PHYTOCHEMICAL SCREENING AND PHARMACOLOGICAL EVALUATION OF *HUMBOLDTIA SANJAPPAE* SASIDH. & SUJANAPAL (LEGUMINOSAE)

Thesis submitted to the
UNIVERSITY OF CALICUT
For the award of the Degree of

DOCTOR OF PHILOSOPHY IN BOTANY
(Under the Faculty of Science)

BY

JAMEEMA SIDHIC

Under the Guidance of
Dr. Satheesh George (Guide)
Dr. Arunaksharan N (Joint-Guide)

CENTRE FOR POST GRADUATE STUDIES AND RESEARCH IN BOTANY
ST. JOSEPH'S COLLEGE (AUTONOMOUS) DEVAGIRI
CALICUT- 673008, KERALA, INDIA

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2025

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This is to certify that the Ph.D. thesis entitled **"PHYTOCHEMICAL SCREENING AND PHARMACOLOGICAL EVALUATION OF *HUMBOLDTIA SANJAPPAE* SASIDH. & SUJANAPAL (LEGUMINOSAE)"** is an authentic record of the original research work accomplished by **Ms. Jameema Sidhic.,** under my supervision and guidance at the Centre for Post Graduate Studies and Research in Botany, St. Joseph's College (Autonomous) Devagiri, Calicut, Kerala and that no part of this thesis has been published earlier for the award of any other degree or diploma. Also certified that the contents in the thesis are subjected to **Plagiarism Check** using the software **iThenticate** and that no text or data is reproduced from other's work.

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10/04/2025

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DECLARATION

I hereby declare that the work presented in the thesis entitled **"PHYTOCHEMICAL SCREENING AND PHARMACOLOGICAL EVALUATION OF *HUMBOLDTIA SANJAPPAE* SASIDH. & SUJANAPAL (LEGUMINOSAE)"** is based on the original work done by me under the guidance of **Dr. Satheesh George** (Guide) and Dr. Arunaksharan N (Joint-Guide) has not been included in any other thesis submitted previously for the award of any degree. The contents of the thesis have undergone plagiarism check using **iThenticate** software at C.H.M.K. Library, University of Calicut, and the similarity index was found within the permissible limit. I also declare that the thesis is free from AI generated contents.

Jameema Sidhic

Place: Devagiri

Date: 10/09/2025

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Jameema Sidhic

ABSTRACT

The present study investigates the phytochemical composition and anti-inflammatory potential of *Humboldtia sanjappae,* focusing on the effect of extraction solvents and plant parts, and employing a multidisciplinary approach encompassing phytochemical analysis, pharmacokinetics, toxicity evaluation, and both *in vitro* and *in vivo* assessments. Ethanol was identified as the most effective extraction solvent, with the bark yielding higher antioxidant activity and phytochemical richness compared to the leaves. The ethanol bark extract underwent LC-MS/MS analysis, revealing 57 phytochemicals categorized into 14 chemical classes, with flavones, phenols, and catechins being predominant. ADMET-based pharmacokinetic predictions indicated favorable drug-likeness, and acute oral toxicity studies confirmed its safety profile (GHS Category 4).

Network pharmacology analysis identified 117 potential inflammation-related targets, with luteolin and CYP19A1 emerging as key nodes. Gene ontology and pathway enrichment analysis revealed the extract's involvement in numerous biological processes and pathways related to inflammation, including PI3K-Akt and AGE-RAGE signaling. In vitro studies using RAW 264.7 macrophages showed dose-dependent inhibition of IL-1β, IL-6, TNF-α, and nitric oxide production, along with downregulation of NF-κB and COX-2 expression. *In vivo* acute and chronic inflammation models in Swiss albino mice demonstrated significant anti-inflammatory effects, confirmed by histopathology, gene expression analysis, and molecular docking studies. Key phytochemicals such as (-)-Epicatechin, 5,7-Dihydroxy-8-prenylflavone, and Neoeriocitrin exhibited strong binding affinities to pro-inflammatory targets.

Overall, the study highlights the therapeutic potential of *H. sanjappae* bark ethanol extract as a natural anti-inflammatory agent, supporting its traditional use and offering promising insights for future drug discovery and nutraceutical development.

സംഗ്രഹം

ഈ പഠനം ഹംബോൾട്ടിയ സഞ്ചപ്പെ സസ്യത്തിന്റെ ഫൈറ്റോകെമിക്കൽ ഘടനയും വീക്കം നിയന്ത്രിക്കാനുള്ള ശേഷിയും പരിശോധിക്കുന്നു. വ്യത്യസ്ത എക്സ്ട്രാക്ഷൻ ലായകങ്ങളും സസ്യഭാഗങ്ങളും അടിസ്ഥാനമാക്കി ഫൈറ്റോകെമിക്കൽ വിശകലനം, ഫാർമക്കോകൈനെറ്റിക് പ്രവചനങ്ങൾ, വിഷാംശം നിരീക്ഷണം, ലബോറട്ടറി അടിസ്ഥാനത്തിലുള്ള (*in vitro*) പരീക്ഷണങ്ങൾ, ജീവികളിൽ നടന്ന (*in vivo*) പരിശോധനകൾ എന്നിവ ഉൾക്കൊള്ളുന്ന സമഗ്രമായ ശാസ്ത്രീയ സമീപനം സ്വീകരിച്ചിരിക്കുന്നു.

ഇലയുമായി താരതമ്യപ്പെടുത്തിയപ്പോൾ പുറംതൊലി ഉയർന്ന ആന്റിഓക്സിഡന്റ് ശേഷിയും ഫൈറ്റോകെമിക്കൽ സമൃദ്ധിയും കാണിച്ചതിനാൽ എഥനോൾ ഏറ്റവും ഫലപ്രദമായ എക്സ്ട്രാക്ഷൻ ലായകമായി തിരിച്ചറിഞ്ഞു. എഥനോൾ പുറംതൊലി എക്സ്ട്രാക്റ്റിന്റെ എൽസി-എംഎസ്/എംഎസ് വിശകലനത്തിൽ 14 രാസക്ലാസുകളിലായി തരംതിരിച്ച 57 ഫൈറ്റോകെമിക്കലുകൾ കണ്ടെത്തി, ഇവയിൽ ഫ്ലേവോണുകൾ, ഫിനോളുകൾ, കാറ്റെച്ചിനുകൾ എന്നിവ പ്രധാനപ്പെട്ടവയായിരുന്നു.

ADMET അടിസ്ഥാനമാക്കിയുള്ള ഫാർമക്കോകൈനെറ്റിക് വിശകലനത്തിൽ, 38 സംയുക്തങ്ങൾ ലിപിൻസ്കിയുടെ നിയമങ്ങൾ പാലിക്കുകയും, മികച്ച മരുന്ന് സാദൃശ്യം കാട്ടുകയും ചെയ്തു. വായിലൂടെ നൽകിയ എക്സ്ട്രാക്റ്റ് ഉപയോഗിച്ച് നടത്തിയ ജീവി അധിഷ്ഠിത വിഷപരീക്ഷണത്തിൽ, ഹംബോൾട്ടിയ സഞ്ചപ്പെ സുരക്ഷിതമാണെന്നും, അതിന്റെ GHS കാറ്റഗറി 4 (LD_{50} >300–2000 mg/kg) എന്ന നിലയിലുള്ളതിനാൽ കൂടുതൽ പഠനങ്ങൾക്ക് യോജ്യമായ ഒട്ടും അപായകരമല്ലാത്ത ഘടകമാണെന്നതും സ്ഥിരീകരിച്ചു.

നെറ്റ്വർക്ക് ഫാർമക്കോളജി വിശകലനത്തിൽ, 117 ഫൈറ്റോകെമിക്കൽ ഘടകങ്ങൾ വീക്കവുമായി ബന്ധപ്പെട്ട ജീനുകൾ ലക്ഷ്യമാക്കിയതായി കണ്ടെത്തി. ല്യൂട്ടോലിൻ പ്രധാനപ്പെട്ട ഫൈറ്റോകെമിക്കൽ ഘടകമായും, CYP19A1 പ്രധാന ലക്ഷ്യജീനായും ഈ വിശകലനം വഴി തിരിച്ചറിഞ്ഞു. ജീൻ ഓന്റോളജി (Gene Ontology) വിശകലനം മുഖേന, PI3K-Akt, AGE-RAGE തുടങ്ങിയ ജീവശാസ്ത്രപാതകളിൽ ഇവയുടെ പങ്കാളിത്തം

തെളിയിച്ചു. RAW 264.7 മാക്രോഫേജ് കോശങ്ങൾ ഉപയോഗിച്ച ലബോറട്ടറി അടിസ്ഥാനത്തിലുള്ള (*in vitro*) പരീക്ഷണങ്ങളിൽ IL-1β, IL-6, TNF-α, നൈട്രിക് ഓക്സൈഡ് ഉൽപാദനം ഡോസ് ആശ്രിതമായി കുറയുകയും, NF-κB, COX-2 എന്നീ മാർക്കറുകളുടെ പ്രകടനം കുറയുകയും ചെയ്തു.

