalarial, anti-cancer and antihypertensive activities.	(Gao et al., 2023)
20.	Isorhamnetin	$C_{16}H_{12}O_7$	Flavone	Tyrosinase inhibitor, anticoagulant, anticancer, anti-inflammatory, antioxidant activities. organ protection and prevention of obesity.	(Gong et al., 2020)
21.	Arecoline	$C_8H_{13}NO_2$	Alkaloid	Anti-helminthic and anti-parasitic activity	(Liu et al., 2016)
22.	Chlorogenic acid	$C_{16}H_{18}O_9$	Phenolic acids (intermediate in the biosynthesis of lignin)	Antioxidant, chemo preventive and carcinogenic inhibitor activities.	(Kim et al., 2023)
23.	Gentisic acid	$C_7H_6O_4$	Phenolic compound	Anti-inflammatory, antirheumatic and antioxidant properties.	(Kim et al., 2023)
24.	Quercetin-3β-D-glucoside	$C_{21}H_{20}O_{12}$	Flavonoid	Antineoplastic, antioxidant and antithrombotic activity, bone density conservation agent, osteogenesis regulator, histamine antagonist, antipruritic drug and geroprotector.	(Kim et al., 2023)

25.	Trifolin	$C_{21}H_{20}O_{11}$	Flavonoid	Antifungal agent	(Kim et al., 2023)
26.	Azelaic acid	$C_9H_{16}O_4$	Fatty acid-Saturated dicarboxylic acid	Antibacterial, antineoplastic and antioxidant activity. Dermatologic drug.	(Kim et al., 2023)
27.	Gallic acid	$C_7H_6O_5$	Phenolic acid	Antioxidant, antineoplastic, anti-inflammatory activity. An astringent, a cyclooxygenase 2 inhibitor.	(Kahkeshani et al., 2019)
28.	Catechol	$C_6H_6O_2$	Phenolic compound	Antibacterial and antifungal effect.	(Kocaçalışkan et al., 2006)
29.	Quercetin	$C_{15}H_{10}O_7$	Flavanol	Antibacterial, anticancer, antineoplastic, antioxidant, chemopreventive, anti-inflammatory and anti-allergy activities. A protein kinase inhibitor, chelator, aurora kinase inhibitor and geroprotector.	(Kim et al., 2023)
30.	16-Hydroxy hexadecanoic acid	$C_{16}H_{32}O_3$	Omega-hydroxy-long-chain fatty acid (component of the plant cuticle)	Protecting plants from radiation and pathogens.	(Kim et al., 2023)
31.	12,13-Dihydroxy octadec-9-enoic acid	$C_{18}H_{34}O_4$	Long-chain fatty acid	Use in treatment of obesity and metabolic diseases.	(Macêdo et al., 2022)
32.	(3beta,9xi)-3-(beta-d glucopyranosyloxy)-14-hydroxycard 20(22)-enolide	$C_{29}H_{44}O_9$	Cardenolide glycoside	Cardioprotective agent.	(Degtyarenko et al., 2007)

33.	Luteolin	C_{15} H_{10} O_6	Tetra hydroxy flavone	Antioxidant, anti-inflammatory, anticancers, antineoplastic activities. Free radical scavenger, vascular endothelial growth factor receptor antagonist, nephroprotective agent, angiogenesis inhibitor, an apoptosis inducer and immunomodulator.	(Kim et al., 2023)
34.	Rutin	C_{27} H_{30} O_{16}	Flavanol glycoside (polyphenolic compound)	Antioxidant and anti-inflammatory activities.	(Kim et al., 2023)
35.	Ursolic acid	C_{30} H_{48} O_3	Triterpenoid	Anti-inflammatory, antioxidant, antiviral, serum lipid-lowering and antineoplastic activities. Promote apoptosis and inhibit cancer cell proliferation.	(Kim et al., 2023)
36.	18-β-Glycyrrhetinic acid	C_{30} H_{46} O_4	Triterpenoid	Immunomodulator, potential expectorant, gastrokinetic and antiulcer activities.	(Hardy et al., 2012; Wang et al., 2017b)

Table 3.8: List of compounds found in AS-GA through OHR-LC-MS analysis with molecular formula, chemical class and reported bioactivities.

Sl. No	Compound name	Molecular formula	Class of compound	Reported bioactivity	Reference
1.	Quinic acid	C_7 H_{12} O_6	Phenolic compound	Antioxidant, antidiabetic, anticancer, antimicrobial, antiviral, aging protective, anti-nociceptive and analgesic effects.	(Benali et al., 2024)
2.	Gentisic acid	C_7 H_6 O_4	Phenolic compound	Anti-inflammatory, antirheumatic and antioxidant properties.	(Kim et al., 2023)

3.	Catechol	$C_6 H_6 O_2$	Phenolic compound	Antibacterial and antifungal effect.	(Kocaçalışkan et al., 2006)
4.	Gallic acid	$C_7 H_6 O_5$	Phenolic compound	Astringent, a cyclooxygenase 2 inhibitor, antioxidant, antineoplastic and anti-inflammatory activities.	(Kahkeshani et al., 2019)
5.	12,13-Dihydroxy octadec-9-enoic acid	$C_{18} H_{34} O_4$	Long-chain fatty acid.	Used in the treatment of obesity and metabolic diseases.	(Macêdo et al., 2022)
6.	Ursolic acid	$C_{30} H_{48} O_3$	Triterpenoid	Anti-inflammatory, antioxidative, antiviral, lipid-lowering and antineoplastic activity. Promote apoptosis and inhibit cancer cell proliferation.	(Kim et al., 2023)
7.	Azelaic acid	$C_9 H_{16} O_4$	Fatty acid-Saturated dicarboxylic acid	Antibacterial, antineoplastic, anti-inflammatory and antioxidant activity. Dermatologic drug.	(Kim et al., 2023)
8.	Quercetin-3β-D-glucoside	$C_{21} H_{20} O_{12}$	Flavonoid	Antineoplastic, antioxidant, antithrombotic, bone density conservation activity. Osteogenesis regulator, histamine antagonist, antipruritic drug and geroprotector.	(Kim et al., 2023)
9.	18-β-Glycyrrhetinic acid	$C_{30} H_{46} O_4$	Triterpenoid	Immunomodulator, antiulcer, potential expectorant and gastrokinetic activities.	(Hardy et al., 2012; Wang et al., 2017b)

10.	Rutin	C_{27} H_{30} O_{16}	Flavonol glycoside. (polyphenolic compound)	Antioxidant and anti-inflammatory activities.	(Kim et al., 2023)
11.	Trifolin	C_{21} H_{20} O_{11}	Flavonoid	Antifungal agent.	(Kim et al., 2023)
12.	Betaine	C_5 H_{11} N O_2	Amino acid derivative	Anti-inflammatory and anticancer activity.	(Guangfu et al., 2018)
13.	(S)-AL 8810	C_{24} H_{31} F O_4	Long-chain fatty acid	Anti -inflammatory and antioxidant activities.	(Sharif & Klimko, 2019)
14.	Rauwolscine	C_{21} H_{26} N_2 O_3	Alkaloid	Central nervous system stimulant, alpha-2-adrenergic blocking activity and anti-depressant	(Kim et al., 2023)
15.	Cinchonine	C_{19} H_{22} N_2 O	Alkaloid	Antimalarial and anti-diabetic activities.	(Boratyński et al., 2019)
16.	Palmitic Acid	C_{16} H_{32} O_2	Long-chain fatty acid	Anti-inflammatory, antioxidant, hypocholesterolemic, pesticide, nematicide, antiandrogenic, hemolytic, 5-Alpha reductase inhibitor, antipsychotic, antibacterial, antifungal and cytotoxic activities.	(Ortega & Campos, 2021)
17.	Scopoletin	C_{10} H_8 O_4	Phenolic compound (coumarin)	Antineoplastic, antidopaminergic, antioxidant, anti-inflammatory and anticholinesterase effects.	(Kim et al., 2023)
18.	DL-Stachydrine	C_7 H_{13} N O_2	Proline derivative	Anti-obesity, anti-fibrotic, anticancer, neuroprotective, anti-inflammatory properties. Cardiovascular system protection.	(Cheng et al., 2020)

19.	3,4-Dihydroxy phenylpropionic acid	$C_9 H_{10} O_4$	Phenolic compound	Antioxidant properties.	(Degtyarenko et al., 2007)
20.	9-Oxo-10(E),12(E)-octadecadienoic acid	$C_{18} H_{30} O_3$	Fatty acid	Anticancer agent and potent PPAR α agonist to decrease triglyceride accumulation.	(Kim et al., 2011; Zhao et al., 2015)
21.	2-Oxindole	$C_8 H_7 NO$	Alkaloid	Antiproliferative property.	(Sharma et al., 2023)
22.	Monolaurin	$C_{15} H_{30} O_4$	Fatty acid-Monoglyceride	Antibacterial, antiviral, antifungal and immunomodulatory activity.	(Barker et al., 2019; Nitbani et al., 2022)
23.	Gelsemine	$C_{20} H_{22} N_2 O_2$	Alkaloid	Anti-inflammatory, anticancer and antioxidant activities.	(Lin et al., 2015)
24.	Quercetin	$C_{15} H_{10} O_7$	Flavanol	Antibacterial, chemopreventive activity, anti-inflammatory, anti-allergy, anticancer, antioxidant, a protein kinase inhibitor and antineoplastic activities. A chelator, Aurora kinase inhibitor and geroprotector.	(Kim et al., 2023)
25.	Kaempferol	$C_{15} H_{10} O_6$	Flavanol	Anti-inflammatory and antioxidant activity, Geroprotector.	(Kim et al., 2023)
26.	Mitragynine	$C_{23} H_{30} N_2 O_4$	Monoterpenoid indole alkaloid.	Analgesic effects.	(Karunakaran et al., 2022)
27.	D-δ-Tocopherol	$C_{27} H_{46} O_2$	Vitamin E	Antioxidant activity.	(Kim et al., 2023)
28.	Arecoline	$C_8 H_{13} NO_2$	Alkaloid	Antihelmintic, ganglionic stimulant, parasympathomimetic activities and a vermifuge.	(Sharma & Anand, 1997)

29.	12-Oxo phytodienoic acid	$C_{18}H_{28}O_3$	Fatty acid (Intermediate in biosynthesis of jasmonic acid)	Defence responses and suppresses neuroinflammation. Anti-inflammatory compound.	(Stelmach et al., 1998; Taki-Nakano et al., 2016)
30.	Trigonelline	$C_7H_7NO_2$	Alkaloid	Hypoglycemic, hypolipidemic, neuroprotective, antimigraine, sedative, memory-improving, antibacterial, anti-diabetic, antiviral and anti-tumor activities.	(Zhou et al., 2012)
31.	Catharanthine	$C_{21}H_{24}N_2O_2$	Monoterpenoid indole alkaloid	Anti-parasitic, vasodilatory, anticancer, antimalarial and antihypertensive activities	(Kim et al., 2023)
32.	N-Acetyldopamine	$C_{10}H_{13}NO_3$	N-acetylated derivative of dopamine.	Reactive intermediate in sclerotization. Sclerotizing agent of the insect cuticle	(Karlson & Sekeris, 1962)

Table 3.9: List of compounds found in FR-NL identified by OHR-LC-MS analysis with molecular formula, chemical class and reported bioactivities.

Sl. No.	**Name of compound**	**Molecular formula**	**Class of compound**	**Reported bioactivity**	**Reference**
1.	Quinic acid	$C_7H_{12}O_6$	Phenolic compound	Antioxidant, antidiabetic, anticancer, antimicrobial, antiviral, aging, protective, anti-nociceptive and analgesic effects.	(Benali et al., 2024)
2.	Quercetin-3β-D-glucoside	$C_{21}H_{20}O_{12}$	Flavonoid	Antineoplastic, antioxidant, antithrombotic, bone density conservation activity. Osteogenesis regulator, histamine antagonist, antipruritic drug and geroprotector.	(Kim et al., 2023)

3.	Rutin	C_{27} H_{30} O_{16}	Flavanol	Antioxidant and anti-inflammatory activities	(Kim et al., 2023)
4.	Azelaic acid	C_9 H_{16} O_4	Fatty acid	Antibacterial, antineoplastic, antioxidant activity. Dermatologic drug.	(Kim et al., 2023)
5.	[5-Hydroxy-3-(hydroxymethyl)-2-oxo-6- Propan-2-ylcyclohex- 3-en-1-yl] 3-methylbutanoate	C_{15} H_{24} O_5	Monoterpenoid	Unknown activity.	-
6.	13S-hydroxy-9Z,11E, 15Z-octadecatrienoic acid	C_{18} H_{30} O_3	Fatty acid	Antiviral activity.	(More et al., 2022)
7.	Miquelianin	C_{21} H_{18} O_{13}	Flavonoid	Antioxidant and antidepressant activities.	(Kim et al., 2023)
8.	6-Gingerol	C_{17} H_{26} O_4	Phenolics	Antineoplastic, antioxidant and anti-inflammatory activity.	(Kim et al., 2023)
9.	Oleic acid	C_{18} H_{34} O_2	Fatty acid	Antioxidant activity.	(Kim et al., 2023)
10.	2',3',4',5,7-	C_{15} H_{10} O_7	Flavone	Antioxidant activity.	(Zhang et al., 2011)

	pentahydroxyflavone				
11.	Nicotinic acid	$C_6 H_5 N O_2$	Alkaloid	Antidote and antilipemic drug. A vasodilator agent, nicotinamidase inhibitor and antihyperlipidemic activity.	(Kim et al., 2023)
12.	5,7-dihydroxy-3,8-dimethoxyflavone	$C_{17} H_{14} O_6$	Flavone	Fungitoxicity.	(Cotoras et al., 2011)
13.	Robinetin	$C_{15} H_{10} O_7$	Flavone	Antifungal, antiviral, antibacterial, anti-mutagenesis and antioxidant activity.	(Birt et al., 1986)
14.	Andrographolide	$C_{20} H_{30} O_5$	Diterpenoid	Anti-HIV, anti-inflammatory and antineoplastic.	(Kim et al., 2023)
15.	Arecoline	$C_8 H_{13} N O_2$	Alkaloid	Anti-helminthic and anti-parasitic activity.	(Liu et al., 2016)
16.	Trigonelline	$C_7 H_7 N O_2$	Alkaloid	Hypoglycemic, hypolipidemic, neuroprotective, antimigraine, sedative memory-improving, antibacterial, anti-diabetic, antiviral and anti-tumor activities.	(Zhou et al., 2012)
17.	10- Hydroxy camptothecin	$C_{20} H_{16} N_2 O_5$	Alkaloid	Anticancer activity.	(Yuan-Gang et al., 2003)
18.	9-hydroxy-10E,12Z-octadecadienoic acid	$C_{18} H_{32} O_3$	Fatty acid	Markers of lipid peroxidation and oxidation.	(Spiteller & Spiteller, 1997)
19.	Nootkatone	$C_{15} H_{22} O$	Sesquiterpenoid	Anti-inflammatory, anticancer, antibacterial, hepatoprotective,	(Jha et al., 2022)

				neuroprotective and cardioprotective activity. Insect repellent.	
20.	9-Oxo-10(E),12(E)-octadecadienoic acid	$C_{18}H_{30}O_3$	Fatty acid	Anticancer agent and potent PPAR α agonist to decrease triglyceride accumulation.	(Kim et al., 2011; Zhao et al., 2015)
21.	5-(4-carboxy-3-methylbutyl)-5,6,8atrimethyl-3-oxo3,4,4a, 5,6,7,8,8a octahydronaphthalene-1-carboxylic acid	$C_{20}H_{30}O_5$	Diterpenoid	Unknown	-
22.	Reserpine	$C_{33}H_{40}N_2O_9$	Alkaloids	Antihypertensive and anti- psychotic activities.	(Kim et al., 2023)
23.	12-Oxo phytodienoic acid	$C_{18}H_{28}O_3$	Fatty acid (Biosynthesis of jasmonic acid)	Defence responses, suppresses neuroinflammation and anti-inflammatory activities.	(Stelmach et al., 1998; Taki-Nakano et al., 2016)
24.	5-Hydroxy-6,7-dimethoxyflavone	$C_{17}H_{14}O_5$	Flavonoid	Antiviral activities.	(Wu et al., 2010)
25.	7- Hydroxy coumarin	$C_9H_6O_3$	Coumarin	Antidiarrheal, antibacterial, antiulcerogenic effects.	(Cruz et al., 2020)
26.	4-Hydroxycoumarin	$C_9H_6O_3$	Coumarin	Antimicrobial and anti-coagulant activity.	(Završnik et al., 2008)
27.	Ouabain	$C_{29}H_{44}O_{12}$	Cardiac glycoside	Anticancer and antiviral activity.	(Carvalho et al., 2022; Du et al., 2021)
28.	Ilicic acid	$C_{15}H_{24}O_3$	Sesquiterpenoid	Anti-inflammatory activity	(Barrero et al., 2008)

29.	Zedoarondiol	C_{15} H_{24} O_3	Sesquiterpenoid.	Cytotoxic, anti-inflammatory and antioxidant activity.	(Anuchapreeda et al., 2018)
30.	Polygodial	C_{15} H_{22} O_2	Sesquiterpene	Antibacterial and antifungal activity. Antifeedant activity against aphids.	(Asakawa et al., 1988; Kubo et al., 2005)
31.	Zerumbone	C_{15} H_{22} O	Sesquiterpenoid.	Anti-inflammatory agent and glioma-associated oncogene inhibitor.	(Kim et al., 2023)
32.	Perillartine	C_{10} H_{15} N O	Monoterpene	Anti-obesity activity.	(Xiao et al., 2001)
33.	Palmitic Acid	C_{16} H_{32} O_2	Fatty acid	Anti-inflammatory, antioxidant, hypocholesterolemic, pesticide, nematicide, antiandrogenic, hemolytic, antipsychotic, antibacterial, antifungal and cytotoxic activities. 5-Alpha reductase inhibitor.	(Ortega & Campos, 2021)

Table 3.10: List of compounds found in FR-GL identified by OHR-LC-MS analysis with molecular formula, chemical class and reported bioactivities.

Sl. No.	Name of compound	Molecular formula	Class of compound	Reported bioactivity	Reference
1.	Quinic acid	C_7 H_{12} O_6	Phenolic compound	Antioxidant, antidiabetic, anticancer, antimicrobial, antiviral, aging protective, anti-nociceptive and analgesic effects.	(Benali et al., 2024)
2.	Quercetin-3β-D-glucoside	C_{21} H_{20} O_{12}	Flavonoid	Antineoplastic, antioxidant, antithrombotic, bone density conservation activity. Osteogenesis regulator,	(Kim et al., 2023)

				histamine antagonist, antipruritic drug and geroprotector.	
3.	Rutin	C_{27} H_{30} O_{16}	Flavanol	Antioxidant and anti-inflammatory activities.	(Kim et al., 2023)
4.	Azelaic acid	C_9 H_{16} O_4	Fatty acid	Antibacterial, antineoplastic, antioxidant activity. Dermatologic drug.	(Kim et al., 2023)
5.	3-O-Feruloyl quinic acid	C_{17} H_{20} O_9	Phenolic compound	Antioxidant activity.	(Liu et al., 2021)
6.	2',3',4',5,7-penta hydroxyflavone	C_{15} H_{10} O_7	Flavone	Antioxidant activity.	(Zhang et al., 2011)
7.	5,7-dihydroxy- 3,8-di methoxyflavone	C_{17} H_{14} O_6	Flavone	Fungitoxicity.	(Cotoras et al., 2011)
8.	Quercetin	C_{15} H_{10} O_7	Flavanol	Antibacterial, chemopreventive, anti-inflammatory, anti-allergy, anticancer, antioxidant, a protein kinase inhibitor, antineoplastic activities. A chelator, Aurora kinase inhibitor and geroprotector.	(Kim et al., 2023)
9.	Robinetin	C_{15} H_{10} O_7	Flavone	Anti-mutagenic, antiradical and anti-promotion properties.	(Birt et al., 1986)

10.	Arecoline	$C_8 H_{13} N O_2$	Alkaloid	Anti-helminthic, antiparasitic activity.	(Liu et al., 2016)
11.	Trigonelline	$C_7 H_7 N O_2$	Alkaloid	Hypoglycemic, hypolipidemic, neuroprotective, antimigraine, sedative, memory-improving, antibacterial, anti-diabetic, antiviral and anti-tumor activities.	(Zhou et al., 2012)
12.	Nicotinic acid	$C_6 H_5 N O_2$	Alkaloid	Antidote and antilipemic drug. A vasodilator agent, nicotinamidase inhibitor and antihyperlipidemic activity.	(Kim et al., 2023)
13.	Andrographolide	$C_{20} H_{30} O_5$	Diterpenoid	Anti-HIV, anti-inflammatory and antineoplastic activities.	(Kim et al., 2023)
14.	10- Hydroxy camptothecin	$C_{20} H_{16} N_2 O_5$	Alkaloid	Anti-tumor activity.	(Xiao et al., 2001)
15.	Nootkatone	$C_{15} H_{22} O$	Sesquiterpenoid	Anti-inflammatory, anticancer, antibacterial, hepatoprotective, neuroprotective and cardioprotective activity. Insect repellent.	(Jha et al., 2022)
16.	12-Oxo phytodienoic acid	$C_{18} H_{28} O_3$	Fatty acid (Intermediate in biosynthesis of jasmonic acid)	Defence responses and suppresses neuroinflammation. Anti-inflammatory compound.	(Stelmach et al., 1998; Taki-Nakano et al., 2016)
17.	Palmitic acid	$C_{16} H_{32} O_2$	Fatty acid	Anti-inflammatory, antioxidant, hypocholesterolemic, pesticide, nematicide, antiandrogenic, hemolytic, antipsychotic, antibacterial, antifungal and cytotoxic activities. 5-Alpha reductase inhibitor.	(Ortega & Campos, 2021)

18.	5-(4-carboxy-3-methylbutyl) - 5,6,8a trimethyl -3- oxo 3,4,4a,5,6,7,8,8a octa hydro naphthane-1-carboxylic acid	C_{20} H_{30} O_5	Diterpenoid	Unknown activity	-
19.	7- Hydroxy coumarin	C_9 H_6 O_3	Coumarin	Antidiarrheal, antibacterial, antiulcerogenic effects and antioxidant capacity.	(Cruz et al., 2020)
20.	4- Hydroxy coumarin	C_9 H_6 O_3	Coumarin	Antimicrobial and anti-coagulant activity.	(Završnik et al., 2008)
21.	15-OxoEDE	C_{20} H_{34} O_3	Fatty acid	Unknown activity.	-
22.	(±)13-HpODE	C_{18} H_{32} O_4	Fatty acid	Unknown activity.	-
23.	Ilicic acid	C_{15} H_{24} O_3	Sesquiterpenoid.	Anti-inflammatory activity.	(Barrero et al., 2008)
24.	Tetranor-12(R)- HETE	C_{16} H_{26} O_3	Fatty acid	Unknown activity.	-
25.	Zedoarondiol	C_{15} H_{24} O_3	Sesquiterpenoid.	Cytotoxic, anti-inflammatory and antioxidant activity.	(Anuchapreeda et al., 2018)
26.	4- Methyl umbelliferyl glucuronide	C_{16} H_{16} O_9	Coumarin	Anti-tumourigenic and antineoplastic agent.	(Kim et al., 2023)
27.	9-hydroxy-10E,12Z-octadecadienoic acid	C_{18} H_{32} O_3	Fatty acid	Markers of lipid peroxidation and oxidation.	(Spiteller & Spiteller, 1997)
28.	13S-hydroxy-9Z,11E,15Z-octadecatrienoic acid	C_{18} H_{30} O_3	Fatty acid	Antiviral activity.	(More et al., 2022)

29.	9-Oxo-10(E),12(E)-octadecadienoic acid	C_{18} H_{30} O_3	Fatty acid	Anticancer agent and potent PPAR α agonist to decrease triglyceride accumulation.	(Kim et al., 2011; Zhao et al., 2015)
30.	Reserpine	C_{33} H_{40} N_2 O_9	Alkaloids	Antihypertensive and antipsychotic activities.	(Kim et al., 2023)
31.	Zerumbone	C_{15} $H_{22}O$	Sesquiterpenoid.	Anti-inflammatory agent and glioma-associated oncogene inhibitor.	(Kim et al., 2023)
32.	Methoxy cinnamaldehyde	C_{10} H_{10} O_2	Flavonoid	Cytoprotective, antimicrobial and anti-invasion activity.	(Van et al., 2022)
33.	Perillartine	C_{10} H_{15} N O	Monoterpene	Anti-obesity activity.	(Xiao et al., 2001)

Table 3.11: List of compounds found in FR-GA identified by OHR-LC-MS analysis with molecular formula, chemical class and reported bioactivities.

Sl. No.	Name of compound	Molecular formula	Class of compound	Reported bioactivity	Reference
1.	2,5- Dihydroxy benzaldehyde	C_7 H_6 O_3	Phenolic compound	Antibacterial, antifungal and antioxidant activity. Tyrosine kinase inhibitor.	(Sahin et al., 2013)
2.	3-Hydroxybenzoic acid	C_7 H_6 O_3	Phenolic compound	Antimicrobial, anti-inflammatory, anticancer and antioxidant.	(Satpute et al., 2018)
3.	4-Hydroxybenzoic	C_7 H_6 O_3	Phenolic	Antioxidant, estrogenic, antimicrobial, antialgal, antimutagenic, antiestrogenic,	(Chaudhary et al., 2013;

	acid		compound	hypoglycemic and anti-inflammatory activities.	Chong et al., 2009)
4.	Quinic acid	$C_7 H_{12} O_6$	Phenolic compound	Antioxidant, antidiabetic, anticancer, antimicrobial, antiviral, anti-nociceptive and analgesic effects.	(Benali et al., 2024)
5.	Azelaic acid	$C_9 H_{16} O_4$	Fatty acid	Antibacterial, antineoplastic and antioxidant activity. Dermatologic drug.	(Kim et al., 2023)
6.	Miquelianin	$C_{21} H_{18} O_{13}$	Flavanol	Antioxidant and antidepressant activities.	(Kim et al., 2023)
7.	Quercetin-3β-D-glucoside	$C_{21} H_{20} O_{12}$	Flavonoid	Antineoplastic, antioxidant, antithrombotic, bone density conservation activity. Osteogenesis regulator, histamine antagonist, antipruritic drug and geroprotector.	(Kim et al., 2023)
8.	Genistein	$C_{15} H_{10} O_5$	Isoflavone	Antineoplastic agent, a tyrosine kinase inhibitor, DNA topoisomerase inhibitor, phytoestrogen and antioxidant activities.	(Kim et al., 2023)
9.	Apigenin	$C_{15} H_{10} O_5$	Flavone	Antineoplastic and chemo-preventive agent. Inhibits the expression of involucrin.	(Kim et al., 2023)
10.	Luteolin	$C_{15} H_{10} O_6$	Flavone	Antioxidant, anti-inflammatory, antineoplastic, nephroprotective, angiogenesis, anticancer, apoptosis inducer and immunomodulating activities..	(Kim et al., 2023)

11.	Caffeic acid	$C_9 H_8 O_4$	Phenolic compound	Glutathione transferase inhibitor, lipoxygenase inhibitor, histone deacetylase inhibitor, antioxidant, anti-inflammatory, anticancer and antineoplastic activities.	(Kim et al., 2023)
12.	Quercetin	$C_{15} H_{10} O_7$	Flavanol	Antibacterial, chemopreventive, anti-inflammatory, anti-allergy, anticancer, antioxidant, a protein kinase inhibitor, antineoplastic activities. A chelator, Aurora kinase inhibitor and geroprotector	(Kim et al., 2023)
13.	9,10-dihydroxy12Z-octadecenoic acid	$C_{18} H_{34} O_4$	Fatty acid	Cytotoxic activity.	(Christopher et al., 2023; Hildreth et al., 2020)
14.	Vanillic acid	$C_8 H_8 O_4$	Phenolic compound	Antioxidant, anti-inflammatory, immuno-stimulating, neuroprotective, hepatoprotective, cardioprotective and antiapoptotic activities.	(Sharma et al., 2020)
15.	3-Hydroxymandelic acid	$C_8 H_8 O_4$	Phenolic compound	Antioxidant properties.	(Parcheta et al., 2022)
16.	Isovanillic acid	$C_8 H_8 O_4$	Phenolic compound	Anti-inflammatory and antibacterial activity.	(Kim et al., 2023)
17.	Homogentisic acid	$C_8 H_8 O_4$	Phenolic compound	Antibacterial activity.	(Nguyen et al., 2018)
18.	Gentisic acid	$C_7 H_6 O_4$	Phenolic compound	Anti-inflammatory, antirheumatic and antioxidant properties.	(Kim et al., 2023)
19.	2,4- Dihydroxy	$C_7 H_6 O_4$	Flavonoid	Anticancer, antiparasite, antibacterial properties and oviposition stimulation in	(Riddick et al., 2019)

	benzoic acid			insects.	
20.	3,5- Dihydroxy benzoic acid	$C_7 H_6 O_4$	Phenolic compound	Antioxidant and antimicrobial activities.	(Kalinowska et al., 2021)
21.	2,3- Dihydroxy benzoic acid	$C_7 H_6 O_4$	Phenolic compound	Antiradical activity.	(Kalinowska et al., 2021)
22.	Rutin	$C_{27} H_{30} O_{16}$	Flavanol	Antioxidant and anti- inflammatory activities.	(Kim et al., 2023)
23.	Homovanillic acid	$C_9 H_{10} O_4$	Fatty acid	Anti-inflammatory agent and marker for metabolic stress.	(Putra et al., 2019)
24.	1,3,7-Trimethyl luricacid	$C_8 H_{10} N_4 O_3$	Alkaloid	Antioxidant activities.	(Bhat et al., 2001)
25.	3-Isopropylmalic acid	$C_7 H_{12} O_5$	Fatty acid	Antioxidant and antimicrobial activities.	(Ricciutelli et al., 2020)
26.	3,4- Dihydroxy phenylacetic acid	$C_8 H_8 O_4$	Phenolic compound	Antioxidant and cytotoxic activities.	(Samsonowicz et al., 2018; Tang et al., 2016)
27.	Myricetin 3-O-galactoside	$C_{21} H_{20} O_{13}$	Flavone	Antinociceptive and anti-inflammatory effects.	(Oliveira et al., 2015)
28.	3-O-Feruloyl quinic acid	$C_{17} H_{20} O_9$	Phenolic compound	Antioxidant activity.	(Liu et al., 2021)
29.	6-Gingerol	$C_{17} H_{26} O_4$	Phenolic compound	Antineoplastic, antioxidant and anti-inflammatory activity.	(Kim et al., 2023)
30.	Phellamurin	$C_{26} H_{30} O_{11}$	Flavonoid	Anti-tumor activity.	(Zhang et al., 2019)
31.	Myricitrin	$C_{21} H_{20} O_{12}$	Flavone	Anti-allergic agent.	(Kim et al., 2023)

32.	Linoleic Acid	$C_{18}H_{32}O_2$	Fatty acid	Anticancer and anti-obesity.	(Pariza et al., 1999)
33.	10-Nitrooleate	$C_{18}H_{33}NO_4$	Fatty acid	Antioxidant and anti-inflammatory actions.	(Narala et al., 2022)
34.	Naringoside	$C_{27}H_{32}O_{14}$	Flavonoid	Antiviral activity.	(Faisal et al., 2021)
35.	Oleic acid	$C_{18}H_{34}O_2$	Fatty acid	Anticancer, Used as an herbicide, insecticide and fungicide.	(Kim et al., 2023)
36.	Didemethoxy curcumin	$C_{19}H_{16}O_4$	Phenolic compound	Alpha-amylase inhibitor.	(Kim et al., 2023)
37.	5,7-dihydroxy-3-(4-hydroxyphenyl)- 4H chromen-4-one	$C_{15}H_{10}O_5$	Flavonoid	Anti-inflammatory activity.	(Gomes et al., 2012)
38.	Galangin	$C_{15}H_{10}O_5$	Flavanols	Growth inhibitor of breast tumor cells, antimicrobial agent, triacylglycerol lipase inhibitor.	(Kim et al., 2023)
39.	Emodin	$C_{15}H_{10}O_5$	Phenolic compound	Role as a tyrosine kinase inhibitor, an antineoplastic agent, anti-inflammatory, signalling, antibiotic, muscle building and anti-angiogenic functions.	(Kim et al., 2023)
40.	5-Hydroxy-6,7-dimethoxyflavone	$C_{17}H_{14}O_5$	Flavonoid	Antiviral activity.	(Wu et al., 2010)
41.	Apigetrin	$C_{21}H_{20}O_{10}$	Flavone	Anti-inflammatory and antibacterial activity.	(Kim et al., 2023)
42.	Kaempferol	$C_{15}H_{10}O_6$	Flavanol	Anti-inflammatory, antioxidant activity	(Kim et al., 2023)

				and geroprotector.	
43.	Fisetin	$C_{15}H_{10}O_6$	Flavonoid	Potential antioxidant, neuroprotective, anti-inflammatory, antineoplastic, anticancer, senolytic and longevity promoting activities.	(Kim et al., 2023)
44.	Cynaroside	$C_{21}H_{20}O_{11}$	Flavone	Antioxidant activity.	(Kim et al., 2023)
45.	Kuromanin	$C_{21}H_{20}O_{11}$	Flavonoids (Anthocyanin)	Antioxidant and neuroprotective activity.	(Winter et al., 2017)
46.	Andrographolide	$C_{20}H_{30}O_5$	Diterpene	Anti-HIV, anti-inflammatory and antineoplastic activities.	(Kim et al., 2023)
47.	Biochanin A	$C_{16}H_{12}O_5$	Isoflavones	Fatty acid amide hydrolase inhibitor, antidiabetic agents, a tyrosine kinase inhibitor and an antineoplastic agent.	(Kim et al., 2023)
48.	Glycitein	$C_{16}H_{12}O_5$	Isoflavone	Anticancer, anti-inflammatory, neuroprotective, protective effects against bone loss, cardiovascular diseases autoimmune diseases and estrogenic activities.	(Shimoda & Hamada, 2010)
49.	3,4-dimethoxy cinnamic acid	$C_{11}H_{12}O_4$	Phenolic compound	Antioxidant, anti-inflammatory and hypolipidemic activities.	(Panagiotis et al., 2023)
50.	5,7-dihydroxy-3-(4-methoxyphenyl)- 4H-chromen-4-one	$C_{16}H_{12}O_5$	Isoflavone	Anticarcinogenic, antidiabetic and antineoplastic properties.	(Kim et al., 2023)
51.	Wogonin	$C_{16}H_{12}O_5$	Flavone	Anti-inflammatory, cyclooxygenase 2 inhibitor, an antineoplastic and	(Kim et al., 2023)

				angiogenesis inhibitor activities.	
52.	Trifolin	$C_{21}H_{20}O_{11}$	Flavonoid	Antifungal agent.	(Kim et al., 2023)
53.	Pelargonidin	$C_{15}H_{10}O_5$	Flavonoid	Anticoagulant, antithrombotic activity, improving blood circulation.	(Ku et al., 2016)
54.	8-Hydroxyquinoline	C_9H_7NO	Phenolic compound	Antibacterial, antifungal and pesticide properties.	(Kim et al., 2023)
55.	Cyanidin	$C_{15}H_{10}O_6$	Flavonoids (Anthocyanin)	Neuroprotective agent and antioxidant activities.	(Kim et al., 2023)
56.	Robinetin	$C_{15}H_{10}O_7$	Flavone	Antifungal, antiviral, antibacterial, anti-mutagenesis and antioxidant activity.	(Birt et al., 1986)
57.	Arecoline	$C_8H_{13}NO_2$	Alkaloid	Anti-helminthic and anti-parasitic activity.	(Liu et al., 2016)
58.	Mulberrin	$C_{25}H_{26}O_6$	Flavone	Antioxidative and anti-inflammatory properties.	(Ye et al., 2022)
59.	Trigonelline	$C_7H_7NO_2$	Alkaloid	Hypoglycemic, hypolipidemic, neuroprotective, antimigraine, sedative, memory-improving, antibacterial, anti-diabetic, antiviral and anti-tumor activities.	(Zhou et al., 2012)
60.	Cafestol	$C_{20}H_{28}O_3$	Diterpene	Apoptosis inducer, a hypoglycemic agent, an angiogenesis inhibitor, an antineoplastic agent, an antioxidant and anti-inflammatory agent.	(Kim et al., 2023)
61.	Chlorogenic acid	$C_{16}H_{18}O_9$	Lignin.	Potential antioxidant, chemo preventive	(Kim et al., 2023)

				and carcinogenic inhibitor.	
62.	Reynoutrin	$C_{20}H_{18}O_{11}$	Flavonoid	Antioxidant and radical-scavenging activity.	(Kim et al., 2023)
63.	Psoralidin	$C_{20}H_{16}O_5$	Coumarin	Anticancer, anti-osteoporotic, anti-inflammatory, anti-vitiligo, antibacterial, antiviral, and antidepressant-like effects.	(Sharifi-Rad et al., 2020)
64.	Maackiain	$C_{16}H_{12}O_5$	Isoflavonoid	Antipyretic and anti-inflammatory effects.	(Huh et al., 2020)
65.	Esculetin	$C_9H_6O_4$	Coumarin	Antioxidant activity.	(Kim et al., 2023)
66.	7-hydroxy-6- methoxy -2H chromen-2-one	$C_{10}H_8O_4$	Coumarin	Antineoplastic, antidopaminergic, antioxidant, anti-inflammatory and anticholinesterase effects.	(Kim et al., 2023)
67.	Nootkatone	$C_{15}H_{22}O$	Sesquiterpenoid	Anti-inflammatory, anticancer, antibacterial, hepatoprotective, neuroprotective and cardioprotective. Insect repellent.	(Jha et al., 2022)
68.	18 alpha-Glycyrrhetic acid	$C_{30}H_{46}O_4$	Triterpenoid	Antiasthmatic, hepatoprotective, anticancer, nephroprotective, antidiabetic, antileishmanial, antiviral, antibacterial, anti-psoriasis, anti-osteoporosis, antiepileptic, antiarrhythmic and anti-inflammatory activities.	(Shinu et al., 2023)
69.	12-Oxo phytodienoic acid	$C_{18}H_{28}O_3$	Fatty acid (Intermediate in biosynthesis of jasmonic acid)	Defence responses, suppresses neuroinflammation and anti-inflammatory activities.	(Stelmach et al., 1998; Taki-Nakano et al., 2016)

70.	10- Hydroxy camptothecin	$C_{20}H_{16}N_2O_5$	Alkaloid	Anticancer bioactivity.	(Yuan-Gang et al., 2003)
71.	Palmitic Acid	$C_{16}H_{32}O_2$	Fatty acid	Anti-inflammatory, antioxidant, hypocholesterolemic, pesticide, nematicide, antiandrogenic, hemolytic, antipsychotic, antibacterial, antifungal and cytotoxic activities. 5-Alpha reductase inhibitor.	(Ortega & Campos, 2021)
72.	7- Hydroxy coumarin	$C_9H_6O_3$	Coumarin	Antidiarrheal, anti-bacterial and antiulcerogenic effects	(Cruz et al., 2020)
73.	4-Hydroxycoumarin	$C_9H_6O_3$	Coumarin	Antimicrobial activity and anti-coagulant activity.	(Završnik et al., 2008)
74.	Nicotinic acid	$C_6H_5NO_2$	Alkaloid	Antidote and antilipemic drug. A vasodilator agent, nicotinamidase inhibitor and antihyperlipidemic activity.	(Kim et al., 2023)
75.	Catechin	$C_{15}H_{14}O_6$	Flavonoid	Antioxidant activities.	(Kim et al., 2023)
76.	3-[4-methyl-1-(2-methyl propanoyl) - 3-oxocyclohexyl] butanoic acid	$C_{15}H_{24}O_4$	Monoterpene	Antiviral and antidiabetic properties.	(Kim et al., 2023)
77.	2- [(2R,4aR,8R,8aR)-8-hydroxy-4a,8-dimethyldecahydronaphthal en-2-yl] prop-2-enoic acid	$C_{15}H_{24}O_3$	Sesquiterpenoid	Anti-inflammatory activity.	(Kim et al., 2023)
78.	Cinchonidine	$C_{19}H_{22}N_2O$	Alkaloid	Anti-malarial, anti-obesity, anticancer, antioxidant, anti-inflammatory and	

				antimicrobial activity.	
79.	Pulegone	$C_{10}H_{16}O$	Monoterpene	Insecticidal, antifeedant, antiparasitical, pesticidal and insect repellent properties.	(Božović & Ragno, 2017)
80.	Reserpine	$C_{33}H_{40}N_2O_9$	Alkaloids	Antihypertensive and antipsychotic activities.	(Kim et al., 2023)
81.	Artemisinin	$C_{15}H_{22}O_5$	Sesquiterpenoid	Antimalarial activity.	(Kim et al., 2023)
82.	2(3H)-Furanone,4-bis [(4-hydroxy-3-methoxy phenyl)methyl]	$C_{20}H_{22}O_7$	Phenolic compound	Hepatoprotective, antileukemic and antifungal activity.	(Yao et al., 2021)
83.	Polygodial	$C_{15}H_{22}O_2$	Sesquiterpenoid	Antibacterial and antifungal activity. Antifeedant activity against aphids.	(Asakawa et al., 1988; Kubo et al., 2005)
84.	Resveratrol	$C_{14}H_{12}O_3$	Lignin	Phytoalexin, an oncogene inhibitor, a geroprotector, antioxidant and has potential chemopreventive activities.	(Kim et al., 2023)
85.	4- Methylumbelliferyl glucuronide	$C_{16}H_{16}O_9$	Coumarins	Anti-tumorigenic and antineoplastic agent.	(Kim et al., 2023)
86.	2-Hydroxycinnamic acid	$C_9H_8O_3$	Phenolic compound	Anti-inflammatory, antioxidant and antimicrobial activities.	(Sova & Saso, 2020)
87.	Ursolic acid	$C_{30}H_{48}O_3$	Triterpenoid	Anti-inflammatory, antioxidant, antiviral, anticancer, serum lipid-lowering and antineoplastic activities.	(Kim et al., 2023)
88.	Tectorigenin	$C_{16}H_{12}O_6$	Isoflavone	Anti-inflammatory and anticancer	(Kim et al., 2023)

				activity.	
89.	Diosmetin	$C_{16}H_{12}O_6$	Flavone	Anticancer, antimicrobial, antineoplastic, cardioprotective, antioxidant, oestrogenic and anti-inflammatory activities.	(Kim et al., 2023)
90.	Zerumbone	$C_{15}H_{22}O$	Sesquiterpenoid	Anti-inflammatory agent and a glioma-associated oncogene inhibitor.	(Kim et al., 2023)
91.	Monolaurin	$C_{15}H_{30}O_4$	Fatty acid	Antibacterial, antiviral, antifungal and immunomodulatory activity.	(Barker et al., 2019; Nitbani et al., 2022)

3.3.3. Other preliminary quantitative analysis

Estimation of chlorophyll content

Fresh samples of normal leaves, gall leaves and galls of *A. scholaris* and *F. racemosa* were examined for the estimation of amounts of chlorophyll a, chlorophyll b, total chlorophyll and carotenoids. The findings were displayed in Table 3.12. The graphical representation of the results of both plants was shown in Plate 3.32: Figure A and B, Plate 3.33: Figure A and B. The gall leaves were found to possess higher chlorophyll content *i.e.* chlorophyll a, chlorophyll b and total chlorophyll than normal leaves of *A. scholaris*. But gall showed much less amount of all pigments than normal leaf and gall leaf. The same observation was found in the case of *F. racemosa*. But carotenoid showed a different pattern, *i.e.* higher in normal leaf, followed by gall leaf and then gall. When it comes to *F. racemosa*, gall leaves appear to have the greatest amount of pigments compared to normal leaf and gall. It was discovered that gall leaves had significantly higher levels of carotenoids than normal leaves. All pigments are substantially less abundant in gall, however among them carotenoids were somewhat more abundant in *F. racemosa* gall than the other tissues.

From these analyses of chlorophyll pigment content of the two plants, it is clear that the gall leaves are rich in photosynthetic pigments compared to normal leaf and gall. Insect induced biochemical and physiological modifications lead to the development of gall, so they are meant for providing shelter and nutrition for the developing insects. Gall tissues were found to contain nutritive cells which act as nutritive sources for the insects. It is clear that gall are not photosynthetically efficient, they are completely developed for the protection and nourishment of the inducer itself. So, they may act as a sink for photoassimilates. Then the surrounding tissue of gall, *i.e.* gall leaves, acts as a source of these photosynthetic products. The chlorophyll content estimation report from these two plants also correlated with the above findings as gall leaf is a richer source of pigments than normal leaf and gall. Gall contained only a trace amount of photosynthetic pigments. Due to the presence of higher pigment content, gall leaves showed a high photosynthetic rate, as a result more sugars are synthesized in the gall leaves and they

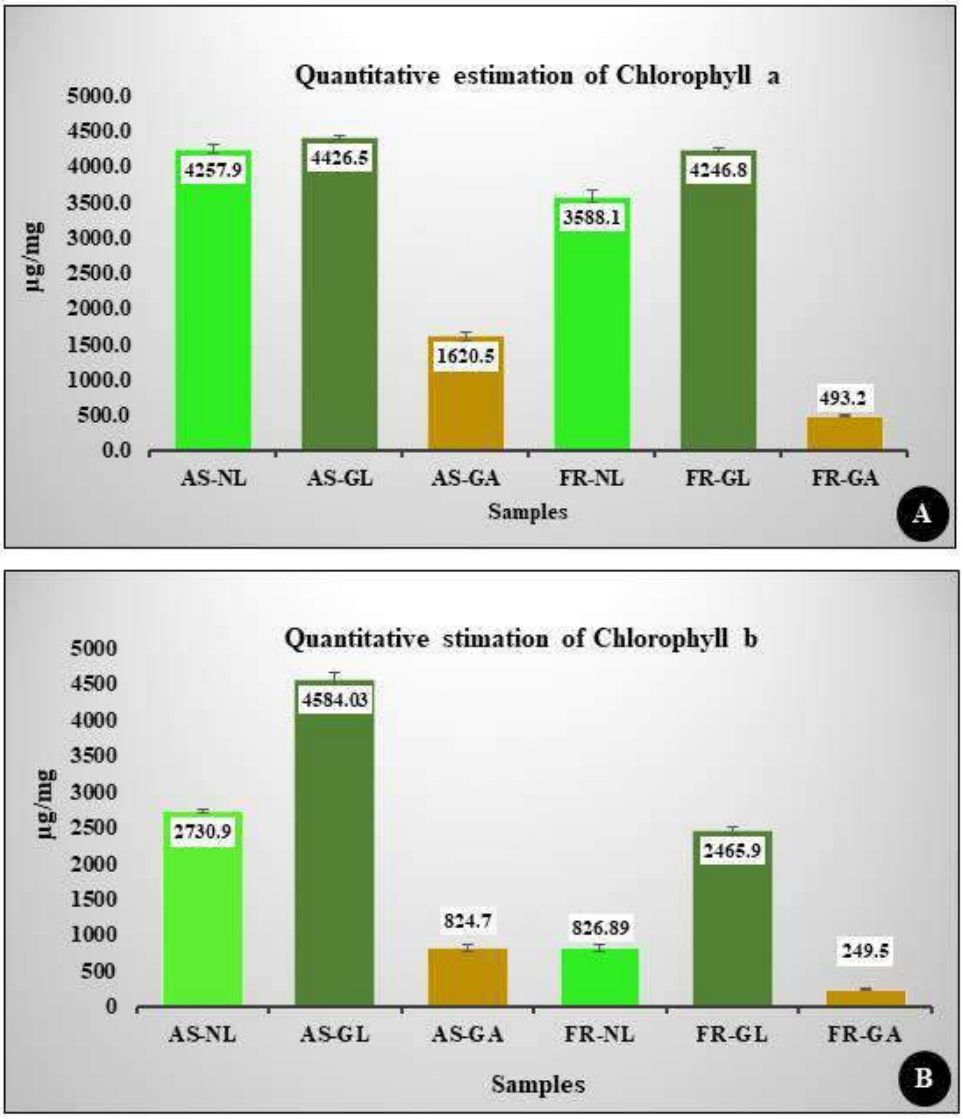
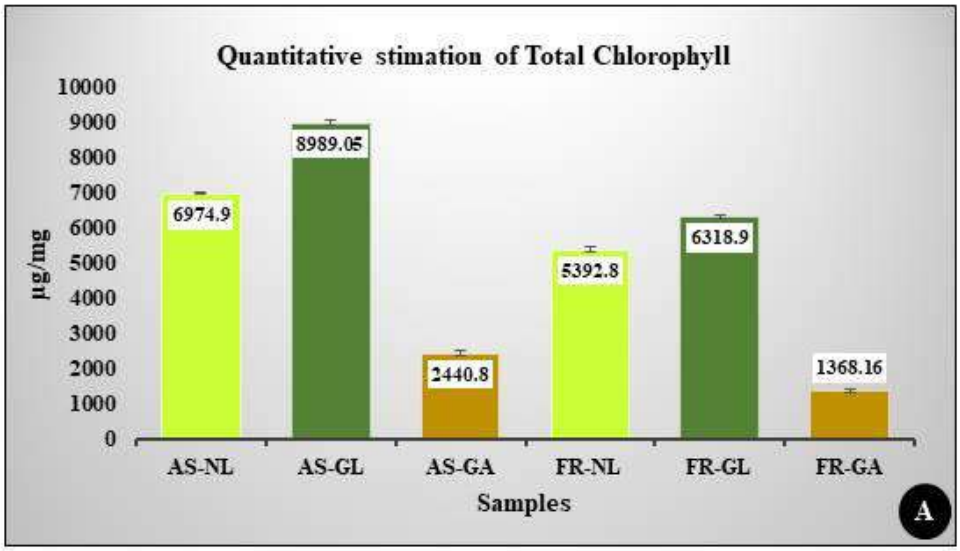


Plate 3.32: Figure A: Results of quantitative estimation of chlorophyll a in normal leaves, gall leaves and galls of *A. scholaris* and *F. racemosa* and Figure B: Results of quantitative estimation of chlorophyll b in normal leaves, gall leaves and leaf galls of *A. scholaris* and *F. racemosa.*



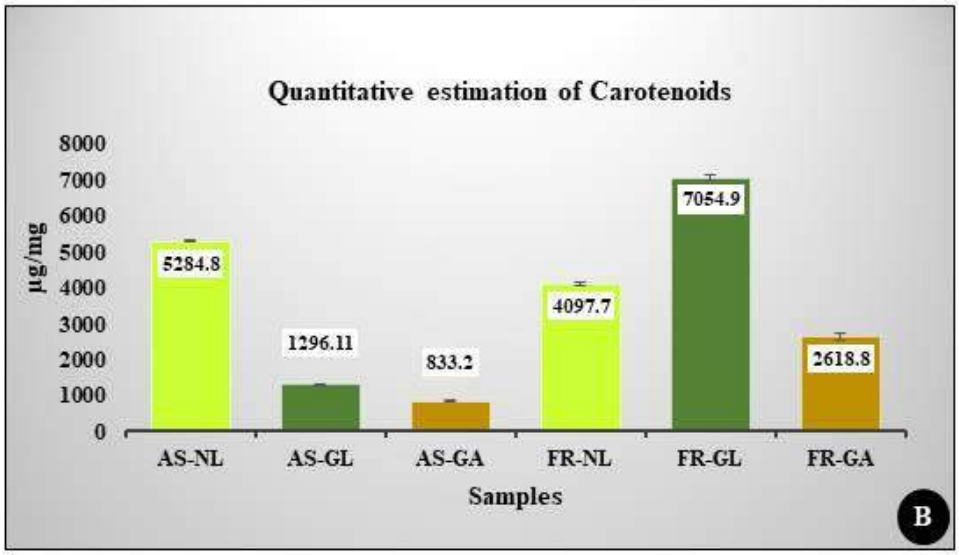


Plate 3.33: Figure A: Results of quantitative estimation of total chlorophyll in normal leaves, gall leaves and leaf galls of *A. scholaris* and *F. racemosa* and Figure B: Results of quantitative estimation of carotenoids in normal leaves, gall leaves and leaf galls of *A. scholaris* and *F. racemosa.*

accumulate in the gall. The adaptive manipulation of insects may be the reason behind increase in chlorophyll content in gall leaves compared to gall and normal leaves.

Several previous studies have demonstrated that the chlorophyll content of galls decreases as the galls mature (Albert et al., 2011; Biswas et al., 2014; Hossain et al., 2017). The net chlorophyll content of fresh ungalled *A. scholaris* leaves was much higher than that of galled leaves. Mesophyll redifferentiation and chloroplast disappearance are the causes of reduction of chlorophyll (Albert et al., 2011; Manna et al., 2018). Ivanova and colleagues in 2022 found that the loss of carotenoids and chlorophylls in the galled regions of the leaf surface caused the amount of pigment per leaf to decrease. The amounts of carotenoid and chlorophyll in the uninfected sections of infected leaves stayed consistent and were comparable to those seen in uninfected leaves of the same species. Thus, they found that a gall mite infection that lowers the chlorophyll content might result in a dramatic reduction in photosynthesis and productivity that is proportionate to the severity of the illness (Ivanova et al., 2022). Kot and colleagues also discovered that the presence of galls on *Quercus robur* infected by Cynipidae species caused a significant decrease of chlorophyll a, b and carotenoids contents (Kot et al., 2018). Huang and associates examined the chlorophyll biosynthesis of five different insect-induced galls made from the leaves of three host plants. They discovered that insect-induced gall may cause the infected leaf to produce fewer photosynthetic pigments, which lowers photosynthetic activity. This allows the plant system to manufacture more chemicals to protect the insects in the gall (Huang et al., 2014).

Table 3.12. Result of quantitative estimation of chlorophyll content in normal leaves, gall leaves and galls of *A. scholaris* and *F. racemosa.*

Plant	Sample	Chlorophyll a (µg/mg)	Chlorophyll b (µg/mg)	Total chlorophyll (µg/mg)	Carotenoids (µg/mg)
A. scholaris	Normal leaf	4257.9 ± 60.3	2730.9 ± 19.7	6974.9 ± 22.2	5284.8 ± 24.2
	Gall leaf	4426.5 ± 19.9	4584.03 ± 98.9	8989.05 ± 60.6	1296.11 ± 28.6
	Gall	1620.49 ± 58.2	824.7 ± 45.8	2440.8 ± 94.9	833.2 ± 28.98
F. racemosa	Normal leaf	3588.1 ± 87.82	826.89 ± 46.76	5392.8 ± 98.16	4097.7 ± 34.29
	Gall leaf	4246.79 ± 20.54	2465.9 ± 46.65	6318.9 ± 49.25	7054.9 ± 67.2
	Gall	493.15± 10.9	249.5 ± 10.6	1368.16 ± 60.4	2618.8 ± 86.2

Estimation of phenylalanine ammonia-lyase (PAL) enzyme activity

The results of PAL enzyme activity analysis in the normal leaves, gall leaves and galls of both plants are displayed in Table 3.13. The analysis of PAL enzyme activity showed a comparable pattern in both plants. The findings made it evident that galls had the highest level of enzyme activity, followed by gall leaves. Gall and gall-associated tissues exhibited higher levels

of enzyme activity than normal healthy tissues. In contrast to *A. scholaris*, the *F. racemosa* gall exhibited greater PAL enzyme activity.

19 Since PAL is a crucial enzyme in the phenylpropanoid pathway, there is a strong correlation between the quantity of phenolic compounds produced and the PAL enzyme activity. Thus, the previously mentioned result will clearly demonstrate the infection with gall inducers has been associated with the increased PAL enzyme activity. Gall will emerge as a result of the inducer attack and this reaction will trigger several defense mechanisms. Activating the production of defensive phytochemicals is one of those mechanisms. Thus, compared to normal tissues, gall and gall leaves have increased enzyme activity. Plate 3.34 displays the results of PAL enzyme activity analysis graphically.

Table 3.13. Result of quantitative estimation of PAL enzyme activity in normal leaves, gall leaves and galls of *A. scholaris* and *F. racemosa*.

Name of plant	Sample	Enzyme activity (unit/ mL)
A. scholaris	Normal leaf	0.02900 ± 0.0017
	Gall leaf	0.1724 ± 0.019
	Gall	0.3261 ± 0.00664
F. racemosa	Normal leaf	0.02295 ± 0.0019
	Gall leaf	0.4549 ± 0.0025
	Gall	0.5937 ± 0.0269

Medda and colleagues in 2020 demonstrated the correlation between phenolic chemicals and PAL enzyme. The PAL enzyme activity of myrtle (*Myrtus communis* L.) leaves and berries was measured. PAL enzyme was found to have a negative association with total polyphenols in berries, as well as a positive correlation with flavonoids and anthocyanins (Medda et al., 2020).

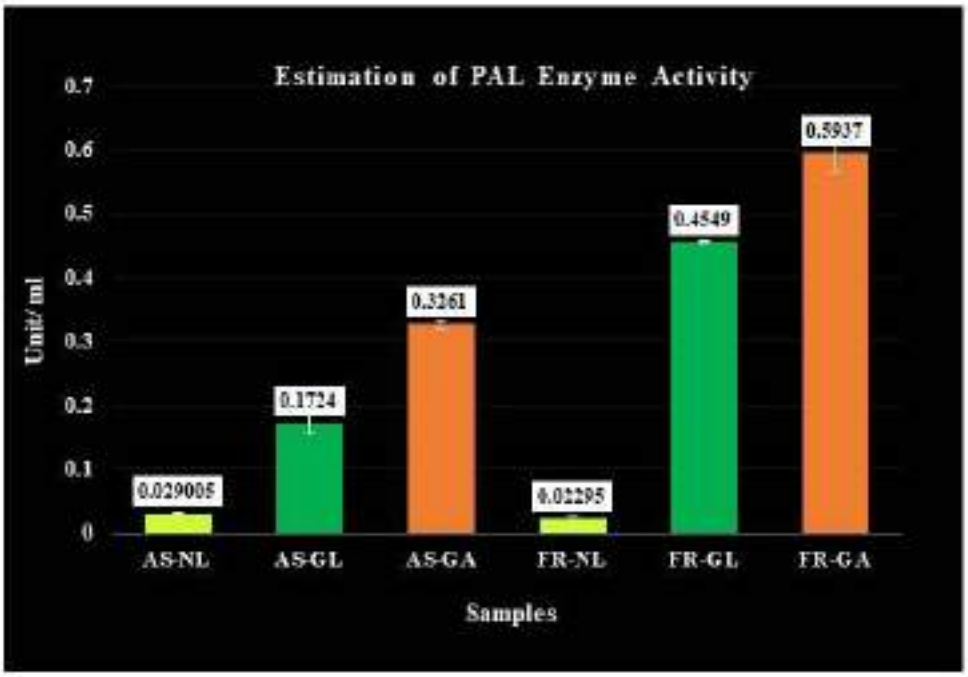


Plate 3.34: The bar graph represents quantitative estimation of PAL enzyme activity in three tissue types - normal leaves, gall leaves and leaf galls of *A. scholaris* and *F. racemosa*. Error bars represent the standard deviation of three replicates (n = 3).

The phenolic content of galls has been found to rise during gall formation. A strong relationship was discovered between the development of gall tissues and phenol production in *A. scholaris* (Biswas et al., 2014; Khurshid et al., 2021). Concentration of phenolic compounds was reported in higher quantities in *F. racemosa* galls compared to leaves (Dsouza & Ravishankar, 2014; Savitha et al., 2013). Our findings also support the previous results of higher PAL enzyme activity in gall and gall associated tissues.

In summary, this study demonstrates that gall formation significantly alters the phytochemical profile of *A. scholaris* and *F. racemosa,* with galled tissues exhibiting higher diversity and concentrations of secondary metabolites, particularly phenolic compounds. These changes likely serve as a defense mechanism against the gall-inducing insects, highlighting the intricate interplay between plant biochemistry and herbivore interactions.

3.4. CONCLUSIONS

In conclusion, this study investigated the impact of gall-inducing psyllids on *A. scholaris* and *F. racemosa,* two plants known to develop leaf galls. By examining the transverse sections, we can identify the alterations in anatomy generated due to gall formation and it was concluded that galls are totally deformed entities. In addition to serving as a source of nourishment, galls also provide a habitat for developing insects. The qualitative phytochemical examination of *A. scholaris* normal leaves, gall leaves and galls revealed that most of the secondary metabolic components including phenolics, alkaloids, flavonoids, saponins and coumarins, had been detected in gall extracts. The GC-MS analysis of methanolic extracts of both plants recognized different phytoconstituents of pharmacological importance. Additionally, it was found that gall and gall-associated tissues possess more phytochemical compounds than normal leaves and each of these compounds is expressed in high concentration in galls. The number of common compounds in normal leaves, gall leaves and galls were lower than the number of unique compounds in each sample.

OHR-LC-MS analysis discovered that compared to normal leaves, the phytochemical content of plant galls were distinct. More phytoconstituents were discovered in gall and gall leaves, and there was also substantial variation in the phenolic contents of gall. It was discovered gall tissues resulting from infection by inducing insects have a significant uniqueness. Based on these findings, it can be stated that gall were morphologically, anatomically and biochemically

distinct and unique structures, which have been produced due to insect-induced alteration in the tissues that they affect. These results also corroborate earlier research showing that infected tissues produce more phytochemical substances, particularly phenolics, as a defensive mechanism. The upcoming chapters will shed insight on the molecular basis of differential phytoconstituent expression as well as provide a better understanding of the gall inducer-host plant interaction.

This study provides a detailed examination of the anatomical and phytochemical alterations induced by gall formation in *A. scholaris* and *F. racemosa*. The results demonstrate that galls are not merely pathological structures but are sites of intense metabolic activity, leading to the accumulation of bioactive compounds with potential therapeutic applications. The findings support the hypothesis that gall induction enhances the production of secondary metabolites, thereby increasing the medicinal value of these plants.

CHAPTER 4 - BIOACTIVITY ANALYSIS OF NORMAL LEAVES, GALL LEAVES AND LEAF GALL EXTRACTS

4.3. RESULTS AND DISCUSSION

4.3.1. Results of antioxidant activity analysis

Reducing power method:

The reducing power of aqueous, hexane and ethanolic extracts from normal leaves, gall leaves and galls of *F. racemosa* and *A. scholaris* was analyzed to assess their antioxidant activity. The reducing power assay measures the ability of extracts to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), with higher absorbance at 700 nm indicating greater antioxidant potential. Ascorbic acid was used as the positive control, tested at five concentrations (20, 40, 60, 80, 100 µg/mL). Results of reducing power analysis of 100 µg/mL aqueous, hexane and ethanolic extracts of *A. scholaris* normal leaves (AS-NL), *A. scholaris* gall leaves (AS-GL) and *A. scholaris* galls (AS-GA) are shown in Plate 4.1: Figure A-I. The photographs of the results of reducing power method of different extracts of *F. racemosa* normal leaves (FR-NL), *F. racemosa* gall leaves (FR-GL) and *F. racemosa* galls (FR-GA) are shown in Plate 4.2: Figure A-I.

Reducing power activity of aqueous extracts

Plate 4.3: Figure A is the graphical representation of the dose-dependent reducing power of aqueous extracts for both plants, highlighting superior activity of AS-GL. Table 4.1 shows the percentage radicle scavenging activity of aqueous extracts of both *A. scholaris* and *F. racemosa.* Among aqueous extracts, FR-GL and AS-GL exhibited higher reducing power than normal leaf and gall extracts. Specifically, AS-GL at 100 µg/mL showed the highest activity among all aqueous extracts. *A. scholaris* extracts generally outperformed *F. racemosa* extracts in aqueous solvent, with AS-NL, AS-GL and AS-GA also showing moderate activity. In contrast, FR-NL and FR-GA had low reducing power, each below 25% at 100 µg/mL, while FR-GL exhibited the highest activity among *F. racemosa* samples (25.4 ± 0.85%). The %RSA of *A. scholaris* and *F. racemosa* gall and gall leaves tissues were significant at the level p value < 0.05.



Plate 4.1: Results of reducing power analysis of *A. scholaris* extracts (100 µg/ml). Figure A-C: shows the reducing power analysis result of aqueous extracts of AS-NL, AS-GL and AS-GA, Figure D-F: represents the result of hexane extracts of AS-NL, AS-GL and AS-GA and Figure G-I: shows the result of ethanol extracts of AS-NL, AS-GL and AS-GA.

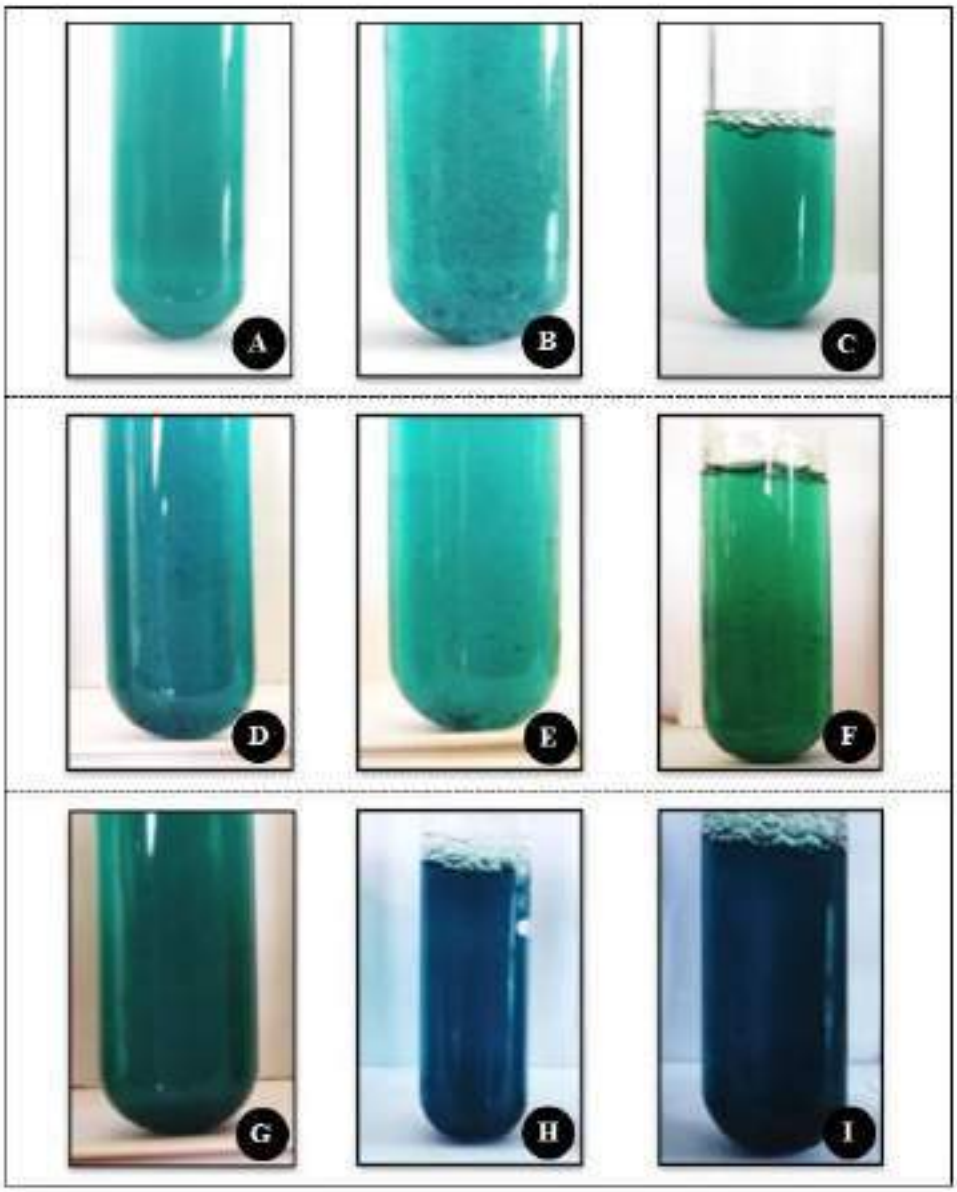


Plate 4.2: Results of reducing power analysis of *F. racemosa* extracts (100 µg/ml). Figure A-C: shows the reducing power of aqueous extracts of FR-NL, FR-GL and FR-GA; Figure D-F: shows reducing power activity of hexane extracts of FR-NL, FR-GL and FR-GA and Figure G-I: shows the result of ethanol extracts of FR-NL, FR-GL and FR-GA.

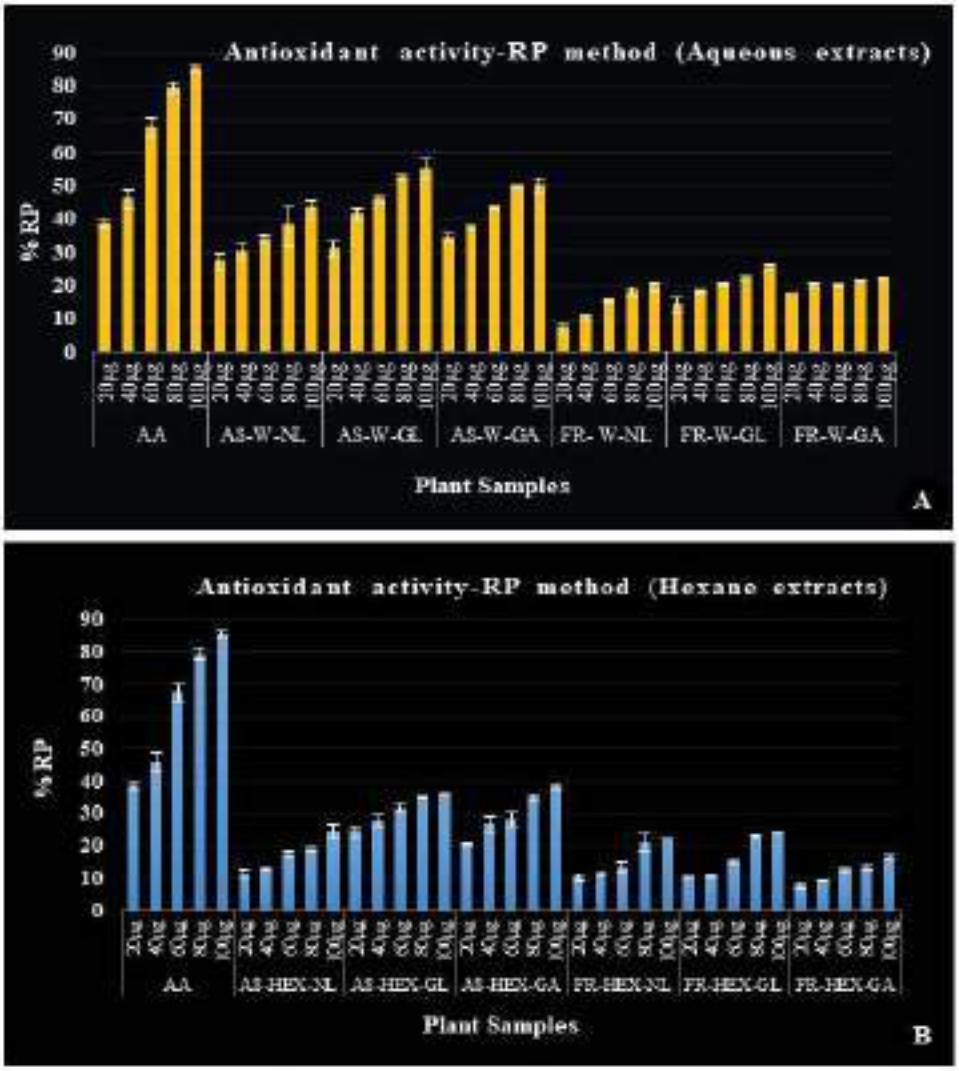


Plate 4.3: Figure A: Graphical representation of antioxidant activity analysis results of aqueous extracts of AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA using reducing power method. Ascorbic acid taken as positive control. Each bar represents reducing power of the sample with SD and Figure B: Graphical representation of results of antioxidant activity analysis of hexane extracts of AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA using RP method. Ascorbic acid taken as positive control. Each bar represents reducing power of the sample with SD.

Table 4.1: The percentage radicle scavenging activity of aqueous extracts of different samples of *A. scholaris* and *F. racemosa* determined using RP method.

Sl. No	Name of sample (Aqueous extracts)	Dilutions of sample used (µg/mL)	%RSA
1.	AS-NL	20	$27 \pm 2.40^{+}$
2.		40	$30.25 \pm 1.91^{+}$
3.		60	$33.85 \pm 1.48^{+}$
4.		80	$37.8 \pm 5.94^{+}$
5.		100	$43 \pm 2.69^{+}$
6.	AS-GL	20	$30.85 \pm 2.26^{*}$
7.		40	$41.45 \pm 1.56^{*}$
8.		60	$46.1 \pm 0.92^{*}$
9.		80	$52.6 \pm 0.78^{*}$
10.		100	$55.0 \pm 3.25^{*}$
11.	AS-GA	20	$34.35 \pm 1.20^{*}$
12.		40	$37.5 \pm 0.64^{*}$
13.		60	$43.5 \pm 0.14^{*}$
14.		80	$49.6 \pm 0.57^{*}$
15.		100	$50.0 \pm 1.98^{*}$
16.		20	$6.9 \pm 1.41^{++}$

17.	FR-NL	40	$10.5 \pm 0.99^{++}$
18.		60	$15.6 \pm 0.42^{++}$
19.		80	$17.9 \pm 1.13^{++}$
20.		100	$19.65 \pm 1.20^{++}$
21.	FR-GL	20	$14.25 \pm 2.33^{++*}$
22.		40	$18.25 \pm 0.21^{++*}$
23.		60	$20.35 \pm 0.35^{++*}$
24.		80	$21.93 \pm 0.89^{++*}$
25.		100	$25.4 \pm 0.85^{++*}$
26.	FR-GA	20	$17.35 \pm 0.21^{++*}$
27.		40	$19.95 \pm 0.49^{++*}$
28.		60	$20.0 \pm 0.21^{++*}$
29.		80	$21.2 \pm 0.21^{++*}$
30.		100	$22.13 \pm 0.49^{++*}$
31.	Ascorbic acid	20	38.6 ± 1.13
32.		40	45.97 ± 2.92
33.		60	67.51 ± 2.67
34.		80	79.24 ± 1.64
35.		100	85.48 ± 0.97

Each value of % RSA represents mean ± standard deviation (n = 3). ++ indicates values significantly different from ascorbic acid (control) at the corresponding concentration at the level p < 0.01. + indicates values significantly different from ascorbic acid (control) at the corresponding concentration p-value < 0.05). *indicates values significantly different from normal leaf extract of each plant at the corresponding concentration (p < 0.05).

Reducing power activity of hexane extracts

Plate 4.3: Figure B illustrates the reducing power of hexane extracts, emphasizing superior performance of *A. scholaris.* The results of antioxidant activity of different samples of *A. scholaris* and *F. racemosa* hexane extracts shown in Table 4.2. Hexane extracts of normal leaves, gall leaves and galls were less active than aqueous extracts overall, but *A. scholaris* samples consistently showed higher reducing power than *F. racemosa* samples. The plant samples such as AS-GL and AS-GA exhibited significantly higher reducing power when compared with AS-NL (p-value < 0.01), with AS-GA at 100 µg/mL demonstrating the highest activity (38.25 ± 0.78%) among hexane extracts. For *F. racemosa*, no clear pattern emerged. FR-GL at 100 µg/mL showed the highest activity among *F. racemosa* samples, same as observed in aqueous extracts, followed by FR-NL, while FR-GA had the lowest reducing power, possibly due to lower solubility of active phenolic compounds in hexane.

Table 4.2: The percentage radicle scavenging activity of hexane extracts of different samples of *A. scholaris* and *F. racemosa* determined using RP method.

Sl. No	Name of sample (Hexane extracts)	Dilutions of sample used (µg/mL)	%RSA
1.	AS-NL	20	$11.5 \pm 1.13^{++}$
2.		40	$12.7 \pm 0.28^{++}$
3.		60	$17.55 \pm 1.06^{++}$
4.		80	$19.15 \pm 0.64^{++}$

5.		100	$24.4 \pm 2.12^{++}$
6.	AS-GL	20	$24.6 \pm 1.70^{+**}$
7.		40	$27.8 \pm 1.56^{+**}$
8.		60	$31.75 \pm 1.34^{+**}$
9.		80	$34.95 \pm 0.35^{+**}$
10.		100	$35.9 \pm 0.28^{+**}$
11.	AS-GA	20	$20.5 \pm 0.14^{+**}$
12.		40	$26.65 \pm 2.47^{+**}$
13.		60	$28.2 \pm 2.26^{+**}$
14.		80	$35.15 \pm 0.64^{+**}$
15.		100	$38.25 \pm 0.78^{+**}$
16.	FR-NL	20	$10.33 \pm 0.95^{++}$
17.		40	$11.45 \pm 0.64^{++}$
18.		60	$13.4 \pm 1.70^{++}$
19.		80	$21.09 \pm 2.52^{++}$
20.		100	$22.28 \pm 0.11^{++}$
21.	FR-GL	20	$10.1 \pm 0.42^{++*}$
22.		40	$10.59 \pm 0.55^{++*}$
23.		60	$15.08 \pm 0.53^{++*}$
24.		80	$22.65 \pm 0.35^{++*}$

25.		100	$24.35 \pm 0.06^{++*}$
26.	FR-GA	20	$7.8 \pm 0.78^{++}$
27.		40	$9.24 \pm 0.06^{++}$
28.		60	$12.6 \pm 0.42^{++}$
29.		80	$13.25 \pm 0.64^{++}$
30.		100	$16.59 \pm 0.87^{++}$
31.	Ascorbic acid	20	38.6 ± 1.13
32.		40	45.97 ± 2.92
33.		60	67.51 ± 2.67
34.		80	79.24 ± 1.64
35.		100	85.48 ± 0.97

Each value of %RSA represents mean ± standard deviation (n = 3). ++ indicates values significantly different from ascorbic acid (control) at the corresponding concentration at the level $p < 0.01$. + indicates values significantly different from ascorbic acid (control) at the corresponding concentration p-value <0.05). ** indicates values significantly different from normal leaves of each plant at the corresponding concentration ($p < 0.01$). *indicates values significantly different from normal leaf extract of each plant at the corresponding concentration ($p < 0.05$).

Reducing power activity of ethanolic extracts

The results of antioxidant activity of different samples of *A. scholaris* and *F. racemosa* ethanolic extracts shown in Table 4.3. Plate 4.4: Figure A graphically represents the reducing power of ethanolic extracts, highlighting the dominance of FR-GL and AS-GA. Ethanolic extracts demonstrated the highest reducing power across all other solvents, reflecting the efficacy

of ethanol in extracting bioactive phenolic compounds. FR-GA and FR-GL exhibited the highest activities, with percentage radical scavenging activity of 60.49% and 51.77% at 100 µg/mL, respectively, significantly outperforming FR-NL ($p < 0.05$). A similar trend was observed in AS-GA at 100 µg/mL showed the highest reducing power (54.41%), followed by AS-GL (36.16%), which was notably lower than AS-GA but still higher than AS-NL. When comparing all extracts, ethanolic extracts had the highest reducing power, followed by aqueous extracts, while hexane extracts showed the lowest activity.

Table 4.3: The percentage radicle scavenging activity of ethanolic extracts of different samples of *A. scholaris* and *F. racemosa* evaluated through RP method.

Sl. No	Name of sample (Ethanolic extracts)	Dilutions of sample used (µg/mL)	%RSA
1.	AS-NL	20	$19.55 \pm 1.77^{++}$
2.		40	$22.8 \pm 0.99^{++}$
3.		60	$24.2 \pm 0.13^{++}$
4.		80	$25.51 \pm 1.12^{++}$
5.		100	$27.35 \pm 0.64^{++}$
6.	AS-GL	20	$20.2 \pm 2.27+^{*}$
7.		40	$25.94 \pm 1.47^{+*}$
8.		60	$27.3 \pm 2.40^{+*}$
9.		80	$31.59 \pm 0.55^{+*}$
10.		100	$36.16 \pm 2.62^{+*}$
11.	AS-GA	20	$26.32 \pm 4.83^{*}$
12.		40	$27.93 \pm 0.60^{*}$

13.		60	33.88 ± 2.79*
14.		80	46.3 ± 3.96*
15.		100	54.41 ± 4.40*
16.	FR-NL	20	14.69 ± 1.12++
17.		40	15.5 ± 0.14++
18.		60	20.54 ± 0.06++
19.		80	27.12 ± 0.62++
20.		100	32.72 ± 2.59++
21.	FR-GL	20	21.47± 0.04+*
22.		40	30.80 ± 1.20+*
23.		60	40.61 ± 1.55+*
24.		80	47.21 ± 0.21+*
25.		100	51.77 ± 1.70+*
26.	FR-GA	20	22.76 ± 1.12*
27.		40	29.64 ± 3.22*
28.		60	34.79 ± 1.21*
29.		80	45.69 ± 1.44*
30.		100	60.49 ± 2.56*
31.	Ascorbic acid	20	38.6 ± 1.13
32.		40	45.97 ± 2.92

33.		60	67.51 ± 2.67
34.		80	79.24 ± 1.64
35.		100	85.48 ± 0.97

Each value of %RSA represents mean ± standard deviation (n = 3). + indicates values significantly different ($p < 0.05$) from ascorbic acid (control) at the corresponding concentration. ++ indicates values significantly different from ascorbic acid (control) at the corresponding concentration ($p < 0.01$).* indicates values significantly different from normal leaves of each plant at the corresponding concentration ($p < 0.05$).