ജീവികളിൽ (*in vivo*) നടത്തിയ തീവ്ര (acute – ക്യാരേജീനൻ പ്രേരിത) വീക്കം, ദീർഘകാലിക (chronic – ഫോർമാലിൻ പ്രേരിത) വീക്കം മോഡലുകളിലും, ഹംബോൾട്ടിയ സഞ്ചപ്പെ എഥനോൾ പുറംതൊലി എക്സ്ട്രാക്റ്റ് ഗണ്യമായ വീക്കം നിയന്ത്രണ ശേഷി തെളിയിച്ചു. ഹിസ്റ്റോപാത്തോളജിക്കൽ പരിശോധനകളും ജീൻ എക്സ്പ്രഷൻ വിശകലനവും ഈ കണ്ടെത്തലുകളെ പിന്തുണച്ചു.

മോളിക്യുലർ ഡോക്കിംഗ് പഠനങ്ങളിൽ, (-)-എപികാറ്റെചിൻ, 5,7-ഡൈഹൈഡ്രോക്സി -8-പ്രൈനൈൽഫ്ലേവോൺ, നിയോറിയൊസിട്രിൻ എന്നിവ COX-1, COX-2, IL-6, IL-1β, TNF-α എന്നീ പ്രധാന വീക്കം സംബന്ധിച്ച മാർക്കറുകളുമായി ശക്തമായ ബന്ധന ശേഷി കാണിച്ചു.

ഈ പഠനം ഹംബോൾട്ടിയ സഞ്ചപ്പെയുടെ എഥനോൾ പുറംതൊലി എക്സ്ട്രാക്റ്റിന്റെ ജൈവസജ്ജതയും അതിന്റെ പ്രകൃതിദത്ത വീക്കം നിയന്ത്രണ ശേഷിയും ശാസ്ത്രീയമായി തെളിയിക്കുന്നു.

ഇതിലൂടെ ഭാവിയിലെ ഔഷധ വികസനത്തിനും ന്യൂട്രാസ്യൂട്ടിക്കൽ തയ്യാറെടുപ്പുകൾക്കുമുള്ള സാധ്യതകൾ ഈ സസ്യത്തിൽ കണക്കു-കൂട്ടാവുന്നതാണ്.

CONTENTS

List of Tables
List of Figures
Abbreviations

Chapter	*Title*	*Page No.*
1	Introduction	1
2	Review of Literature	7
3	Materials and Methods	26
4	Results and Discussion	43
5	Summary and Conclusion	118
6	Recommendations	126
7	References	127
8	Annexure I: Publications.	163

LIST OF TABLES

Table No	Title
Table 1	Medicinal plants and their traditional uses
Table 2	Different classes of terpenes and their role in healthcare
Table 3	Phytochemical Constituents Identified in Different *Humboldtia* Species
Table 4	Procedure for preliminary phytochemical analysis
Table 5	Treatment Groups and Conditions
Table 6	Preliminary phytochemical analysis of different extracts
Table 7	Rf Values and their detection wavelengths
Table 8	Comparison of Maximum Rf and Area Percentage of different solvent extract of bark at 254 nm
Table 9	Comparison of Maximum Rf and Area Percentage of different solvent extract of bark at 366 nm
Table 10	Comparison of Maximum Rf and Area Percentage of different solvent extract of leaf and bark at 550 nm
Table 11	Total Phenolics and flavonoid content of different extracts
Table 12	Antioxidant potential of different extracts
Table 13	Identified Phytochemicals in ethanolic extract of *Humboldtia sanjappae* bark detected by LC-MS/MS
Table 14	Pharmacological properties of compounds identified from HBA
Table 15	ADMET properties of the identified phytochemical compounds of HBA
Table 16	Body weights of marked animals over the toxicity studies
Table 17	Correlation Analysis of Body Weight Changes During the Study
Table 18	Common targets associated with phytochemical and inflammation
Table 19	Anti-inflammatory activity of HBA against LPS-induced activation of RAW 264.7 cells vs. standard aspirin (1 mM)

Table 20	Change in paw thickness over time (mm) in the acute inflammation model
Table 21	Mean change in paw volume over time (mL) of acute inflammation model
Table 22	Mean change in paw thickness over time (mm) of chronic inflammation model
Table 23	Mean change in paw volume over time (mL) of chronic inflammation model
Table 24	Molecular docking and binding energy analysis of phytochemicals targeting key inflammatory proteins
Table 25	Histopathology observation comparison of two different inflammation models

LIST OF FIGURES

Figure No.	Title
Figure 1	Major classes of phytochemicals
Figure 2	Methodological Flowchart
Figure 3	*Humboldtia sanjappae*, Western Ghats of Kerala (10.04829˚ N, 76.8399˚ E), Ernakulam District
Figure 4	Soxhlet extraction of *H. sanjappae* bark and leaf using ethanol-water mixtures
Figure 5	Calibration curve of gallic acid showing linearity between concentration (µg) and absorbance at 550 nm
Figure 6	Calibration curve of quercetin showing linearity between concentration (µg) and absorbance at 520 nm
Figure 7	Workflow for evaluating the anti-inflammatory effects
Figure 8a	Experimental design for carrageenan-induced acute inflammation in mice
Figure 8b	Experimental design for formalin-induced chronic inflammation in mice
Figure 9	Bar chart representing yield percentage of different extracts
Figure 10	Heat map showing intensity of phytochemicals between samples
Figure 11	HPTLC plates of different extracts of *H. sanjappae*
Figure 12	HPTLC chromatogram of different extracts of *H. sanjappae*
Figure 13	Distribution of total number of Rf values detected at three wavelengths
Figure 14	Comparison of Rf and area percentages at different detection wavelengths
Figure 15	Variation in the total number of Rf values among extracts
Figure 16	Bar graph showing variations in phenolic and flavonoid concentrations
Figure 17	Antioxidant potential of samples expressed in µg/mL
Figure 18	Correlation heatmaps: TPC, TFC, and antioxidant activities

Figure No.	Title
Figure 19	Workflow of LC-MS/MS analysis, ADMET evaluation, and toxicity studies
Figure 20	Total ion chromatogram by LC-MS/MS of ethanolic bark extract
Figure 21a	Phytochemicals: Flavones and Phenols
Figure 21b	Phytochemicals: Catechins, Terpenoids, and Tannins
Figure 21c	Phytochemicals: Betalains, Benzopyrans, Pyridine derivatives, Coumarins, Glycosides, etc.
Figure 21d	Phytochemicals: Betalains, Benzopyrans, Pyridine derivatives, and Steroid lactones
Figure 22	Percentage distribution of different phytochemical classes
Figure 23	Pharmacokinetic properties of various compounds
Figure 24	CYP substrate/inhibitor distribution among identified compounds
Figure 25	Distribution of renal OCT2 substrates in HBA
Figure 26	Frequency of phytochemicals associated with specific toxicity types
Figure 27	Distribution of total clearance values and the number of compounds in HBA
Figure 28	Effects of administering HBA at different doses
Figure 29	Graph of body weight changes over days
Figure 30	Venn diagram of disease and phytochemical target genes.
Figure 31	Compound-target network
Figure 32a & b	Protein-protein interaction networks of target genes.
Figure 33	Top 15 Gene Ontology (GO): gene count by category.
Figure 34	Top 15 GO categories by fold enrichment.
Figure 35a–t	KEGG pathway maps for the 20 most significant pathways.
Figure 36	Clustering tree of pathways based on gene overlap.
Figure 37	Pathway enrichment network of biological processes.
Figure 38	Anti-inflammatory activity of HBA against LPS-induced cytokine production.
Figure 39	Effect of HBA on NO levels in LPS-induced RAW 264.7 cells.

Figure No.	Title
Figure 40	Expression of NF-κB and COX2 genes in LPS- and HBA-treated macrophages.
Figure 41	Paw thickness changes in acute inflammation (right hind paw).
Figure 42	Paw thickness changes over time in acute inflammation model.
Figure 43	Paw volume changes over time in acute inflammation model.
Figure 44	Histological sections after 6h treatment in acute inflammation model.
Figure 45	Expression of inflammatory markers under acute inflammation.
Figure 46	Paw thickness changes in chronic inflammation (right hind paw)
Figure 47	Paw thickness changes over time in chronic inflammation model.
Figure 48	Changes in the paw volume over time in the chronic inflammation model.
Figure 49	Histological examination of paw tissue sections after 5 days of treatment in chronic inflammation model.
Figure 50	The expression of various inflammatory markers under chronic inflammation.
Figure 51	Ligand protein interaction diagram of COX-1 V/S (-)-Epicatechin.
Figure 52	Ligand protein interaction diagram of COX-2 V/S 5,7-dihydroxy-8-prenylflavone.
Figure 53	Ligand protein interaction diagram of IL-6 V/S Cinnamtannin A2.
Figure 54	Ligand protein interaction diagram of IL-6 V/S Cinnamtannin A2.
Figure 55	Ligand protein interaction diagram of IL-6 V/S Cinnamtannin A2.
Figure 56	The probable mechanism of the inhibition and reduction in inflammation.