Evaluation of antioxidant potency based on IC_{50} values

To quantify antioxidant potency, IC_{50} values (the concentration required to achieve 50% reducing power) were calculated (Table 4.4 and Plate 4.4: Figure B). Ethanolic extracts of FR-GL and FR-GA exhibited the lowest IC_{50} values (78.8 ± 2.53 µg/mL and 66.4 ± 1.13 µg/mL, respectively), indicating high potency. For *A. scholaris*, the ethanolic extract of galls had the lowest IC_{50} (88.2 ± 8.33 µg/mL), followed by AS-GL (175.9 ± 0.27 µg/mL). Aqueous extracts showed moderate IC_{50} values (e.g., AS-GL: 99 ± 5.66 µg/mL), while hexane extracts had the highest IC_{50} values (e.g., AS-NL: 269.6 ± 6.05 µg/mL), reflecting lower potency. Ascorbic acid, the positive control, had an IC_{50} of 39 ± 0.95 µg/mL, serving as a benchmark for comparison.

ANOVA confirmed significant differences in %RSA among normal leaf extract to gall leaf and gall extracts of *A. scholaris* and *F. racemosa*. Then Tukey's HSD tests were done to find the significant levels of each sample. Except aqueous extract of *A. scholaris* ($p < 0.05$), all other extracts of *A. scholaris* showed significance at $p < 0.01$ against normal leaf extracts. In the case of *F. racemosa*, except ethanolic extract, all other extracts showed $p < 0.01$ against FR-NL. When the ascorbic acid (standard) at the corresponding concentration tested for significance against different samples and found that except AS-GL and AS-GA (aqueous extracts) and ethanolic extract of AS-GA, all other extracts showed significance at the level $p < 0.05$. In the case of *F. racemosa*, all other samples and extracts, except hexane exhibited $p < 0.05$. According to Tukey's HSD tests, all of the *F. racemosa* extracts were noticeably more effective than those

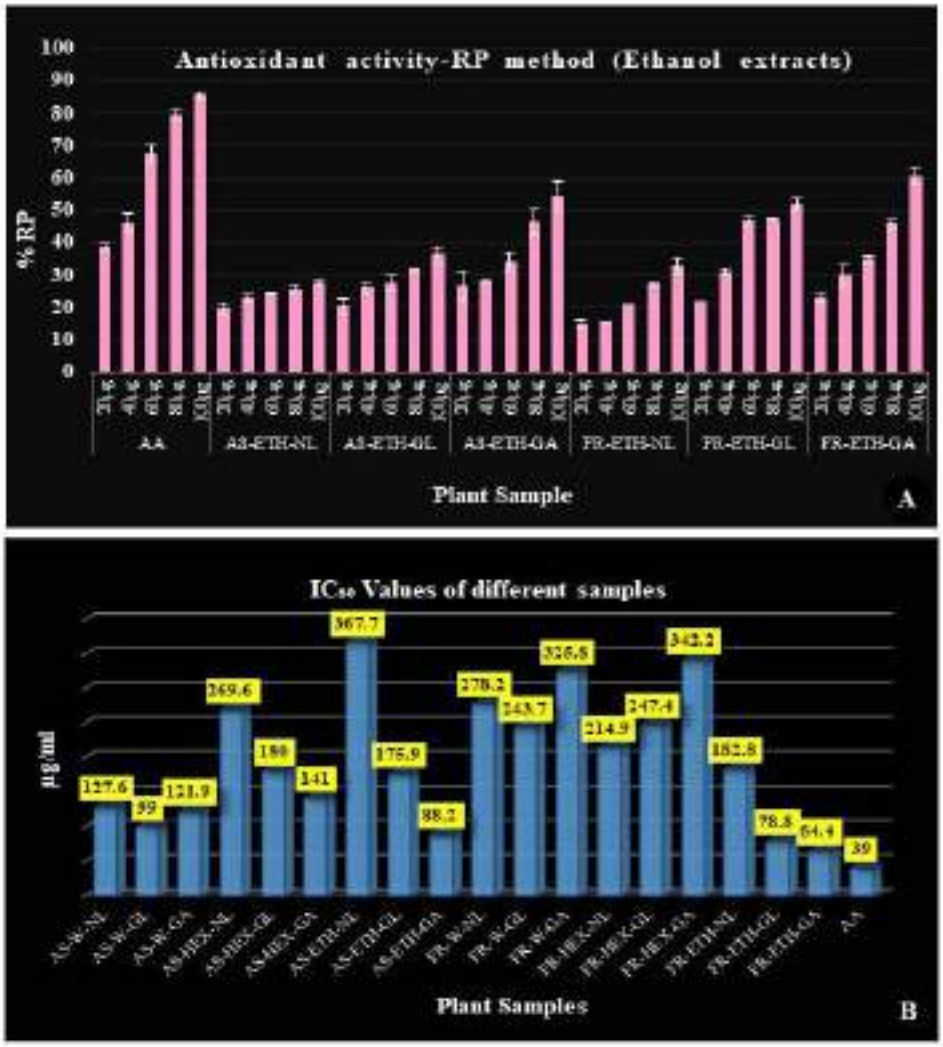


Plate 4.4 : Figure A: Graphical representation of results of antioxidant activity analysis of ethanolic extracts of AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA using RP method. Ascorbic acid taken as positive control. Each bar represents reducing power of the sample with standard deviation and Figure B: IC_{50} values obtained by RP assay using aqueous, hexane and ethanolic extracts of different samples of *A. scholaris* and *F. racemosa*.

of *A. scholaris* ($p < 0.05$). The greater antioxidant capacity of galls compared to normal tissues is indicated by the higher IC_{50} values of normal leaf and gall leaf extracts across *A. scholaris*. In *F. racemosa*, the gall-leaf extract is more potent than the normal leaf and gall extract, as indicated by its lower IC_{50} values.

Table 4.4: IC_{50} values of *A. scholaris* and *F. racemosa* extracts of normal leaves, gall leaves and leaf galls analysed through RP method

Sl. No	Plant sample	IC_{50} (µg/mL)
1.	AS-NL (Aqueous)	127.6 ± 5.87
2.	AS-GL (Aqueous)	99 ± 5.66
3.	AS-GA (Aqueous)	121.9 ± 3.67
4.	AS-NL (Hexane)	269.6 ± 6.05
5.	AS-GL (Hexane)	180 ± 2.29
6.	AS-GA (Hexane)	141 ± 1.08
7.	AS-NL (Ethanol)	367.7 ± 8.33
8.	AS-GL (Ethanol)	175.9 ± 0.27
9.	AS-GA (Ethanol)	88.2 ± 8.33
10.	FR-NL (Aqueous)	278.2 ± 3.37
11.	FR-GL (Aqueous)	243.7 ± 3.53
12.	FR-GA (Aqueous)	325.8 ± 1.97
13.	FR-NL (Hexane)	214.9 ± 8.94
14.	FR-GL (Hexane)	247.4 ± 0.80
15.	FR-GA (Hexane)	342.2 ± 8.38

16.	FR-NL (Ethanol)	182.8 ± 7.67
17.	FR-GL (Ethanol)	78.8 ± 2.53
18.	FR-GA (Ethanol)	66.4 ± 1.13
19.	Ascorbic acid (AA)	39 ± 0.95

Each value represents mean ± Standard deviation (n = 3)

Results of DPPH radical scavenging assay:

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay evaluated the antioxidant activity of aqueous, hexane and ethanolic extracts of normal leaves, gall leaves and galls from *A. scholaris* and *F. racemosa*. The assay measures the ability of extracts to neutralize DPPH radicals, with percentage radical scavenging activity (%RSA) calculated. Results are presented below by solvent type, comparing AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA. Plate 4.5 showing the results of DPPH radical scavenging activity of ethanol, aqueous and hexane extracts of AS-NL, AS-GL and AS-GA in different dilutions of extracts (0.25, 0.5, 0.75 and 1 mg/ml). Plate 4.6 showing the radical scavenging activity of ethanol, aqueous and hexane extracts of FR-NL, FR-GL and FR-GA in different dilutions of extracts.

DPPH radical scavenging activity of aqueous extracts

Table 4.5 shows the radical scavenging activity percentage exhibited by different samples of *A. scholaris* and *F. racemosa* aqueous extracts. Plate 4.7: Figure A illustrates the dose-dependent %RSA of aqueous extracts of both plants, with error bars representing standard deviations. Aqueous extracts of *A. scholaris* showed higher %RSA than *F. racemosa* across all samples. For AS-GL and AS-GA at 1 mg/mL exhibited comparable and high %RSA (42.8% and 43.7%, respectively), significantly outperforming AS-NL (13.18%, $p < 0.01$). In contrast, *F. racemosa* aqueous extracts displayed low %RSA, with FR-GA at 1 mg/mL achieving the highest activity (30.03%), followed by FR-NL (19.54%) and FR-GL (13.03%). The superior performance of *A. scholaris* aqueous extracts suggests greater water solubility of active compounds, likely phenolics in *A. scholaris* compared to *F. racemosa*.

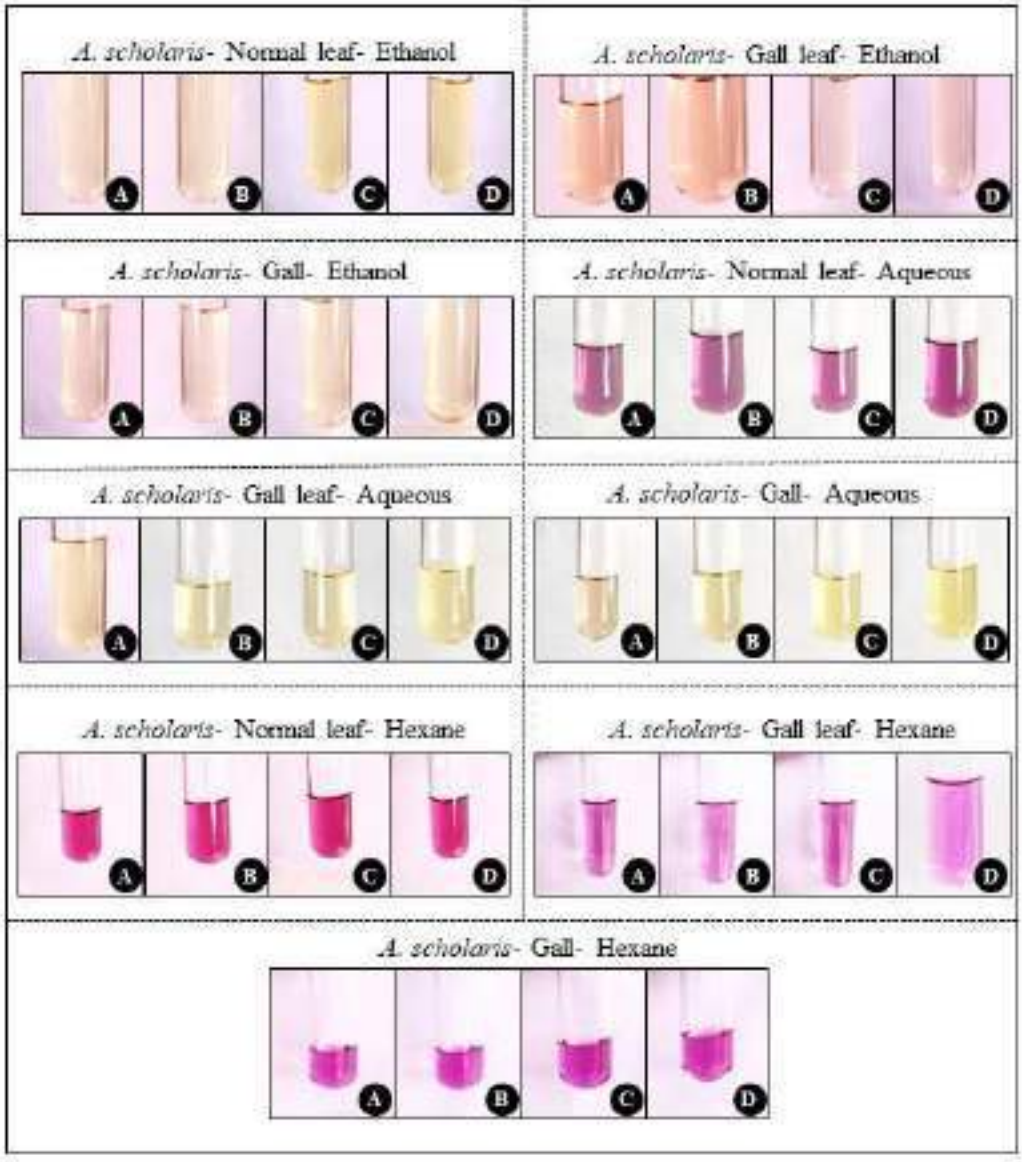


Plate 4.5: Photographs showing the results of DPPH radical scavenging activity of ethanol, aqueous and hexane extracts of *A. scholaris* -normal leaf, gall leaf and gall in different dilutions of extracts (A: 2.5 µL, B: 5 µL, C: 7.5 µL and D: 10 µL).

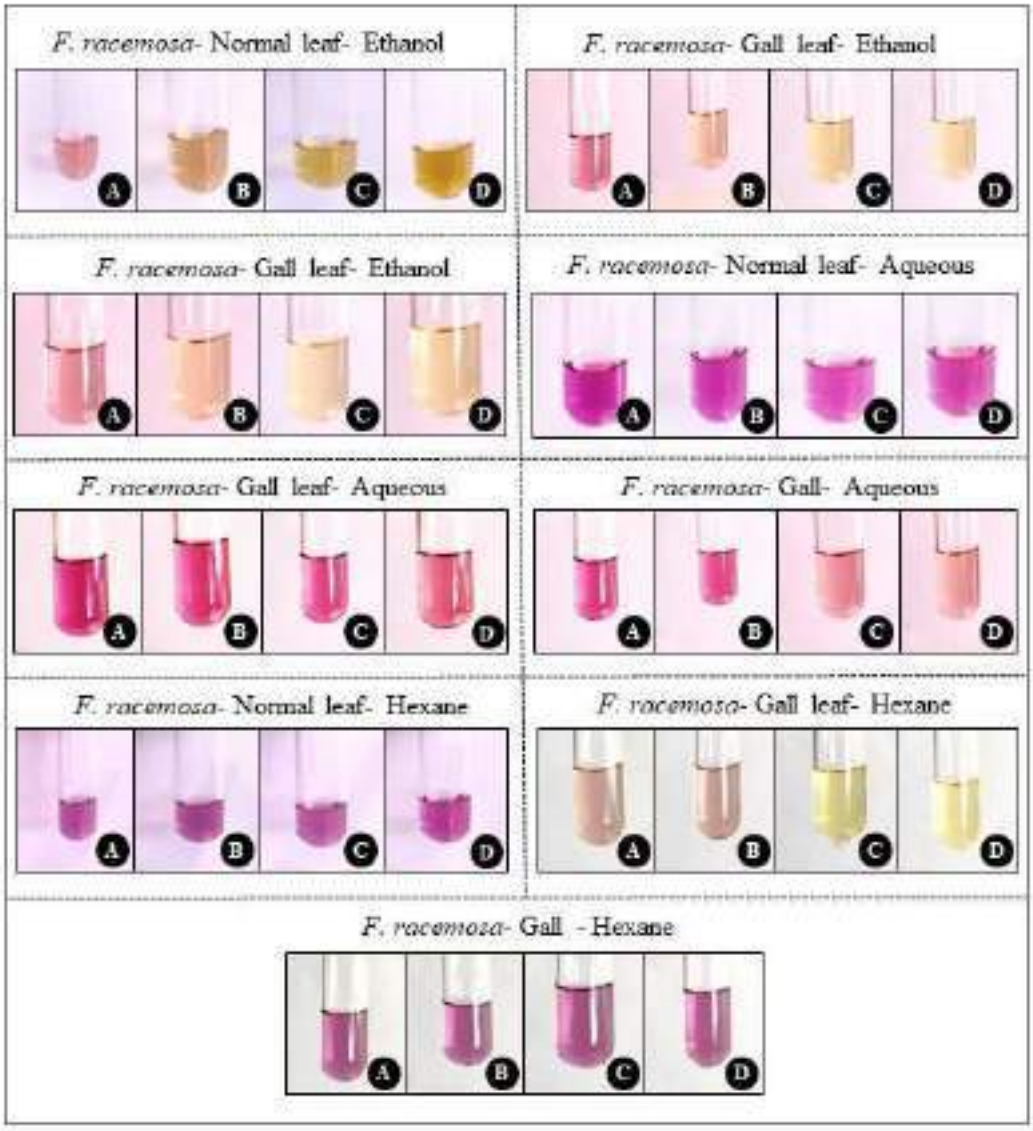


Plate 4.6: Photographs showing the results of DPPH radical scavenging activity of ethanol, aqueous and hexane extracts of *F. racemosa*- normal leaf, gall leaf and gall in different dilutions of extracts (A: 2.5 µL, B: 5 µL, C: 7.5 µL and D: 10 µL).

Table 4.5: Radical scavenging activity percentage (%RSA) of aqueous extracts of different samples of *A. scholaris* and *F. racemosa* compared with Ascorbic acid (standard) at different concentrations through the DPPH method.

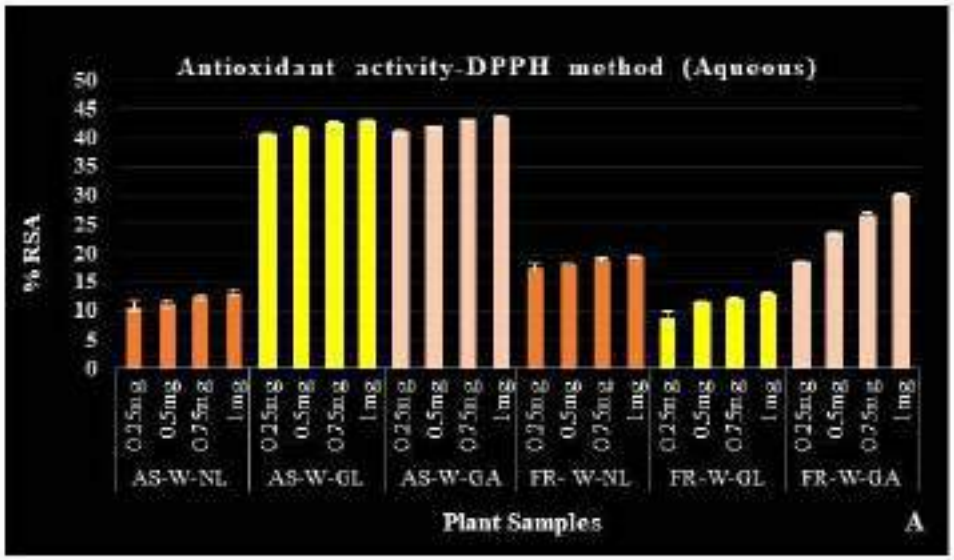
Sl. No	Name of sample (Aqueous extract)	Dilutions of sample used (mg/mL)	%RSA
1.	AS-NL	0.25	$10.82 \pm 0.88^{++}$
2.		0.5	$11.27 \pm 0.71^{++}$
3.		0.75	$12.52 \pm 0.38^{++}$
4.		1	$13.18 \pm 0.52^{++}$
5.	AS-GL	0.25	$40.62 \pm 0.28^{++**}$
6.		0.5	$41.60 \pm 0.33^{++**}$
7.		0.75	$42.54 \pm 0.19^{++**}$
8.		1	$42.82 \pm 0.37^{++**}$
9.	AS-GA	0.25	$41.07 \pm 0.44^{**}$
10.		0.5	$41.87 \pm 0.11^{**}$
11.		0.75	$43.02 \pm 0.07^{**}$
12.		1	$43.75 \pm 0.34^{**}$
13.	FR-NL	0.25	$17.61 \pm 0.45^{++}$
14.		0.5	$18.12 \pm 0.10^{++}$
15.		0.75	$18.99 \pm 0.25^{++}$
16.		1	$19.54 \pm 0.10^{++}$
17.	FR-GL	0.25	$8.91 \pm 1.20^{++**}$

18.		0.5	$11.39 \pm 0.31^{++**}$
19.		0.75	$12.01 \pm 0.40^{++**}$
20.		1	$13.03 \pm 0.30^{++**}$
21.	FR-GA	0.25	$18.45 \pm 0.23^{++*}$
22.		0.5	$23.25 \pm 0.41^{++*}$
23.		0.75	$26.64 \pm 0.42^{++*}$
24.		1	$30.03 \pm 0.42^{++*}$
25.	Ascorbic acid	0.025	47.70 ± 0.03
26.		0.05	47.84 ± 0.06
27.		0.075	47.95 ± 0.07
28.		0.1	48.21 ± 0.06

Each value of %RSA represents mean ± standard deviation (n = 3). ++ indicates values significantly different from ascorbic acid (control) at the corresponding concentration ($p < 0.01$). * indicates values significantly different from normal leaves of each plant at the corresponding concentration ($p < 0.05$). ** indicates values significantly different from the normal leaf of each plant at the corresponding concentration ($p < 0.01$).

DPPH radical scavenging activity of hexane extracts

The results of antioxidant activity of different samples of *A. scholaris* and *F. racemosa* hexane extracts determined using DPPH assay is shown in Table 4.6. Plate 4.7: Figure B represents the %RSA of hexane extracts, highlighting superior activity of FR-GA. Hexane extracts of *F. racemosa* generally outperformed *A. scholaris*, with FR-GA at 1 mg/mL showing the highest %RSA (37.18%), followed by FR-GL (35.13%) and FR-NL (29.54%). For *A. scholaris*, a sequential increase in %RSA was observed from AS-NL (18.38%) to AS-GL



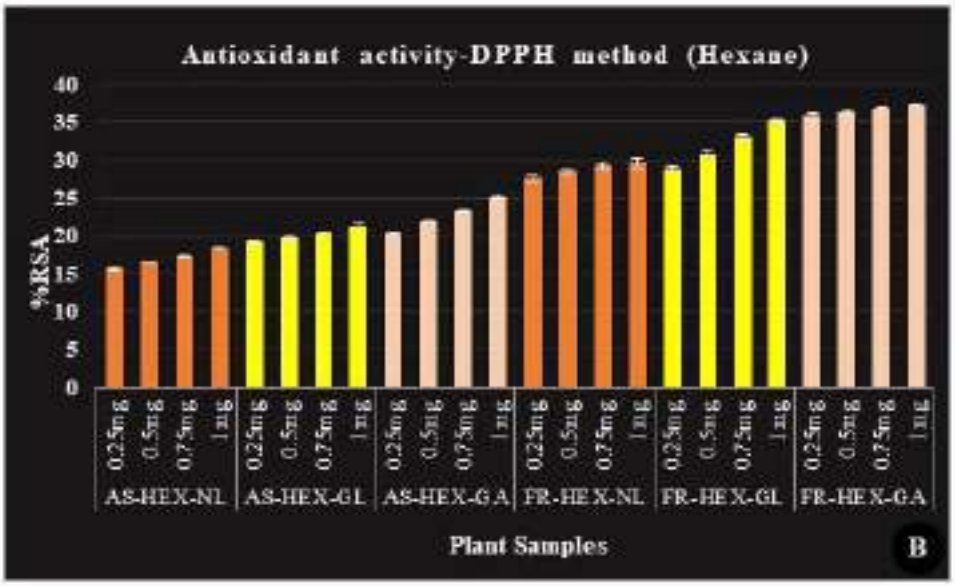


Plate 4.7: Figure A: Graphical representation of results of DPPH antioxidant activity analysis of aqueous extracts of AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA. Each bar represents the mean % RSA of a sample with standard deviation and Figure B: Graphical representation of results of antioxidant activity of hexane extracts by DPPH method. Each bar represents the mean % RSA of a sample with standard deviation.

(21.34%) to AS-GA (25.12%) at 1 mg/mL, indicating higher activity in gall tissues and the differences were significant at $p < 0.001$. The lower %RSA of *A. scholaris* hexane extracts compared to *F. racemosa* suggests that the active compounds of *F. racemosa* are more effectively extracted in non-polar solvents.

Table 4.6: The percentage radicle scavenging activity of hexane extracts of different samples of *A. scholaris* and *F. racemosa* analysed through DPPH method.

Sl. No	Name of sample (Hexane extract)	Dilutions of sample used (mg/mL)	%RSA
1.	AS-NL	0.25	$15.61 \pm 0.28^{++}$
2.		0.5	$16.56 \pm 0.00^{++}$
3.		0.75	$17.3 \pm 0.09^{++}$
4.		1	$18.38 \pm 0.15^{++}$
5.	AS-GL	0.25	$19.23 \pm 0.22^{++**}$
6.		0.5	$19.81 \pm 0.20^{++**}$
7.		0.75	$20.44 \pm 0.05^{++**}$
8.		1	$21.34 \pm 0.20^{++**}$
9.	AS-GA	0.25	$20.41 \pm 0.15^{++**}$
10.		0.5	$21.87 \pm 0.19^{++**}$
11.		0.75	$23.23 \pm 0.01^{++**}$

12.		1	25.12 ± 0.26^{++**}
13.	FR-NL	0.25	27.59 ± 0.45^{++}
14.		0.5	28.43 ± 0.13^{++}
15.		0.75	29.12 ± 0.49^{++}
16.		1	29.54 ± 0.61^{++}
17.	FR-GL	0.25	28.73 ± 0.40^{++*}
18.		0.5	30.8 ± 0.41^{++*}
19.		0.75	32.96 ± 0.17^{++*}
20.		1	35.13 ± 0.17^{++*}
21.	FR-GA	0.25	35.77 ± 0.29^{++**}
22.		0.5	36.26 ± 0.29^{++**}
23.		0.75	36.8 ± 0.09^{++**}
24.		1	37.18 ± 0.09^{++**}
25.	Ascorbic acid	0.025	47.70 ± 0.03
26.		0.05	47.84 ± 0.06
27.		0.075	47.95 ± 0.07
28.		0.1	48.21 ± 0.06

Each value of %RSA represents mean ± standard deviation (n = 3). ++ indicates values significantly different from ascorbic acid at the corresponding concentration (p< 0.01). * indicates values significantly different from normal leaves at corresponding concentration (p < 0.05). ** indicates values significantly different from the normal leaf of each plant at the particular concentration (p < 0.01).

DPPH radical scavenging activity of ethanolic extracts

The results of antioxidant activity of different samples of *A. scholaris* and *F. racemosa* ethanolic extracts shown in Table 4.7. Plate 4.8: Figure A illustrates the %RSA of ethanolic extracts, emphasizing the dominance of gall leaves and galls. Ethanolic extracts exhibited the highest %RSA across both plants, reflecting the efficacy of ethanol in extracting bioactive phenolics. FR-GL and FR-GA at 1 mg/mL showed comparable and high %RSA (43.24% and 44.53%, respectively), significantly outperforming FR-NL (33.99%) with p-value < 0.05. Similarly, AS-GL and AS-GA at 1 mg/mL achieved 44.9% and 46.68% RSA%, respectively, surpassing AS-NL (31.04%). The near-equivalent %RSA of *F. racemosa* and *A. scholaris* ethanolic extracts from gall tissues suggests presence of potent antioxidants.

Table 4.7: The percentage radicle scavenging activity of ethanolic extracts of different samples of *A. scholaris* and *F. racemosa* analysed through DPPH method.

Sl. No	Name of sample (Ethanol)	Dilutions of sample used (mg/mL)	%RSA
1.	AS-NL	0.25	$26.56 \pm 0.39^{++}$
2.		0.5	$27.96 \pm 0.02^{++}$
3.		0.75	$30.56 \pm 0.06^{++}$
4.		1	$31.04 \pm 0.17^{++}$
5.	AS-GL	0.25	$41.74 \pm 0.14^{++**}$
6.		0.5	$42.47 \pm 0.22^{++**}$

7.		0.75	$43.92 \pm 0.39^{++**}$
8.		1	$44.91 \pm 0.18^{++**}$
9.	AS-GA	0.25	$42.07 \pm 0.15^{+**}$
10.		0.5	$44.86 \pm 0.26^{+**}$
11.		0.75	$45.67 \pm 0.03^{+**}$
12.		1	$46.68 \pm 0.21^{+**}$
13.	FR-NL	0.25	$31.03 \pm 0.62^{++}$
14.		0.5	$33.21 \pm 0.33^{++}$
15.		0.75	$33.61 \pm 0.24^{++}$
16.		1	$33.99 \pm 0.36^{++}$
17.	FR-GL	0.25	$31.50 \pm 0.69^{++*}$
18.		0.5	$36.91 \pm 0.61^{++*}$
19.		0.75	$41.78 \pm 0.37^{++*}$
20.		1	$43.24 \pm 0.15^{++*}$
21.	FR-GA	0.25	$35.85 \pm 0.17^{+**}$
22.		0.5	$37.70 \pm 066^{+**}$
23.		0.75	$41.52 \pm 0.60^{+**}$
24.		1	$44.53 \pm 0.30^{+**}$
25.	Ascorbic acid	0.025	47.70 ± 0.03
26.		0.05	47.84 ± 0.06

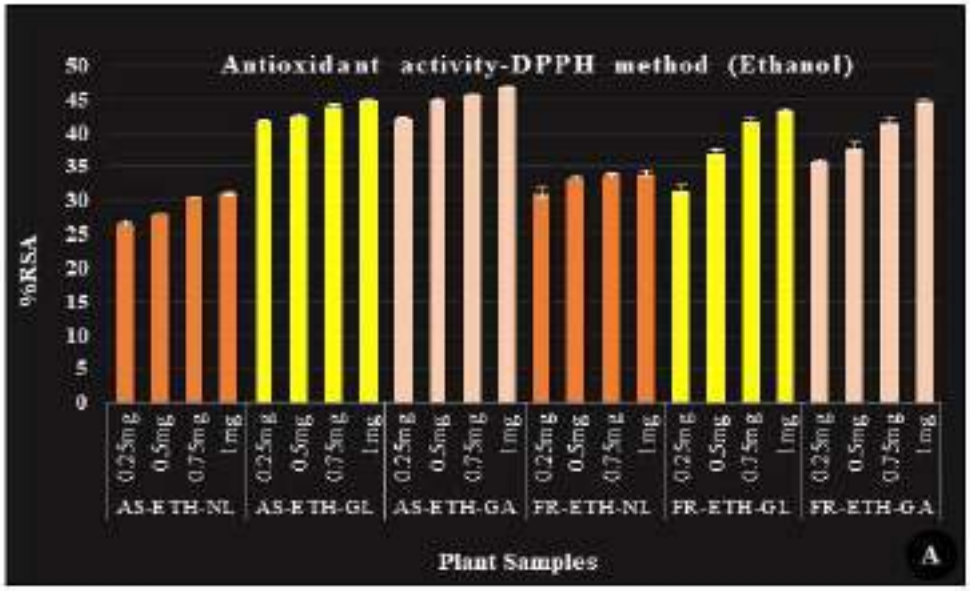
27.		0.075	47.95 ± 0.07
28.		0.1	48.21 ± 0.06

Each value of %RSA represents mean ± standard deviation (n = 3). + indicates values significantly different from ascorbic acid (standard) at the corresponding concentration ($p < 0.05$). ++ indicates values significantly different from ascorbic acid at the particular concentration ($p < 0.01$). * indicates values significantly different from normal leaves of the particular plant at the corresponding concentration ($p < 0.05$). ** indicates values significantly different from the normal leaf extract of each plant at the corresponding concentration ($p < 0.01$).

Evaluation of antioxidant potency based on IC_{50} values

To quantify antioxidant potency, IC_{50} values were calculated (Table 4.8, Plate 4.8: Figure B). Ethanolic extracts of FR-GL exhibited the lowest IC_{50} (1.35 ± 0.02 mg/mL), followed by FR-GA (1.47 ± 0.05 mg/mL) and AS-GA (1.51 ± 0.03 mg/mL), indicating high potency. AS-GL ethanolic extracts had an IC_{50} of 2.18 ± 0.23 mg/mL. Aqueous extracts of FR-NL showed the highest IC_{50} (12.70 ± 2.49 mg/mL), reflecting low potency, followed by AS-NL aqueous extracts (12.22 ± 1.75 mg/mL) and FR-NL hexane extracts (10.34 ± 5.64 mg/mL). Ascorbic acid, the positive control, had an IC_{50} of 0.38 ± 0.03 mg/mL. These results confirm that ethanolic extracts of gall associated tissues have the highest antioxidant capability.

ANOVA confirmed significant differences in %RSA among normal leaf extract to gall leaf and gall extracts of *A. scholaris* and *F. racemosa*. Tukey's HSD tests revealed that all the extracts of *F. racemosa* significantly more potent than *A. scholaris* ($p < 0.05$). In the case of *F. racemosa*, except hexane and ethanolic extracts of FR-GL, all other extracts showed $p < 0.01$ when compared with FR-NL. When the ascorbic acid (standard) at the corresponding concentration tested for significance against different samples and found that except ethanolic extracts of AS-GA ($p < 0.05$) and FR-GA ($p < 0.05$), all other samples and extracts exhibited $p < 0.01$.



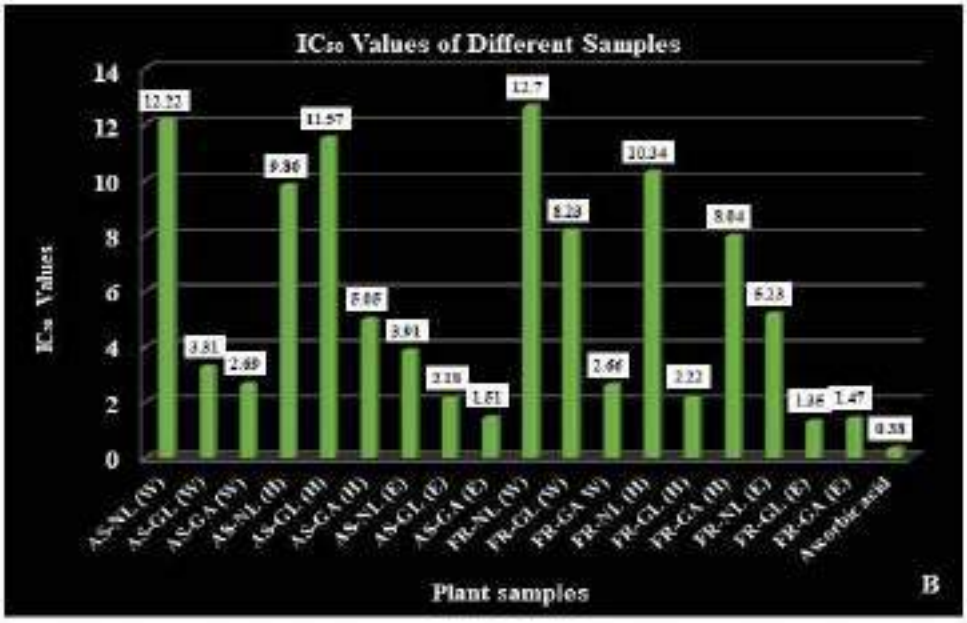


Plate 4.8: Figure A: Graphical representation of antioxidant activity of ethanolic extracts of AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA. Each bar represents the mean %RSA of a sample with standard deviation and Figure B: IC_{50} values of different samples of *A. scholaris* and *F. racemosa* determined using DPPH radical scavenging assay.

Table 4.8: IC_{50} values of normal leaves, gall leaves and leaf galls of *A. scholaris* and *F. racemosa* extracts determined using DPPH method.

Sl. No	Plant sample	IC_{50} (mg/mL)
1.	AS-NL (Aqueous)	12.22 ± 1.75
2.	AS-GL (Aqueous)	3.31 ± 0.03
3.	AS-GA (Aqueous)	2.69 ± 0.05
4.	AS-NL (Hexane)	9.86 ± 1.2
5.	AS-GL (Hexane)	11.57 ± 1.37
6.	AS-GA (Hexane)	5.05 ± 0.40
7.	AS-NL (Ethanol)	3.91 ± 0.35
8.	AS-GL (Ethanol)	2.18 ± 0.23
9.	AS-GA (Ethanol)	1.51 ± 0.03
10.	FR-NL (Aqueous)	12.70 ± 1.49
11.	FR-GL (Aqueous)	8.23 ± 1.42
12.	FR-GA (Aqueous)	2.66 ± 0.03
13.	FR-NL (Hexane)	10.34 ± 1.64
14.	FR-GL (Hexane)	2.22 ± 0.10
15.	FR-GA (Hexane)	8.04 ± 1.33
16.	FR-NL (Ethanol)	5.23 ± 0.34
17.	FR-GL (Ethanol)	1.35 ± 0.02
18.	FR-GA (Ethanol)	1.47 ± 0.05

19.	Ascorbic Acid	0.38 ± 0.03

Each value represents mean ± standard deviation (n = 3)

Ethanolic extracts of AS-GA exhibited the highest antioxidant activity through this study, with an IC_{50} of 1.95 ± 0.65 mg/mL, followed by AS-GL (2.04 ± 0.43 mg/mL) and AS-NL (3.64 ± 0.74 mg/mL). Ganjewala and Gupta reported that methanol extracts of *A. scholaris* leaves, follicles and latex scavenged DPPH free radicals, with leaf extracts showing 70-80% activity (Ganjewala & Gupta, 2013). Bopche and colleagues (2023) found a concentration-dependent antioxidant activity in *A. scholaris* methanolic leaf extracts, with an IC_{50} of 77.67 µg/mL compared to 26.72 µg/mL for ascorbic acid (Bopche et al., 2023). Antony and colleagues in 2011 reported an IC_{50} of 2.83 mg/mL for *A. scholaris* aqueous leaf extracts, higher than our ethanolic gall extracts, indicating greater potency (Antony et al., 2011). Shanmugapriya and Jayanthi found IC_{50} values of 60.9 µg/mL for *A. scholaris* methanolic crude extracts and 41.64 µg/mL for purified samples (Shanmugapriya & Jayanthi, 2019). Arulmozhi and colleagues (2010) noted that dichloromethane and ethyl acetate fractions of *A. scholaris* ethanolic leaf extracts were effective DPPH scavengers (Arulmozhi et al., 2010). In this study, the reducing power (RP) method confirmed that ethanolic extracts had the highest inhibitory action, with galls showing the greatest activity.

Previous studies compared the antioxidant properties of *F. racemosa* bark and leaves. At a concentration of 200 µg/mL, *F. racemosa* leaves had a scavenging activity of 48.55 ± 2.670%, while bark had a scavenging activity of 73.46 ± 1.455% (Sultana et al., 2014). Since FR-NL, FR-GL and FR-GA have different phytochemical profiles, the antioxidant qualities of these samples were examined in the current study. Previous research revealed that an ethanolic extract of *F. racemosa* leaves produced a considerable antioxidant capability. It was revealed using a number of techniques, including H_2O_2 scavenging activity assay, FRAP assay etc. (Yadav et al., 2019). In this work, the RP method and DPPH assay were performed on aqueous, hexane and ethanolic extracts of different *F. racemosa* samples and it was discovered that the ethanolic extracts had the highest levels of antioxidant activity.

Sumi and colleagues (2016) reported an IC_{50} of 10.29 µg/mL for *F. racemosa* methanolic leaf extracts by DPPH assay and in case of reducing power assay IC_{50} of 45.564 µg/mL (Sumi et al., 2016). According to the RP assay, the reducing capacity of various samples varied with dose and the gall ethanolic extracts had the highest antioxidant activity when compared to other extracts. Based on a study by Bagyalakshmi and colleagues, toluene extracts of *F. racemosa* leaves had the strongest antioxidant activity, with an IC_{50} of 2.35 ± 0.41 µg/mL and an inhibition percentage of 88.5%. Nearly the same percentages of inhibition were displayed by the ethanol and ethyl acetate extracts (54% and 56%, respectively) (Bagyalakshmi et al., 2019). Londhe and colleagues reported an IC_{50} of 47.96 µg/mL for *F. racemosa* ethanolic leaf extracts, with increasing reducing power at higher concentrations (Londhe et al., 2012). Through DPPH assay, the present work discovered that the FR-GL ethanolic extracts possessed significant antioxidant activity with an IC_{50} value of 1.32 ± 0.11 mg/mL and FR-GA extracts displayed an IC_{50} of 1.38 ± 0.18 mg/mL. Since ascorbic acid had an IC_{50} of 0.38 ± 0.09 mg/mL, it may be concluded that FR-GA and FR-GL showed substantial activity when compared to ascorbic acid.

4.3.2. Results of antibacterial activity analysis

The antibacterial activity of crude aqueous, hexane and ethanolic extracts prepared from normal leaves, gall leaves and galls of *A. scholaris* and *F. racemosa* were evaluated against four pathogenic bacteria: *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*. Preliminary screening tested three dilutions (10 µL, 20 µl, 30 µL) from a 200 mg/mL stock concentration. Aqueous and hexane extracts of all samples from both plants showed no activity against any of the four bacteria at these dilutions. Ethanolic extracts, however, exhibited activity against *S. aureus*, *B. subtilis* and *E. coli*, but not against *P. aeruginosa*, across various dilutions. Consequently, further analysis focused on ethanolic extracts at four dilutions: 10 µL (2000 µg), 20 µL (4000 µg), 30 µL (6000 µg) and 40 µL (8000 µg). Streptomycin (10 µg/disc) served as the positive control. Plate 4.9 shows the antibacterial activity of controls (streptomycin- positive control and ethanol-negative control) against *B. subtilis* (Figure A), *E. coli* (Figure B), *P. aeruginosa* (Figure C) and *S. aureus* (Figure D).

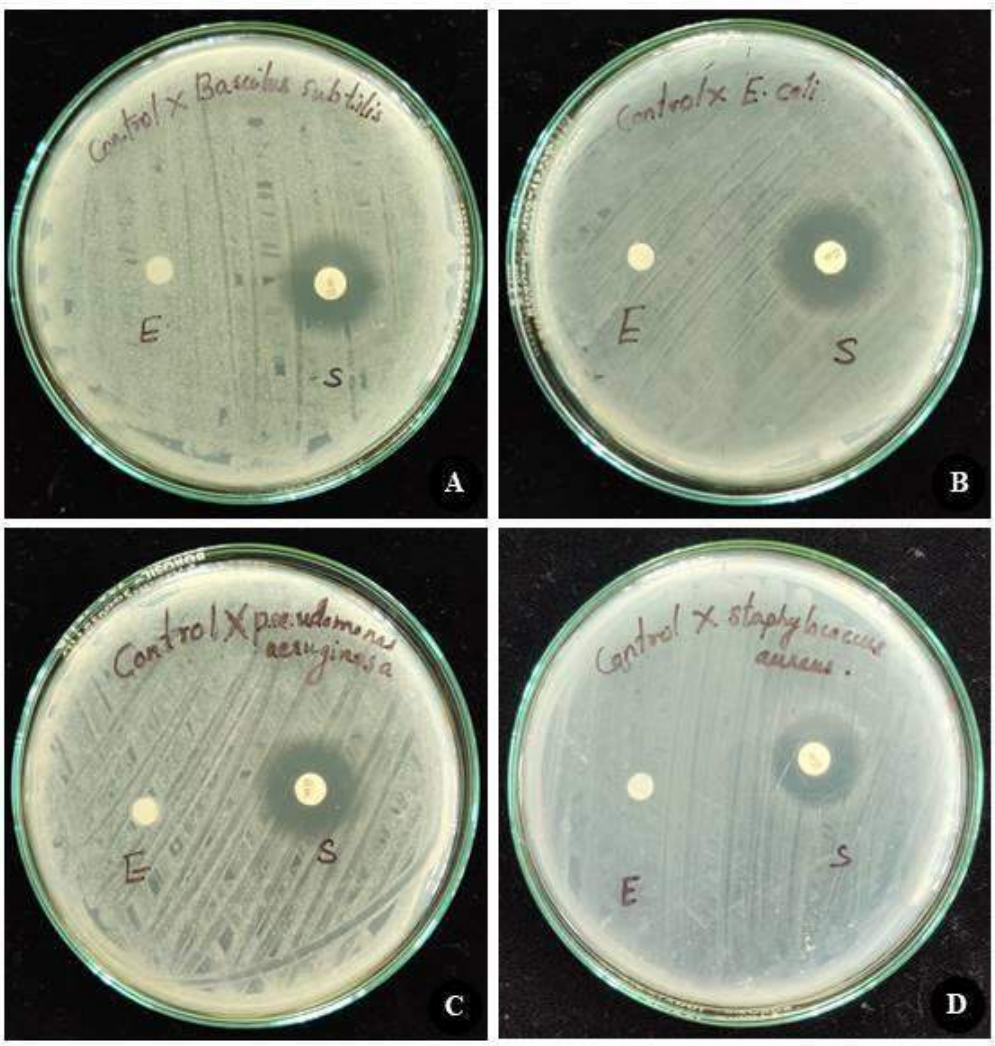


Plate 4.9: Antibacterial analysis of controls. The activity of ethanol (negative control) and streptomycin (positive control) was tested against four tested bacterial strains. Figure A: *Bacillus subtilis*, Figure B: *Escherichia coli*, Figure C: *Pseudomonas aeruginosa* and Figure D: *Staphylococcus aureus*. The figures demonstrate streptomycin exhibiting standard antibacterial activity, while ethanol shows no inhibition of bacterial growth.

Antibacterial activity of *A. scholaris*

Antibacterial analysis performed against ethanolic extracts of *A. scholaris* is shown in Plate 4.10: Figure A shows the zone of inhibition produced by AS-NL, AS-GL and AS-GA against *B. subtilis*, Figure B, is the image showing the zone of inhibition produced by AS-NL, AS-GL and AS-GA against *E.coli* and Figure C represents the antibacterial activity of AS-NL, AS-GL and AS-GA against *S. aureus*. The results of antibacterial activity analysis against *E. coli*, *B. subtilis* and *S.aureus* with the average of inhibition zone (mm) produced with standard deviation (SD) is represented in Table 4.9. The antibacterial activity of an antibacterial drug (streptomycin) was shown in Table 4.11.

Ethanolic extracts of AS-NL, AS-GL and AS-GA showed varying antibacterial activity against *S. aureus, B. subtilis* and *E. coli*. AS-NL at 10 µL (2000 µg) exhibited no activity against any bacteria, while at 20 µL and above, it was active against *E. coli* (e.g., 8.66 ± 0.7 mm at 20 µL) and *B. subtilis* (e.g., 12.25 ± 0.25 mm at 40 µL), but not *S. aureus*. AS-GL at 10 µL showed activity only against *B. subtilis* (10 ± 0.50 mm), but displayed concentration-dependent activity against *B. subtilis* across all dilutions, peaking at 40 µL (13.5 ± 1.0 mm). AS-GL also showed activity against *E. coli* at 30 µL (10.25 ±0.25 mm) and 40 µL (12.66 ± 0.29 mm) and against *S. aureus* at 40 µL (10.5 ± 0.5 mm). AS-GA was inactive at 10 µL but showed activity against *E. coli* and *B. subtilis* at 20 µL and above, with the highest inhibition zone against *E. coli* at 40 µL (14.33 ± 0.29 mm). AS-GA also exhibited activity against *S. aureus* at 40 µL (14.16 ± 0.58 mm). Overall, AS-GA at 40 µL demonstrated the highest activity among *A. scholaris* extracts, particularly against *E. coli*.

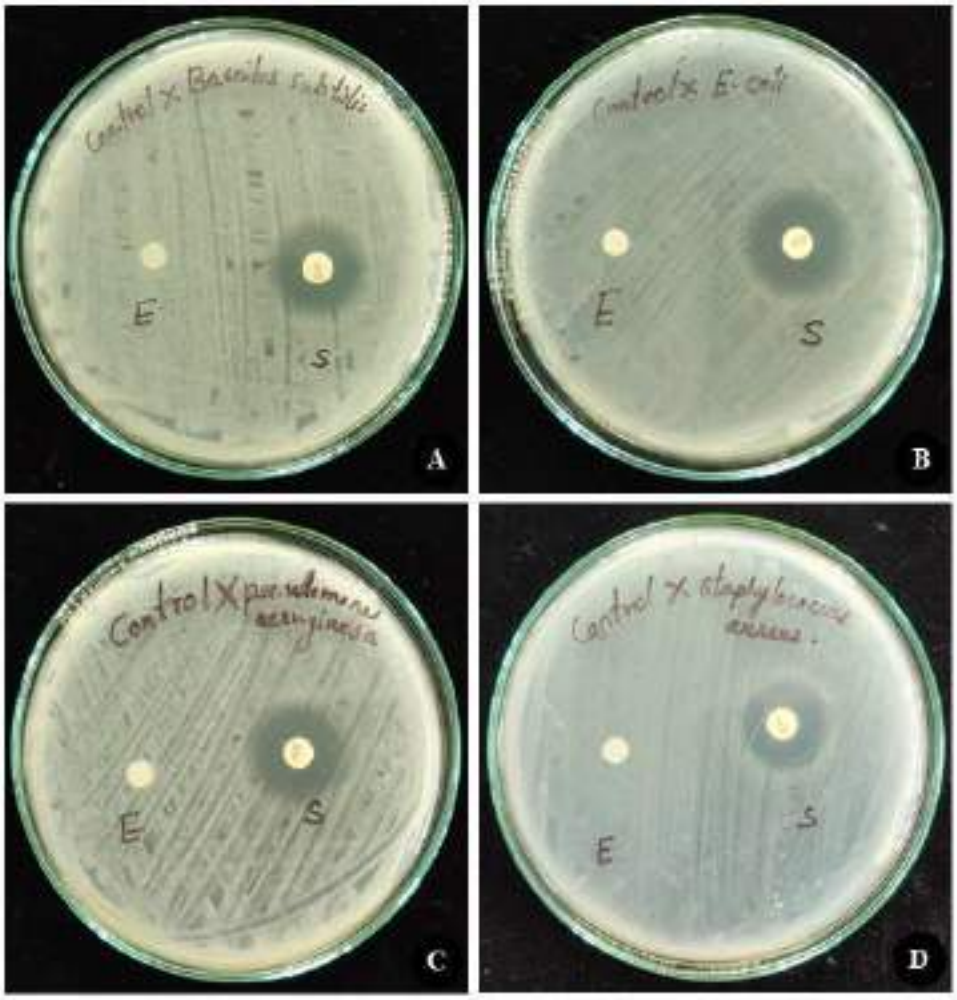


Plate 4.9: Antibacterial analysis of controls. The activity of ethanol (negative control) and streptomycin (positive control) was tested against four tested bacterial strains. Figure A: *Bacillus subtilis*, Figure B: *Escherichia coli*, Figure C: *Pseudomonas aeruginosa* and Figure D: *Staphylococcus aureus*. The figures demonstrate streptomycin exhibiting standard antibacterial activity, while ethanol shows no inhibition of bacterial growth.

Table 4.9: Antibacterial activity of AS-NL, AS-GL and AS-GA with the average zone of inhibition (mm) produced with SD calculated in 4 different dilutions (10µL, 20µL, 30µL and 40µL).

Sample	Bacteria	10 µL (mm)	20 µL (mm)	30 µL (mm)	40 µL (mm)
AS-NL	*E. coli*	-	$8.66 \pm 0.7^{++}$	$9.33 \pm 0.29^{++}$	$10.5 \pm 0.50^{++}$
	B. subtilis	-	$10 \pm 0.5^{++}$	$11.25 \pm 0.25^{++}$	$12.25 \pm 0.25^{++}$
	S.aureus	-	-	-	-
AS-GL	*E. coli*	-	-	$10.25 \pm 0.25^{++}$	$12.66 \pm 0.29^{++**}$
	B. subtilis	$10 \pm 0.50^{++**}$	$11 \pm 0.5^{++}$	$12 \pm 0.5^{++}$	$13.5 \pm 1.0^{++}$
	S.aureus	-	-	-	$10.5 \pm 0.5^{++**}$
AS-GA	*E. coli*	-	$9 \pm 0.1^{++*}$	$11.16 \pm 0.29^{++**}$	$14.33 \pm 0.29^{++**}$
	B. subtilis	-	$10.08 \pm 0.52^{++}$	$12.33 \pm 1.26^{++}$	$13.5 \pm 0.50^{++}$
	S.aureus	-	-	-	$14.16 \pm 0.58^{++**}$

Values represent mean ± standard deviation (n = 3). ++ indicates values significantly different from Streptomycin (standard antibacterial drug) at the level of $p < 0.01$. * indicates values significantly different from the normal leaf extract of *A. scholaris* at the corresponding concentration at $p < 0.05$. ** indicates values significantly different ($p < 0.01$) from the normal leaf of *A. scholaris* at the corresponding concentration.

Antibacterial activity of *F. racemosa*

Antibacterial analysis performed against ethanolic extracts of *F. racemosa* (Plate 4.10: Figure D) produced zones of inhibition against *B. subtilis*. Plate 4.10: Figure E represents the photograph showing the zone of inhibition produced by FR-NL, FR-GL and FR-GA against *E. coli* and Figure F shows the activity of FR-NL, FR-GL and FR-GA against *S. aureus.* The results of antibacterial activity analysis of *F. racemosa* extracts against *E. coli*, *B. subtilis* and *S. aureus* with the average of inhibition zone (mm) produced with standard deviation (SD) is represented in Table 4.10. The antibacterial activity of an antibacterial drug, the control (streptomycin) was shown in Table 4.11. The results of all extracts and samples of both plants were represented as heat map in Plate 4.11.

Ethanolic extracts of FR-NL, FR-GL and FR-GA exhibited concentration-dependent antibacterial activity against *S. aureus*, *B. subtilis* and *E. coli*. FR-NL showed the lowest activity, with no inhibition at 10 µL, activity against *B. subtilis* at 20 µL (9.1 ± 0.1 mm) and 30 µL (10.25 ± 0.25 mm) and activity against both *B. subtilis* (11 ± 1.0 mm) and *S. aureus* (11.5 ± 0.50 mm) at 40 µL. FR-GL was highly active, showing activity against *E. coli* (9.25 ± 0.25 mm) and *S. aureus* (11.5 ± 0.87 mm) at 10 µL and against all three bacteria at higher dilutions. At 40 µL, FR-GL produced the highest inhibition zones: 17.25 ± 0.25 mm (*E. coli*), 15.66 ± 0.29 mm (*B. subtilis*) and 17.16 ± 0.58 mm (*S. aureus*). FR-GA showed activity against *S. aureus* at 10 µL (10.83 ± 0.29 mm), but required higher concentrations for *E. coli* and *B. subtilis* (e.g., 10.25 ± 1.25 mm for *E. coli* at 30 µL). At 40 µL, FR-GA produced inhibition zones of 17.16 ± 0.14 mm (*S. aureus*), 13.25 ± 0.25 mm (*E. coli*) and 13.83 ± 1.26 mm (*B. subtilis*), which were lower than FR-GL zones for *E. coli* and *B. subtilis*. FR-GL at 40 µL exhibited the highest antibacterial activity among all samples and dilutions.

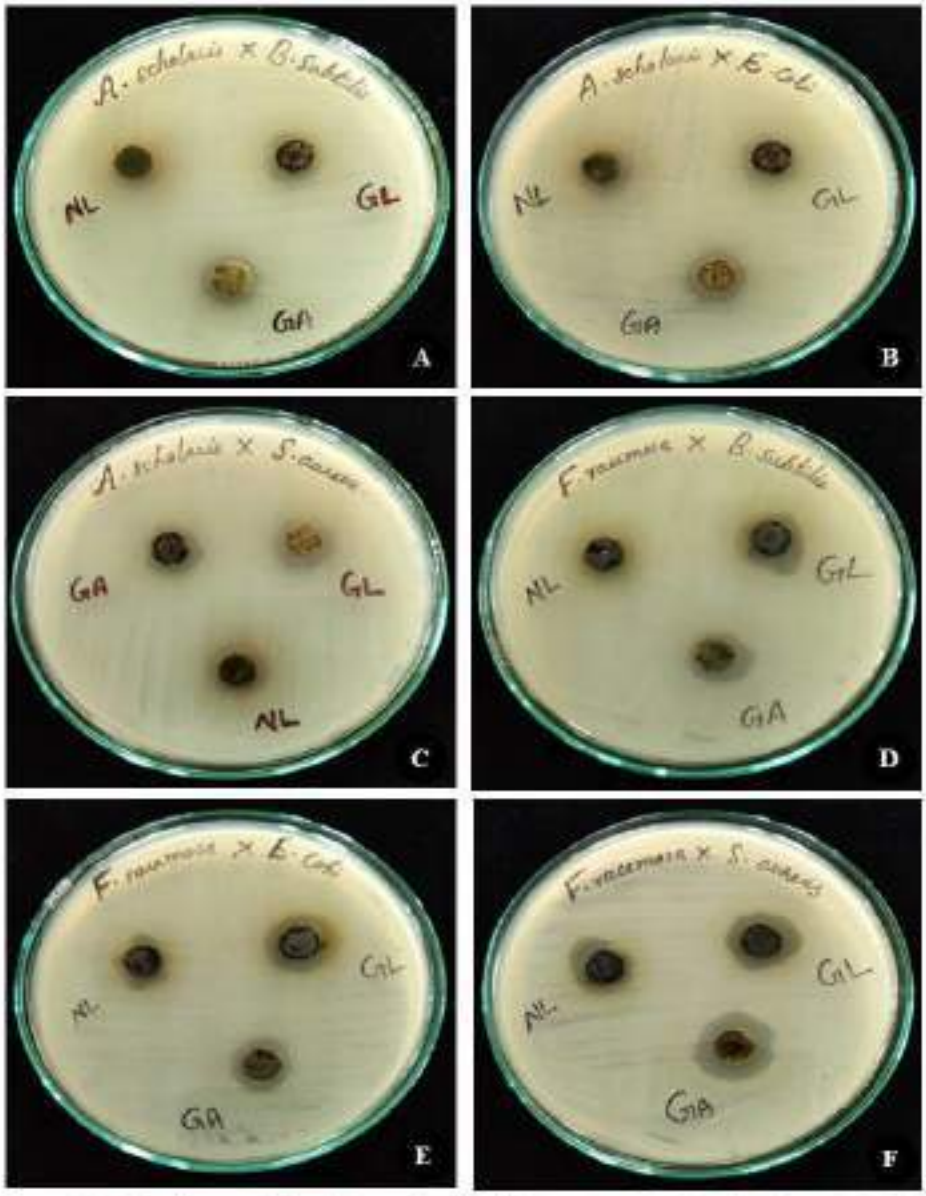


Plate 4.10: Antibacterial activity of ethanolic extracts of *A. scholaris* and *F. racemosa*. Figure A-C: Zones of inhibition produced by AS-NL, AS-GL and AS-GA against *B. subtilis*, *E. coli* and *S. aureus*, respectively and Figure D-F: Zones of inhibition of FR-NL, FR-GL and FR-GA against *B. subtilis*, *E. coli* and *S. aureus*, respectively.

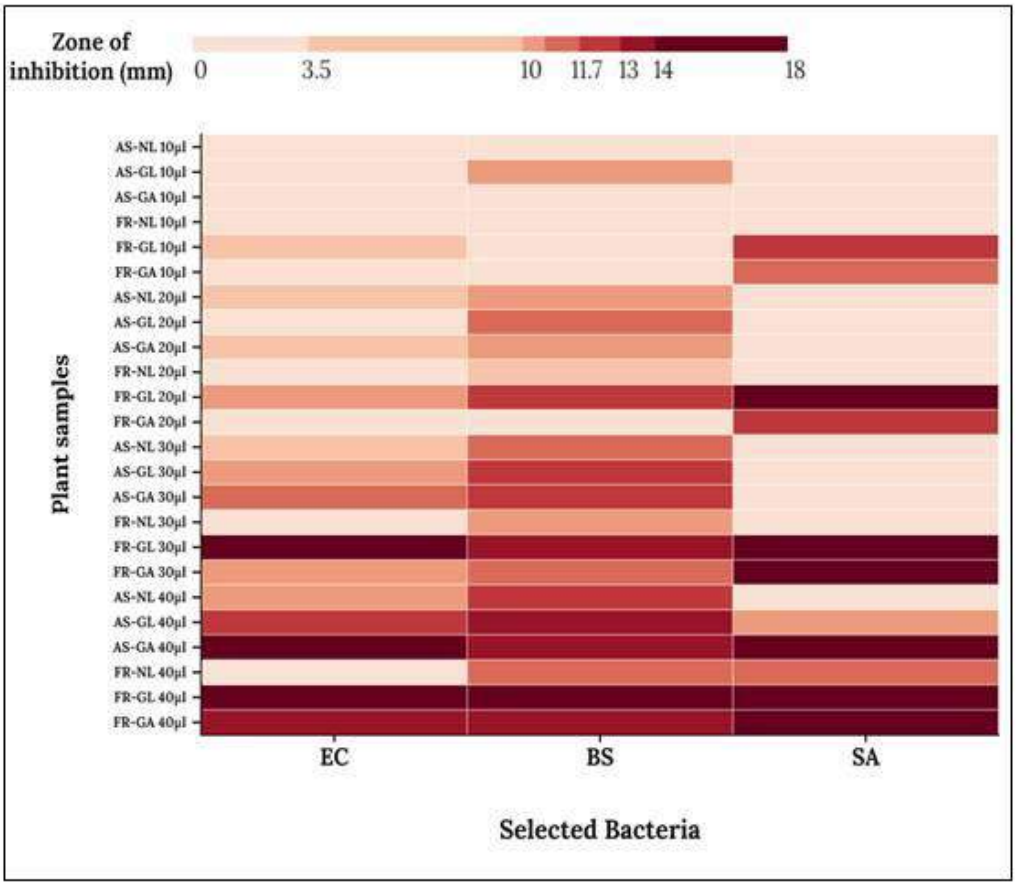


Plate 4.11: Heat maps showing results of antibacterial analysis: The results of antibacterial analysis of AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA ethanolic extracts in 4 different dilutions with the zone of inhibition (mm) produced. Light colours indicates no activity and the increase of darkness of colours indicates increase in inhibition zone against *Staphylococcus aureus* (SA), *Bacillus subtilis* (BS) and *Escherichia coli* (EC).

Table 4.10: Antibacterial activity of FR-NL, FR-GL and FR-GA with the average zone of inhibition (mm) produced with SD calculated in 4 different dilutions (10 µL, 20 µL, 30 µL and 40 µL).

Sample	Bacteria	10 µL (mm)	20 µL (mm)	30 µL (mm)	40 µL (mm)
FR-NL	*E. coli*	-	-	-	-
	B. subtilis	-	$9.1 \pm 0.1^{++}$	$10.25 \pm 0.25^{++}$	$11 \pm 1.0^{++}$
	S.aureus	-	-	-	$11.5 \pm 0.50^{++}$
FR-GL	*E. coli*	$9.25 \pm 0.25^{++**}$	$10.5 \pm 0.5^{++**}$	$14 \pm 0.5^{++**}$	$17.25 \pm 0.25^{++**}$
	B. subtilis	-	$12.67 \pm 0.29^{++**}$	$13 \pm 0.5^{++**}$	$15.66 \pm 0.29^{++**}$
	S.aureus	$11.5 \pm 0.87^{++**}$	$14 \pm 1.0^{++**}$	$15.83 \pm 0.29^{++**}$	$17.16 \pm 0.58^{++**}$
FR-GA	*E. coli*	-	-	$10.25 \pm 1.25^{++**}$	$13.25 \pm 0.25^{++**}$
	B. subtilis	-	-	$10.66 \pm 0.29^{++}$	$13.83 \pm 1.26^{++*}$
	S.aureus	$10.83 \pm 0.29^{++**}$	$12.33 \pm 0.29^{++**}$	$16.33 \pm 0.29^{++**}$	$17.16 \pm 0.14^{++**}$

Values represent mean ± standard deviation (n = 3). ++ indicates values significantly different from Streptomycin (standard) at the level of $p < 0.01$. * indicates values significantly different from the normal leaf extract of *F. racemosa* at the corresponding concentration at $p < 0.05$. ** indicates values significantly different ($p < 0.01$) from the normal leaves of *F. racemosa* at the corresponding concentration.

Table 4.11: The average zone of inhibition produced by streptomycin (+ve control) against all tested organisms with SD.

Control	Concentration	Bacteria	Average inhibition zone (mm)
Streptomycin disc	10µg	*E. coli*	21 ± 1.00
	10µg	*B. subtilis*	22.25 ± 0.75
	10µg	*S. aureus*	24.16± 0.29

ANOVA was done for finding the statistical significance of the obtained results and confirmed significant differences in inhibition zones among extracts and dilutions ($p < 0.05$). Tukey's HSD tests for comparing both plants it is found that, FR-GL at 40 µL (8000 µg) consistently produced the largest inhibition zones against *S. aureus* (17.16 ± 0.58 mm), *E. coli* (17.25 ± 0.25 mm) and *B. subtilis* (15.66 ± 0.29 mm), outperforming *A. scholaris* extracts ($p < 0.01$). AS-GA at 40 µL was the most active among extracts of *A. scholaris*, particularly against *E. coli* (14.33 ± 0.29 mm). Tukey's HSD tests indicated that FR-GL ($p < 0.01$) and FR-GA ($p < 0.05$) at 40 µL were significantly more potent than other samples. Streptomycin (positive control), produced inhibition zones of 21 ± 1.00 mm (*E. coli*), 22.25 ± 0.75 mm (*B. subtilis*) and 24.16 ± 0.29 mm (*S. aureus*), serving as a benchmark.

The current investigation found that only ethanolic extracts of *A. scholaris* effective against *S. aureus, B. subtilis* and *E. coli*, but not *P. aeruginosa*. In this study, AS-GA ethanolic extracts produced inhibition zones of 14.33 ± 0.29 mm against *E. coli* and 14.16 ± 0.58 mm against *S. aureus*, while AS-NL showed no activity against *S. aureus* at any tested dose. Khyade and colleagues (2009) reported that methanol extracts *A. scholaris* of leaves exhibited broad-spectrum antibacterial activity against Gram-positive and Gram-negative bacteria, while petroleum ether extracts showed no inhibition, aligning with our findings for ethanolic extracts (Khyade et al., 2009). Khan and colleagues (2003) evaluated *A. scholaris* crude methanolic extracts and fractions (petrol, dichloromethane, ethyl acetate, butanol) and found that butanol fractions were highly active against bacteria, but no fractions inhibited the tested fungus (Khan et al., 2003). Misra and colleagues noted that methanol and isopropanol extracts of *A. scholaris* leaves were active against *S. aureus*, supporting the efficacy of polar solvents (Misra et al., 2011).

Bonvicini and colleagues found that ethanolic extracts of *A. scholaris* exhibited antimicrobial activity against reference strains and clinical isolates, including methicillin-resistant *S. aureus* (Bonvicini et al., 2014). Bagheri and colleagues (2020) reported that methanol and isopropanol extracts of *A. scholaris* leaves showed stronger activity against Gram-positive bacteria than Gram-negative ones (Bagheri et al., 2020). From the present study it was clear that different extracts of *A. scholaris* were active against both Gram-positive and Gram-negative bacteria. Labaran and colleagues (2024) observed large inhibition zones against *S. aureus* and *E. coli* with *A. scholaris* leaf extracts, noting greater susceptibility of *S. aureus* (Labaran et al., 2024). The crude ethanolic extracts of *A. scholaris* leaves produced inhibition zones of 14 mm against *S. aureus* and 13 mm against *E. coli*, with petroleum ether showing no activity (Ara et al., 2020). Mushtaq and colleagues in 2023 reported that *S. aureus* growth was suppressed with increasing concentrations of *A. scholaris* leaf extracts (Mushtaq et al., 2023), a trend observed in our study as antibacterial activity increased from 10 µL to 40 µL.

In the preliminary screening, the petroleum ether extract of *F. racemosa* leaves was discovered to be more active than the others against *S. aureus*, *B. subtilis*, *P. aeruginosa* and *E. coli* in earlier investigations. As a result, it was subjected to further examination with different concentrations. The concentration of 350 mg/mL was shown to be the most efficient against *S. aureus* (Mandal et al., 2000b). Similar to that, in order to identify the best solvent, three extracts were first screened in this study. Ethanolic extracts were found to be the most efficient against the tested organisms based on that assessment. Further analysis was conducted using various ethanolic extract dilutions. Thilagavathi and Kathiravan (2017) reported minimal antibacterial activity of *F. racemosa* ethanolic leaf extracts, with moderate efficacy against *S. aureus* and *E. coli* but no activity against *P. aeruginosa*, consistent with our findings (Thilagavathi & Kathiravan, 2017). The present investigation also came to the same conclusion that there was no activity against *P. aeruginosa.*

Kumari and colleagues evaluated the antibacterial efficacy of *F. racemosa* leaf and gall extracts in 2020. The Dichloromethane fraction of crude extract inhibited *E. coli* and *P. aeruginosa* with zone of inhibition of 19.71 ± 0.31 mm and 19.75 ± 0.11 mm, respectively. The crude extract of *F. racemosa* leaf and galls with ethyl acetate showed the highest inhibition with 19.50 ± 0.19 mm zone. Hexane fraction of crude extract produced a zone of inhibition of 18.50 ±

0.23 against *S. aureus* (Kumari et al., 2020). A study conducted by Hosain and colleagues revealed that methanolic extract of *F. racemosa* leaves has poor activity against Gram-positive and Gram-negative bacteria. The extracts produced a zone of inhibition of 8 mm for *S. aureus* and 9 mm for *B. subtilis* (Hosain et al., 2011). The current investigation came to the same conclusion that normal leaves of *F. racemosa* were less effective at inhibiting bacterial growth. At low concentrations, ethanolic extracts of FR-NL did not suppress bacterial growth, however at higher concentrations; they formed 11 mm and 11.5 mm zones of inhibition against *B. subtilis* and *S. aureus*. There is no effect found on *E. coli* proliferation.

Bagyalakshmi and colleagues (2019) found that toluene extracts of *F. racemosa* leaves inhibited *Staphylococcus* spp., while methanolic and aqueous extracts were ineffective against various bacteria (Bagyalakshmi et al., 2019). Mpala and colleagues in 2019 reported no antibacterial efficacy of *F. racemosa* methanolic and aqueous leaf extracts (Mpala et al., 2019). In contrast, our results showed that ethanolic extracts of FR-GL and FR-GA were highly effective, with FR-NL also exhibiting some activity. Phytochemical analysis revealed high concentrations of phenolic and flavonoid components in *F. racemosa* leaves and galls, likely responsible for the observed bioactivity.

4.3.3. Results of antifungal activity analysis

Antifungal assay of *C. albicans* by agar-well diffusion method:

The antifungal properties of various extracts from *A. scholaris* and *F. racemosa* were evaluated against *C. albicans* using the agar-well diffusion method. A stock solution of 100 mg/mL was used to prepare extracts of normal leaves, gall leaves and galls. For initial screening, hexane, ethanolic and aqueous extracts were tested at 20 µL and 40 µL. Plates showing a zone of inhibition were considered positive, while those without were considered negative. Itraconazole, an antifungal agent used as positive control for the assay. Table 4.12 shows antifungal activity against *C.albicans* by all the extracts of ethanol in different concentrations with the zone of inhibition in mm and IC_{50} values of the extracts. Plate 4.12: Figure A shows the zone of inhibition of positive control Itraconazole and Figure B represents a photograph of plate showing the activity of negative control. Hexane and aqueous extracts of all samples of *A. scholaris* produced negative results. Plate 4.12: Figure C shows the anticandidal activity of AS-NL, AS-

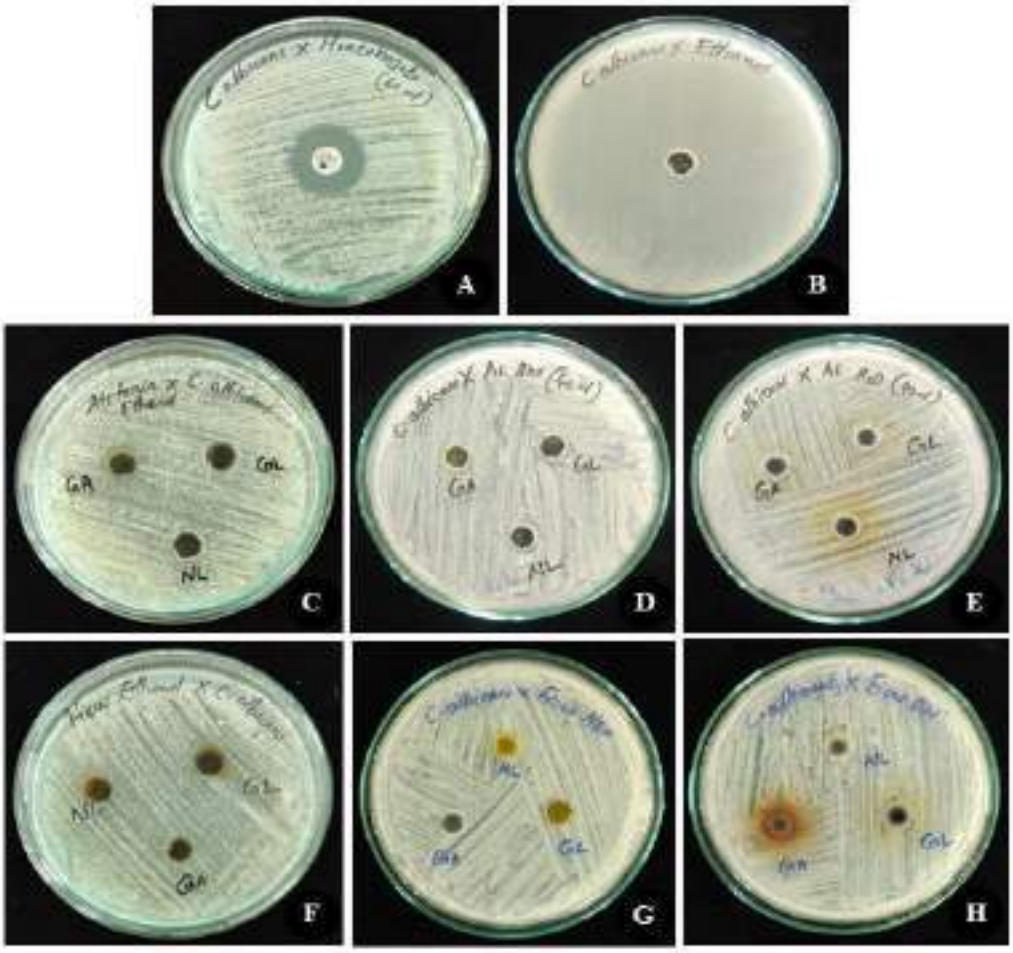


Plate 4.12: Anticandidal activity of *A. scholaris* and *F. racemosa*. Figure A: positive control (Itraconazole), Figure B: negative control, Figure C: activity of AS-NL, AS-GL and AS-GA ethanolic extracts, Figure D: no activity for AS-NL, AS-GL and AS-GA hexane extracts, Figure E: AS-NL, AS-GL and AS-GA aqueous extracts (no activity), Figure F: zone of inhibition of FR-NL, FR-GL and FR-GA ethanolic extracts, Figure G: FR-NL, FR-GL and FR-GA hexane extracts (no activity) and Figure H: no activity for FR-NL, FR-GL and FR-GA aqueous extracts.

GL and AS-GA ethanolic extracts, Figure D shows the activity of hexane extracts, no activity obtained for AS-NL, AS-GL and AS-GA hexane extracts and Figure E represents the activity of aqueous extracts of different samples of *A. scholaris*. In the case of *A. scholaris* only ethanolic extracts showed antifungal activity. In the case of *F. racemosa* hexane and aqueous extracts have no activity against *C. albicans* (Plate 4.12: Figure G and Figure H respectively). Only ethanolic extracts exhibited anticandidal activity, consequently ethanolic extracts were selected for further analysis at different dilutions prepared from a 150 mg/mL stock solution. Representative photographs showing the antifungal activity of ethanolic extracts of *F. racemosa* were shown in Plate 4.12: Figure F.

The results of the analysis showed that every extract exhibited concentration-dependent effectiveness against *C. albicans*. Highest anticandidal activity was possessed by FR-GL in 60 µL dilution, it showed a zone of inhibition of 13.2 ± 0.14 mm, followed by AS-GA (60 µL) with zone of inhibition of 12.45 ± 0.21 mm. The inhibition zone produced by FR-GA (60 µL) and AS-GL (60 µL) was 9.35 ± 0.35 mm and 9.05 ± 0.35 mm, respectively. AS-NL and FR-NL extracts had the least amount of efficacy against *C. albicans* in a 60 µL dilution. After comparing all of the dilutions, it was discovered that FR-GL exhibited the greatest activity, directly proportional with the extract concentrations. The results of antifungal activity against *C. albicans* were compared with the Itraconazole (60 µL), which showed 24.5 ± 2.8 mm inhibition zone.

FR-GL exhibits comparable antifungal activity at higher concentrations of extracts. IC_{50} values were calculated for all extracts. FR-GL had the lowest IC_{50} (0.534 mL), indicating the highest potency, followed by AS-GA (0.548 mL). FR-NL had the highest IC_{50} (3.051 mL), indicating the lowest potency. The IC_{50} value of Itraconazole (0.178 mL) was significantly lower, reflecting its superior efficacy. Plate 4.14: Figure A shows the graphical representation of the antifungal activity of ethanol extracts of different samples of *A. scholaris* and *F. racemosa* against C. *albicans.*

The statistical analysis confirmed significant differences in anticandidal activity ($p < 0.05$). All the dilutions of all three extracts showed a significant difference from the control, Itraconazole. Tukey's HSD test revealed that FR-GL at 60 µL produced significantly larger inhibition zones than all other extracts ($p < 0.01$), followed by AS-GA. AS-GA at 60 µL was

significantly more effective than AS-GL and AS-NL ($p < 0.01$). FR-GA and AS-GL showed similar activity at 60 µL, with significant difference ($p < 0.05$). FR-NL and AS-NL exhibited the lowest activity, significantly lower than AS-GA ($p < 0.01$). These results confirm that FR-GL is the most potent extract, followed closely by AS-GA.

Table 4.12: Results of antifungal activity of ethanolic extracts against *C. albicans* using AS-NL, AS-GL, AS-GA, FR-NL, FR-GL and FR-GA along with antifungal drug Itraconazole. Zone of inhibition represented in mm with SD. IC_{50} values of all the samples presented in mm.

Sl. No	Sample name	Inhibition zone (mm) of 20 µL dilution	Inhibition zone (mm) of 40 µL dilution	Inhibition zone (mm) of 60 µL dilution	IC_{50} (mL)
1	AS-NL	$7.25 \pm 0.07^{++}$	$7.6 \pm 0.42^{++}$	$8.125 \pm 0.25^{++}$	1.973
2	AS-GL	$8.1 \pm 0.28^{++**}$	$8.85 \pm 0.35^{++**}$	$9.05 \pm 0.35^{++**}$	1.784
3	AS-GA	$9.4 \pm 0.14^{++**}$	$11.7 \pm 0.57^{++**}$	$12.45 \pm 0.21^{++**}$	0.548
4	FR-NL	$8.15 \pm 0.07^{++}$	$8.45 \pm 0.21^{++}$	$8.7 \pm 0.28^{++}$	3.051
5	FR-GL	$11.225 \pm 0.11^{++**}$	$12.55 \pm 0.35^{++**}$	$13.2 \pm 0.14^{++**}$	0.534
6	FR-GA	$8.25 \pm 0.35^{++}$	$9.257 \pm 0.11^{++*}$	$9.35 \pm 0.35^{++*}$	1.532
7	Itraconazole	16.01 ± 1.8	21.39 ± 1.06	24.5 ± 2.8	0.178

Values represent mean ± standard deviation (n = 3). ++ indicates values significantly different from Itraconazole (+ve control) at the level of $p < 0.01$. * indicates values significantly different from the normal leaf extract of the particular plant at the corresponding concentration at $p < 0.05$. ** indicates values significantly different ($p < 0.01$) from the normal leaf extract of the corresponding plant at the respective concentration.

iThenticate Page 148 of 209 - Integrity Submission Submission ID trn:oid:::3618:121855597

Antifungal assay of *A. niger* by food poisoning method

Table 4.13 shows the percentage growth inhibition produced by different ethanolic extracts of *A. scholaris* and *F. racemosa* against *A. niger* along with positive control Itraconazole. Antifungal activity of ethanolic extracts of *A. scholaris* and *F. racemosa* against *A. niger* is shown in Plate 4.13. The Figure A is the photograph showing the growth of hyphae in PDA medium with Itraconazole, Figure B shows the growth of *A. niger* hyphae in medium with ethanol, Figure C is the growth of hyphae in medium with AS-NL extract, Figure D shows the growth of hyphae in medium with AS-GL ethanolic extract, Figure E represents the growth of hyphae in medium with AS-GA extract, here maximum inhibition in growth can be seen with a growth inhibition percentage of 84.89 ± 1.22. Figure F and G shows the growth of hyphae in medium with FR-NL extract and FR-GL extract respectively. Among all the extracts tested, the highest inhibition of growth exhibited by FR-GL extract, with growth inhibition percentage of 90.41 ± 2.20. Figure H shows the growth of hyphae in medium with FR-GA extract. Plate 4.14: Figure B represents a bar diagram showing the average growth inhibition percentages of all extracts with standard deviation.

All other extracts except ethanol have no activity against all the tested microorganisms, so only ethanolic extracts of *A. scholaris* and *F. racemosa* were selected to analyse antifungal activity against *A. niger.* All the three samples of both plants were used to screen the antifungal activity by food poisoning method. The study is limited only to preliminary screening with 2 concentrations. 100 mg/mL stock solutions of all extracts were prepared. 1 mL from the stock is used for first screening and doesn't show any inhibition of mycelial growth. Then concentration increased upto 500 mg and activity was found. The average mycelial growth of treated plates was compared with control. The results indicate that lower concentration of all the extracts have no action against *A. niger*. Here antifungal drug Itraconazole (50 mg/mL stock concentration) was also tested for activity against *A. niger*.