ABBREVIATION

ADMET	:	Absorption, Distribution, Metabolism, Excretion, and Toxicity
BBB	:	Blood-Brain Barrier
BP	:	Biological Process
CC	:	Cellular Component
CNS	:	Central Nervous System
COX-1	:	Cyclooxygenase-1
COX-2	:	Cyclooxygenase-2
DAVID	:	Database for Annotation, Visualisation, and Integrated Discovery
ELISA	:	Enzyme-Linked Immunosorbent Assay
FBS	:	Foetal Bovine Serum
FDR	:	False Discovery Rate
$FeCl_3$	:	Ferric Chloride
GC-MS	:	Gas Chromatography Coupled with Mass Spectrometry
GO	:	Gene Ontology
HPLC	:	High-Performance Liquid Chromatography
HPTLC	:	High-Performance Thin Layer Chromatography
IL	:	Interleukins
IL-1β	:	Interleukin-1 Beta
IL-6	:	Interleukin-6
KEGG	:	Kyoto Encyclopaedia of Genes and Genomes
LC-MS/MS	:	Liquid Chromatography Tandem Mass Spectrometry
LPS	:	Lipopolysaccharide
MF	:	Molecular Function
mg/kg	:	Milligram Per Kilogram

mL	:	Millilitre
mM	:	Millimolar
MM-GBSA	:	Molecular Mechanics-Generalized Born Surface Area
MS	:	Mass Spectrometry
MTD	:	Maximum Tolerated Dose
nm	:	Nanometre
NO	:	Nitric Oxide
NPAA	:	Non-Protein Amino Acids
OECD	:	Organization for Economic Cooperation and Development
OH	:	Hydroxyl
PDB	:	Protein Data Bank
P-gp	:	P-Glycoprotein
psi	:	Pounds Per Square Inch
qRT-PCR	:	Quantitative Reverse Transcription Polymerase Chain Reaction
Rf	:	Retention Factor
TCM	:	Traditional Chinese Medicine
TFC	:	Total Flavonoid Content
TNF-α	:	Tumor Necrosis Factor-Alpha
TPC	:	Total Phenolic Content
TPTZ	:	2,4,6-Tripyridyl-S-Triazine
UV	:	Ultraviolet
UV-Vis	:	Ultraviolet-Visible
VDs	:	Volume of Distribution
WHO	:	World Health Organization
µg	:	Microgram

Chapter 1

INTRODUCTION

1.1 Herbal Practice and Traditional Medicine

1.2 Plant-Based Modern Medicine

1.3 Targeted and Untargeted Metabolomic Studies

1.4 Application of *in silico*, *in vitro* and *in vivo* approaches in phytochemical based therapeutic research

1.5 Research Problem

1.6 Objectives of the Study

1.1 Herbal Practice and Traditional Medicine

Traditional medicine, particularly herbal medicine, has been used for thousands of years to prevent and treat various illnesses (Pan *et al.*, 2014; Zhou *et al.*, 2019). For centuries, traditional medicine has been a fundamental component of healthcare systems, particularly in countries where modern pharmaceuticals are either expensive or unavailable. Numerous cultures, such as those practicing Ayurveda, Traditional Chinese Medicine (TCM), and Indigenous healing practices, depend on medicinal plants to alleviate a variety of maladies (Patwardhan & Mashelkar, 2009; Sucher, 2013).

While the use of herbs in traditional medicine is often based on cultural beliefs and traditional evidence (Brazier & Levine, 2003) recent research has shown that many herbal remedies are effective in treating certain health conditions (Fasinu *et al.*, 2012; Mousavi *et al.*, 2020). Despite the scientific evidence supporting the use of certain herbs in traditional medicine, it is important to note that not all herbal remedies are safe or effective (Pal & Shukla, 2003; WHO, 1991). Some herbs can interact with various medications and can cause adverse reactions (Ekor, 2014; Hussin, 2002), and there is a lack of regulation and standardization in the production and labelling of herbal supplements (Sardesai, 2002; Zhang *et al.*, 2012).

The scientific background of herbal medicine involves studying the active compounds within plants and their effects on the body. Plants contain a variety of chemicals such as alkaloids, flavonoids, and terpenes, which can have medicinal properties (Bourgaud *et al.*, 2001). Researchers have identified and isolated many of these compounds and have studied their effects on the body (Jones & Kinghorn, 2005; Puttaswamy *et al.*, 2020; Soleymani *et al.*, 2020).

1.2 Plant-Based Modern Medicine

The significance of phytochemical research is underscored by the fact that modern pharmaceuticals have been derived from traditional medicinal plants. For centuries, aspirin, which is derived from willow bark, has been employed to alleviate pain and inflammation (Sneader, 2000). Quinine, which was initially employed by indigenous South American communities to alleviate fevers, was subsequently recognised as a critical antimalarial medication (Kayser, 2024). Artemisinin which is derived from

Artemisia annua and has its origins in traditional Chinese medicine, is now a fundamental component of malaria treatment (Guo, 2016; Krungkrai & Krungkrai, 2016; Li *et al.*, 2005), Morphine, which is derived from the opium poppy, has a long record of use in the treatment of pain (Mani & Dhawan, 2011). The Madagascar periwinkle, which has been traditionally used to treat a variety of maladies, was found to contain vinca alkaloids, such as vinblastine and vincristine (Iskandar & Iriawati, 2016). These alkaloids are now important components in the treatment of childhood cancers. Taxol, a chemotherapeutic agent that is derived from the yew tree, is another innovative development that has evolved from traditional medicine (Slichenmyer & Von Hoff, 1991; Wall & Wani, 1996). These examples underscore the significance of investigating medicinal plants for potential therapeutic applications.

1.3 Targeted and Untargeted Metabolomic Studies

Advancements in analytical techniques have transformed phytochemical research, enabling both targeted and untargeted metabolomic investigations (Pandohee *et al.*, 2023).

Untargeted Metabolomics is a methodology that offers comprehensive analysis of all detectable metabolites in a sample, without of prior knowledge of their identities (Liang *et al.*, 2018). Mass Spectrometry (MS) facilitates untargeted metabolomics through extensive metabolite profiling (Cui *et al.*, 2018). High-Performance Thin Layer Chromatography (HPTLC) is a versatile chromatographic technique that can be applied to untargeted metabolomic studies, providing a comprehensive fingerprint of the phytochemical composition of plant extracts (An *et al.*, 2024; Audoin *et al.*, 2014). HPTLC's ability to visualize and compare metabolite patterns across different samples makes it a valuable tool for understanding plant metabolic diversity (Makasana *et al.*, 2016; Vázquez *et al.*, 2023). Likewise, LC-MS/MS is used for untargeted metabolomics, enabling the comprehensive profiling of plant metabolites with high sensitivity and specificity (Naz *et al.*, 2014).

In contrast, targeted metabolomics focuses on the quantification and analysis of specific known metabolites based on prior knowledge. This approach is used to validate the presence and concentration of bioactive compounds in plant extracts

(Liang *et al.*, 2018; Zhou *et al.*, 2022). Biochemical assays such as total phenolic content (TPC) and total flavonoid content (TFC) assays also fall under targeted metabolomics as they quantify specific classes of phytochemicals (Lyu *et al.*, 2022).

1.4 Application of *in silico, in vitro* and *in vivo approaches* in phytochemical based therapeutic research.

Medicinal plants possess various secondary metabolites with potential therapeutic uses, although their efficacy and safety require scientific validation (Pal & Shukla, 2003; WHO, 1991). Researchers may accelerate drug development and enhance plant-based therapies for clinical use by integrating *in silico, in vitro* and *in vivo* studies.
Computational methodologies have transformed pharmaceutical discovery, serving as vital tools at every stage of the drug development. Researchers may speed up drug development and enhance plant-based therapies for clinical use by integrating computational, laboratory, and animal model studies. These strategies help reduce costs and improve the efficiency of identifying and developing novel pharmaceuticals (Huang *et al.*, 2021; Sliwoski *et al.*, 2014). The computational techniques mainly involve molecular docking and pharmacokinetic studies. Molecular docking helps in the discovery of the interaction of phytochemicals with biological targets, such as proteins or enzymes (Pagadala *et al.*, 2017). Pharmacokinetic studies identify molecular characteristics that contribute to bioactivity. Which include, ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) analysis that predicts drug-like properties of the compound (Jia *et al.*, 2020). All these *in silico* approaches speedup the phytochemical research in perspective of therapeutic aspects.