Table 4.13: Percentage growth inhibition against *A. niger* shown by ethanolic extracts of different samples of *A. scholaris* and *F. racemosa* along with SD.

Sl. No.	Sample	Growth inhibition percentage

iThenticate Page 148 of 209 - Integrity Submission Submission ID trn:oid:::3618:121855597

1.	Control (Itraconazole)	93.47 ± 0.90
2.	AS-NL	$10.15 \pm 1.60^{++}$
3.	AS-GL	$16.80 \pm 4.53^{++*}$
4.	AS-GA	$84.89 \pm 1.22^{++**}$
5.	FR-NL	$17.09 \pm 4.98^{++}$
6.	FR-GL	$90.41 \pm 2.20^{+**}$
7.	FR-GA	$74.91 \pm 2.31^{++**}$

Each value of growth inhibition percentage represents mean ± standard deviation (n = 3). + indicates values significantly different from Itraconazole (+ve control) at the level of $p < 0.05$. ++ indicates values significantly different from Itraconazole ($p < 0.01$). * indicates values significantly different from normal leaf samples of a particular plant ($p < 0.05$). ** indicates values significantly different from the normal leaf of the corresponding plant at the level $p < 0.01$.

The statistical analysis revealed that all the extracts of *A. scholaris* and *F. racemosa* exhibited significant antifungal activity when compared to the control drug, Itraconazole ($p < 0.01$). Among the tested extracts, the ethanolic extract of gall leaves of *F. racemosa* (FR-GL) demonstrated the highest inhibitory effect against *A. niger*, showing 90.41 ± 2.20% growth inhibition. This was followed by the gall extract of *A. scholaris* (AS-GA), which exhibited 84.89 ± 1.22% inhibition. Statistical evaluation indicated that all extracts, except AS-GL, showed highly significant antifungal activity ($p < 0.01$), while AS-GL also exhibited a significant level of inhibition ($p < 0.05$). These results suggest that gall-associated tissues, particularly in *F. racemosa*, possess more potent antifungal constituents compared to normal leaf extracts.

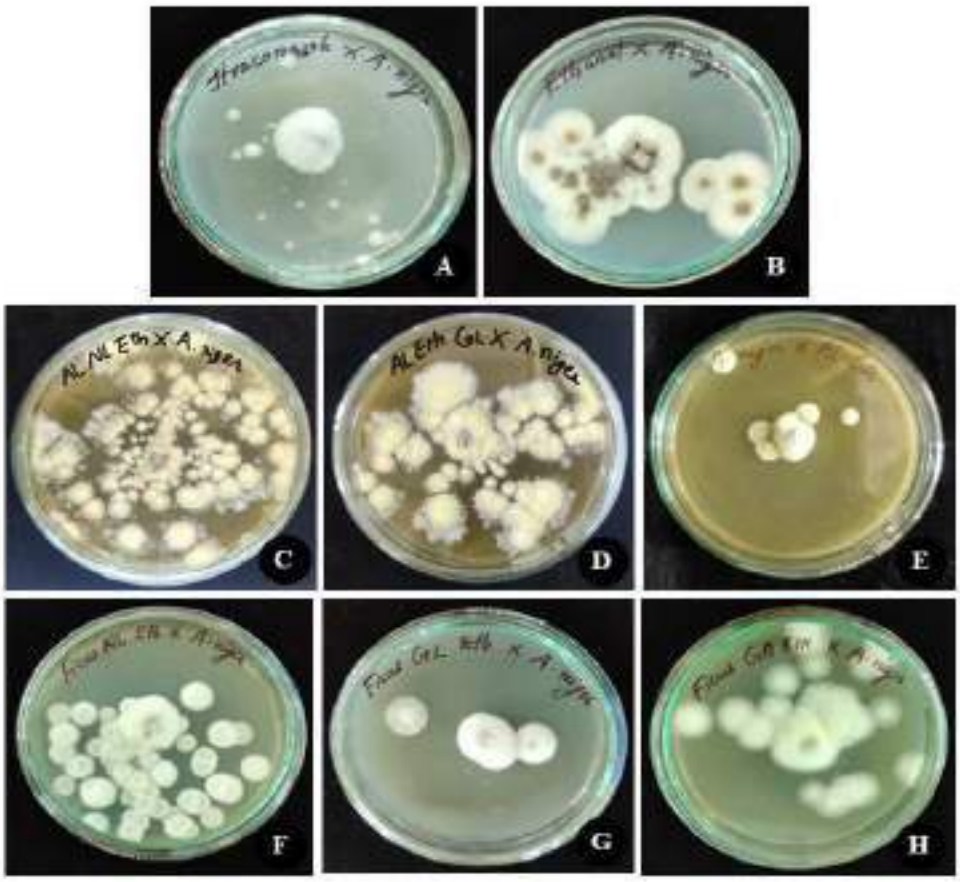
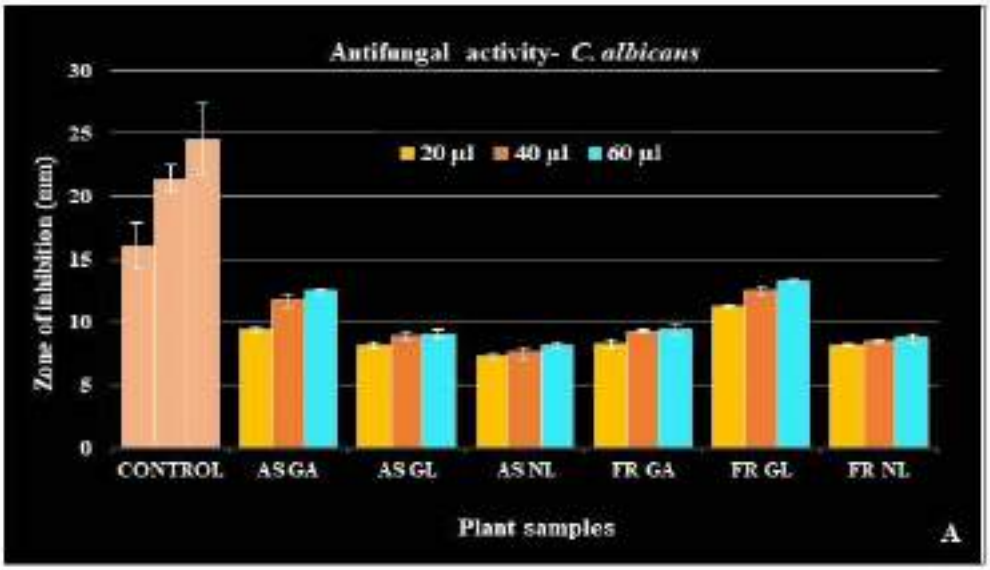


Plate 4.13: Antifungal activity of ethanolic extracts of *A. scholaris* and *F. racemosa* against *A. niger*. Figure A: Growth of hyphae in medium with Itraconazole, Figure B: Growth of hyphae in medium with ethanol, Figure C: Growth of hyphae AS-NL extract, Figure D: Growth in medium with AS-GL extract, Figure E: Growth of hyphae in AS-GA extract, Figure F: Growth of hyphae with FR-NL extract, Figure G: Growth in medium with FR-GL extract and Figure H: Growth of hyphae in medium with FR-GA extract.



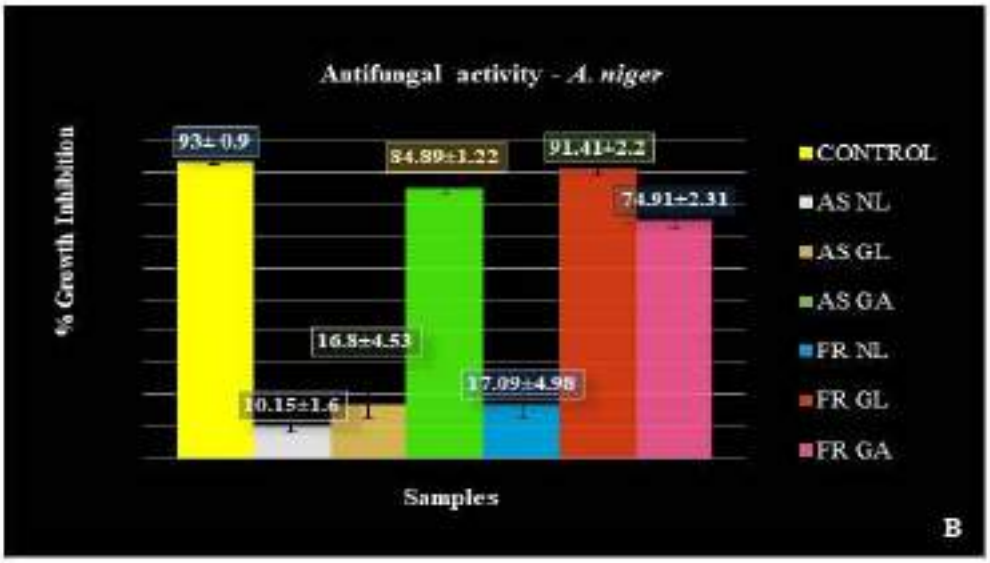


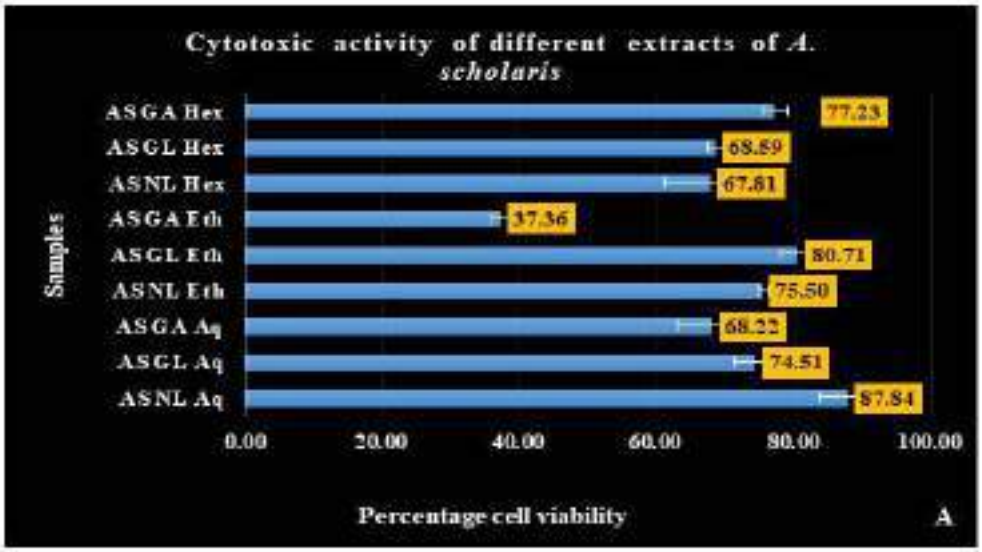
Plate 4.14: Graphical representation of antifungal activity. Figure A: Antifungal activity of ethanolic extracts of *A. scholaris* and *F. racemosa* against *C. albicans*. The X-axis represents different plant samples and positive control, while the Y-axis shows inhibition zones (mm) and Figure B: Percentage growth inhibition of ethanolic extracts *of A. scholaris* and *F. racemosa* against *A. niger*, with Itraconazole as the positive control. The X-axis represents different samples and the Y-axis shows growth inhibition (%).

Bonvicini and colleagues found that ethanolic extracts of *A. scholaris* exhibited antifungal activity against *C. albicans* (Bonvicini et al., 2014). The isopropanol extracts of *A. scholaris* leaves are highly effective against pathogenic fungi (Bagheri et al., 2020). Another study proved that at all doses, the ethanolic leaf extract of *F. racemosa* exhibited moderate activity against *Rhizopus* and no activity against *A. flavus* (Thilagavathi & Kathiravan, 2017). In the present investigation, FR-GL at 60 µL dilution demonstrated a 13.2 ± 0.14 mm zone of inhibition against *C. albicans*, while AS-GA at 60µL demonstrated 12.45 ± 0.21 mm zone of inhibition. FR-GL found to inhibit *A. niger* growth by 9%. In 2023, Mishra and colleagues investigated the bactericidal properties and found the methanol extract showed the greatest activity with zone of inhibition against *S. aureus* (23.42 mm), *C. albicans* (26.74 mm) and *A. niger* (25.78 mm) (Mishra et al., 2023).

4.3.4. Results of cytotoxic activity analysis

Preliminary screening for cytotoxic assay

The cytotoxic activity of aqueous, hexane and ethanolic extracts (250 mg/mL) from *A. scholaris* and *F. racemosa* was evaluated using the MTT assay on 3T3 cell lines. For AS-GA ethanolic extracts showed the highest cytotoxic activity, with a cell viability of 37.36%, significantly lower than other AS-NL and AS-GL (Plate 4. 15: Figure A). For FR-GL ethanolic extracts demonstrated the highest cytotoxicity, with a cell viability of 35.62% (Plate 4. 15: Fig B). The preliminary screening results were shown in Table 4.14. Initial screening determined cell viability percentages, revealing that ethanolic extracts of galls AS-GA and FR-GL exhibited the highest cytotoxicity.



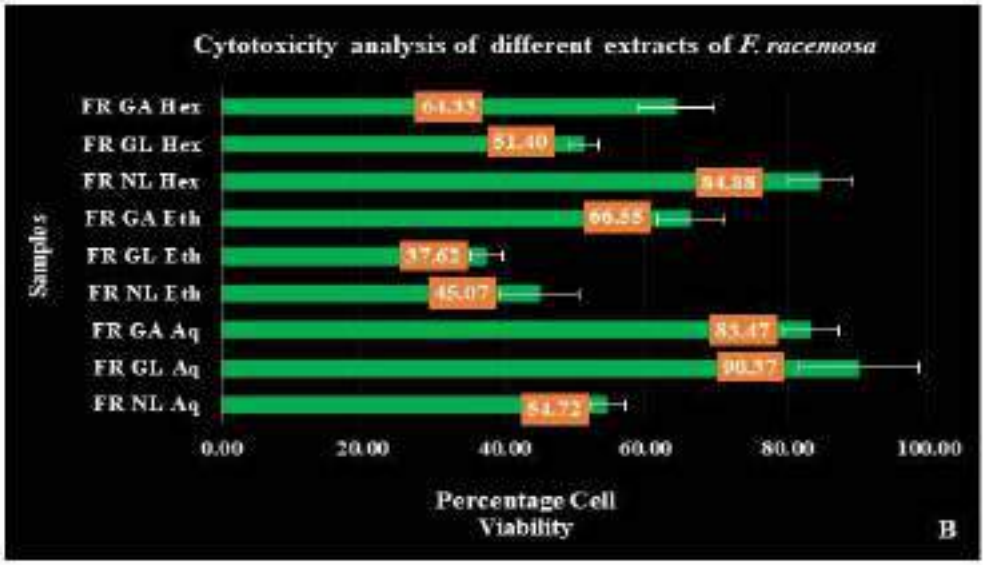


Plate 4.15: Figure A: Cell viability percentage of *A. scholaris* extracts- AS-NL, AS-GL and AS-GA showing mean values with standard deviation, indicating their effect on cell viability and Figure B: Cytotoxic activity of *F. racemosa* extracts-FR-NL, FR-GL and FR-GA represented as cell viability percentage with standard deviation

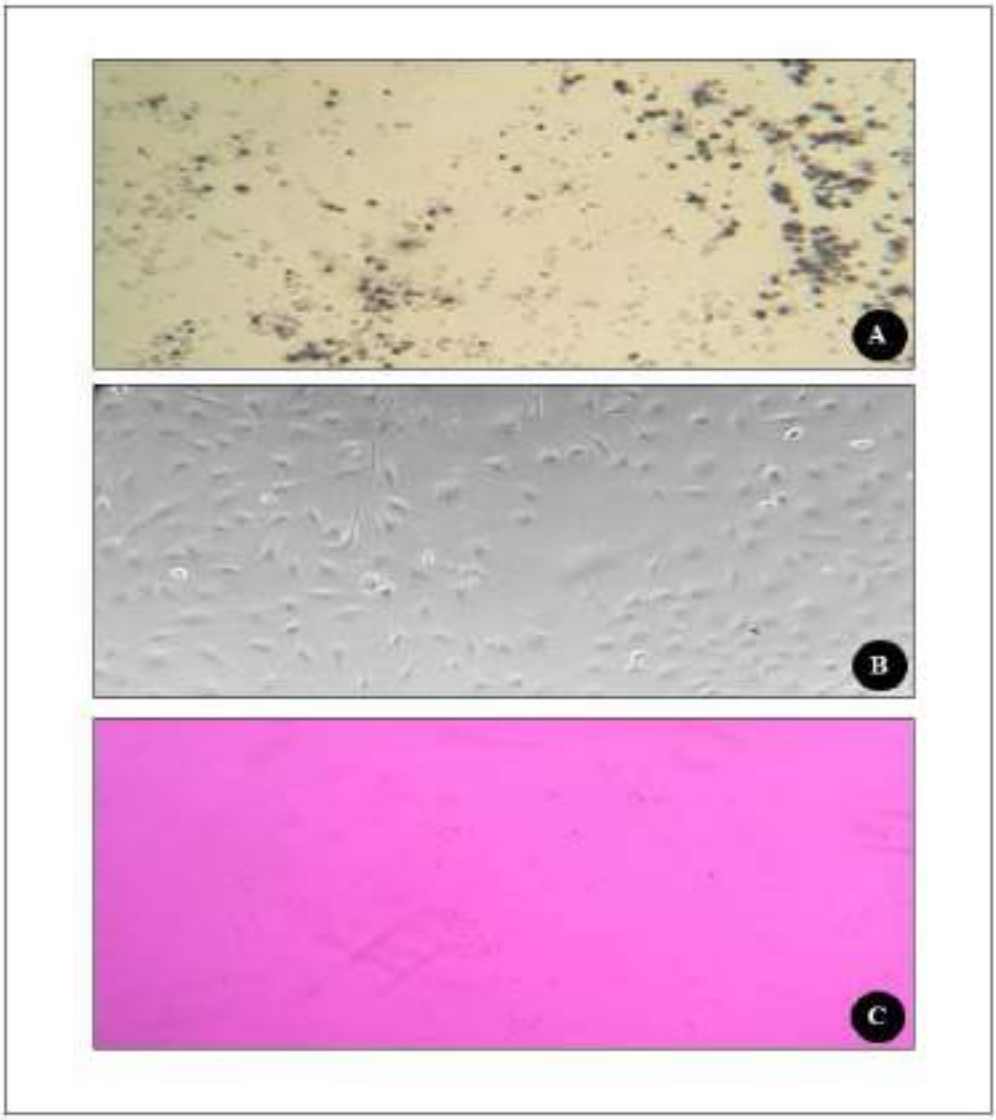


Plate 4.16: Microscopic representation of 3T3 cells. Figure A: Image of untreated 3T3 cells used for cytotoxicity assay, Figure B: Image of 3T3 cells after treatment, showing the formation of formazan crystals indicating metabolically active cells and Figure C: Photograph of MTT-treated 3T3 cells after addition of solubilisation reagent showing the developed color intensity used for viability measurement.

Table 4.14: Percentage cell viability of aqueous, ethanolic and hexane extracts of *A. scholaris* and *F. racemosa*.

Sl. No.	Extract (250 mg/mL)	Name of sample	Cell viability percentage
1.	Aqueous	AS-NL	87.84 ± 3.97
2		AS-GL	74.51 ± 3.09*
3.		AS-GA	68.22 ± 5.30*
4.		FR-NL	54.72 ± 2.46
5.		FR-GL	90.37 ± 8.46**
6.		FR-GA	83.47 ± 3.93**
7.	Ethanol	AS-NL	75.50 ± 0.62
8.		AS-GL	80.71 ± 2.73
9.		AS-GA	37.36 ± 1.43**
10.		FR-NL	45.07 ± 5.60
11.		FR-GL	35.62 ± 2.32*
12.		FR-GA	66.55 ± 4.80*
13.	Hexane	AS-NL	67.81 ± 6.71
14.		AS-GL	68.59 ± 1.13
15.		AS-GA	77.23 ± 1.79*
16.		FR-NL	84.88 ± 4.54

17.		FR-GL	51.40 ± 2.00**
18.		FR-GA	64.33 ± 5.44*

Each value of percentage of cell viability represents mean ± standard deviation (n = 3). * indicates the value of percentage of cell viability, which is significantly different from percentage of cell viability of the normal leaf samples of the particular plant ($p < 0.05$). ** indicates the value of percentage of cell viability, which is significantly different from percentage of cell viability of the normal leaf samples of the particular plant at the level of significance $p < 0.01$.

For finding statistical significance, ANOVA was conducted to assess differences in cell viability across concentrations with a significance level ($p < 0.05$). Tukey's HSD post hoc test was conducted to compare cell viability percentages between normal leaves, gall leaves and galls samples for *A. scholaris* and *F. racemosa* across aqueous, ethanol and hexane extracts. Significant differences ($p < 0.05$) were observed in aqueous extract and ethanolic extracts of both plants and also in hexane extract of *F. racemosa*. No significant differences were found in hexane extract of *A. scholaris*.

Concentration-dependent MTT assay

Based on the preliminary results, ethanolic extracts of AS-GA and FR-GL were selected for concentration-dependent MTT analysis. 3T3 cells treated with DMSO served as controls, while cells treated with test solutions at concentrations of 50, 100, 200, 300 and 400 mg/mL were analyzed. Absorbance was measured at 570 nm and 630 nm using a microplate reader and cell viability percentages were calculated (Table 4.15). The MTT assay results showed that cell viability decreased as extract concentration increased, indicating a concentration-dependent increase in cytotoxicity. Plate 4.16: Figure A is the image of untreated 3T3 cells used for cytotoxicity assay, Figure B shows the microscopic photograph of 3T3 cells after treatment, showing the formation of formazan crystals indicating metabolically active cells and Figure C is the photograph of MTT treated 3T3 cells after addition of solubilisation reagent showing the developed color intensity used for viability measurement. Representation of a culture plate

showing formazan crystals represented in Plate 4.17: Figure A. The percentage cell viability of each concentration of both extracts was represented graphically in Plate 4.17: Figure B.

15 The viability percentage of each dilution of extracts (50 mg/ml, 100 mg/ml, 200 mg/ml, 300 mg/ml and 400 mg/ml) were calculated. It showed that as the concentration increases the cytotoxic effect of extract also increases. 50 mg/mL ethanolic extracts of AS-GA showed 80 ± 0.65% of cell viability and the same dilutions of FR-GL ethanol extracts showed maximum cell viability of 97.71 ± 2.54%. For dilutions 100 mg/mL and 200 mg/mL of extracts of *A. scholaris* showed 49.44 ± 2.91% and 40.89 ± 2.69% cell viability respectively. 34.15 ± 4.14% and 15.60 ± 5.8% cell viability was shown by 300 mg/mL and 400 mg/mL of extracts. In the case of FR-GL ethanol extracts, 100 mg/mL and 200 mg/mL dilutions displayed 89.43 ± 2.11% and 79.27 ± 3.94% cell viability respectively. The percentage cell viability lowers upto 9.90 ± 5.15% for concentration 400 mg/ml. IC_{50} value was calculated for ethanolic extracts of AS-GA (IC_{50}-170.6 ± 4.72 mg/mL). The ethanolic extracts of FR-GL exhibited an IC_{50} value of 258.8 ± 8.47 mg/mL.

Table 4.15: Concentration dependent cell viability assay results of AL-GA and FR-GL showing the cell viability percentage of 50-500 mg/mL of sample concentrations.

Sl. No	Concentration of sample (mg/mL)	AL-GA (%cell viability)	FR-GL (%cell viability)	Triton X-100 (control) (%cell viability)
1.	50 mg/mL	$80.00 \pm 0.65^{++}$	$97.71 \pm 2.54^{++}$	0.00
2.	100 mg/mL	$49.44 \pm 2.91^{++}$	$89.43 \pm 2.11^{++}$	0.00
3.	200 mg/mL	$40.89 \pm 2.69^{++}$	$79.27 \pm 3.94^{++}$	0.00
4.	300 mg/mL	$34.15 \pm 4.14^{++}$	$27.83 \pm 0.31^{++}$	0.00
5.	400 mg/mL	$15.60 \pm 5.80^{++}$	$9.40 \pm 5.15^{++}$	0.00

Each value of percentage cell viability represents mean ± SD (n = 3). ++ indicates values significantly different from Triton X-100 (+ve control) at the level of $p < 0.01$.

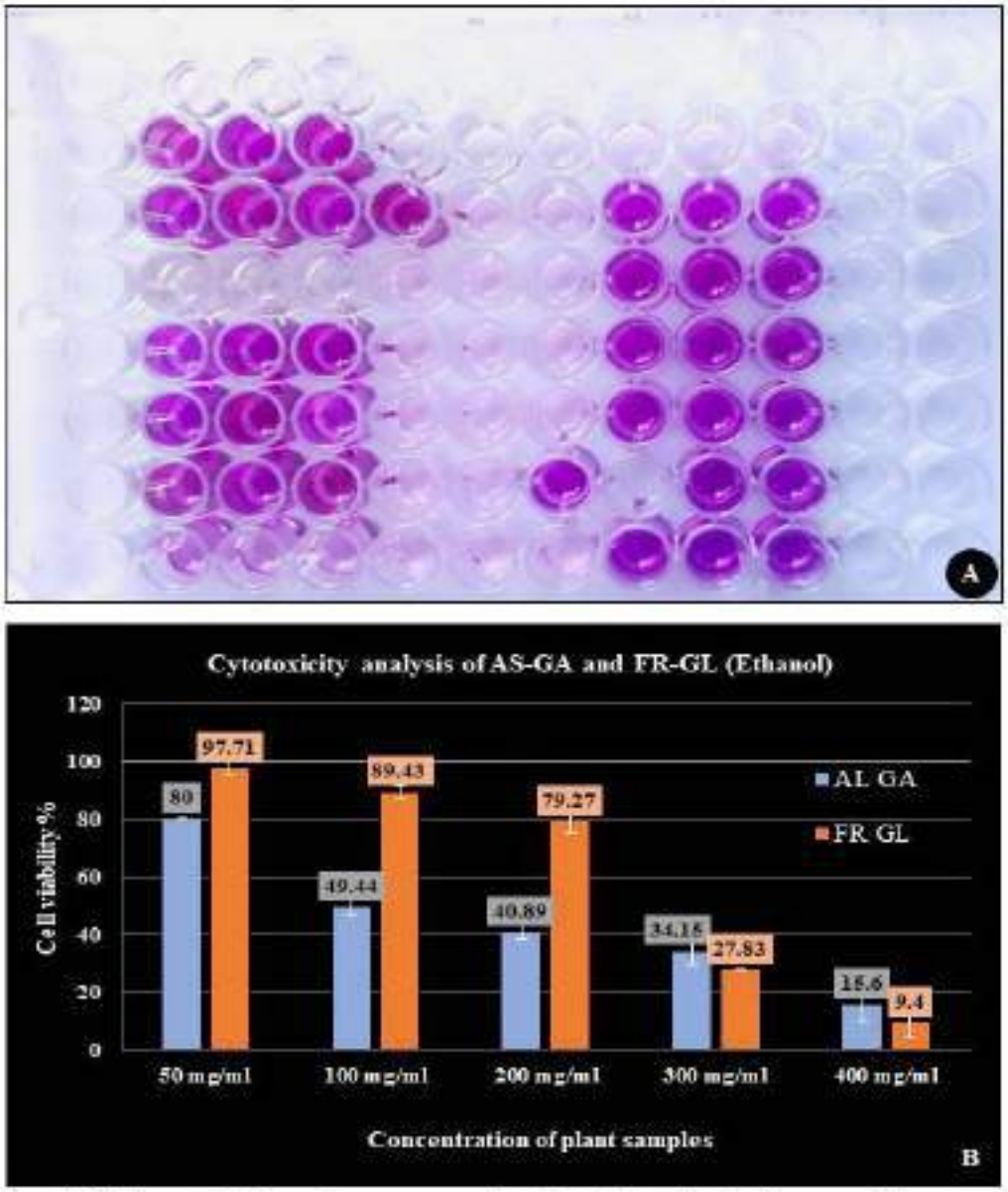


Plate 4.17: Figure A: shows the representation of a culture plate having purple formazan crystals and Figure B: The graph showing the results of cell viability percentage of etanolic extracts of *A. scholaris* gall (AS-GA) and *F. racemosa* gall leaf (FR-GL) using MTT assay.

Comparing the two extracts, AS-GA exhibited higher cytotoxicity than FR-GL at lower concentrations (50-200 mg/mL). At 50 mg/mL, AS-GA had a cell viability of 80.00 ± 0.65% compared to FR-GL 97.71 ± 2.54%. At 100 mg/mL, AS-GA cell viability (49.44 ± 2.91%) was significantly lower than FR-GL (89.43 ± 2.11%). However, at higher concentrations (400 mg/mL), both extracts showed comparable cytotoxicity, with cell viabilities of 15.60 ± 5.80% (AS-GA) and 9.40 ± 5.15% (FR-GL). The steeper decline in cell viability for AS-GA at lower concentrations suggests greater potency, corroborated by its lower IC_{50} value. From the results it was discovered that compared to normal leaves of the selected plants the cytotoxicity was higher in the gall associated portions. In the case of AS-GA, it displayed a higher cytotoxic effect than other parts, but in the case of *F. racemosa,* the leaf portion in which gall resides *i.e.* gall leaves showed maximum cytotoxicity. From these observations it can be found that gall induction on the leaves of both selected plants will result in greater content of bioactive chemicals in those portions of plants. These bioactive compounds may result in the different biological activities shown by the plants.

4 The statistical analysis confirmed significant differences in cytotoxic activity ($p < 0.01$). Tukey's HSD post-hoc test indicated that cell viability at 50 mg/mL and 100 mg/mL differed significantly between AS-GA and FR-GL ($p < 0.01$), with AS-GA showing greater cytotoxicity. All the tested concentrations showed significant cytotoxicity at $p < 0.01$. The concentration-dependent decrease in cell viability was statistically significant for both extracts ($p < 0.01$).

18 In 2023, Shanmugapriya and coworkers used the MTT assay to examine whether *A. scholaris* leaf extract induced apoptosis in the breast cancer cell lines MCF-7 and MDA-MB 231. They reported that in breast cancer cell lines, treatment with *A. scholaris* extract significantly increased the rate of apoptosis (Shanmugapriya et al., 2023). Itam and colleagues employed the brine shrimp lethality test method in their study to examine the cytotoxic activities of *A. scholaris* leaf extract. They discover that the death rate of *Artemia salina* larvae rises in concordance with the extract concentration. With an IC_{50} value of 883.28 µg/mL, methanol extract exhibited highest cytotoxicity, followed by ethyl acetate extract with IC_{50} 954.72 µg/mL (Itam et al., 2018). Sarkar and colleagues reported an LC_{50} of 61.85 µg/mL for *A. scholaris* leaf extracts against brine shrimp (Sarkar et al., 2021). The MTT technique was used in this study to assess the cell viability of 3T3 cell lines in different samples of aqueous, hexane and ethanol

extracts. Based on the initial screening conducted, ethanolic extracts of AS-GA exhibit much higher cytotoxic activity than aqueous and hexane extracts. Concentration-dependent cytotoxic action was demonstrated by gall ethanolic extracts.

In 2016, Bagheri and associates found that *A. scholaris* hexane extracts caused 100% cell death in Dalton's lymphoma ascites cells at 500 µg/mL, while methanolic extracts lacked cytotoxicity. However, at lower concentrations, its cytotoxicity was less pronounced in the aqueous extracts of *A. scholaris* leaves, benzene and isopropanol exhibited minimal action at the higher dosage (Bagheri et al., 2016). Ethyl acetate fraction of *A. scholaris* had the highest cytotoxicity activity against brine shrimp with LD_{50} values 944.730 ppm after 6 h treatment (Ara et al., 2020). Based on the current investigation, the highest cytotoxicity was detected at 500 mg/mL of AS-GA, with cell viability of -4.80 ± 2.66% and an IC_{50} of 170.6 ± 4.72 mg/mL. In an earlier investigation, the cytotoxicity was also examined on a mammalian cell line and it was discovered that at the tested dilutions, the extracts had no effect on the proliferation of mammalian cells (Bonvicini et al., 2014). The present study does not give information regarding the cytotoxic activity of extracts in cancer or mammalian cell lines. The results of this experiment can only be interpreted as a basic cell viability assessment of extracts.

Gorla and Shankar (2016) analysed *F. racemosa* ethanolic and hexane extracts for cytotoxicity with XTT assay resulted in moderate anticancer activities against A549 and MDA-MB-435S cell lines. At a dosage of 100 µg/mL, *F. racemosa* hexane extract inhibited cell growth more effectively than ethanol extracts (Gorla & Shankar, 2016). A study published in 2021 disclosed anticancer properties of various parts of *F. racemosa*. MTT analysis of the anticancer effects on DLA tumour cells revealed a concentration-dependent antitumorigenic effect (Poongothai & Annapoorani, 2021). In 2011, Hosain and colleagues investigated the cytotoxic potential of *F. racemosa* leaf extracts. LC_{50} Value of methanol extract of *F. racemosa* leaves was 0.467 µg/mL which indicates significant activity (Hosain et al., 2011). In this experiment, gall leaves ethanolic extracts were further examined at five different concentrations. The outcomes indicated that the cytotoxic efficiency increased with increase in concentration of extracts. Cell viability at a 400 mg/mL concentration of FR-GL is 9.40%. The IC_{50} value for FR-GL was 258.8 ± 8.47 mg/mL.

iThenticate Page 161 of 209 - Integrity Submission Submission ID trn:oid:::3618:121855597

4.3.5. Results of anti-inflammatory activity analysis

Anti-inflammatory activity of FR-GL extracts were analysed using protein denaturation inhibition assay in five different dilutions of sample.The results were shown in Table 4.16. The extract (FR-GL) exhibited dose-dependent inhibition of protein denaturation, indicating notable anti-inflammatory potential. At the highest concentration (5 µg/µL), the extract showed 34.33% protection, compared to 80.23% by the standard drug, Diclofenac sodium. These results suggest that FR-GL possesses moderate anti-inflammatory activity. The photographs of the results were shown in Plate 4.18: Figure A- Figure G.

Table 4.16: Anti-inflammatory activity of five different dilutions of FR-GL extracts, using protein denaturation inhibition assay.

Sl. No.	Dilutions of FR-GL extract (µg/µL)	Percentage of protection
1.	1	$17.37 \pm 0.03^{**}$
2.	2	$22.21 \pm 0.47^{**}$
3.	3	$28.04 \pm 0.64^{**}$
4.	4	$31.63 \pm 1.75^{**}$
5.	5	$34.33 \pm 0.30^{**}$
6.	Diclofenac sodium (standard)	80.23 ± 0.20

Each value of percentage of protection represents mean ± standard deviation (n = 3). ** indicates values of percentage of protection, that significantly different from the percentage of protection value of standard - Diclofenac sodium at $p < 0.01$.

Statistical analysis (ANOVA) followed by Tukey's HSD post hoc test revealed a significant ($p < 0.01$) concentration-dependent increase in inhibition of protein denaturation from 17.37 ± 0.03% at 1 µg/µL to 34.33 ± 0.30% at 5 µg/µL. All dilutions of FR-GL extract showed significantly lower inhibition of protein denaturation compared to the standard Diclofenac sodium (80.23 ± 0.20%).

iThenticate Page 161 of 209 - Integrity Submission Submission ID trn:oid:::3618:121855597

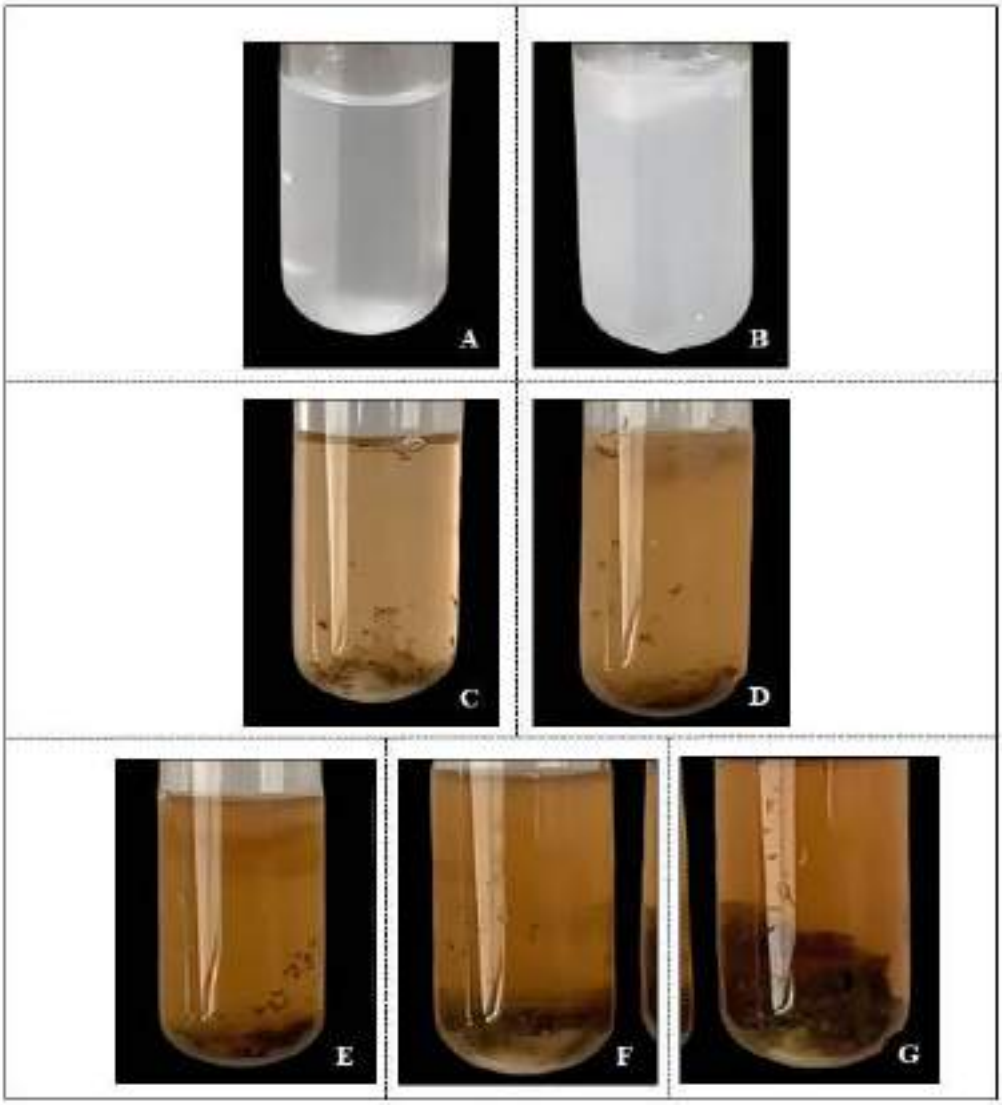


Plate 4.18: Anti-inflammatory activity analysis by protein denaturation inhibition assay. Figure A: Control assay showing untreated protein sample, Figure B: Activity of standard drug (Diclofenac sodium) showing inhibition of protein denaturation and Figure C-G: Anti-inflammatory activity of *F. racemosa* gall leaf (FR-GL) ethanolic extract at concentrations of 1, 2, 3, 4 and 5 µg/µL, respectively, showing progressive inhibition of protein denaturation with increasing extract concentration.

The present findings corroborate earlier reports highlighting the anti-inflammatory potential of *F. racemosa*. The *in vitro* assays provide supporting evidence for the anti-inflammatory efficacy of *F. racemosa*. The bark extracts showed a concentration-dependent inhibition of protein denaturation, a mechanism closely associated with the prevention of inflammatory processes. Interestingly, hot water extracts were more effective than cold water extracts, and their inhibitory potential even surpassed that of standard reference drugs such as Prednisolone and Ibuprofen at lower concentrations (Dharmadeva et al., 2018). This highlights the role of extraction methods in maximizing bioactive compound availability and effectiveness. In our study, the FR-GL extract at a concentration of 5 µg/µL provided 34.33% protection against protein denaturation, although the standard Diclofenac sodium exhibited a much higher protection rate of 80.23%.

In addition to *in vitro* studies, the carrageenan, serotonin, histamine and dextran-induced rat paw edema models, leaf extracts at 200 and 400 mg/kg demonstrated significant anti-inflammatory activity, with the maximum inhibition observed at 400 mg/kg (30.4 - 33.9% reduction in paw edema after 3 h) (Mandal et al., 2000a). These outcomes indicate that *F. racemosa* possesses broad-spectrum activity against different mediators of inflammation, suggesting both antihistaminic and antiserotonergic properties in addition to its inhibitory effect on carrageenan-induced edema. Similarly, intraperitoneal administration of the extract at a lower dose (20 mg/100 g) was also reported to suppress carrageenan-induced acute inflammation, with notable effects at later phases (3rd, 5th and 7th h), thus confirming the consistency of its anti-inflammatory action across different experimental models (Mehta et al., 2012). This suggests that *F. racemosa* exhibits measurable anti-inflammatory activity.