It is crucial to conduct *in vitro* studies to validate predictions from computational analyses. These assays span from simple biochemical assays to cell line studies. Those studies give an early stage identification of the therapeutic potential of the phytochemicals at controlled environment (Allen *et al.*, 2005; Gomez-Lechon *et al.*, 2008). The *in vitro* study includes cell-based assays to evaluate cytotoxicity, antioxidant, anti-inflammatory, and antimicrobial activities (Adan *et al.*, 2016; Kotha *et al.*, 2022; Truong *et al.*, 2019). Enzyme inhibition studies are major *in vitro* analysis which assess the ability of phytochemicals to regulate disease-related enzymes (Ghanbari *et al.*, 2006; Grochowski *et al.*, 2017). Pharmaceutical companies

continue to promote *in vitro* models to reduce reliance on animal testing in drug discovery and development. However, they recognise that additional progress is required to enhance the current state of readiness of the models (Stresser *et al.*, 2024).

Even though *in silico* and *in vitro* screening are valuable tools in research *in vivo* studies still has its role due to its accurate results. Because, they can more or less replicate the complex physiological interactions that occur within a living organism. Use of animals for studies has not happened recently; if we check the history, we would understand that the initial science days were Lavoisier and Pasteur, (Oparin, 1957) who studied the origin of life using animal models. Choosing an appropriate animal model is essential, as not all models are effective due to diverse limitations. The optimal model must accurately reflect human physiology and pathology to guarantee consistent and outcomes (Mukherjee *et al.*, 2022). Murine models are important for drug research, but they need to be used in a logical and structured way, starting with toxicology (Talmadge *et al.*, 2007). Pre-clinical drug testing relies on animal models, which are useful tools before clinical studies. To ensure that they can be used safely and effectively in humans, they are needed to test pharmacokinetic parameters like drug effectiveness, safety, and toxicity (Belma *et al.*, 2019). A lot of study on crude extracts uses animals to test the effects, safety, and possible benefits of natural or plant-based compounds. These studies help scientists figure out how the extract works in a live system, such as how it is absorbed, what side effects it might have, and how well it works overall. This is significant because it gives important details before human testing on people, which guarantees that the extract is safe and useful for medical purposes.

1.5 Research problem

Ethnomedicinal plants are valuable sources of drug candidates, yet many of these plants, in the Western Ghats, remain underexplored. *Humboldtia*, a genus within the Leguminosae family, thrives in the biodiversity of the Western Ghats, Kerala, India, and holds significant ethnobotanical importance. However, many *Humboldtia* species remain understudied in terms of their biological efficacy, while some lack scientific validation for their traditional uses. Significantly, more than half of the drugs utilized worldwide in modern pharmaceuticals have their origins in natural sources.

Significantly, more than half of the drugs utilized worldwide in modern pharmaceuticals have their origins in natural sources (Sidhic *et al.*, 2023).

Humboldtia, a genus belonging to the Leguminosae family (Azani *et al.*, 2017), has become a subject of great interest in recent years due to the diverse range of phytocompounds and medicinal potential found within its various species (Surendran *et al.*, 2021). Among the four actively studied species, *H. brunonis, H. vahliana, H. unijuga, and H. laurifolia,* significant amounts of phytochemicals such as phenolics, tannins, phlobatannins, saponins, terpenoids, anthraquinones, and steroids have been identified (Kumar *et al.*, 2012; Leela & Pillai, 2005; Nair *et al.*, 2018; Samaraweera *et al.*, 1983).

These compounds have demonstrated diverse pharmacological properties such as antibacterial, anticancer, antioxidant, anti-inflammatory, anticonvulsant, and antidepressant effects. Notably the Kani tribes have traditionally used *Humboldtia unijuga* for treating various ailments (Vijayan *et al.*, 2007). Recent studies have identified the anticancer properties of Erythrodiol-3-acetate (HU-1) and 2,4-di-tert-butyl phenol (HU-2) found within the plant (Nair *et al.*, 2018).

Humboldtia sanjappae, a medium-sized tree species, has yet to be studied for its phytochemistry and pharmacology, making it a fascinating research topic to explore. Examining the phytochemistry of *H. sanjappae* is crucial for discovering its bioactive constituents, which may enhance its therapeutic qualities. Through the analysis of its chemical composition, the prospective therapeutic agents of pharmacological importance can be identified. Examining the plant's pharmacology, further support its traditional applications and aids in identifying the active chemicals responsible for its observed effects. Moreover, *H. sanjappae* is indigenous to the Western Ghats, a biodiversity hotspot that is becoming endangered by habitat destruction and climate change (Lo Seen *et al.*, 2010; Raghavan *et al.*, 2016). Recognising the medical potential of the plant not only facilitates drug discovery but also underscores the necessity for conservation initiatives to safeguard this ecologically and pharmaceutically important species.

The current study looks at the phytochemical and pharmacological possibilities of *H. sanjappae* in a broad way. To explore its bioactive molecules and uncover its

chemical diversity, both untargeted and targeted metabolomics will be employed. *In silico* ADMET analysis will assess drug-likeness and pharmacokinetic properties, while network pharmacology will help predict molecular targets and underlying mechanisms of action. *In vivo* studies will also confirm the safety and effectiveness of key bioactive chemicals, giving us a full picture of their therapeutic potential. This integrative approach bridges the gap between chemical composition and pharmacological relevance of *H. sanjappae*.

1.6 Objectives of the study

1. Phytochemical evaluation of *H. sanjappae* using various chromatographic and spectroscopic techniques.

2. Pharmacological evaluation of *H. sanjappae* using *in silico, in vitro and in vivo* models.

Chapter 2

REVIEW OF LITERATURE

2.1 Ethnobotany and its Relevance.

2.2 Bioactive Compounds or Secondary Metabolites.

2.3 Classes of Phytochemicals.

2.4 Phytopharmacology and Techniques for the Characterisation of Phytochemicals.

2.5 Phytochemicals for Mitigating Inflammation*: in silico*, *in vitro* and *in vivo* Studies.

2.6 Phytochemical and Pharmacological Studies of Genus *Humboldtia*

2.1 Ethnobotany and its relevance

Since the dawn of civilization, people have used plants for various purposes. In the health care systems of many poor and underdeveloped countries, the traditional medicine system based on herbal cures has long been important and this traditional herbal medicine system has become increasingly significant in developed nations (Ganesan & Xu, 2017). Although the practice has existed for centuries the term ethnobotany was first introduced by Harshberger in 1985 in the Philadelphia Evening Telegraph. Over the time the term ethnobotany has been interpreted in various ways, but it is now nearly commonly understood to refer to the direct link between humans and plants (Sharma & Kumar, 2011).

Throughout the course of human evolution, they have possessed specific pharmacological knowledge of the medicinal characteristics of plants. In various cultures and for thousands of years, medicinal plants have been used as traditional therapies for various human ailments. They still serve as the primary source of medication in developing nations' rural areas (Chitme *et al.*, 2004). It is estimated that traditional medicine is used to treat approximately 80% patients in these countries (Kim, 2005).

Natural substances and molecules that are biologically active have been isolated and identified throughout human history, leading to the development of novel treatments and the advancement of the medical and pharmaceutical industries (Pye *et al.*, 2017). The domains, ethnopharmacology and ethnobotany are closely related. The role of ethnobotany is to verify traditional uses of medicinal plants by creating a drug to cure patients. Hence, ethnobotanical approach is a body of work that includes contributions from pharmacology, chemistry, and botany. So major branch of ethnobotany is ethnopharmacology, which is the interdisciplinary scientific examination of the biologically active drugs that are traditionally utilised (Süntar, 2020).

Ethnobotanists scientifically explore traditional uses, cultural meanings, and potential medicinal properties. This invaluable knowledge paves the way for conserving and sustainably utilizing plant resources, while offering promising avenues for new medicine and treatment discoveries. Some example of medicinal plants and their traditional uses are given in the Table 1**.**