In this study a preliminary analysis was conducted to evaluate the antibacterial, antifungal, antioxidant and cytotoxic activities of aqueous, hexane and ethanolic extracts from *A. scholaris* and *F. racemosa*. The results demonstrated that ethanolic extracts were the most effective across all tested activities, particularly those from AS-GA and FR-GL. So, FR-GL extracts were analysed by separation of different compounds using TLC (Chapter 5). The same sample is used for detection of anti-inflammatory activity.

iThenticate Page 164 of 209 - Integrity Submission Submission ID trn:oid:::3618:121855597

4.4. CONCLUSIONS

According to the studies of the bioactive properties of the tissues of *A. scholaris* and *F. racemosa*, the gall and tissues related to galls exhibit increased activity. Bioactivity of plant extracts is due to the presence of various phytochemical components present in them. As mentioned in the preceding chapter, galls and gall leaves have substantially more secondary metabolites than normal leaves. There is another finding that the bioactivity may vary between various extracts, which could be explained by variable degrees of solubility of the active components in the solvents utilized. The antimicrobial properties of extracts may be related to the presence of antimicrobial components including alkaloids, flavonoids, saponins, tannins and phenolics. Many bioactive phytoconstituents may be present in the plant samples, which could explain the reducing power ability of extracts. As mentioned above, galls and gall leaves extracts contain greater quantity and number of bioactive substances, such as phenols, flavonoids, tannins, etc., which are tumour inhibitors, scavengers of free radicals, inducers of apoptosis and promoters of cell differentiation. The FR-GL extract possessed greater activity than all other samples in terms of antioxidant, antibacterial, antifungal and cytotoxic activities. FR-GL was tested and proved to possess moderate anti-inflammatory activity. Gall and gall associated tissues of both plants have higher activity than healthy leaves. So, it can be concluded that gall or gall associated tissues have much greater phytoconstituents and thus significant bioactivity. Additionally in future exploring the ecological roles of gall-induced bioactivity and comparing these plants with other gall-forming species could generalize the findings, contributing to phytopharmacology and sustainable drug development.

iThenticate Page 164 of 209 - Integrity Submission Submission ID trn:oid:::3618:121855597

CHAPTER 5 - ISOLATION OF BIOACTIVE FRACTIONS RESPONSIBLE FOR POTENT BIOLOGICAL ACTIVITY

5.3. RESULTS AND DISCUSSION

5.3.1. Selection of most bioactive sample

All the extracts of *A. sholaris* and *F. racemosa* were evaluated for their antioxidant potential using the RP and DPPH radical scavenging assays. The obtained results from both assays were statistically analyzed to compare the antioxidant efficiency among the samples. Selection of the most active extracts was based on the following criteria: (i) percentage radical scavenging activity (ii) lowest IC_{50} values in both RP and DPPH assays, indicating higher antioxidant potency; (iii) consistent performance across both assays and (iv) statistically significant difference ($p < 0.05$) compared to the other samples. The highest antioxidant activity was observed in FR-GL (ethanol) and FR-GA (ethanol) based on these parameters.

Based on the antibacterial screening, the selection of the most active sample was carried out by considering the zone of inhibition (mm) against three bacterial strains (*E. coli*, *B. subtilis* and *S. aureus*). Among the samples of *A. scholaris*, AS-GA ethanolic extracts exhibited the largest inhibition zones across all tested bacteria (*E. coli*: 14.33 mm; *B. subtilis*: 13.5 mm; *S. aureus*: 14.16 mm) and was therefore considered the most active among samples of *A. scholaris*. In the case of *F. racemosa*, FR-GL (ethanol) showed the highest antibacterial activity with maximum zones of inhibition recorded for *E. coli* (17.25 mm), *B. subtilis* (15.66 mm) and *S. aureus* (17.16 mm), surpassing both FR-NL and FR-GA extracts. Comparing the most active extracts of both plants, FR-GL demonstrated the overall strongest antibacterial potential, indicating it as the most potent sample. Similarly, in antifungal assays, AS-GA demonstrated substantial activity against *C. albicans* and *A. niger*, with maximum inhibition zones of 12.45 mm (IC_{50} - 0.548 mL) and 84.89% growth inhibition respectively. FR-GL again outperformed all other extracts, showing inhibition zones of 13.2 mm against *C. albicans* (IC_{50} - 0.534 mL), 90.41% growth inhibition against *A. niger*, closely approaching the standard antifungal drug, Itraconazole. Overall, across both antibacterial and antifungal assays, FR-GL emerged as the most potent extract.

The cytotoxic potential of aqueous, ethanolic and hexane extracts of *A. scholaris* and *F. racemosa* was evaluated based on percentage cell viability. Among the *A. scholaris* extracts, AS-GA showed significant cytotoxicity, particularly in the ethanolic extract, with a cell viability of 37.36%. In the case of *F. racemosa*, FR-GL demonstrated the highest cytotoxic effect overall, with the ethanolic extract exhibiting the lowest cell viability of 35.62%. In concentration dependent cytotoxic assays using FR-GL (ethanol) and AS-GA (ethanol), FR-GL showed a more gradual reduction in cell viability from lower concentrations (97.71 ± 2.54% at 50 mg/mL) to higher concentration (79.27 ± 3.94% at 200 mg/mL). In AS-GA (80.00 ± 0.65% at 50 mg/mL and 40.89 ± 2.69% at 200 mg/mL), while both reached comparable cytotoxicity at 400 mg/mL (9.40 ± 5.15% for FR-GL and 15.6 ± 5.8% for AS-GA). Overall, FR-GL shows the highest cytotoxic potential, effectively lowering cell viability at concentrations relevant for biological activity.

FR-GL demonstrated consistently higher activity across all tested assays, and statistical analysis verified its significant potency ($p < 0.05$), making it the preferred extract for subsequent fractionation and purification.

5.3.2. Optimization of chromatographic conditions of FR-GL plant extracts

The objective of TLC using pre-coated silica plates was to optimize a solvent system to effectively separate the most bioactive sample (FR-GL) into multiple compounds. Through systematic trials of various solvent systems, an optimal mobile phase of hexane:acetone in an 8:2 ratio was determined, which successfully resolved seven distinct compounds. Plate 5.1: Figure A represents TLC analysis of FR-GL on pre-coated silica gel plate, shown under visible light, Figure B shows the TLC image under UV 254 nm and Figure C represents TLC analysis of FR-GL on pre-coated silica gel plate visible under UV 365 nm.

5.3.3. Preparative TLC analysis

The separation of FR-GL extract was done on plates prepared using silica gel G and water, shown in Plate 5.1: Figure D. The TLC analysis of FR-GL extract in hexane: acetone (8:2) resulted in seven bands. For each band Rf values vere calculated. The following Table 5.1 shows the Rf values of different bands separated from the FR-GL extract. The plant extract spotted in a

plate and develops in a chamber saturated with solvent shown in Plate 5.1: Figure E and the plant extract separated into seven bands inside the chamber shown in Figure F. TLC plate images under visible light, under UV (254 nm) and under UV (365nm) were shown in Plate 5.2: Figure A, Figure B and Figure C respectively. The separated bands were scraped off (Plate 5.3: Figure A) and dissolved using ethyl acetate. Then the filtered samples were stored in glass containers for future use. The seven samples separated and collected were shown in Plate 5.3: Figure B.

Table 5.1: The retention factor values of the bands produced through preparative TLC analysis of FR-GL extract.

Sl. No	Band number	Retention factor
1.	Band 1	0.05
2.	Band 2	0.07
3.	Band 3	0.13
4.	Band 4	0.19
5.	Band 5	0.23
6.	Band 6	0.27
7.	Band 7	0.37

Kumar and colleagues performed HPTLC analysis of *F. racemosa* leaves and resolved bands for quercetin and kaempferol (Kumar et al., 2019). TLC of leaf extract in ethyl acetate and acetone reports three spots for various phytochemicals (Ganatra et al., 2012). FR-GL extract analysed by TLC in the present work produced 7 distinct bands for various phytochemicals. *F.*

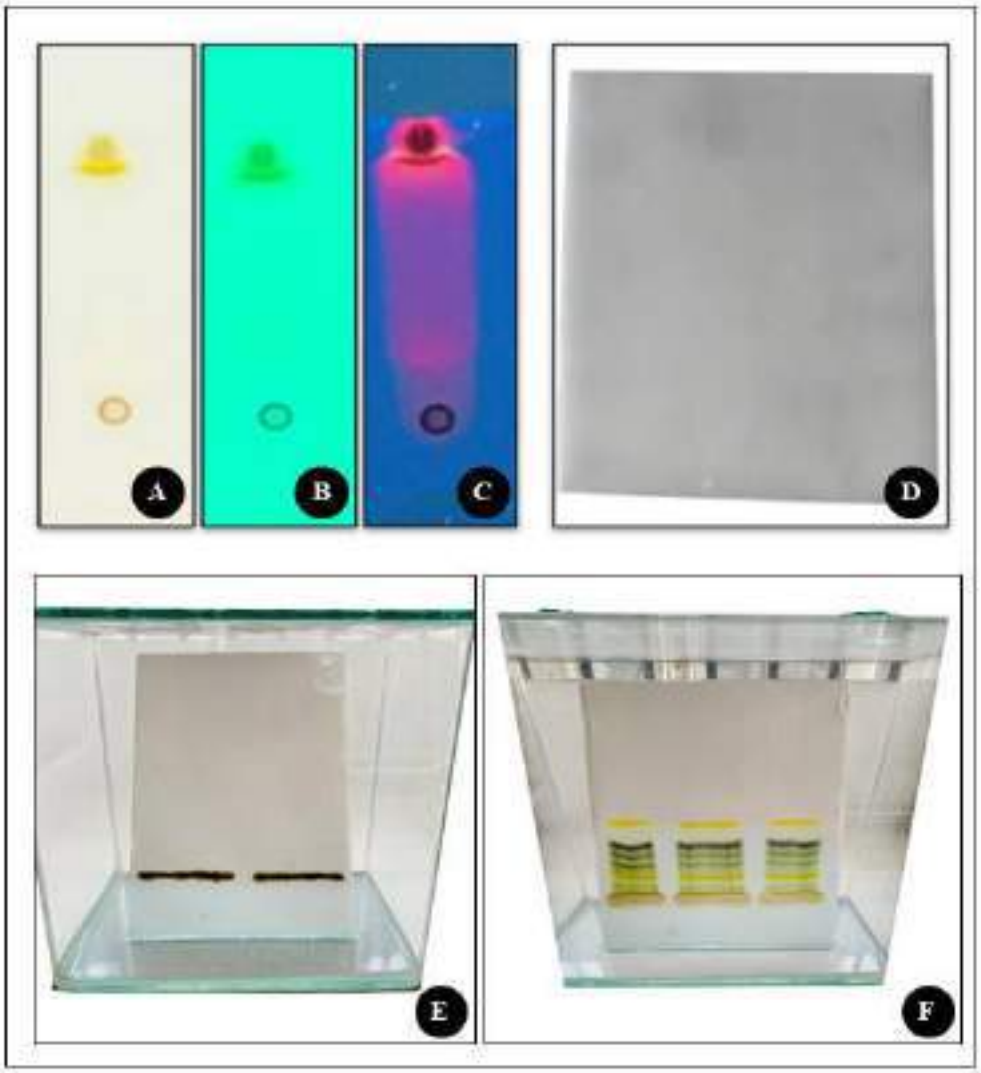


Plate 5.1: Figure A: TLC analysis of FR-GL using pre-coated silica gel plate, shown under visible light, Figure B: Pre-coated silica gel plate for separation of FR-GL under UV 254 nm, Figure C: TLC on pre-coated silica gel plate visible under UV 365 nm, Figure D: TLC plate prepared using silica gel G and water is drying at room temperature, Figure E: FR-GL extracts spotted in the plate and developing in chamber saturated with solvent and Figure F: Separation of the extract into distinct bands inside the chromatographic chamber.

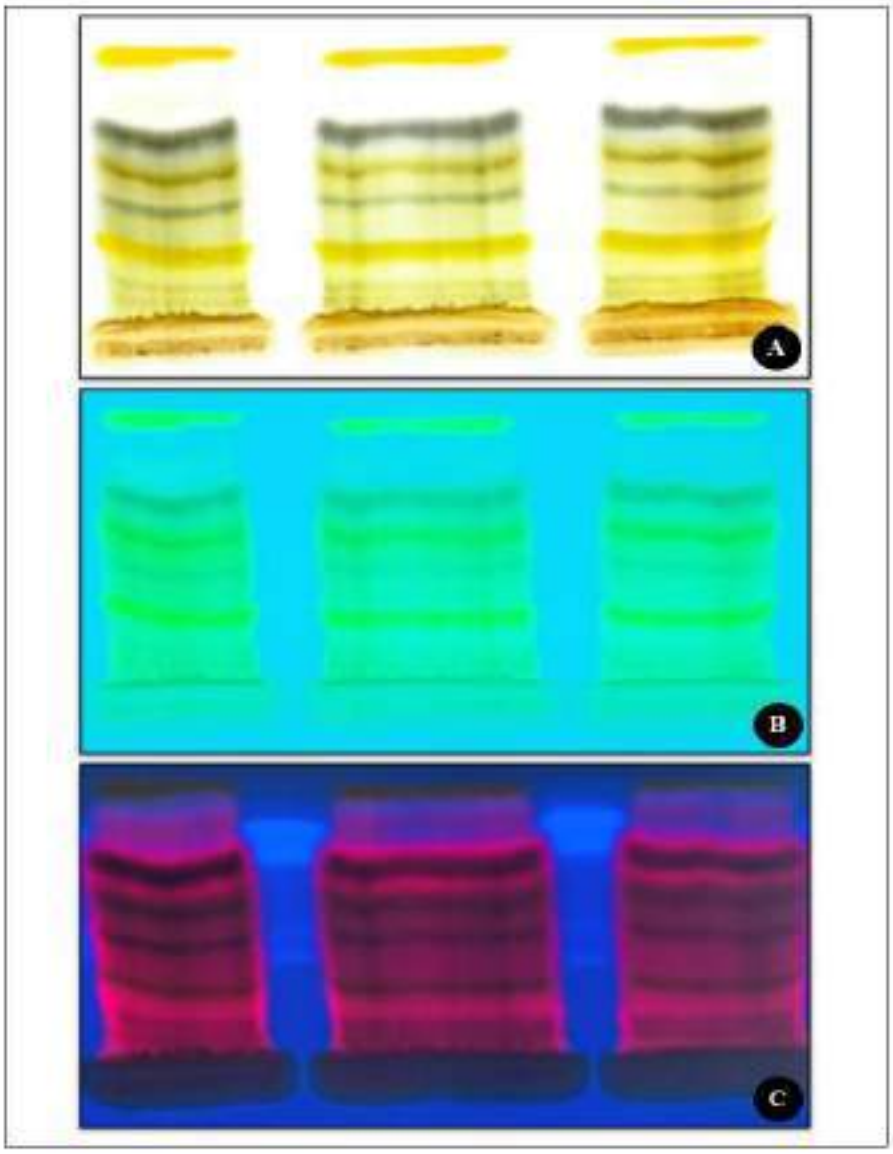


Plate 5.2: Figure A: TLC separated plate images under visible light, Fig B: TLC plate showing separated bands under UV light of 254 nm and Figure C: TLC separated plate images under UV light (365nm).

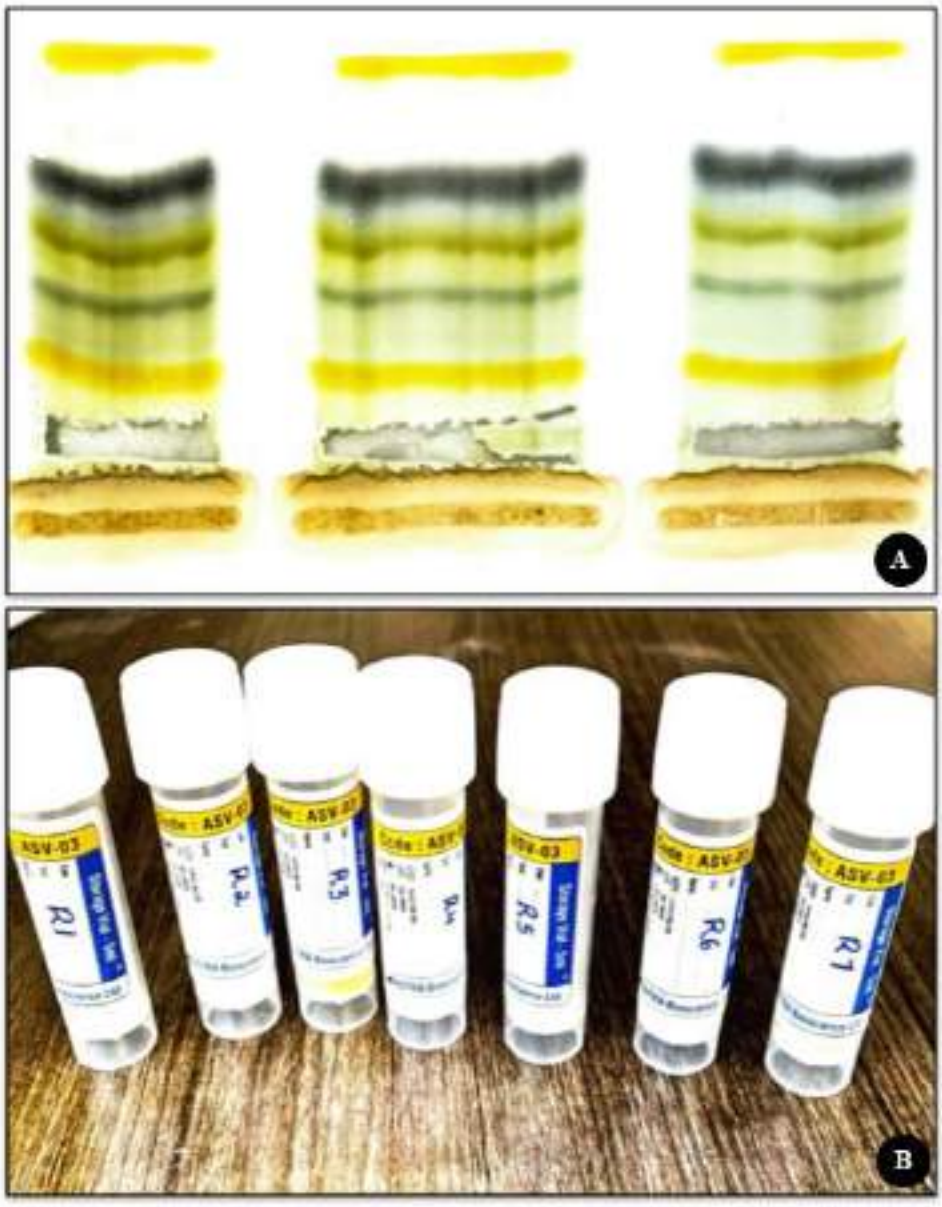


Plate 5.3: Figure A: Scraping off and separation of compounds from the silica gel plate and Figure B: Seven distinct compounds isolated from FR-GL extract, labeled as R1–R7.

racemosa stem bark were analysed by TLC technique in reported work and found that stem bark and small branches have similar phytochemical profile (Verma et al., 2015). Stem bark was also analysed by Solanki and Bhavsar in 2015 proved that alcoholic fraction were rich with highly polar compounds such as polyphenols, flavonoids, alkaloids, etc., than aqueous extract (Solanki & Bhavsar, 2017). In 2021, TLC was performed on fruit extract of *F. racemosa* separated 3 different compounds (Anand et al., 2021).

5.3.5. Results of antibacterial activity assay of separated and purified fractions

As mentioned in the previous section, preparative thin-layer chromatography yielded seven distinct bands, designated as compounds C1, C2, C3, C4, C5, C6 and C7. These compounds were tested for antibacterial activity using the disc diffusion method against *S. aureus, B. subtilis* and *E. coli* at two concentrations (20 µL and 80 µL), with ethyl acetate as the negative control.

Against *S. aureus*, only compound C1 was active, producing an inhibition zone of 10 mm at 20 µL, with no increase in activity at 80 µL (10 mm), suggesting a plateau in efficacy. The resulted plate images showing the activity were represented in Plate 5.4: Figure A and Figure B represent the activity of 20 µL and 80 µL respectively. Against *E. coli*, compounds C5 and C7 were active. At 20 µL, C5 produced an inhibition zone of 11 mm (Figure C), which increased to 15 mm at 80 µL (Figure D), indicating concentration-dependent activity. C7 produced an inhibition zone of 11.5 mm at 20 µL (Figure C), with a slight increase to 12 mm at 80 µL (Figure E), suggesting limited concentration dependence. Compounds C2, C3, C4 and C6 showed no activity against any of the tested bacteria at either concentration. None of the compounds (C1-C7) exhibited activity against *B. subtilis* at either concentration, producing no inhibition zones (Figure F).

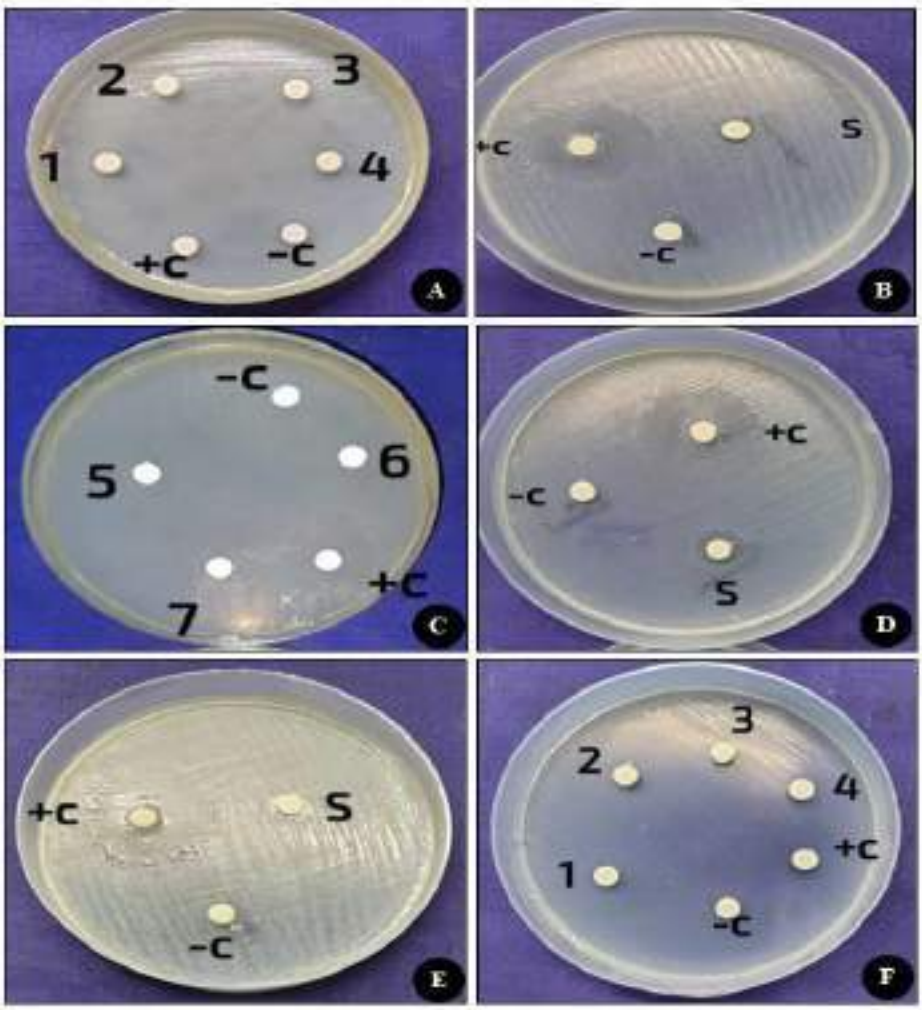


Plate 5.4: Antibacterial activity of TLC separated products: Figure A: Antibacterial activity of C1 (20 µL) against *S. aureus*, Figure B: Zone of inhibition produced by C1 (80 µL) sample (S) against *S. aureus*, Figure C: Antibacterial activity shown by C5 (20 µL) and C7 (20 µL) against *E.coli*, Figure D: Zone of inhibition produced by C5 (80 µL) sample (S) against *E. coli* along with positive and negative controls, Figure E: Antibacterial activity shown by C7 (80 µL) sample (S) against *E. coli* and Figure F: No activity detected by any TLC separated compounds against *B. subtilis*.

The results were summarized in Table 5.2, indicating that C1, C5 and C7 are the primary active compounds in FR-GL ethanolic extract, with selective activity against *S. aureus* and *E. coli*. The highest zone of inhibition was produced by the compound C5 (15 mm), so this fraction of FR-GL was selected for identification through NMR technology. The selective activity of these compounds suggests that FR-GL antibacterial efficacy is driven by specific phenolic or flavonoid components, warranting further chemical characterization. The lack of replicate measurements due to limited extract quantities and time constraints is a limitation and future studies should include triplicate analyses to confirm reproducibility. Plate 5.5: Figure A shows the heat map of the results of antibacterial activity of different fractions obtained.

No previous studies have reported the bioactivity of fractions from galls or other parts of *F. racemosa*. In contrast, the bioactive fractions of stem bark extract of *Ficus ovata* are reported to exhibit notable antimicrobial potential against a range of fungal and bacterial species. The crude extract and fractions 2 and 4, showed broad-spectrum activity, while other fractions displayed more selective effects, highlighting the differential distribution of bioactive constituents within the extract (Kuete *et al.*, 2009). Kuete and colleagues in 2011 did vacuum flash chromatography of *Ficus polita* roots and yielded five fractions (FPR1-FPR5). Among these, fractions FPR1 and FPR2 exhibited broad-spectrum antimicrobial activity, effectively inhibiting the growth of all eight tested microorganisms, including *Salmonella typhi*, *E. coli* and *C. albicans* (Kuete *et al.*, 2011). Here, this study separated 7 distinct compounds from the gall leaves of the *F. racemosa* plant and among the compound 3 were active against the tested bacteria.

Table 5.2: Antibacterial activity analysis of the separated products of FR-GL, showing zone of inhibition in mm produced by 2 different dilutions of compound.

Bacteria tested	**Compound**	**Zone of inhibition (mm) of 20 µL sample**	**Zone of inhibition (mm) of 80 µL sample**
Bacillus subtilis	C1	No activity	No activity
	C2	No activity	No activity
	C3	No activity	No activity

	C4	No activity	No activity
	C5	No activity	No activity
	C6	No activity	No activity
	C7	No activity	No activity
Staphylococcus aureus	C1	10	10
	C2	No activity	No activity
	C3	No activity	No activity
	C4	No activity	No activity
	C5	No activity	No activity
	C6	No activity	No activity
	C7	No activity	No activity
Escherichia coli	C1	No activity	No activity
	C2	No activity	No activity
	C3	No activity	No activity
	C4	No activity	No activity
	C5	11	15
	C6	No activity	No activity
	C7	11.5	12

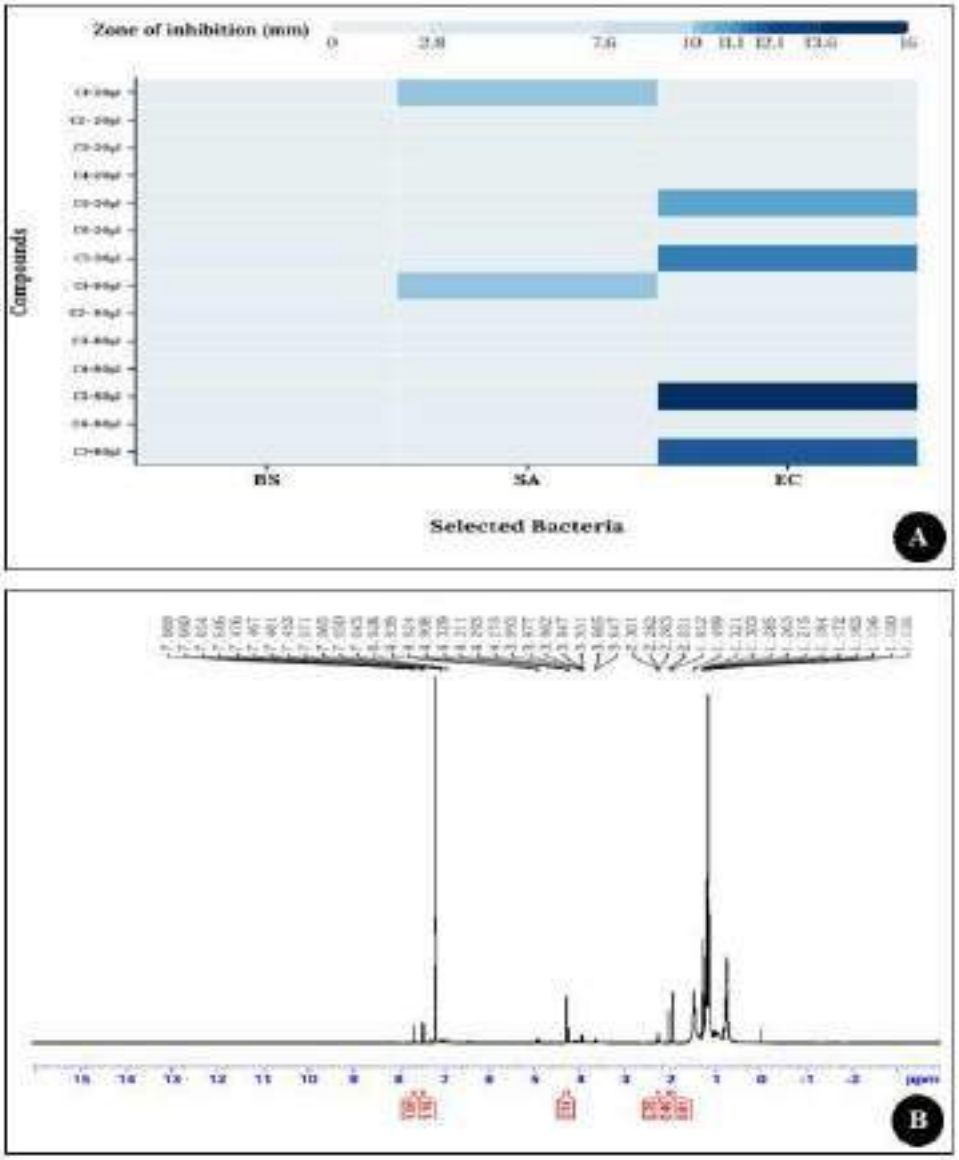


Plate 5.5: Figure A: Heat map representing the antibacterial activity of seven fractions separated from FR-GL ethanolic extract using preparative TLC technique (BS- *B. subtilis*, SA- *S. aureus* and EC- *E. coli*) and Figure B: The 1H NMR spectrum obtained from analysis of the compound C5 in methanol.

5.3.6. Results of Nuclear magnetic resonance (NMR) spectrometry analysis

The selected bioactive fraction (C5) was subjected to proton nuclear magnetic resonance (^{1}H NMR) spectroscopic analysis to identify the functional groups and structural features of the compound. The sample was dissolved in methanol and analyzed at 400 MHz. The obtained spectrum (Plate 5.6: Figure B) displayed several smaller peaks between δ 3.3-3.9 ppm, attributed to methanol solvent protons and trace impurities. Minor signals observed in the δ 1.0-2.8 ppm region suggest the presence of aliphatic protons, while weak peaks between δ 7.0-7.7 ppm indicate possible aromatic proton environments. However, the absence of distinct, well-resolved peaks characteristic of a single compound implies that the fraction likely contains a mixture of components or impurities rather than a pure metabolite. Therefore, the NMR data were insufficient for definitive structure elucidation. Further purification and complementary analyses are required for the identification of the active component.

5.4. CONCLUSIONS

Optimized TLC conditions for FR-GL using pre-coated silica plates and then preparative TLC was done. The sample was separated into of seven different compounds using preparative TLC. The separated fractions were purified and subjected to antibacterial assays to identify the most active fraction. Among these, the fraction labeled as C5 exhibited the highest antibacterial activity against the selected bacterial strains. The C5 fraction was subsequently analyzed using ^{1}H NMR spectroscopy and due to the absence of distinct, well-resolved peaks characteristic of a single compound implies that the fraction likely contains a mixture of components or impurities rather than a pure metabolite, but a mixture of structurally related components. Therefore, the NMR data were insufficient for definitive structure elucidation.

CHAPTER 6 - EXPRESSION PROFILING OF GENES INVOLVED IN THE SECONDARY METABOLIC PATHWAY FROM DIFFERENT SAMPLES

6.3. RESULTS AND DISCUSSION

RNA quality evaluation for qRT-PCR analysis

High-quality RNA was essential for accurate qRT-PCR analysis of genes in normal leaves, galled leaves and galls of *A. scholaris* and *F. racemosa*, to quantify gall-induced gene upregulation. RNA quality was assessed for 12 samples per plant species (4 each from normal leaf, gall leaf and gall) using UV-visible spectrophotometry (NanoDrop, Thermo Fisher) to measure the 260:280 ratio. All samples exhibited ratios between 1.8 and 2.1 (mean ± SD: 1.95 ± 0.08 for samples of *A. scholaris*, 1.97 ± 0.07 for tissues of *F. racemosa*), indicating minimal protein or phenolic contamination. RNA integrity was further verified by 1.2% agarose gel electrophoresis, with samples showing intact 28S and 18S rRNA bands (28S:18S ratio ~2:1), confirming no degradation.

All samples that met quality criteria were selected for further analysis ensuring reliable cDNA synthesis and qRT-PCR results. These rigorous checks validated the suitability of RNA for downstream analysis. The gel image of RNA of normal leaves, gall leaves and galls of *A. scholaris* is shown in Plate 6.3: Figure A. RNA from normal leaves, gall leaves and galls of *F. racemosa* is shown in Figure B, Figure C and Figure D respectively.

Primer design and synthesis

Primers for qRT-PCR amplification of DAHPS, EPSPS, CS, PAL, CHI and DFR were designed to ensure specific and efficient gene expression analysis in gall-induced tissues. The sequences of primers used for synthesis of qRT-PCR primers were given in Table 6.5.

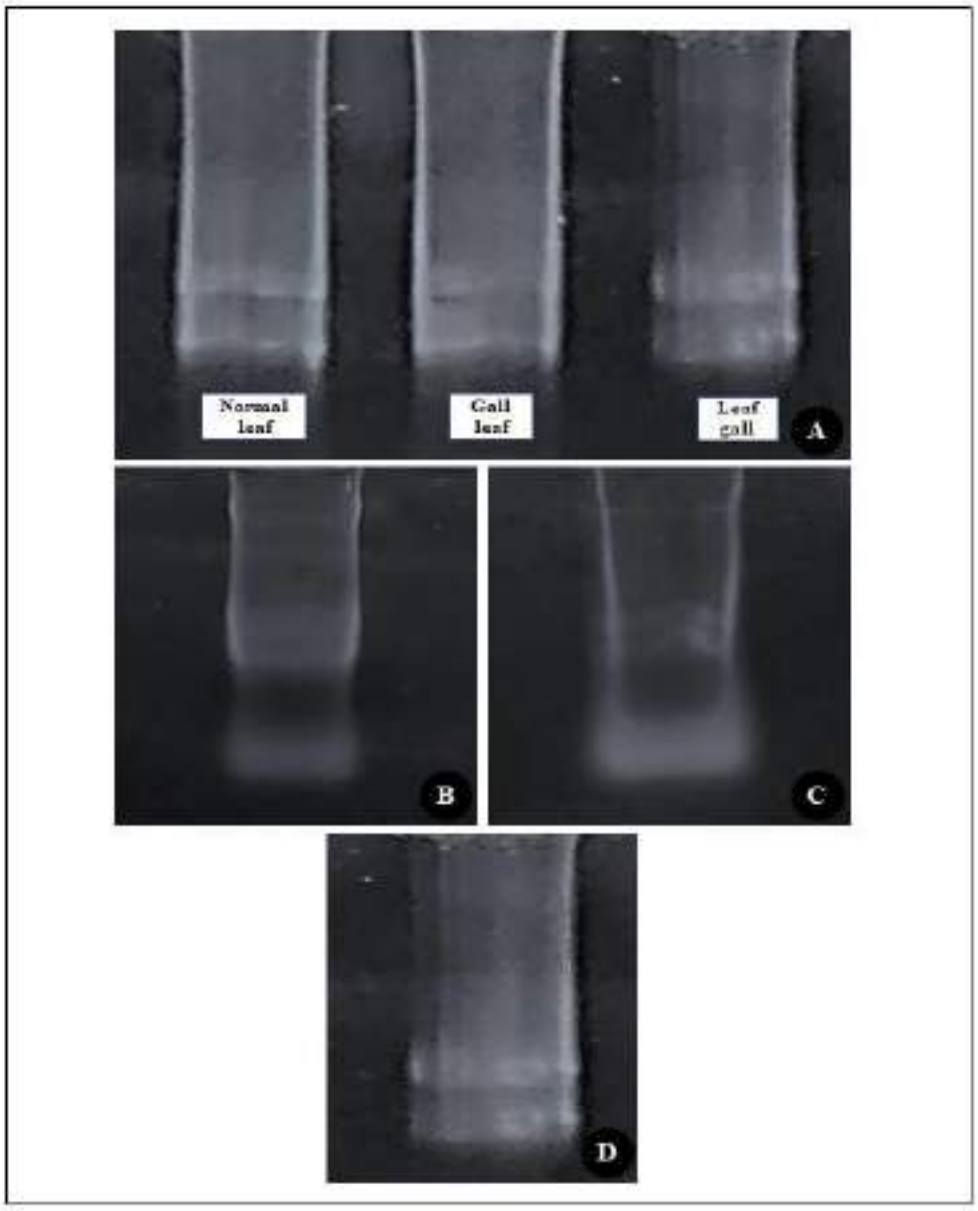


Plate 6.3: Extraction of RNA from different samples. Figure A: RNA from AS-NL, AS-GL and AS-GA, Figure B: RNA isolated from FR-NL, Figure C: RNA isolated from FR-GL and Figure D: RNA isolated from FR-GA.

Table 6.5: List of forward and reverse primer sequences of selected genes used in this study with their respective product sizes (bp).

Sl. No.	Gene	Product size (bp)	Forward sequence (5′ to 3′)	Reverse sequence (5′ to 3′)
1	DAHPS	268	GCCGTCCATTCCGCCGATCC	AGCTCCTAGCCTCACCGGCA
2	EPSPS	104	AAAAAACCCACCTTCCTTCC	TCCCTTCTCCTGCTTTCTC
3	CS	136	CCATCTCCACCTGTCAAAATC	TACAACCAACACCTCCTCC
4	PAL	105	CCGGTGGTGAAGCTCGGTGG	GGCCCGGGCTGACTCAGAGA
5	CHI	104	CGGTGGCGCAGGTGTGAGAG	TGCCGGCGAGTGAAGACACG
6	DFR	182	GCAGACAATGGACAAGACAAAG	CAGGGAACCAAGAAGCAAAC

Primer efficiency curve

The standard curve for primer efficiency analysis of each gene is attached in Plate 6.4 and 6.5. From the standard curves primer efficiency was calculated. All the six qRT-PCR primers synthesised have been found to have efficiency between 90-100%.