Table 1: Medicinal plants and their traditional uses

Sl. No.	Plant	Used for	Reference
1.	*Aloe vera*	Skin conditions, digestive issues, and wound healing	(Kumar & Yadav, 2014; Maenthaisong *et al.*, 2007)
2.	*Arnica montana*	Pain relief and wound healing	(Iannitti *et al.*, 2016)
3.	*Withania somnifera*	Used as an adaptogen and for stress relief	(Priyanka *et al.*, 2020; Singh *et al.*, 1982)
4.	*Ocimum basilicum*	Digestive issues and as an anti-inflammatory	(Ch *et al.*, 2015; Rodrigues *et al.*, 2016)
5.	*Cimicifuga racemose*	Menopausal symptoms and menstrual cramps	(Leach & Moore, 2012; Lieberman, 1998)
6.	*Calendula officinalis*	Wound healing and as an anti-inflammatory	(Arora *et al.*, 2013; Parente *et al.*, 2012)
7.	*Uncaria tomentosa*	Immune support and as an anti-inflammatory	(Aguilar *et al.*, 2002; Chauhan *et al.*, 2015)
8.	*Matricaria chamomilla*	Digestive issues and as a sleep aid	(Beniaich *et al.*, 2022; El Mihyaoui *et al.*, 2022)
9.	*Salvia hispanica*	Digestive issues and as an anti-inflammatory	(Grancieri *et al.*, 2019; Melo *et al.*, 2019)
10.	*Cinnamomum zeylanicum*	Blood sugar control and as an anti-	(Han & Parker, 2017; Imani *et al.*, 2017;

		inflammatory	Kumar & Kumari, 2019)
11.	*Syzygium aromaticum*	Toothaches and as an anti-inflammatory	(Ghamari *et al.*, 2017; Khanal *et al.*, 2021; Nikoui *et al.*, 2017)
12.	*Symphytum officinale*	Wound healing and as an anti-inflammatory	(Isabela *et al.*, 2023; Mulkijanyan *et al.*, 2009)
13.	*Taraxacum officinale*	Liver detoxification and as a diuretic	(Mahboubi & Mahboubi, 2020)
14.	*Echinacea purpurea*	Immune support and as an anti-inflammatory	(Kumar & Ramaiah, 2011; O'neill *et al.*, 2002)
15.	*Sambucus nigra*	Immune support and as an anti-inflammatory	(Santin *et al.*, 2022; Schön *et al.*, 2021; Waswa *et al.*, 2022)
16.	*Foeniculum vulgare*	Digestive issues and as a diuretic	(Badgujar *et al.*, 2014; Jadid *et al.*, 2023)
17.	A*llium sativum*	Immune support and as an anti-inflammatory	(Tudu *et al.*, 2022)
18.	*Zingiber officinale*	Digestive issues and as an anti-inflammatory	(Anosike *et al.*, 2009; Mashhadi *et al.*, 2013; Nikkhah Bodagh *et al.*, 2019)
19.	*Ginkgo biloba*	Cognitive support and as an anti-inflammatory	(Curtis-Prior *et al.*, 1999; Shahrajabian *et al.*, 2022; Singh *et al.*, 2019)
20.	*Panax ginseng*	Used as an adaptogen and for cognitive support	(Oliynyk & Oh, 2013; Provino, 2010; Smith *et al.*, 2014)
21.	*Centella asiatica*	Cognitive support and wound healing	(McDougall Jr *et al.*, 2005; Mgonja *et al.*,

			2022; Sabaragamuwa *et al.*, 2018)
22.	*Crataegus oxyacantha*	Cardiovascular support and as an anti-inflammatory	(Li & Wang, 2011; Wu *et al.*, 2020)
23.	*Cannabis sativa*	Pain relief and as an anti-inflammatory	(Kongkadee *et al.*, 2022; Vigil *et al.*, 2020)
24.	*Ocimum sanctum*	Stress relief and as an anti-inflammatory	(Güez *et al.*, 2017; Kamelnia *et al.*, 2023; Mequanint *et al.*, 2011; Singh & Agrawal, 1991)
25.	*Aesculus hippocastanum*	Circulation support and as an anti-inflammatory	(Gallelli, 2019)

2.2 Bioactive compounds or secondary metabolites

Bioactive compounds from plants are commonly called phytoconstituents, they are naturally occurring substances present in plants and enhance human health in different ways (Hasler & Blumberg, 1999). All live plant cells contain the same primary metabolites, which perform fundamental life functions such as cell division, growth, storage, respiration, and reproduction (Fernie & Pichersky, 2015). However, secondary plant metabolites, often referred to as phytoconstituents, are produced from primary metabolites under the influence of environmental stressors like as light, temperature, and other metals with the aid of several metabolic pathways (Ali, 2014). Secondary metabolite development is highly particular for the plant family (Bhatla *et al.*, 2018). Plants from different families produce a wide range of secondary metabolites with a variety of medicinal uses (Alamgir, 2018; Kandar, 2021). Secondary metabolites are not directly involved in the plant's growth and development, but instead serve functions such as defence against predators or attraction of pollinators (Divekar *et al.*, 2022; Harborne, 2007; Stevenson *et al.*, 2017). Generally speaking, phytochemicals are plant compounds that shield plant cells from possible hazards such as pollution, stress, dehydration, harmful UV rays, and insects or microorganisms attack (Gibson *et al.*, 1998).

Secondary metabolites are defined as organic substances that did not directly contribute to an organism's healthy function, growth, or reproduction (Zuckerkandl & Pauling, 1965). Although tens of thousands of secondary metabolites have been chemically identified, it is still unclear what are the biological functions of the majority of these compounds (Croteau *et al.*, 2000; Gershenzon & Dudareva, 2007). Secondary metabolites, which are prevalent in medicinal plants, are principally responsible for bioactivity. As a result, medicinal plants are a plentiful source of bioactive phytochemicals with a variety of bioactivities and the potential to treat a wide range of diseases (Aye *et al.*, 2019; Jain *et al.*, 2019; Kabera *et al.*, 2014).

Secondary metabolites are derived from primary metabolites and are synthesized through various modifications such as methylation, glycosylation, and hydroxylation. They possess a more intricate structural composition and side chains compared to primary metabolites (Figueiredo *et al.*, 2008). Based on the biosynthetic pathway,

plant metabolites were classified into three major categories (Figure 1): (i) phenolic compounds (consisting of simple sugars and benzene rings), (ii) terpenes and steroids (composed primarily of carbon and hydrogen), and (iii) nitrogen-containing compounds (Saxena *et al.*, 2013; Taiz *et al.*, 2015).

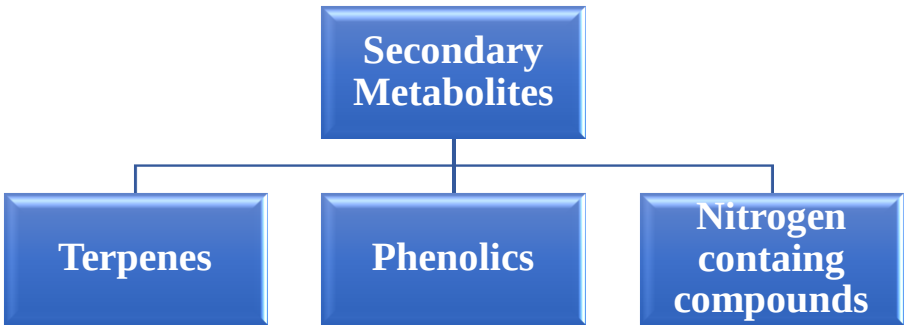

Figure 1: Major classes of phytochemicals (Taiz *et al.*, 2015)

2.2.1 Terpenes

Terpenes are the largest class of secondary metabolites. Tea, thyme, cannabis, Spanish sage, and citrus fruits such as lemon, orange, and mandarin are among the common plant sources of terpenes (Cox-Georgian *et al.*, 2019). Most of the terpene compounds are insoluble in water (Taiz *et al.*, 2015). These compounds are synthesized from the five-carbon (C_5) precursor units isoprene units (Nagel *et al.*, 2019). Hence, terpenes are classified by the number of C_5 units they contain. Table 2 represents different classes of terpenes and their role in healthcare.

Like many other natural chemicals, isoprenoids exhibit biological properties that have been used to treat and prevent human diseases (Wang *et al.*, 2005). Many terpenes have been shown to have anti-inflammatory properties, which could help reduce pain and swelling in the body (Guimarães *et al.*, 2014). Curcumin is one of the terpenes that were widely utilized in natural folk medicine due to its anti-inflammatory (Basnet & Skalko-Basnet, 2011; Zheng *et al.*, 2020) antioxidant (Weber *et al.*, 2005) and anticancer properties (Vallianou *et al.*, 2015). Other anti-inflammatory terpenes include beta-caryophyllene, myrcene, beta-pinene and limonene (Araruna *et al.*, 2020; Rufino *et al.*, 2015; Sain *et al.*, 2014; Santos *et al.*, 2022). According to Rufino *et al.* (2015), myrcene and limonene can stop human chondrocytes from producing nitric

oxide when IL-1 is present. Some research suggests that terpenes such as beta-caryophyllene has anti-cancer properties.

Table 2: Different classes of terpenes and their role in healthcare

Number of C_5 units	Number of Carbons	Classes of terpenes	Example	Effects	References
2	10	Monoterpenes	Pinene	Anti-inflammatory	(Bae *et al.*, 2012)
3	15	Sesquiterpenes	Artimisine	Anti-malarial	(Guo, 2016)
4	20	Diterpenes	Jatrophanes	anti-cancer	(Pešić *et al.*, 2011)
5	25	Sesterpenes	Ophiobolin	Induce Apoptosis	(Yang *et al.*, 2012)
6	30	Triterpenes	Lupeol	Anti-cancer, Anti-inflamamtory	(Saleem, 2009)
8	40	Tetraterpenes	Carotenoids	Anti-inflammatory, Prevent alzheimer's	(Ciccone *et al.*, 2013; Kabir *et al.*, 2022)
>8	>40	Polyterpenes	Rubber	Cell cycle arrest and trigger apoptosis	(Furuya *et al.*, 2017)

Sain *et al.* (2014) reported that, the process of apoptosis in lymphoma and neuroblastoma cells was triggered by Beta caryophyllene through the modulation of 15-LOX (an upstream target) which resulted in the down-regulation of genes that prevent apoptosis and up-regulation of genes that promote apoptosis. Some terpenes have been shown to have anxiolytic effects, meaning they may help reduce anxiety

and stress levels (Jiménez-Ferrer *et al.*, 2017). Examples of anxiolytic terpenes include linalool (Souto-Maior *et al.*, 2011) and limonene (de Almeida *et al.*, 2014).