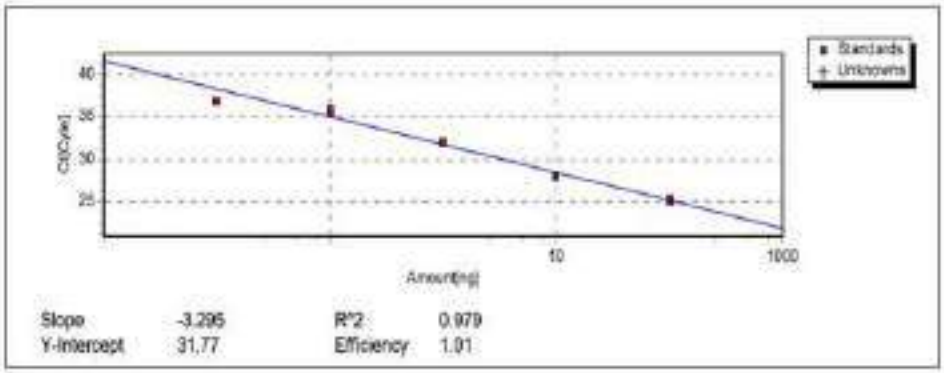
Gene expression analysis using qRT-PCR

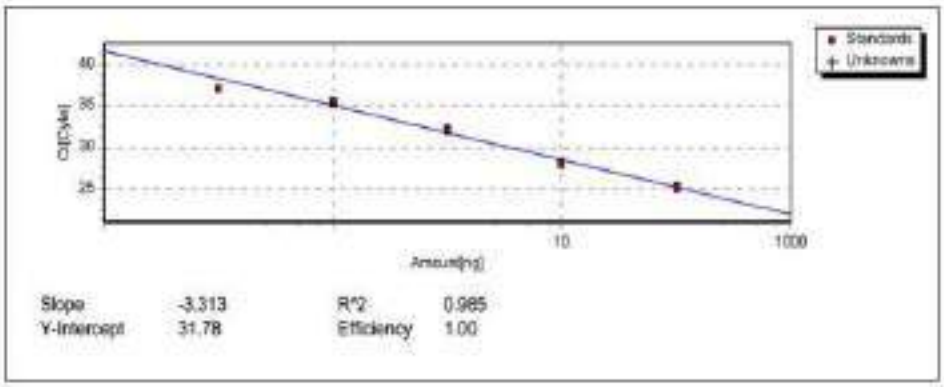
Plants have different systems of defence to resist the attacks of insects and pathogens, which are triggered by a diverse range of biotic stimuli. One of the defensive reactions triggered by gall inducer attack is the production of different secondary metabolites with disease resistance properties. Phenolic compounds have the capacity to defend against the pathogen in various ways; it may act as phytoalexins or as ROS scavengers, physical barriers or pathogenesis-related (PR) proteins or signal molecules that induce other defence related pathways. Due to the antioxidative characteristics of these compounds, which reduce cell membrane peroxidation and protect plant cells from the harmful impacts of oxidative stress, plants growing in stressful environments are able to biosynthesize more phenolic compounds than plants growing in normal conditions. It has been discovered through OHR-LC-MS that phenolics in gall leaves and galls of

A. scholaris are 16 and 11 respectively. *A. scholaris* normal leaves have only 8 different phenolic compounds. In the normal leaves and gall leaves of *F. racemosa* found to contain 12 and 13 phenolic compounds respectively. 66 different phenolic compounds having various significant biological activities are identified in galls of *F. racemosa*.

Gall induction by *Pauropsylla* species triggers robust defense responses in *A. scholaris* and *F. racemosa*, marked by increased phenolic biosynthesis, a key mechanism for resisting insect attack. GC-MS and OHR-LC-MS analysis quantified phenolic compounds in normal leaves, galled leaves and galls, revealing distinct profiles. These findings, consistent with phytochemical profiles explained in the Chapter 3, underscore the role of phenolics as phytoalexins, antioxidants and structural barriers, contributing to the antimicrobial and antioxidant activities reported in Chapter 4. The marked phenolic increase in *F. racemosa* galls suggests a stronger metabolic reprogramming, potentially driven by differential gene expression.

Altered biosynthesis of phenolics is regulated by the increased activities of various key enzymes of phenolic biosynthetic pathways. This study focuses on 6 genes (DAHPS, EPSPS, CS, PAL, CHI and DFR) from shikimic acid pathway and phenylpropanoid pathway that are involved in the production of different classes of phenolic compounds; through qRT-PCR. Plant defence mechanisms involve alterations in gene expression mediated by several signalling pathways in response to herbivores, pathogens and mechanical injuries. Plant defensive genes are triggered by signalling molecules, which in turn causes the plant to become resistant to biotic and abiotic stressors. The shikimic acid pathway converts PEP and E4P to chorismate, the precursor of the aromatic amino acid tryptophan, phenylalanine and tyrosine, via seven metabolic steps. The majority of secondary phenolic compounds are produced from phenylalanine through the action of several enzymes. The deamination of phenylalanine to cinnamic acid controls the formation of phenolic compounds such as lignin, flavonoids, pigments, phytoalexins and benzoic acid derivatives like salicylic acid. The pathway leads to the generation of flavonoids, flavanones, anthocyanins, catechins and procyanidins.





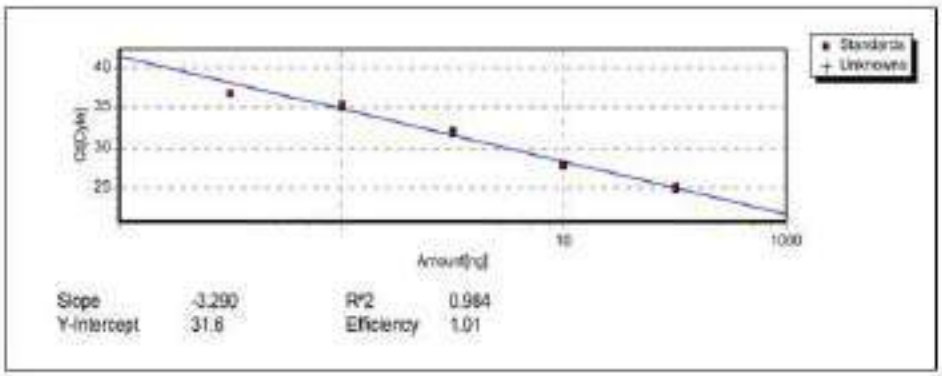
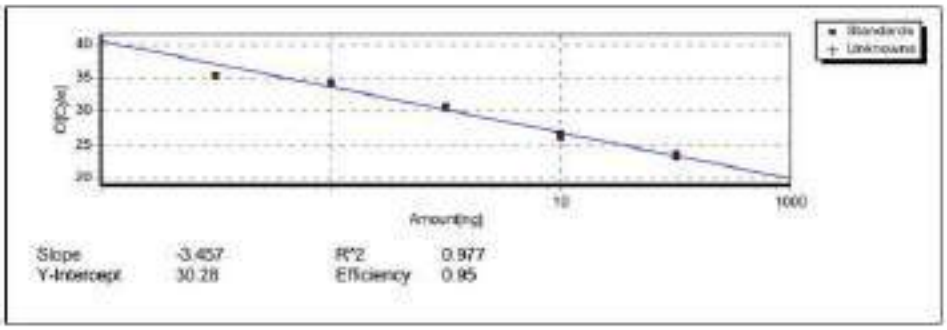
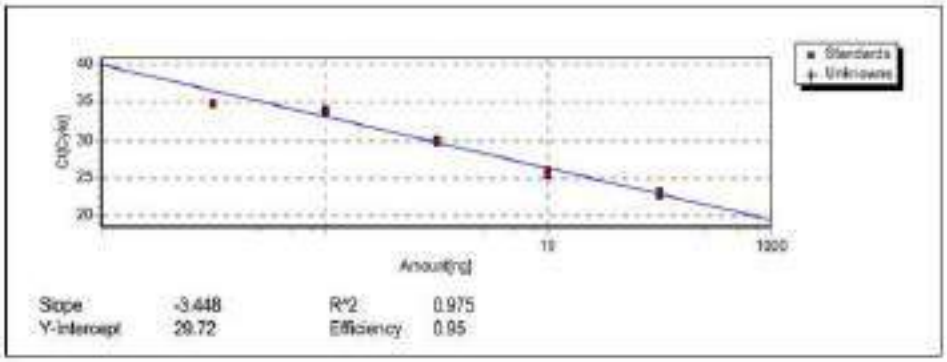


Plate 6.4: Primer efficiency analysis. Figure A: Standard curve for primer efficiency analysis of DAHPS gene primer, Figure B: Standard curve of EPSPS gene primer and Figure C: Standard curve of CS gene primer.





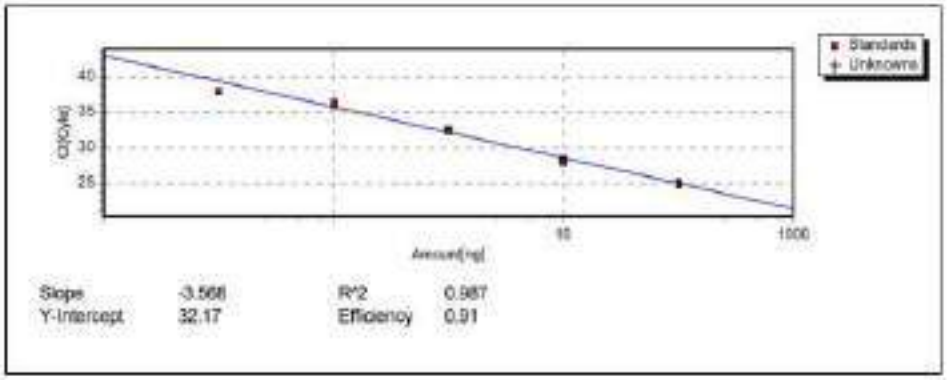


Plate 6.5: Primer efficiency analysis. Figure A: Standard curve for primer efficiency analysis of PAL gene primer, Figure B; Primer efficiency curve of CHI gene and Figure C: Primer efficiency analysis of DFR gene primer.

6.3.1. Gene expression profile of *A. scholaris*

qRT-PCR analysis quantified the expression of 3-Deoxy- D-arabino- heptulosonate-7-phosphate synthase (DAHPS), EPSP synthase (EPSPS), Chorismate synthase (CS), Phenylalanine ammonia-lyase (PAL), Chalcone-flavanone isomerase (CHI) and Dihydroflavonol 4-reductase (DFR) in galled leaves and galls of *A. scholaris*. Normal leaves served as the control, and their fold gene expression was normalized to 1. These genes, integral to the shikimic acid and phenylpropanoid pathways, exhibited differential expression in response to gall induction. Table 6.6 represents the data of fold gene expression in different samples of *A. scholaris.* A representation of the qRT-PCR analysis outcome file showing fluorescent profile of gene expression of *A. scholaris* is presented in Plate 6.6: Figure A.

DAHP synthase gene expression

In *A. scholaris,* the expression of genes involved in the shikimic acid and phenylpropanoid pathways exhibits distinct responses to gall-inducing biotic stress caused by psyllids, such as *P. tuberculata*. The present study provides novel insights into DAHPS gene expression (Plate 6.7: Figure A). The DAHPS gene, known to be upregulated under biotic and abiotic stresses, showed higher expression in the gall leaves with a fold gene expression (FGE) of 3.01 ± 0.39 and exhibited higher expression, approximately a 2-fold increase relative to the control. In the galls, the FGE is 6.14 ± 1.40. Notably, the DAHPS expression in gall leaves of *A. scholaris* was higher than that of all other genes (EPSPS, CS, PAL, CHI, DFR), underscoring its significant induction under biotic stress. These findings highlight the tissue-specific and species-specific regulation of DAHPS in response to biotic stress produced by gall inducer.

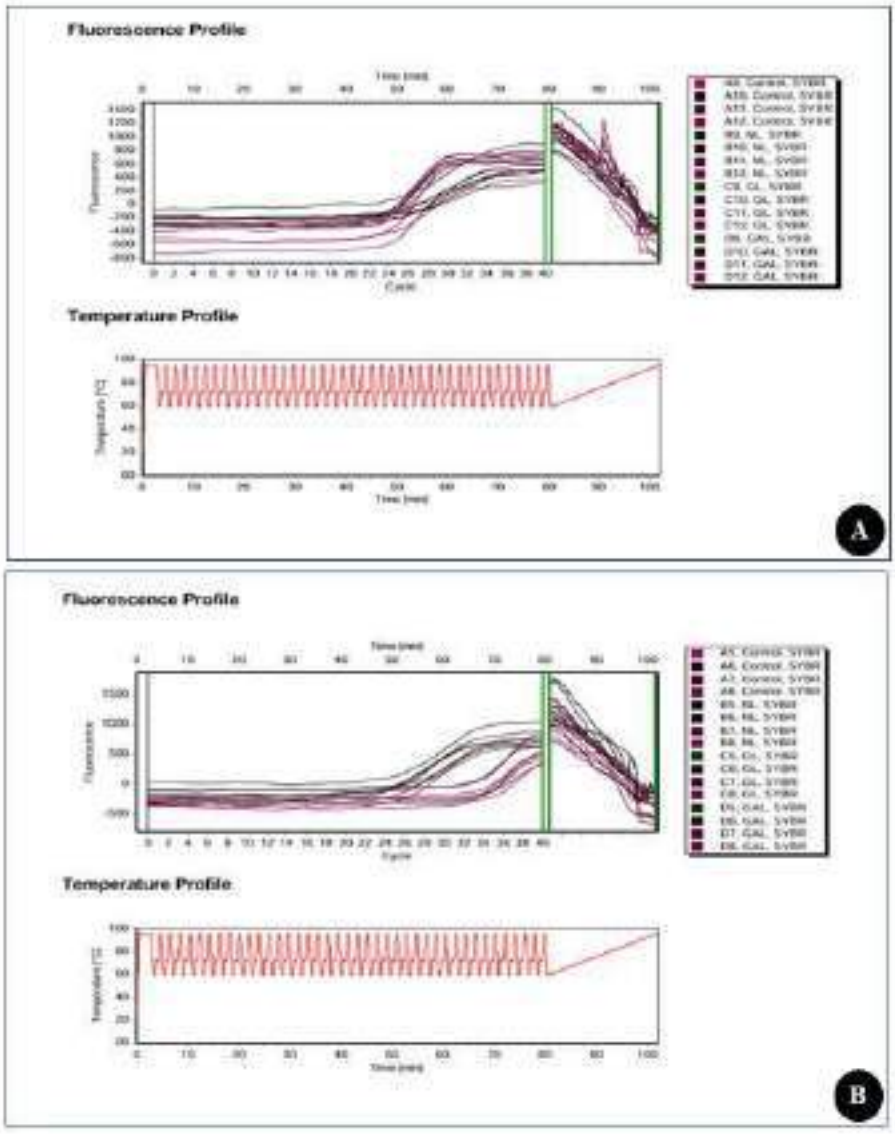


Plate 6.6: Figure A: Representation of qRT-PCR analysis outcome file of *A. scholaris*, showing fluorescent profile of gene expression and Figure B: Representation of qRT-PCR analysis outcome file of *F. racemosa*, showing fluorescent profile of gene expression.

DAHPS gene expression is often upregulated in response to biotic and abiotic stresses. In Sitka spruce (*Picea sitchensis*), the DAHPS gene was induced by mechanical wounding and herbivory from spruce budworms (*Choristoneura occidentalis*) and white pine weevils (*Pissodes strobi*) in bark and shoot tips (Ralph et al., 2006b). Similarly, wounded carrots (*Daucus carota*) subjected to water stress exhibited a 20-fold increase in DAHPS gene expression (Becerra-Moreno et al., 2015). This study also supports these findings of stronger activation of DAHPS as observed in other plants like Sitka spruce (*P. sitchensis*) under herbivory stress (Ralph et al., 2006b), potentially supporting the production of defense-related secondary metabolites via the shikimate pathway. This increase implies greater carbon allocation to aromatic amino acids, which are precursors for phenolic biosynthesis, though the low FGE suggests a potential rate-limiting step in the pathway.

EPSP synthase gene expression

The present study investigated the effects of insect-induced gall formation on gene expression of EPSP synthase in the leaves of *A. scholaris.* EPSPS facilitating chorismate production in the pathway. The galls of *A. scholaris* exhibited a striking 21.52 ± 1.98-fold increase in galls, but only 2.53 ± 0.21 in gall leaves, reflecting gall-specific activation (Plate 6.7: Figure B).

Wounding stress influences phytochemical production in strawberry (*Fragaria* × *ananassa*) fruits, leading to differential phytochemistry and altered gene expression. Wounding significantly increased the levels of phenolic compounds, total soluble sugars and certain phenylpropanoids. The EPSPS gene, involved in the shikimate pathway, showed increased expression in fruits from plants wounded 14 days before harvest, with 6.25 ±1.03 fold increase in expression (Ibanez et al., 2019). Ralph and colleagues in 2006 also reported the upregulation of EPSPS gene in poplar (*Populus* spp.) leaves subjected to herbivory by forest tent caterpillars (*Malacosoma disstria*) (Ralph et al., 2006a). The EPSPS gene was significantly upregulated in the galls with an FGE of 21.52 ± 1.98, aligning with findings in wounded strawberry (*Fragaria* × *ananassa*) fruits where EPSPS expression increased (Ibanez et al., 2019).

Chorismate synthase (CS) gene expression

The CS gene expression in response to gall formation in *A. scholaris* was also investigated in this study. CS catalyzing the final step in the shikimic acid pathway. The CS gene was significantly more upregulated in galls, than gall leaves. In the present analysis it was found upregulated in galls (18.19 ± 1.78) with minimal gall leaves expression (1.81 ± 0.37), aligning with precursor accumulation (Plate 6.7: Figure C).

In broccoli (*Brassica oleracea*), differential gene expression was analyzed in heads subjected to two wounding intensities using qRT-PCR: floret cuts and chopping. The CS gene was significantly upregulated in chopped samples, exhibiting both early and late responses, indicating enhanced chorismate production to support defense pathways (Torres-Contreras et al., 2018). Similarly, in poplar (*Populus* spp.), herbivory by forest tent caterpillars (*Malacosoma disstria*) induced the expression of the CS gene, alongside DAHPS and EPSPS, reflecting activation of the shikimate pathway (Ralph et al., 2006a). Similarly, the CS gene exhibited an 18.19 ± 1.78-fold increase in galls, indicating enhanced chorismate production, a critical step in secondary metabolite biosynthesis, as seen in broccoli under wounding stress (Torres-Contreras et al., 2018) and poplar, due to herbivory by forest tent caterpillars (Ralph et al., 2006a). According to these results, gall induction triggers the defensive mechanism of the plant, which probably increases the production of chorismate. These findings, which show tissue-specific and species-specific expression patterns in response to gall formation, emphasize the crucial role of CS in the shikimate pathway under biotic stress.

Phenylalanine ammonia-lyase (PAL) gene expression

PAL is a key phenylpropanoid enzyme converting phenylalanine to trans-cinnamic acid, it is essential for phenylpropanoid biosynthesis. The gene showed similar upregulation in galls (18.74 ± 1.46) and gall leaves expression of FGE 2.02 ± 0.50, supporting increased phenolic synthesis. The expressions were matching the expression level of the CS gene in the same tissue. These findings suggest that gall-inducing biotic stress significantly enhances PAL-mediated phenolic biosynthesis in *A. scholaris* (Plate 6.7: Figure D).

In wounded carrots *(Daucus carota*), a 14-fold increase in PAL gene expression was observed, resulting in elevated phenolic compound levels during storage (Becerra-Moreno et al., 2015). In the resistant citrus species *Poncirus trifoliata*, PAL gene expression was activated during the early stages of infection by *Phytophthora parasitica,* suggesting a role in limiting pathogen invasion (Boava et al., 2011). Similarly, in the resistant rice cultivar 'Kasalath', PAL enzyme activity and transcript levels increased significantly in response to infestation by the small brown plant hopper, correlating with elevated levels of defensive phenolic compounds, such as tannins and quinones (Duan et al., 2014). In grapevine (*Vitis vinifera*), PAL gene expression differed between susceptible and resistant leaves infected with the host pathogen *Plasmopara viticola,* but not in non-host interactions with *Pseudoperonospora cubensis* (Kortekamp, 2006). This robust induction mirrors observations in wounded carrots and resistant citrus, where PAL activation led to elevated phenolic compound levels (Becerra-Moreno et al., 2015; Boava et al., 2011). According to these findings, herbivory-induced responses and wounds are frequently linked to upregulated PAL gene expression. Its function in gall-induced defense mechanisms is highlighted by the notable induction of PAL in *A. scholaris,* which most likely promotes the synthesis of phenolic compounds to fight insect induced stress.

Chalcone-flavanone isomerase (CHI) gene expression

qRT-PCR analysis found that *A. scholaris* gall formation activates the expression of the CHI gene. CHI is a critical gene for flavonoid production. It displayed the highest expression in gall (25.22 ± 2.21), with modest gall leaves induction (1.48 ± 0.53), indicating a strong gall-driven flavonoid response (Plate 6.7: Figure E).

Microarray gene expression profiling in Sitka spruce (*Picea sitchensis)* revealed differential expression of genes across functional categories, including plant defense, octadecanoid and ethylene signaling, transport, secondary metabolism and transcriptional regulation, in response to feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*). Real-time PCR analysis confirmed that flavonoid biosynthesis genes, such as chalcone synthase (CHS) and CHI, were upregulated following herbivory, highlighting the role of phenolic secondary metabolites in conifer defense against insects and pathogens (Ralph et al., 2006b). In transgenic rice (*Oryza sativa*), stress-inducible CHI

expression was significantly higher than in wild-type plants, with 1.63 to 3.83-fold increases under drought stress, 2.72 to 3.95-fold under salt stress and 1.81 to 3.46-fold under cold stress. These transgenic lines exhibited elevated total phenolic and flavonoid content and outperformed control plants under drought, salt and cold stress, demonstrating role of CHI in abiotic stress tolerance (Jayaraman et al., 2021). The CHI gene, involved in flavonoid biosynthesis, was upregulated in both galls (FGE: 25.22 ± 2.21) and gall leaves (FGE: 1.48 ± 0.53), consistent with its role in stress-induced flavonoid production in transgenic rice under abiotic stress (Jayaraman et al., 2021) and also with biotic stress tolerance by increased phenolic production in Sitka spruce (Ralph et al., 2006b). The substantial elevation of CHI in *A. scholaris* during gall development confirms its role in phenolic compound production as a defense against biotic stress caused by insects.

Dihydroflavonol 4-reductase (DFR) gene expression

The DFR gene is a significant part of the flavonoid pathway, involved in anthocyanin and flavanol synthesis. The present qRT-PCR analysis demonstrated the gene was significantly upregulated in galls (8.97 ± 1.18) but downregulated in gall leaves (0.45 ± 0.12), suggesting metabolite channeling to gall tissues. *A. scholaris* showed a lower DFR expression in galls than the results seen in *F. racemosa* in the current investigation, indicating species-specific responses to gall-inducing biotic stress (Plate 6.7: Figure F).

In transgenic tobacco (*Nicotiana tabacum*) overexpressing DFR and anthocyanidin reductase (ANR), elevated levels of alkaloids and flavonols conferred resistance against the tobacco leaf cutworm (*Spodoptera litura*) (Kumar et al., 2013). Abiotic stresses also influence DFR expression. Drought stress induced DFR gene expression in potato (*Solanum tuberosum*) tubers (André et al., 2009) and wheat (*Triticum aestivum*) leaves (Ni et al., 2020), suggesting a role in mitigating environmental stress through flavonoid accumulation. The lower expression in *A. scholaris* suggests species-specific metabolic priorities. DFR showed an FGE of 8.97 ± 1.18 in galls, contributing to defense against insect-induced gall formation, as seen in resistant *Camellia oleifera* under anthracnose stress (Yang et al., 2022).

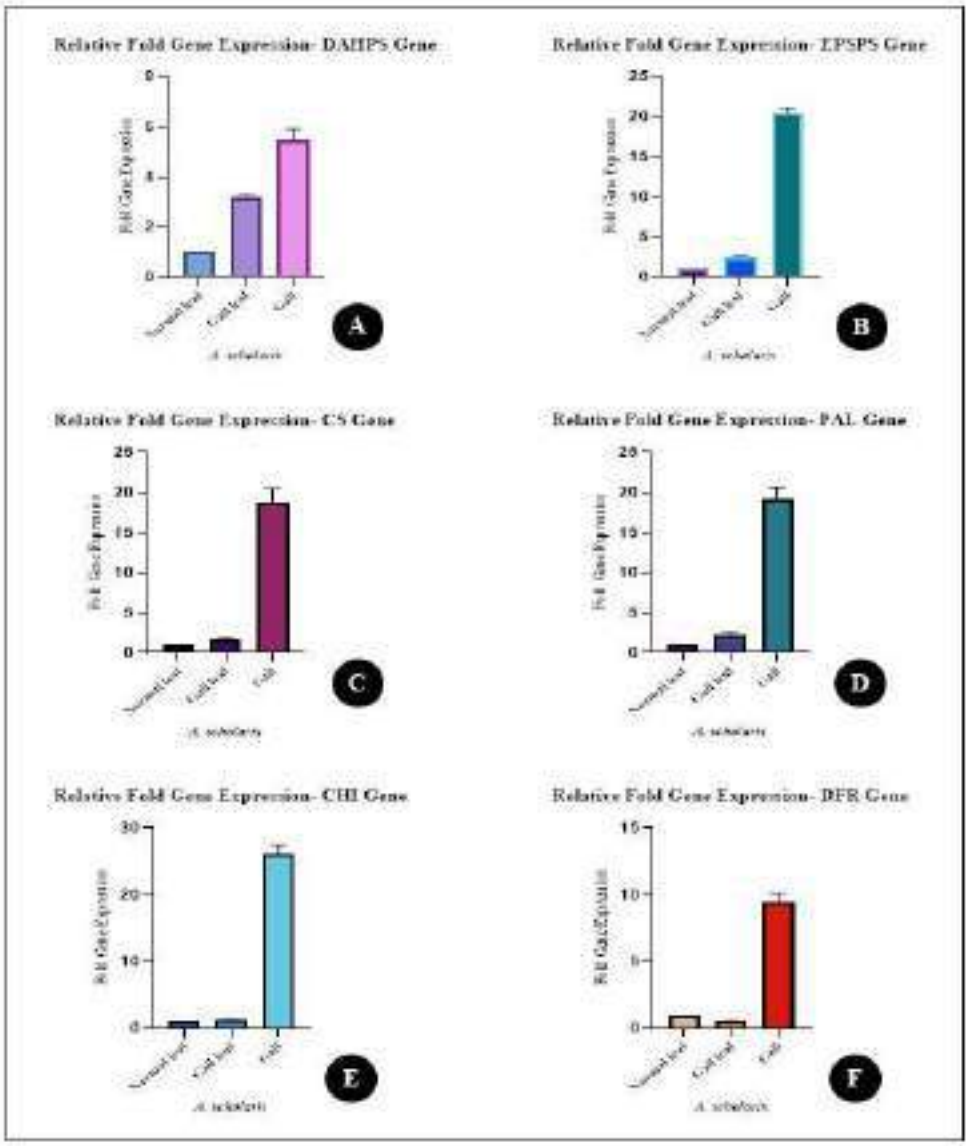


Plate 6.7: qRT-PCR expression analysis- Figure A-F: Relative fold gene expression (FGE) of DAHPS, EPSPS, CS, PAL, CHI and DFR genes in gall leaves and galls compared to control (normal leaves) in *A. scholaris*.

ANOVA revealed significant differences in the relative fold gene expression levels across the three tissues ($p < 0.01$), indicating that gall induction markedly influences the transcriptional activity of key genes associated with secondary metabolite biosynthesis. The subsequent Tukey's HSD test confirmed a consistent expression trend of GA > GL > NL for all the analyzed genes, signifying that gene expression was highest in galls, moderate in gall leaves and lowest in normal leaves. Among the six genes analyzed, DAHPS and EPSPS showed significant upregulation in gall leaves compared to normal leaves, whereas CS, PAL, CHI and DFR exhibited non-significant differences. However, all genes were significantly upregulated in galls relative to the control at level of $p < 0.01$. This pronounced upregulation of secondary metabolism-related genes in gall tissues demonstrates that gall formation triggers enhanced biosynthetic activity, likely as part of a complex plant insect interaction. The elevated expression of these genes reflects a metabolic shift toward increased synthesis of phenolic and flavonoid compounds, which may serve defensive, structural or signaling roles during gall development. Graphical representation comparative analysis of the relative expression of all the selected genes in *A. scholaris* is provided as a bar diagram in Plate 6.9: Figure A.

Table 6.6: Relative fold gene expression levels of selected genes in *A. scholaris* normal leaves (control), gall leaves and galls, with values expressed as mean ± SD.

Sl. No	Name of gene	Relative fold gene expression in *A. scholaris*		
		Normal leaves (Control)	Gall leaves	Galls
1	DAHPS	1	$3.01 \pm 0.39^{**}$	$6.14 \pm 1.40^{**}$
2	EPSPS	1	$2.53 \pm 0.21^{**}$	$21.52 \pm 1.98^{**}$
3	CS	1	1.81 ± 0.37	$18.19 \pm 1.78^{**}$
4	PAL	1	2.02 ± 0.5	$18.74 \pm 1.46^{**}$

5	CHI	1	1.48 ± 0.53	25.22 ± 2.21**
6	DFR	1	0.45 ± 0.12	8.97 ± 1.18**

Each value of relative fold gene expression represents mean ± standard deviation (n = 3). **indicates values significantly different from normal leaves (control) at $p < 0.01$.

6.3.2. Gene expression profile of *F. racemosa*

qRT-PCR analysis of *F. racemosa* revealed pronounced upregulation of DAHPS, EPSPS, CS, PAL, CHI and DFR in galls induced by *P. depressa*, with normal leaves as the control (FGE = 1). The mean FGE of three different experiments calculated with standard deviation (Table 6.7). A representative output file of the analysis performed using the Eppendorf Mastercycler ep Gradient Realplex 2, with cDNA of *F. racemosa* as the template, is shown in Plate 6.6: Figure B.

DAHP synthase gene expression

The current study sheds new light on DAHPS gene expression in response to biotic stress generated by gall-inducing insects in *F. racemosa*. The expression study revealed that galls had significantly higher expression than normal leaves and gall leaves. In contrast, gall leaves expression has been found to be lower than the control. In galls, DAHPS expression was significantly higher in *F. racemosa* (29.84 ± 0.52), indicating stronger activation in *F. racemosa*. Conversely, in gall leaves the FGE < 1. The elevated expression in *F. racemosa* gall is linked to the higher production of defense-related secondary metabolites through shikimic acid pathway (Plate 6.8: Figure A).

In broccoli Torres-Contreras and colleagues found the differential expression of DAHPS also. Like other genes, wounding stress upregulated DAHPS expression also (Torres-Contreras et al., 2018). Additionally, poplar (*Populus* spp.) leaves showed increased DAHPS expression in response to herbivory by forest tent caterpillars, *Malacosoma disstria* (Ralph et al., 2006a). In contrast, strawberry fruit did not show altered DAHPS expression when subjected to wounding 7 or 14 days before harvest (Ibanez et al., 2019). As aromatic amino acids are required for protein synthesis and normal functioning of plants, DAHPS genes are constitutively transcribed and translated to produce enzymes. However, stress generated by gall inducers activates DAHPS and

boosts their transcript level, enhancing the secondary metabolic pathway and producing a significant amount of phytochemicals. When compared to all other genes investigated, DAHPS showed a lesser surge in expression in galls. This study also indicates a stronger activation of the shikimate pathway in *F. racemosa*, potentially linked to defense-related secondary metabolite production, as observed in poplar (*Populus* spp.) under herbivory by *M. disstria* (Ralph et al., 2006a).

EPSP synthase gene expression

The current study shows that the EPSPS gene was significantly upregulated in response to gall-inducing biotic stress, with a FGE greater than 30 in the galls. The results showed strong upregulation in gall with FGE of EPSPS 30.26 ± 1.88 and low in gall leaves expression (0.57 ± 0.04) (Plate 6.8: Figure B).

These findings align with previous observations of herbivory-induced activation of secondary metabolism genes in poplar. Microarray transcriptome profiling of poplar (*Populus* spp.) leaves subjected to herbivory by forest tent caterpillars, *M. disstria* revealed differential expression of genes across multiple functional classes, including stress response, secondary metabolism, photosynthesis, transport, octadecanoid and ethylene signaling, transcriptional regulation and signaling pathways. These genes were either upregulated or downregulated, reflecting a complex metabolic reprogramming (Ralph et al., 2006a). These results highlight the role of wounding and herbivory in modulating secondary metabolism, with EPSPS playing a key role in the shikimate pathway to support the biosynthesis of defense-related compounds. The upregulation of EPSPS in *F. racemosa* underscores the conserved response to biotic stress across plant species. The EPSPS gene was highly upregulated in galls with an FGE of 30.26 ± 1.88, reflecting its critical role in the shikimic acid pathway, similar to findings in strawberry fruits wounded 14 days before harvest (Ibanez et al., 2019).

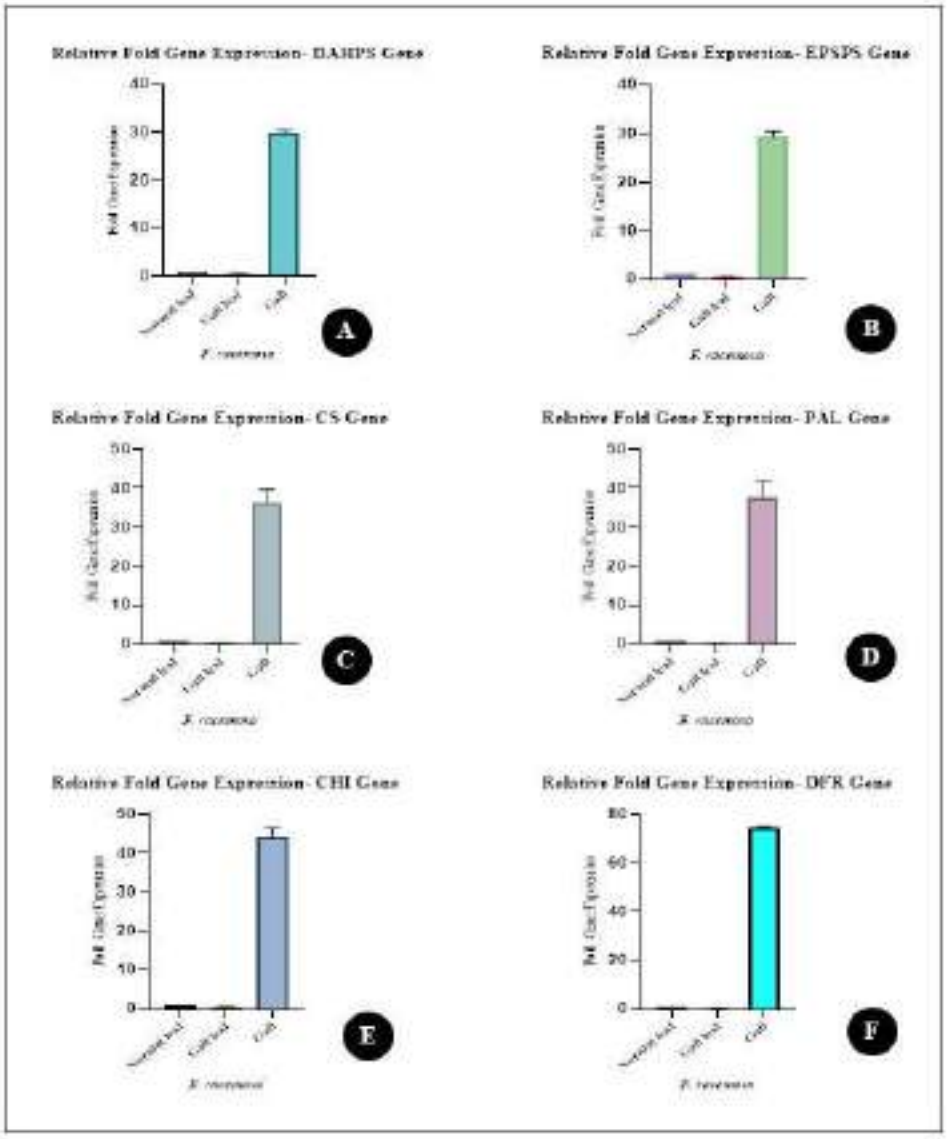


Plate 6.8: qRT-PCR analysis. Figure A-F: Relative expression levels of DAHPS, EPSPS, CS, PAL, CHI and DFR genes in gall leaves and galls of *F. racemosa* expressed as fold change compared to normal leaves (control).

Chorismate synthase (CS) gene expression

This study examined the expression of the CS gene in response to gall formation in *F. racemosa.* Compared to gall leaves, galls showed a much higher upregulation of the CS gene facilitating chorismate synthesis, showed strong upregulation (35.16 ± 3.70), suggesting localized precursor production (Plate 6.8: Figure C).

Torres-Contreras and colleagues reported that differential CS gene expression was observed in *Brassica oleracea* in response to chopping injuries and floret cuts, which elevated the synthesis of chorismate to support defense pathways as same as seen in DAHPS (Torres-Contreras et al., 2018). Likewise, the herbivory of *M. disstria* on poplar (*Populus* spp.) resulted in the expression of the CS gene, which indicates the activation of the shikimate pathway (Ralph et al., 2006a). The CS gene showed a 35.16 ± 3.70-fold increase in galls, supporting enhanced chorismate production for secondary metabolism, as reported in broccoli under wounding stress (Torres-Contreras et al., 2018). These findings suggest that gall-inducing biotic stress activates the plant defense system, likely enhancing chorismate production to support secondary metabolite biosynthesis. These results highlight the critical role of CS in the shikimic acid pathway under biotic and abiotic stress, with tissue-specific and species-specific expression patterns in response to gall formation.

Phenylalanine ammonia-lyase (PAL) gene expression

The expression of the PAL gene in response to gall development in *F. racemosa* was examined in the current study. The expression in gall leaves was 0.37 ± 0.04-fold, which is much less than normal (control) leaf. In *F. racemosa,* about 37.77 ± 4.13-fold increase in PAL expression was observed in galls, reflecting insect-induced activation of defense-related pathways (Plate 6.8: Figure D).

Microarray gene expression profiling in Sitka spruce (*Picea sitchensis*) revealed upregulation of PAL genes in response to insect feeding or mechanical wounding, contributing to large-scale transcriptomic changes (Ralph et al., 2006b). In broccoli, wounding stress induced the upregulation of five PAL-encoding genes (Torres-Contreras et al., 2018). In hybrid poplar (*Populus* spp.), PAL gene expression was also activated following infection by forest tent caterpillars, *M. disstria* and in Strawberry, a 1.85-fold increase in PAL expression was observed

in response to wounding (Ibanez et al., 2019; Ralph et al., 2006a). The current study results also indicate a strong defense response through phenolic compound production, consistent with observations in resistant rice cultivars under plant hopper infestation (Duan et al., 2014).

However, not all biotic stresses induce PAL expression. In soybean (*Glycine max*) infected with *Rhizoctonia solani*, PAL gene expression remained unaffected (Chen et al., 2009). Similarly, cDNA array analysis of rice cultivars (resistant B5 and susceptible MH63) infested by the piercing-sucking insect *Nilaparvata lugens* showed downregulation of the PAL gene, contradicting typical patterns of PAL induction under biotic stress (Zhang et al., 2004). These results underscore the context-specific regulation of PAL gene expression, with upregulation commonly associated with wounding and herbivory-induced defense responses, but not universal across all plant-pathogen or plant-insect interactions. The significant induction of PAL in *F. racemosa* highlights its role in gall-induced defense mechanisms, likely contributing to the production of phenolic compounds to counter insect stress.

Chalcone-flavanone isomerase (CHI) gene expression

This study demonstrates that CHI gene expression is activated during gall development in *F. racemosa*. The CHI gene, involved in flavonoid synthesis exhibited the second-highest FGE in galls among the studied genes, with an FGE of 44.36 ± 2.16, indicating significant induction due to gall-forming biotic stress by *P. depressa*. In gall leaves, CHI expression was also less than the control like other genes (Plate 6.8: Figure E).

In *Gossypium barbadense* (sea island cotton), which is highly susceptible to Fusarium wilt, seedlings overexpressing CHI genes showed enhanced resistance to the disease, underscoring protective role of CHI against pathogens (Zu et al., 2023). Additionally, in the lawn grass *Eremochloa ophiuroides*, wounding and methyl jasmonate treatments increased CHI transcript levels and total anthocyanin content in shoots, further linking CHI to stress-induced flavonoid production (Chung et al., 2019). The CHI gene expression was the second-highest among the studied genes, aligning with its stress-induced expression in *E. ophiuroides* under wounding (Chung et al., 2019). These findings highlight the critical role of CHI in flavonoid-mediated defense and stress responses across diverse plant species. The significant upregulation of CHI in *F. racemosa* during gall formation suggests its involvement in producing phenolic

compounds to counter insect-induced biotic stress, consistent with its stress-inducible expression in other systems.

Dihydroflavonol 4-reductase (DFR) gene expression

The current study investigated DFR gene expression in response to gall formation and found that the expression is significantly higher in galls, with a 74.70-fold increase compared to all other genes studied, showing a markedly greater level of expression. The DFR expression observed in gall is the highest among studied genes (Plate 6.8: Figure F).

In Sitka spruce (*Picea sitchensis*), feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) upregulated DFR gene expression, highlighting its importance in flavonoid metabolism for conifer defense (Ralph et al., 2006b). In a resistant variety of *Camellia oleifera*, increased flavonoid accumulation was associated with anthracnose caused by *Colletotrichum gloeosporioides*, driven by the overexpression of flavonoid biosynthetic genes, including DFR. This overexpression likely enhances resistance to anthracnose (Yang et al., 2022).

However, DFR expression is not universally induced by biotic stress. In hybrid poplar (*Populus* spp.), cDNA microarray analysis showed no significant change in DFR gene expression in response to poplar leaf rust (*Melampsora medusae*) infection (Miranda et al., 2007). Similarly, cDNA array analysis of rice cultivars (resistant B5 and susceptible MH63) infested by the brown plant hopper (*Nilaparvata lugens*) revealed downregulation of DFR genes. Among 17 flavonoid pathway genes analyzed, two in B5 and five in MH63 were downregulated following insect damage, indicating a context-specific response (Zhang et al., 2004). Notably, in this study, the DFR gene displayed the highest upregulation in galls with an FGE of 74.70 ± 1.14, underscoring its critical role in flavonoid-mediated defense against gall-inducing insects, as seen in transgenic tobacco resistant to leaf cutworms (Kumar et al., 2013).

These findings suggest that DFR plays a significant role in flavonoid-mediated defense against insect-induced gall formation, particularly in *F. racemosa.* These findings highlight the context-specific regulation of DFR gene expression, with significant upregulation in response to certain biotic and abiotic stresses, but not others (e.g., leaf rust, plant hopper infestation). The

pronounced induction of DFR in *F. racemosa* galls underscores its critical role in flavonoid-mediated defense against gall-inducing insects.

The expression profiles of all the studied genes across the different samples were statistically analyzed to determine the level of significance in their differential expression. ANOVA revealed that the observed differences in relative fold expression in galls were statistically significant ($p < 0.01$) in all cases, indicating that the variations in transcript abundance among the tissues were not due to random variation but reflected genuine biological responses. The subsequent Tukey's HSD test confirmed that the gall induction and associated physiological changes leads to a measurable effect on gene expression patterns. Among the six genes analyzed in *F. racemosa*, none of the genes showed significant changes in expression in gall leaves compared to normal leaves $p > 0.05$, as indicated by the absence of significant differences in their relative fold expression values. However, all six genes (DAHPS, EPSPS, CS, PAL, CHI and DFR) exhibited strong and statistically significant upregulation in galls relative to normal leaves $p < 0.01$, suggesting that gall induction triggers a pronounced transcriptional activation of the shikimic acid and phenylpropanoid pathway genes. The activation of these genes is likely a part of the biochemical response of the plant to the infection caused by the gall-inducing insect *P. depressa*. Such differential gene expression indicates that *F. racemosa* enhances the synthesis of key secondary metabolites as a defense strategy to counter the stress induced by insect infestation. Graphical representation comparative expression of all selected genes in *F. racemosa* is provided as a bar diagram in Plate 6.9: Figure B.

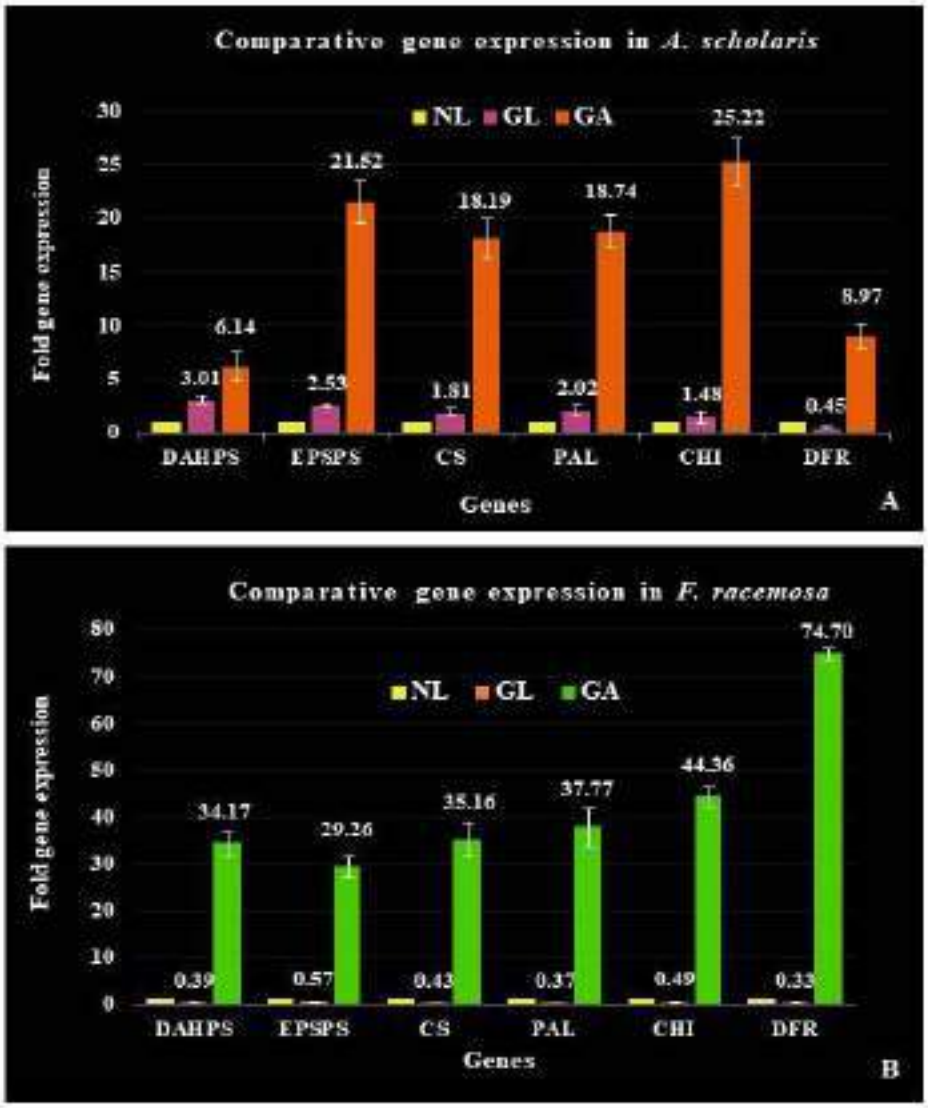


Plate 6.9: Comparative analysis of gene expression: Figure A: Fold gene expression of 6 selected genes in *A. scholaris* normal leaves (control), gall leaves and leaf galls and Figure B: Fold gene expression in *F. racemosa* normal leaf (control), gall leaves and leaf galls.

Table 6.7: Relative fold gene expression levels of six selected genes in *F. racemosa* normal leaves, gall leaves and galls, with values expressed as mean ± SD.

Sl. No	Name of gene	Relative fold gene expression in *F. racemosa*		
		Normal leaves (Control)	Gall leaves	Galls
1	DAHPS	1	0.39 ± 0.19	29.84 ± 0.52**
2	EPSPS	1	0.57 ± 0.04	30.26 ± 1.88**
3	CS	1	0.43 ± 0.08	35.16 ± 3.70**
4	PAL	1	0.37 ± 0.04	37.77 ± 4.13**
5	CHI	1	0.49 ± 0.14	44.36 ± 2.16**
6	DFR	1	0.35 ± 0.09	74.70 ± 1.14**

Each value of relative fold gene expression represents mean ± standard deviation (n = 3). **indicates values significantly different from normal leaves (control) at $p < 0.01$.

Comparison of gene expression

Compared to *F. racemosa*, the *A. scholaris* exhibits lower FGE in galls, particularly for DFR, reflecting species-specific responses to gall inducers. The moderate upregulation in *A. scholaris* possibly due to less aggressive induction of *P. tuberculata* compared to *P. depressa.* The downregulation of DFR in gall leaves suggests resource allocation to galls, a strategy to localize defense compounds. Higher FGE in galls of *F. racemosa* (DFR: 74.70) compared to *A. scholaris* (DFR: 8.97) reflects a more intense response to *P. depressa*. This may stem from

greater phenolic diversity of *F. racemosa*, driven by DFR and CHI. The consistent downregulation of gall leaves in *F. racemosa* contrasts with moderate upregulation in gall leaves of *A. scholaris*, suggesting species-specific differences in defense strategies. The large range of phenolics in *F. racemosa* leaf galls compared to healthy leaf tissue also reflect the stress tolerance established due to the activity of the insect *P. depressa.*

This study showed that gall induction consistently increased the expression of all of the selected genes in leaf galls of both plants. Genes with low transcript abundance can be considered as a limited step in the pathway, whereas genes with high expression can be regarded as highly active enzymes in the system. Plants growing in stressed environments could biosynthesize more phenolic chemicals than plants living under normal conditions. As a result, the concentration of phenols in plant tissue serves as an excellent indicator of plant stress resistance.

Plants undergo significant metabolic reprogramming in response to herbivore interactions, activating both direct and indirect defense mechanisms that place substantial demands on primary metabolism. This reprogramming often involves a trade-off between growth-related processes and defense responses. Specifically, transcripts associated with photosynthesis are significantly downregulated, while those involved in stress responses, wound repair, pathogen defense and the transport of carbon and nitrogen for defense are markedly upregulated. For instance, in *Nicotiana attenuata*, herbivory by *Manduca sexta*, a moth induces an inverse relationship between photosynthesis and defense-related gene expression, prioritizing defense over photosynthetic activity (Hermsmeier et al., 2001). A similar pattern was observed in *Sorghum bicolor* during infestation by the phloem-feeding greenbug aphid (*Schizaphis graminum*), where defense gene upregulation coincided with reduced expression of photosynthetic genes (Zhu-Salzman et al., 2004). Likewise, in *Picea sitchensis* (Sitka spruce), mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reduced the expression of photosynthesis-related genes in shoot tips and bark (Ralph et al., 2006b). These studies collectively demonstrate that plants reallocate metabolic resources from primary functions, such as photosynthesis to defense mechanisms under herbivore stress. This metabolic shift underscores the adaptive plasticity of plants, enabling them to prioritize survival and defense over the growth when faced with stressors.

Collectively, such transcriptional reprogramming highlights the dynamic molecular adaptability of plants, where gene expression patterns shift rapidly upon herbivore or pathogen attack. This molecular shift promotes the enhanced synthesis of defensive phytochemicals, ensuring effective protection against biotic stress.

Comparative gene expression in gall leaves of *A. scholars* and *F. racemosa* (Plate 6.10)

In *A. scholaris*, all genes except DFR showed a fold gene expression (FGE) of greater than 1, indicating upregulation. The genes CS and CHI exhibited an FGE between 1 and 2, suggesting a moderate increase in expression. The genes EPSPS and PAL showed an FGE between 1 and 3, indicating a relatively higher upregulation compared to CS and CHI. The gene DAHPS displayed an FGE greater than 3, representing the highest level of upregulation observed among all the genes studied. In contrast, the gene DFR had an FGE of 1 or less, indicating no significant upregulation. In *A. scholaris,* all genes except DFR showed FGE greater than 1, indicating upregulation. In *F. racemosa,* all six genes exhibited FGE less than 1, indicating downregulation in gall leaf tissue.

Comparative gene expression in gall of *A. scholars* and *F. racemosa* (Plate 6.11)

In gall tissue, *F. racemosa* displayed higher FGE for all genes compared to *A. scholaris.* In *A. scholaris*, the genes DAHPS and DFR showed a FGE of less than 10. The genes CS and PAL exhibited an FGE of less than 20. The genes EPSPS and CHI displayed an FGE of less than 30. In *F. racemosa*, the gene DAHPS had an FGE of less than 30, representing the lowest among all the genes studied in *F. racemosa*. The genes EPSPS, CS and PAL showed an FGE of less than 40. The gene CHI exhibited an FGE of less than 50. The gene DFR showed the highest expression with an FGE of 74.70 ± 1.14.

This study elucidates the tissue-specific and species-specific regulation of shikimic acid and phenylpropanoid pathway genes (DAHPS, EPSPS, CS, PAL, CHI and DFR) in *F. racemosa* and *A. scholaris.* In the galls of *F. racemosa* exhibited significantly higher FGE for all genes compared to *A. scholaris.* This marked upregulation in *F. racemosa* galls, driven by *P. depressa* infestation, reflects enhanced transcription of secondary metabolic pathway genes, leading to elevated phenolic and flavonoid levels that strengthen defense against insect stress. In contrast,

iThenticate Page 202 of 209 - Integrity Submission Submission ID trn:oid:::3618:121855597

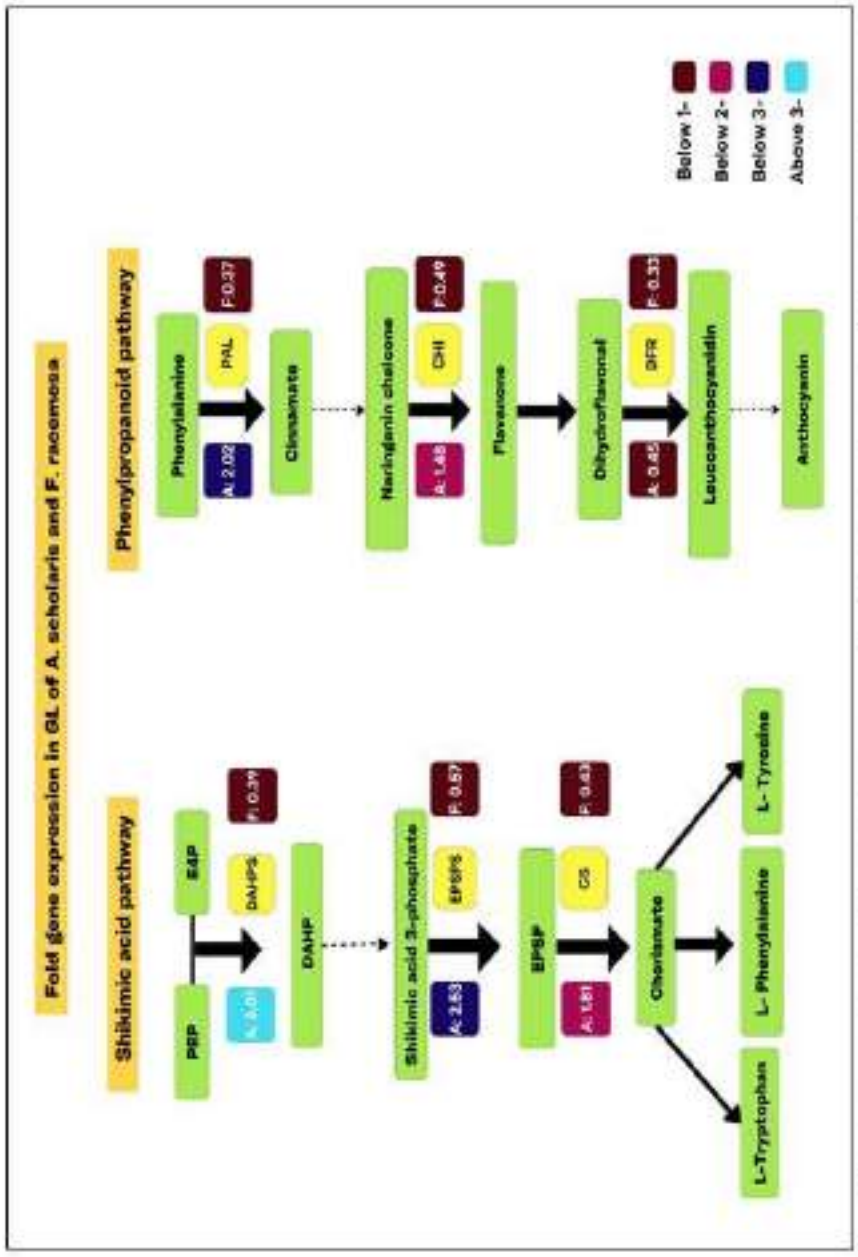


Plate 6.10: Gene expression in gall leaves: Fold gene expression (FGE) of selected genes in gall leaves of *A. scholaris* denoted with the letter 'A' and FGE of selected genes in gall leaves of *F. racemosa* represented with letter 'F'. Different colored boxes indicated with the range of FGE. Dotted lines indicate multiple steps.

iThenticate Page 202 of 209 - Integrity Submission Submission ID trn:oid:::3618:121855597

iThenticate Page 203 of 209 - Integrity Submission Submission ID trn:oid:::3618:121855597

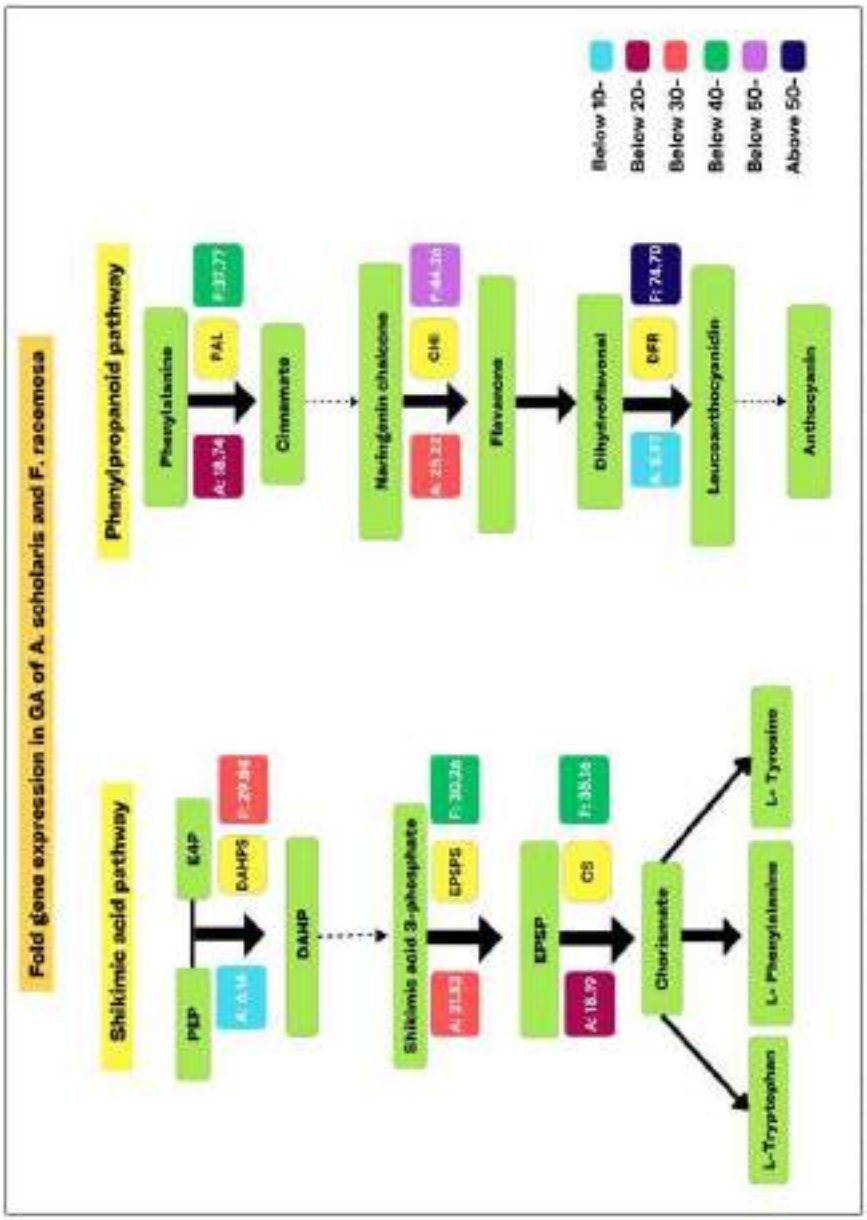


Plate 6.11: Gene expression in galls: FGH of selected genes in galls of *A. scholaris* denoted with the letter 'A' and FGH of selected genes in galls of *F. racemosa* represented with 'F'. Different colored boxes indicated with the range of FGH. Dotted lines indicate multiple steps.

iThenticate Page 203 of 209 - Integrity Submission Submission ID trn:oid:::3618:121855597

A. scholaris showed higher FGE in gall leaves (higher than control) compared to *F. racemosa* (all genes FGE < 1), indicating differential metabolic priorities between species.

The low FGE (<1) for all genes in *F. racemosa* gall leaves indicates no significant upregulation of defense-related pathways in the leaf tissue without gall. In gall leaves, unaffected by galling, *F. racemosa* maintains baseline metabolism, prioritizing growth and photosynthesis over defense gene expression, as seen in other systems under non-stressed conditions (Mithöfer & Boland, 2012; Taiz & Zeiger, 2010). This tissue-specific response aligns with studies showing herbivore-induced gene expression confined to attacked tissues, with unattacked tissues exhibiting minimal defense gene induction (War et al., 2012). Additionally, *F. racemosa* may allocate resources to gall tissues to support insect-derived structures, suppressing systemic defense gene expression in gall leaves to minimize metabolic costs (Mithöfer & Boland, 2012; Nabity et al., 2009).

However, earlier chapters revealed higher levels of bioactive chemicals and bioactivity in *F. racemosa* gall leaves compared to galls, despite the lack of gene upregulation. This paradox may be attributed to translocation of these compounds from other tissues (galls), maintaining defense response without requiring active gene expression during galling stress (Mithöfer & Boland, 2012; Schwachtje et al., 2006). Alternatively, post-transcriptional regulation or enzyme activity in gall leaves could sustain bioactive compound levels, bypassing the need for transcriptional upregulation (Ng et al., 2018; Staiger et al., 2013). These findings highlight the adaptive strategies of *F. racemosa* and *A. scholaris* in balancing growth and defense, with *F. racemosa* employing localized gall-specific responses. The study provides valuable insights into plant-insect interactions and secondary metabolite regulation, with implications for understanding defense mechanisms in gall-forming systems.

iThenticate Page 205 of 209 - Integrity Submission Submission ID trn:oid:::3618:121855597

6.4. CONCLUSIONS

The molecular processes that take place within the cell or tissue will determine its physiological and biochemical properties. Normal leaves, gall leaves and galls of *A. scholaris* and *F. racemosa* were analysed using qRT-PCR, which demonstrated that the gall induction influenced the expression of genes. It will generate a distinct phytochemical profile in tissues of gall leaves and galls compared to normal or healthy leaf tissues. The infection by *P. tuberculata* on leaves of *A. scholaris* leads to the induction of genes in the pathway to a higher level up to 25-fold in galls. All the genes except DFR showed activation to a lesser extent in gall leaves also.

The insect *P. depressa* infected *F. racemosa* leaves also alter the transcript levels of genes of secondary metabolic pathway. The induction of gene expression leads to higher phenolic levels in *F. racemosa* galls also. There was no evidence of upregulation of gene expression in the gall leaves of *F. racemosa.* However, in the earlier chapters, it was discovered that the bioactive chemicals and bioactivity of the gall leaves was higher. Therefore, it can be explained by the possibility that phytoconstituents get synthesized in greater quantities in gall tissues and may be translocated or diffused to the surrounding leaf tissues. It may be a defensive mechanism shown by the plant. A different possibility might be post-transcriptional or post-translational regulation, in which changes in enzyme activity occur without variations in mRNA levels. The increased production of transcript in gall can be considered as a defensive action against gall inducers. The presence of a large number of phenolic compounds in galls and the rich transcript profile of important genes involved in phenolic biosynthesis will shed light on phenolic-mediated stress tolerance in these plants.

Thus gall and gall associated tissues have higher phytoconstituents and greater bioactivity. A comprehensive study of the phenylpropanoid and shikimic acid pathway, which is triggered by external stimuli, at the genic level can be used to enhance the therapeutic industrial production of plant-based formulations. It can also be used in plant breeding to improve the useful traits in crops like disease resistance and quality improvements. The results can be useful for further studies in the field of development of plants based drugs with much higher medicinal properties and bioactivities.

iThenticate Page 205 of 209 - Integrity Submission Submission ID trn:oid:::3618:121855597

CHAPTER 7 - SUMMARY

This study elucidates the complex plant responses to the infection by gall-forming insects, emphasizing the integration of molecular and biochemical data to understand secondary metabolism dynamics in insect-induced galls. Comparative analysis of biomolecules in healthy leaves, galled leaves and galls provides insights into gall development processes. Insect-induced galls are recognized as sites of enhanced secondary metabolism, prompting the investigation of two gall-forming systems: *Pauropsylla tuberculata*-induced leaf galls on *Alstonia scholaris* and *Pauropsylla depressa*-induced leaf galls on *Ficus racemosa*. The study initially compared phytochemical profiles between normal (healthy) leaf tissues and insect-induced galls. Standard phytochemical assays revealed significantly higher levels of phenolics, flavonoids and other bioactive compounds in galls and galled leaves compared to normal leaves, indicating that gall formation triggers defense signaling and elevates bioactive defense compound production through upregulated secondary metabolic pathways.

To explore the relationship between biochemical changes and molecular pathway activation during gall development, key genes in the shikimate and phenylpropanoid pathways were analyzed using quantitative real-time PCR (qRT-PCR). The genes included 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), chorismate synthase (CS), phenylalanine ammonia-lyase (PAL), chalcone-flavanone isomerase (CHI) and dihydroflavonol 4-reductase (DFR). Results showed significant upregulation of these genes in galls compared to normal leaves, with *F. racemosa* galls exhibiting higher fold gene expression (FGE) than *A. scholaris* galls. This transcriptional activation correlates with the observed phytochemical accumulation, confirming that gall induction enhances secondary metabolism. In contrast, gall leaves showed significant biochemical differences but minimal gene expression changes compared to normal leaves, with *F. racemosa* gall leaves exhibiting FGE < 1 for all genes and *A. scholaris* gall leaves showing low FGE. This suggests that phytochemical accumulation in gall leaves may result from systemic translocation of metabolites from galls rather than local gene expression, as insects manipulate host tissues to act as nutrient sinks while plants redistribute defense compounds to adjacent uninfected tissues.

The medicinal potential of plants is closely tied to their bioactive phytochemical content. Using standard bioactivity assays, this study compared the antibacterial, antifungal, antioxidant and cytotoxic properties of normal leaves, galled leaves and galls in both species. Galls and galled leaves exhibited significantly higher bioactivity than healthy leaves, attributed to elevated secondary metabolite concentrations. These findings align with reports of galls being used in traditional medicine and highlight their potential in phytotherapy fields such as pharmacology, ethnomedicine and drug discovery. The results also support the validation of traditional medicinal practices, the development of sustainable natural products and applications in ecological and agricultural contexts.

This study enhances understanding of the complex reprogramming of plant defense and metabolic pathways triggered by gall-inducing insects. It elucidates the molecular basis for physiological, morphological and biochemical changes during gall formation, demonstrating how insects manipulate plant tissues for their benefit while plants adapt by enhancing defense compound production and distribution. The results underscore the plasticity of plant tissues and the role of galls as unique microenvironments that reflect intricate ecological interactions. Galls serve as valuable models for studying inducible secondary metabolism, offering insights into plant-insect interactions and the potential for novel therapeutic agent discovery.

CHAPTER 8 - RECOMMONDATIONS

Based on the study of structural, phytochemical and molecular characteristics of leaf galls of *Alstonia scholaris* and *Ficus racemosa*, the following recommendations can be suggested for further research and practical applications in the field of phytochemistry and plant biotechnology. The recommendations aim to deepen the understanding of gall-induced metabolic changes and explore their ecological and therapeutic implications.

1. More research into the phytochemical substances responsible for increased bioactivity in galls and galled leaves is required. This can be accomplished with modern techniques like high-resolution mass spectrometry. These techniques will make it possible to identify new chemicals. Such findings could have important phytopharmacological applications.
2. Transcriptomic and proteomic analyses can be used to identify all differentially expressed genes. These approaches help to understand the biochemical and molecular mechanisms behind the increased production of secondary metabolites during gall formation. They also provide insights into how gall-inducing insects change plant metabolism. This knowledge can support the development of strategies for metabolic engineering.
3. To explore the ecological role of gall formation in plant defense and its evolutionary significance, field studies should be conducted to assess how gall formation affects plant fitness, herbivory rates and interactions with other organisms. Comparative studies across different populations or species could reveal geographic or species-specific variations. These investigations will help clarify the adaptive significance of gall formation and its role in plant-insect co-evolution.
4. Metabolomics and genomics should be used to compare the phytochemical profiles and bioactivities of galled and non-galled tissues across different species and populations of *Alstonia* and *Ficus*. This approach will help identify species-specific or population-specific traits that influence gall-induced metabolic changes. Additionally, studies on other gall-inducing species should be conducted to determine whether these effects are specific to *Pauropsylla* species.

5. To advance the application of gall-induced bioactive compounds from *A. scholaris* and *F. racemosa* in drug discovery and phytopharmacology, screen the identified compounds for therapeutic potential. Conduct *in vitro* studies using cell culture models to assess efficacy and safety. If promising, proceed with additional *in vivo* validations or preclinical trials to confirm effects. This could develop novel natural products or drugs, backing traditional uses of these plants.
6. To understand the impact of environmental factors on gall formation and secondary metabolism in *A. scholaris* and *F. racemosa*, research should assess how climate change influences these processes. Ecological studies suggest that climate variations could alter gall frequency and plant responses, affecting their medicinal properties. Long-term field studies under diverse environmental conditions should be conducted to evaluate these effects.
7. Future research should focus on exploiting upregulated secondary metabolite genes found in gall tissues for targeted metabolic engineering. These genes, critical for the medicinal properties of plants, offer opportunities to enhance phytochemical production in whole plants or cultured cells. Genetic engineering techniques should be used to upregulate these genes in target or related species. Plant cell cultures can enable scalable, consistent production of high-value metabolites *in vitro*.
8. To promote the conservation and sustainable use of *A. scholaris* and *F. racemosa*, efforts should focus on raising awareness. These plants, vital for their ecological and medicinal value, hold significant potential for sustainable utilization. Collaboration with local communities and organizations is recommended to develop effective management plans for their responsible use.

In conclusion, these recommendations provide a strategic framework for advancing research on *A. scholaris* and *F. racemosa*, harnessing their gall-induced bioactive potential for pharmaceutical innovation and ensuring their continued ecological and medicinal value for future generations.

CHAPTER 9 – REFERENCES

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INDEX

A. niger, 31, 110, 111, 141, 142, 143, 158
abiotic stresses, 20, 21, 32, 33, 165, 182, 183, 192
agar-well diffusion, 110, 138
Alkaloids, 14, 24, 37, 53, 54, 55, 56, 83, 87, 95, 202, 206, 212
Alstonia scholaris, 8, 16, 17, 19, 23, 35, 39, 40, 103, 164, 198, 200, 202, 203, 204, 205, 206, 207, 209, 211, 212, 214, 215, 216, 217, 219, 220, 221, 223, 225, 226, 227, 228, 231, 235
amino acid synthesis, 23
antibacterial, 9, 11, 14, 19, 23, 29, 30, 37, 61, 62, 65, 74, 79, 80, 82, 83, 84, 85, 86, 89, 90, 91, 92, 93, 94, 104, 105, 108, 109, 132, 133, 134, 136, 137, 138, 150, 151, 154, 156, 157, 160, 161, 163, 165, 199, 204, 213, 216, 217, 219, 220, 221
antibacterial activity, 30, 132, 133, 134, 135, 156, 158, 161, 163
anticandidal activity, 139, 140
Antifungal activity, 30, 73, 109, 138, 141, 208, 225, 227
anti-inflammatory activity, 16, 32, 64, 73, 74, 76, 82, 90, 113, 149, 150, 151, 207, 209, 220, 232
antioxidant, 7, 9, 11, 14, 19, 23, 28, 29, 36, 37, 61, 62, 63, 64, 65, 68, 69, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 103, 104, 105, 107, 114, 116, 118, 121, 123, 125, 127, 129, 131, 150, 151, 154, 157, 165, 181, 199, 202, 203, 204, 205, 206, 211, 213, 214, 217, 218, 223, 224, 225, 226, 227, 228, 234
Apocynaceae, 17, 23, 202, 206
Aspergillus niger, 31, 109, 211, 231
auxin, 12, 13, 14, 22
bioactive compounds, 9, 15, 16, 19, 23, 38, 102, 104, 147, 164, 165, 198, 200, 203, 215, 228
Bioactivity, 7, 16, 104, 151, 154, 214, 222, 224
biosynthetic pathways, 14, 16, 37, 164, 181
biotic stresses, 15, 34, 166, 190
C. albicans, 31, 110, 138, 139, 140, 143, 158, 161
Candida albicans, 30, 109, 211
Carotenoid, 46
cDNA microarray, 33, 192, 225
cell viability, 106, 112, 143, 144, 145, 146, 147, 148, 158
cellular hypertrophy, 13
Chalcone-flavanone isomerase, 34, 173, 182, 185, 191

CHI, 34, 167, 168, 173, 180, 181, 182, 185, 187, 188, 191, 193, 194, 195, 196, 198
chlorophyll, 24, 27, 36, 45, 49, 51, 52, 97, 98, 213, 216
Chlorophyll a, 45, 98
Chlorophyll b, 45, 98
Chorismate synthase, 34, 166, 173, 182, 184, 189
cytokinins, 12, 13, 14, 22
Cytokinins, 22
Cytotoxic activity, 31, 89, 106, 111, 143, 208
cytotoxicity, 31, 111, 143, 145, 147, 148, 154, 158, 230
DAHPS, 33, 166, 167, 168, 173, 180, 181, 182, 183, 184, 187, 188, 189, 193, 195, 196, 198
DFR, 34, 167, 168, 173, 180, 181, 182, 186, 187, 188, 191, 192, 193, 194, 195, 196, 197, 198
Dihydroflavonol 4-reductase, 34, 182, 186, 191
DPPH, 29, 105, 107, 123, 124, 125, 126, 127, 128, 130, 131, 157, 214
DPPH radical scavenging activity, 29, 107, 123, 125, 127
E. coli, 30, 132, 133, 134, 135, 136, 137, 138, 157, 160, 161
egg albumin denaturation, 32, 106, 232
EPSPS, 33, 166, 167, 168, 173, 180, 181, 182, 183, 184, 187, 188, 189, 193, 195, 196, 198, 211
Escherichia coli, 30, 108, 132, 162
expression profile, 182, 188
Ficus racemosa, 8, 16, 18, 19, 25, 35, 39, 40, 56, 103, 164, 198, 200, 202, 203, 204, 207, 209, 211, 213, 215, 217, 218, 219, 220, 221, 223, 224, 225, 226, 228, 229, 230, 231, 232, 233
flavonoids, 8, 11, 15, 19, 23, 24, 25, 26, 32, 36, 37, 38, 41, 42, 55, 56, 57, 60, 66, 67, 68, 69, 70, 100, 101, 105, 151, 160, 165, 166, 167, 182, 198, 214, 232, 233
fold gene expression, 182
foreign organisms, 11
free radical scavenging, 29, 105, 107, 123, 125, 127, 154, 157, 203
gall inducer, 8, 12, 36, 49, 102, 180, 182
gall induction, 7, 9, 12, 14, 15, 16, 21, 22, 23, 27, 35, 36, 68, 70, 102, 103, 147, 164, 165, 167, 169, 182, 184, 187, 193, 194, 197, 198, 211, 232, 233
Gall induction, 12, 13, 21, 33, 36, 181
gall leaves, 9, 40, 41, 44, 53, 54, 56, 57, 58, 59, 60, 61, 65, 66, 67, 68, 69, 70, 97, 98, 99, 100, 101, 103, 110, 114, 116, 122, 123, 127, 130, 132, 138, 142, 144, 147, 148, 151, 161, 170, 179, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 193, 194, 195, 196, 197, 198

gall physiology, 19
GC-MS, 7, 8, 26, 28, 43, 44, 57, 58, 59, 101, 181, 202, 203, 205, 212, 215, 218, 223, 224, 225, 228, 229, 230, 231
gene expression, 9, 14, 16, 32, 33, 34, 36, 38, 164, 167, 168, 169, 172, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 209, 210, 213, 214, 215, 217, 218, 219, 220, 225, 233, 234
gene expression profiling, 167, 168
Gram-negative bacteria, 30, 105, 108, 136, 137, 138, 156
Gram-positive bacteria, 30, 137
growth inhibition percentage, 141, 142
herbal formulations, 9, 15
herbal medicines, 11, 103
herbivores, 12, 15, 20, 21, 33, 34, 36, 37, 104, 181, 220, 233
herbivory, 27, 70, 169, 183, 184, 185, 188, 189, 190, 191, 194, 200, 205, 221, 227
hyperplasia, 13, 35, 47
IC_{50} values, 32, 121, 122, 129, 130, 131, 139, 140, 157
indole-3-acetic acid, 12
inducer, 12, 17, 20, 21, 23, 33, 48, 71, 77, 89, 93, 97, 99
insects, 8, 11, 12, 13, 14, 19, 20, 21, 23, 26, 33, 35, 38, 40, 47, 48, 52, 66, 67, 68, 73, 90, 97, 98, 101, 103, 164, 165, 180, 185, 188, 192, 198, 199, 200, 209, 211, 212, 213, 225
larvae, 21, 52, 147
larval chamber, 13, 50, 51, 52
medicinal plants, 7, 8, 15, 16, 19, 23, 34, 35, 47, 104, 152, 164, 166, 168, 202, 210, 215, 220, 221, 229, 232
metabolic engineering, 16, 166, 169, 200, 201
metabolomic profile, 27
molecular alterations, 12, 15
mRNA, 169, 197, 210
MTT, 31, 106, 111, 112, 143, 145, 147, 148
MTT assay, 106, 143, 145, 147
normal leaves, 7, 8, 14, 26, 33, 34, 35, 39, 40, 41, 44, 45, 47, 52, 53, 54, 56, 57, 58, 59, 60, 62, 65, 66, 67, 68, 70, 97, 98, 99, 100, 101, 103, 114, 116, 120, 122, 123, 125, 127, 129, 130, 132, 135, 138, 144, 147, 151, 164, 169, 170, 179, 181, 187, 188, 193, 198, 199
Nuclear Magnetic Resonance, 153, 206
nutrient sinks, 13, 14, 22, 199
Nutrition hypothesis, 21, 27
nutritive region, 13, 52
OHR-LC-MS, 7, 8, 44, 65, 66, 67, 68, 69, 70, 71, 73, 78, 81, 84, 88, 101, 181
PAL, 34, 46, 99, 100, 167, 168, 173, 180, 181, 182, 184, 185, 187, 188, 190, 193, 195, 196, 198, 220, 227

Pauropsylla, 8, 10, 14, 24, 25, 48, 49, 164, 165, 167, 168, 181, 198, 200, 205, 210, 216, 223, 225, 229
Pauropsylla depressa, 8, 25, 49, 198, 210, 229
Pauropsylla tuberculata, 8, 24, 48, 198, 205, 216, 223, 225
pharmacological properties, 11, 19
pharmacology, 11, 199, 216, 218
phenolics, 8, 13, 15, 19, 21, 22, 23, 24, 25, 26, 27, 28, 32, 36, 37, 38, 41, 42, 55, 57, 66, 67, 68, 69, 70, 101, 104, 123, 127, 151, 164, 165, 166, 167, 170, 181, 194, 198, 202
Phenolics, 14, 37, 54, 56, 70, 74, 82, 165
phenylalanine, 15, 34, 37, 46, 99, 166, 181, 184, 198, 204, 208, 209
Phenylalanine ammonia-lyase, 34, 46, 167, 182, 184, 190
phenylpropanoid pathway, 15, 22, 46, 99, 166, 167, 181, 193, 196, 207
photosynthesis, 14, 22, 34, 45, 98, 189, 194, 196, 210, 221
phytochemical analyses, 16
phytochemical compounds, 11, 14, 28, 55, 57, 65, 69, 101, 104, 164, 228
phytochemicals, 16, 20, 25, 27, 28, 32, 36, 44, 53, 59, 70, 99, 103, 160, 188, 195, 211
phytoconstituents, 25, 27, 28, 41, 53, 70, 101, 151, 197
phytopharmacology, 9, 16, 103, 151, 165, 200, 202
Plant galls, 11, 19, 20, 35, 38
plant-parasite relationship, 11
Pseudomonas aeruginosa, 30, 108, 132
PTLC, 153, 154
qRT-PCR, 8, 9, 164, 168, 169, 177, 178, 179, 180, 181, 182, 184, 185, 186, 188, 197, 198, 207
Qualitative phytochemical analysis, 41
Quantitative phytochemical analysis, 43, 57, 65, 66, 67, 68, 69
reducing power activity, 29
reducing power assay, 29, 114, 131
RNA-seq, 33
S. aureus, 30, 132, 133, 134, 135, 136, 137, 138, 143, 157, 160, 161
secondary metabolic pathways, 14, 32, 33, 198
secondary metabolism, 8, 9, 15, 33, 166, 167, 169, 185, 187, 189, 190, 198, 199, 201, 209
secondary metabolites, 8, 11, 14, 15, 17, 19, 20, 21, 26, 28, 36, 37, 38, 40, 41, 60, 68, 101, 102, 103, 104, 105, 106, 151, 164, 165, 166, 180, 183, 185, 188, 193, 200, 202, 208, 212, 214, 217, 220, 234
shikimic acid pathway, 15, 33, 166, 181, 184, 188, 189, 190, 197
sink hypothesis, 22
Staphylococcus aureus, 30, 108, 132, 162

stress-response, 14
therapeutic potential, 9, 19, 26, 34, 36, 38, 107, 165, 166, 201, 234
therapeutic properties, 14, 15, 104
Thin-layer chromatography, 152
traditional medicine, 15, 19, 199
transcriptome profiling, 33, 189
Transcriptomic studies, 15
zone of inhibition, 110, 132, 133, 134, 135, 136, 138, 139, 143, 154, 156, 157, 161