There are many medical applications for terpenes, but antiplasmodial activity stands out because it works in a way that is similar to that of the widely used antimalarial medication chloroquine (Cox-Georgian *et al.*, 2019). Fever and malaria have long been treated with the herb *Artemisia annua* in Chinese traditional medicine. Chinese scientists were capable of extracting the active component from the leafy parts of *Artemisia annua*. It has been demonstrated that the substance, known as qinghaosu (QHS) or artemisinin, may have the ability to alleviate the symptoms of malaria (Li *et al.*, 2005; White, 2008). Artemisinin-based combination therapies are now considered the first-line treatment for *Plasmodium falciparum* malaria, as recommended by the World Health Organization. The discovery of artemisinin dates back to 1971 when a Chinese medical scientist named Youyou Tu identified the antimalarial properties of qinghaosu derived from the traditional Chinese qinghao plant, for which she was awarded the Nobel Prize in 2015 (Krungkrai & Krungkrai, 2016). Similary, Paclitaxel is one of the most successful terpenes available in the market today (Efferth *et al.*, 2008). Taxol, which is a natural product obtained from the bark of the Pacific Yew (*Taxus brevifolia*) tree, is a terpenoid. This is an anti-cancer medication that enhances polymerization and stabilization of tubulin to microtubules and disrupts the mitotic spindle. Clinical trials have demonstrated that taxol is efficacious in treating patients with refractory ovarian cancer, breast cancer, malignant melanoma, and possibly other solid tumors (Slichenmyer & Von Hoff, 1991).

2.2.2 Phenolics

Phenolics are a broad class of natural compounds that are found in a variety of plants, including fruits, vegetables, grains, herbs, and spices (Asif, 2015). They are characterized by their aromatic ring structure, which is composed of one or more hydroxyl (OH) groups attached to the ring (Lin & Xing, 2008). Phenolics play important roles in plant growth and development (Wagay *et al.*, 2020), as well as in defense against environmental stressors such as UV radiation, pests, and pathogens (Kumar *et al.*, 2020; Tuladhar *et al.*, 2021).

Phenolics are classified into several subclasses based on their chemical structure, including flavonoids, phenolic acids, stilbenes, lignans, and coumarins (Vuolo *et al.*, 2019). Flavonoids are the most abundant and diverse subclass of phenolics, and they include compounds such as quercetin, kaempferol, and catechins. Phenolic acids include compounds such as ferulic acid, gallic acid, and caffeic acid (García-Mediavilla *et al.*, 2007; Latos-Brozio & Masek, 2019; Shen *et al.*, 2022).

Phenolics have been found to possess a wide range of biological activities, including antioxidant (Zeb & Zeb, 2021), anti-inflammatory (García-Mediavilla *et al.*, 2007), anti-carcinogenic, cardioprotective, and neuroprotective properties (Ozcan *et al.*, 2014). They are thought to exert these effects by scavenging free radicals and reactive oxygen species (Mathew *et al.*, 2015), modulating gene expression and signal transduction pathways, and regulating enzymatic activities (Costa *et al.*, 2012; Ferhi *et al.*, 2019).

For example, resveratrol, a phenolic compound has been shown to have cardioprotective effects by reducing oxidative stress and inflammation, as well as improving blood lipid levels (Bradamante *et al.*, 2004; Cho *et al.*, 2017). Another phenolic compound, curcumin, found in turmeric, has been shown to have anti-inflammatory and anti-cancer properties, as well as potential benefits for brain health (Bhat *et al.*, 2019; Boroumand *et al.*, 2018) .

Overall, phenolics are a diverse and fascinating class of compounds with a wide range of biological activities and potential health benefits. As research in this area continues to grow, it is likely that we will discover even more interesting and useful applications for these compounds in the future.

2.2.3 Nitrogen containing compounds

Substances found in plants that have nitrogen atoms in their chemical structure are known as nitrogen-containing secondary metabolites. These substances function as pigments, flavouring agents, signalling molecules, and defence against pests, diseases, and environmental stresses in plants, among other things (Singh, 2018). There are several kinds of phytochemicals that contain nitrogen, including as alkaloids, glucosinolates, cyanogenic glycosides, and non-protein amino acids. They can

sometimes be harmful to humans and also exhibit a surprising diversity of pharmacological activity, often in spectacular ways (Crozier *et al.*, 2006; Souto *et al.*, 2011).

Alkaloids are a broad collection of chemical compounds found in nature, primarily composed of basic nitrogen atoms. Alkaloids frequently induce important physiological effects on humans and other animals, and many are utilised in medicine, either as pharmaceuticals or as the foundation for drug creation (Kukula-Koch & Widelski, 2017; Manske & Holmes, 2014; Pelletier, 1983). Common alkaloids are caffeine, nicotine, morphine, and quinine. They can have many impacts on the body, such as activating the central nervous system, acting as analgesics, or even acting as toxins in high concentrations. Plants with alkaloids have been utilised for therapeutic and recreational reasons for ages. The research on alkaloids remains significant in areas including pharmacology, medicine, and botany (Ashihara *et al.*, 2008; del Rayo Camacho *et al.*, 2000; Gorrod & Wahren, 1993; Stork, 1960)

Amygdalin, the initial cyanogenic glycoside discovered in a plant, was extracted from bitter almonds in 1830 (Robiquet, 1830). Over 2500 plant species are currently identified as containing cyanogenic glycosides (Vetter, 2000). Amygdalin is associated with many pharmacological actions (He *et al.*, 2020). Like cyanogenic glycosides, Glucosinolates - distinct categories of nitrogen-containing phytochemicals present in different plants. They possess a wide range of pharmacological properties that could have impact for human health (Miękus *et al.*, 2020; Traka, 2016).

Non-protein amino acids (NPAAs) as it indicates, it will not incorporate into the protein. These amino acids are responsible for plant defence. Certain plant families, such as legumes and grasses, have elevated quantities of non-protein amino acids, which are linked to insect resistance (Huang *et al.*, 2011; Seifikalhor *et al.*, 2019). These unique molecules find their way into commonly consumed foods like alfalfa (containing canavanine) and lentils (with homoarginine). Interestingly, some NPAAs even sneak in from unintentionally harvested wild plants alongside crops. However, not all NPAAs are created equal. While some are harmless, others can pose health risks. For instance, certain Lathyrus species harbor neurotoxic oxalyl-amino acids. These toxins can potentially travel up the food chain through animal intermediates,

raising concerns. Furthermore, the growing interest in herbal medicines in the Western world introduces another avenue for exposure to NPAAs. Careful research and understanding of these compounds are crucial to ensure safe consumption of both traditional and modern plant-based products (Nunn *et al.*, 2010).

2.3 Phytopharmacology and techniques for the characterisation of phytochemicals

Phytopharmacology, is a branch of pharmacology that deals with the study of the active compounds present in plants and their effects on living organisms. Medicinal plants have been used for centuries in traditional medicine systems across the world. As we discussed above, they contain a diverse range of chemical compounds, such as alkaloids, flavonoids, terpenoids, and phenolic compounds, which have therapeutic properties and can be utilized for the treatment of various diseases and health conditions (Ahmed *et al.*, 2024).

Medicinal plants are rich sources of bioactive compounds. These compounds have specific physiological effects on the human body (Altemimi *et al.*, 2017). For instance, alkaloids like morphine and quinine, found in opium poppy and cinchona tree respectively, have potent analgesic and anti-malarial properties (Achan *et al.*, 2011; Guo *et al.*, 2018). Flavonoids, present in various fruits and vegetables, exhibit antioxidant and anti-inflammatory activities (Maleki *et al.*, 2019; Pan *et al.*, 2010; Wang *et al.*, 2019). Terpenoids, found in essential oils of many plants, have antimicrobial and antiviral properties (Astani *et al.*, 2010; Guimarães *et al.*, 2019).

Chromatographic techniques are essential instruments for the separation, purification, and quantification of these compounds (Bajpai *et al.*, 2016). To maintain consistency and quality in biological tests, extracts can be standardized using High-Performance Liquid Chromatography (HPLC). This method identifies and measures key marker compounds and uses chemical fingerprints to ensure reliable analysis of herbal products (Lazarowych & Pekos, 1998).

Gas chromatography coupled with mass spectrometry (GC-MS) is widely used for analysing volatile constituents (Ranjan *et al.*, 2023), including essential oils (Arora & Pandey-Rai, 2014; Pérez G *et al.*, 2011). Phytochemical profiling and fingerprinting

are also facilitated by the use of other chromatographic technique, High-Performance Thin Layer Chromatography (HPTLC), which simplify the screening of numerous plant samples in pharmacological trials (Gökbulut, 2021; Jain *et al.*, 2014).

Spectroscopic techniques facilitate the structural elucidation and characterisation of isolated compounds, thereby complementing chromatography. Ultraviolet-Visible (UV-Vis) spectroscopy is frequently employed to ascertain the total phenolic and flavonoid content of plant extracts, which can be attributed to their antioxidant potential (Ghasemzadeh *et al.*, 2011; Urbano *et al.*, 2006). The chemical constitution of active compounds is confirmed through, Mass spectrometry (MS), particularly when combined with liquid chromatography (LC-MS/MS), is employed for the precise determination of mass and fragmentation analysis. LC-MS/MS data can be employed to monitor the bioavailability and metabolism of phytochemicals in animal plasma and tissues, thereby offering valuable insights into their therapeutic potential and systemic behaviour (J. Wang *et al.*, 2024). LC-MS/MS is a highly sensitive and specific analytical technique that is essential for phytochemical-based pharmacological research. This method is essential for the qualitative and quantitative analysis of complex plant extracts, as it combines the physical separation capabilities of liquid chromatography (LC) with the potent mass analysis capability of tandem mass spectrometry (MS/MS) (Arlee *et al.*, 2024; Mu *et al.*, 2025).

2.4 Phytochemicals for mitigating Inflammation: *in silico, in vitro* and *in vivo* studies

Inflammation is a critical immune system response; while it was previously believed that inflammation was linked to immune system diseases, it is now recognised that inflammation is indicative of several kinds of diseases (Ahmed, 2011; Schmid-Schönbein, 2006).

According to researches, persistent, unresolved inflammation is associated with a variety of health complications and may even contribute to the development of cancer (Allavena *et al.*, 2008). Although there are numerous anti-inflammatory drugs, their long-term use is frequently restricted by substantial side effects. Therefore, there is an increasing interest in plant-based remedies, which are becoming more effective and safer alternatives for managing various forms of inflammation (Gooch *et al.*, 2007;

Nikolaou *et al.*, 2013). However, the conversion of these naturally derived compounds from promising laboratory discoveries to clinically approved treatments necessitates a rigorous and multi-step validation process. New researches are currently being conducted to establish the efficacy and safety of phytochemicals through detailed phytochemical analysis, *in silico, in vitro* and *in vivo*.

In silico approaches play a pivotal role in the early stages of phytochemical research by enabling the prediction of interactions between plant-derived compounds and specific biological targets. These computational techniques significantly reduce the time and cost associated with drug discovery and help prioritize the most promising candidates for further investigation (Ekins *et al.*, 2007; Ojuka *et al.*, 2023). Computational or *in silico* methods are valuable tools. They allow us to simulate almost every step of drug discovery. With their help, we can make faster and smarter decisions (Swaan & Ekins, 2005). Scientists usually deal with a vast amount of information in phytochemical research, which might lead to drug discovery. To speed up the process, they need shortcuts or guiding principles. These help them find out the most promising targets and molecules. One such method is network pharmacology analysis (Fatokun *et al.*, 2024; Noor *et al.*, 2022).

Network pharmacology is a modern approach used to identify active compounds and potential molecular targets from a wide range of herbal formulations or individual herbs (Chandran *et al.*, 2016; Shao & Zhang, 2013). For example, Zhishi and Zhiqiao are commonly used in traditional Chinese medicine (TCM) to treat gastrointestinal and inflammatory disorders. Despite widespread use, the mechanisms underlying their anti-inflammatory effects were not fully understood. Network pharmacology analysis, shown that Zhishi and Zhiqiao reduce inflammation by targeting multiple pathways, blocking inflammatory signals, restoring metabolic balance, and delivering active compound (Zhao *et al.*, 2018). The bioactive constituents of *Lantana camara* L., a plant that has been traditionally used for its medicinal properties, were comprehensively profiled in the study conducted by El-Banna *et al.* (2022) using UPLC-MS/MS-based metabolomics. In order to clarify the anti-inflammatory effects, the authors implemented molecular docking and network pharmacology analyses. This method has identified critical inflammation-related targets, such as cytokines, enzymes, and signalling proteins, and has mapped the interactions between *L.*

camara's metabolites and these targets. The binding affinity of bioactive compounds to inflammatory mediators, such as COX-2, TNF-α, or NF-κB pathway proteins, was further validated through molecular docking, which confirms their mechanistic function in inflammation modulation. Similarly, Khairy *et al.* (2023) investigated immunomodulatory plants by first screening their extracts *in vitro* for anti-inflammatory activity, followed by computational analyses to identify key bioactive compounds and their molecular targets, such as cytokines (TNF-α, IL-6) and signaling pathways (NF-κB, MAPK).

The multi-target mechanisms of plant-derived compounds were uncovered by network pharmacology, which was helpful in phytochemical research. This was demonstrated in studies on berberine and *Salvia miltiorrhiza* (Cui *et al.,* 2020) . It contributed to the prediction and validation of the interaction between berberine and critical inflammatory targets and signalling pathways, such as NF-κB, MAPK, and STAT1, thereby demonstrating its potential to treat autoimmune and inflammatory disorders. In the same a manner, network pharmacology has identified critical targets, including JUN, IL-6, and TNF for Tanshinone I and STAT3 and AKT1 for Cryptotanshinone from *Salvia miltiorrhiza*. This information provides a deeper understanding of their roles in modulating inflammation via TLR and JAK-STAT pathways (Cui *et al.*, 2020; K. Wang *et al.*, 2024).

Network pharmacology and molecular docking are two of the best *in silico* methods used in phytochemical study. They work together to figure out how bioactive compounds from plants work. Recent studies have demonstrated the power of combining network pharmacology and molecular docking to elucidate the mechanisms of phytochemicals. For instance, Lu *et al.* (2025) employed network pharmacology to predict that tectoridin, a bioactive compound from *Pueraria lobata*, could mitigate ischemic stroke by modulating oxidative stress, inflammation, and autophagy pathways. Molecular docking further revealed strong binding affinities between tectoridin and key targets in the TLR4/MYD88/NF-κB and PI3K/Akt/mTOR pathways, findings later validated by *in vitro* and *in vivo* studies showing reduced infarct volume and improved neurological function. Similarly, research on *Anisodus tanguticus* utilized network pharmacology to identify anti-inflammatory and analgesic mechanisms, including EGFR and IL-17 pathway inhibition. Molecular docking

confirmed interactions between its active compounds (e.g., fabiatrin and scopolin) and target proteins, with molecular dynamics simulations reinforcing the stability of these bindings (Tan *et al.*, 2025). Another study on *Lycium barbarum* highlighted its potential in combating inflammation and bone loss, with docking analyses proving strong binding to pro-inflammatory mediators like IL-1β, IL-6, and TNF-α (Ma *et al.*, 2025). Together, these examples illustrate how integrating network pharmacology with molecular docking provides a robust framework for predicting, validating, and optimizing the therapeutic potential of phytochemicals.

Although *in silico* studies offer a valuable starting point, *in vitro* and cell line experiments are essential for the verification of bioactivity. The discovery of phytochemical-based therapeutics is speeded up by the integration of computational and experimental approaches; however, translational challenges remain. For instance, in the case of *Ailanthus excelsa*, the chloroform fraction rich in caftaric acid and other phenolics not only showed strong binding affinities in docking studies but also demonstrated significant cytotoxic and anti-inflammatory activity in cell-based assays and carrageenan-induced paw edema models (Sapkal *et al.*, 2023). Similarly, non-prenylated stilbenoids confirmed their predicted COX-2 and 5-LOX inhibition by attenuating LPS-induced inflammatory responses and reducing TNF-α expression in THP-1 cells. *In vitro* experiments with quercetin, predicted through network pharmacology and docking to target PI3K/Akt pathway, validated its anti-inflammatory activity by reducing cytokine production and inhibiting Akt phosphorylation in RAW 264.7 macrophages (Leláková *et al.*, 2019). Comparable results were observed for quercetin from *Bacopa monnieri*, where its inhibitory effect on iNOS and COX-2 expression in LPS-stimulated macrophages confirmed its docking-based target predictions (Jeyasri *et al.*, 2022).

While *in silico* and *in vitro* studies offer mechanistic insights into the pharmacological potential of phytochemicals, *in vivo* studies in murine models are essential for validating their therapeutic efficacy, bioavailability, and safety in a whole-organism context. The translational potential of plant-derived compounds has been further bolstered by the recent demonstration of their anticancer, anti-inflammatory, and immunomodulatory effects in mouse models. For example, the ethanolic extract of *Melilotus indicus* demonstrated substantial anticancer properties in an induced skin

cancer model. This resulted in a significant improvement in skin architecture and the downregulation of carcinogenic markers, including CEA and TNF-α (Bashir *et al.*, 2022). It is important to note that the combination of coumarin and quercetin exhibited superior anticancer activity in comparison to individual regimens, indicating a synergistic interaction. In the same vein, *Spondias mangifera* fruit extract exhibited potent anti-arthritic effects in a Complete Freund's Adjuvant (CFA)-induced arthritis model, substantially reducing paw oedema, TNF-α, and IL-6 levels (Khalid *et al.*, 2021). These results underscore the significance of phytochemicals in the regulation of inflammatory pathways, which is supported by *in silico* docking results that identified critical interactions with TNF-α and COX-2.

Additionally, coumarin derivatives, including braylin, demonstrated dose-dependent anti-inflammatory and antinociceptive effects in CFA-induced paw inflammation. These derivatives suppressed pro-inflammatory cytokines (IL-1β, TNF-α, IL-6) and increased anti-inflammatory TGF-β (Espirito-Santo *et al.*, 2017). The study proposed mechanisms that are mediated by glucocorticoid receptors, which were further corroborated by *in silico* docking. Furthermore, the traditional medicinal use of *Plectranthus rugosus* extracts and oleanolic acid was further supported by their significant inhibition of pro-inflammatory cytokines (IL-6, TNF-α) and nitric oxide (NO) in murine models, without any cytotoxicity (Mir *et al.*, 2024).

2.5 Phytochemical and pharmacological studies of genus *Humboldtia*

The genus *Humboldtia* (Leguminosae) comprises nine identified species, predominantly endemic to the Western Ghats of India (Surendran *et al.*, 2021). These plants possess significant value in traditional medicine systems, with indigenous communities employing various parts for therapeutic applications. *Humboldtia* has been utilised in traditional medicine, with extracts from its bark and leaves being used to treat various health issues, including gastrointestinal ulcers, neurological disorders such as epilepsy, gynaecological problems like menstrual irregularities, and urogenital ailments. It also helps in manging wounds, stimulate gastric function and soothe irritated mucous membranes of urinary tract (Asirvatham & Yesudanam, 2017; Nagabhushan & Raveesha, 2015; Trivedi, 2009). Ethnobotanical studies conducted in the Shiradi and Bisle Ghats regions of Karnataka reveal that local populations utilize

the leaves and bark of *H. brunonis* for alleviating symptoms of arthritis and diabetes (Kumar *et al.*, 2012).

Investigators have found that the bark of *H. vahliana* can help with a number of health problems, such as stomach ulcers, leprosy, liver problems, and seizure problems (Leela & Pillai, 2005). Kani people who live in the Agasthyamala biosphere have used *H. unijuga*, which they call "*palakan*," for a long time to treat headaches, Varicella infections, and snakebites (Nair *et al.*, 2018; Vijayan *et al.*, 2007). New research has shown that *H. vahliana* can be used for more therapeutic purposes, especially in managing disorders that cause seizures (Asirvatham & Yesudanam, 2018).

The Table 3 lists the main phytochemicals found in four species of the genus *Humboldtia* and shows how they might be useful in medicine. Researchers have looked into *H. brunonis* a lot and found several different secondary metabolites in it. These include steroids, flavonoids, alkaloids, cardiac glycosides, phenols, terpenoids, anthraquinones, flavonoids, phlobatannins, and terpenoids (Kumar *et al.*, 2014; Kumar *et al.*, 2012; Nagabhushan & Raveesha, 2015; Pavithra *et al.*, 2013; Sheik & Chandrashekar, 2014; Sindhu *et al.*, 2014). These chemicals are known to have healing properties, like being antioxidants, killing microbes, and reducing inflammation. Two chemicals, 2,4-di-tert-butyl phenol and erythrodiol-3-acetate, were found in *H. unijuga*. These chemicals have been linked to anti-inflammatory and anticancer properties (Nair *et al.*, 2018). Researchers discovered that *H. laurifolia* has a lot of unique flavonoids and triterpenoids, such as (2R,3R)-3,5,7,3',5'-pentahydroxyflavone, 3α-methoxyfriedelan, and other bioactive compounds like apigenin and lupeol, which suggests that it has strong antioxidant and cytotoxic potential (Samaraweera *et al.*, 1983). Furthermore, *H. vahliana* showed a wide range of phytochemicals, including sitosterol-3-O-β-D-glucopyranoside, β-sitosterol, apigenin, and others such as alkaloids, glycosides, tannins, flavonoids, phenolics, and carbohydrates. This supports its traditional use in treating neurological and inflammatory disorders (Asirvatham & Yesudanam, 2017; Asirvatham & Yesudanam, 2018; Leela & Pillai, 2005). Taking all of this information together shows a strong basis for further drug research within the genus.

Table 3: Phytochemical Constituents Identified in Different *Humboldtia* Species

Species	Identified Phytochemicals	References
H. brunonis	Tannins, phlobatannins, saponins, terpenoids, anthraquinones, steroids, flavonoids, glycosides, alkaloids, cardiac glycosides, phenols	(Kumar *et al.*, 2014; Kumar *et al.*, 2012; Nagabhushan & Raveesha, 2015; Pavithra *et al.*, 2013; Sheik & Chandrashekar, 2014; Sindhu *et al.*, 2014)
H. unijuga	2,4-di-tert-butyl phenol, erythrodiol-3-acetate	(Nair *et al.*, 2018)
H. laurifolia	(2R,3R)-3,5,7,3',5'-pentahydroxyflavone, 3α-methoxyfriedelan, o-acetyloleanolic aldehyde, apigenin, sitosterol, lupeol, sitosteryl ester	(Samaraweera *et al.*, 1983)
H. vahliana	Sitosterol-3-O-β-D-glucopyranoside, β-sitosterol, apigenin, 3α-methoxyfriedelan, alkaloids, cardiac glycosides, flavonoids, tannins, phenolics, carbohydrates	(Asirvatham & Yesudanam, 2017; Asirvatham & Yesudanam, 2018; Leela & Pillai, 2005)

Though *Humboldtia* is a taxonomic diverse genus with nine recognised species, only four have been studied for their phytochemical content: *H. brunonis, H. unijuga, H. laurifolia, and H. vahliana.* On the other hand, species like *H. bourdilloni, H. decurrens, H. sanjappae, H. trijuga,* and *H. ponmudiana* have not been studied much in terms of the chemicals that constitute them. This big research lacuna shows how important it is to test all plant members of the genus for phytochemicals to fully understand their medical and pharmaceutical potential.

The genus *Humboldtia*, abundant in various phytochemicals including flavonoids, terpenoids, and phenolic compounds, possesses considerable yet underutilised potential for anti-inflammatory drug development. Although initial phytochemical analyses indicate potential therapeutic benefits, extensive pharmacological validation is still lacking. Significantly, *in silico* studies are absent within the genus, and *in vitro* research has been performed solely on a limited number of species mainly *H.*

brunonis and *H. vahliana* which exhibit confirmed antioxidant and antimicrobial properties. Remarkably, there is an almost complete absence of *in vivo* studies, with *H. unijuga* being the only exception in which anticancer activity was assessed. Considering the recognised role of inflammation in chronic diseases, a systematic investigation of *Humboldtia*'s anti-inflammatory properties using integrated *in silico*, *in vitro*, and *in vivo* methodologies is imperative.

H. sanjappae represents a significant research gap among the studied species. Despite its phylogenetic closeness to pharmacologically active relatives, this endemic species lacks fundamental phytochemical characterisation and bioactivity testing. The minimal existing research has concentrated exclusively on taxonomy, neglecting its therapeutic potential altogether. This oversight is especially troubling due to the species' limited distribution and conservation status, which increases the likelihood of losing potentially valuable genetic material before its medicinal properties can be recorded. This study seeks to address these significant gaps through an extensive phytochemical screening and pharmacological assessment of *H. sanjappae*. The research aims to identify novel compounds or therapeutic leads that may aid in drug discovery or the conservation-driven valorisation of native flora by integrating contemporary analytical methods with *in vitro* and *in vivo* bioassays.

Chapter 3

MATERIALS AND METHODS

3.1	Plant collection, Authentication and Extraction
3.2	Extract Yield
3.3	Preliminary Phytochemical Analysis
3.4	High Performance Thin Layer Chromatography
3.5	Quantitative Phytochemical Analysis
3.6	Antioxidant Activity
3.7	Liquid Chromatography–Mass Spectrometry (LC-MS/MS) Profiling
3.8	Drug Likeness and *In-silico* ADMET Prediction
3.9	Acute Oral Toxicity Evaluation Using Animal Models
3.10	Potential Target Screening of Active Compounds and Inflammation
3.11	Effect of HBA on Lipopolysaccharide-Induced Anti-Inflammatory Activity in Macrophages
3.12	*In vivo* Evaluation of Anti-inflammatory Potential Using Animal Model
3.13	Molecular Docking and MM-GBSA Analysis of Identified Phytochemicals from HBA Against Inflammatory Targets
3.14	Statistical Analysis

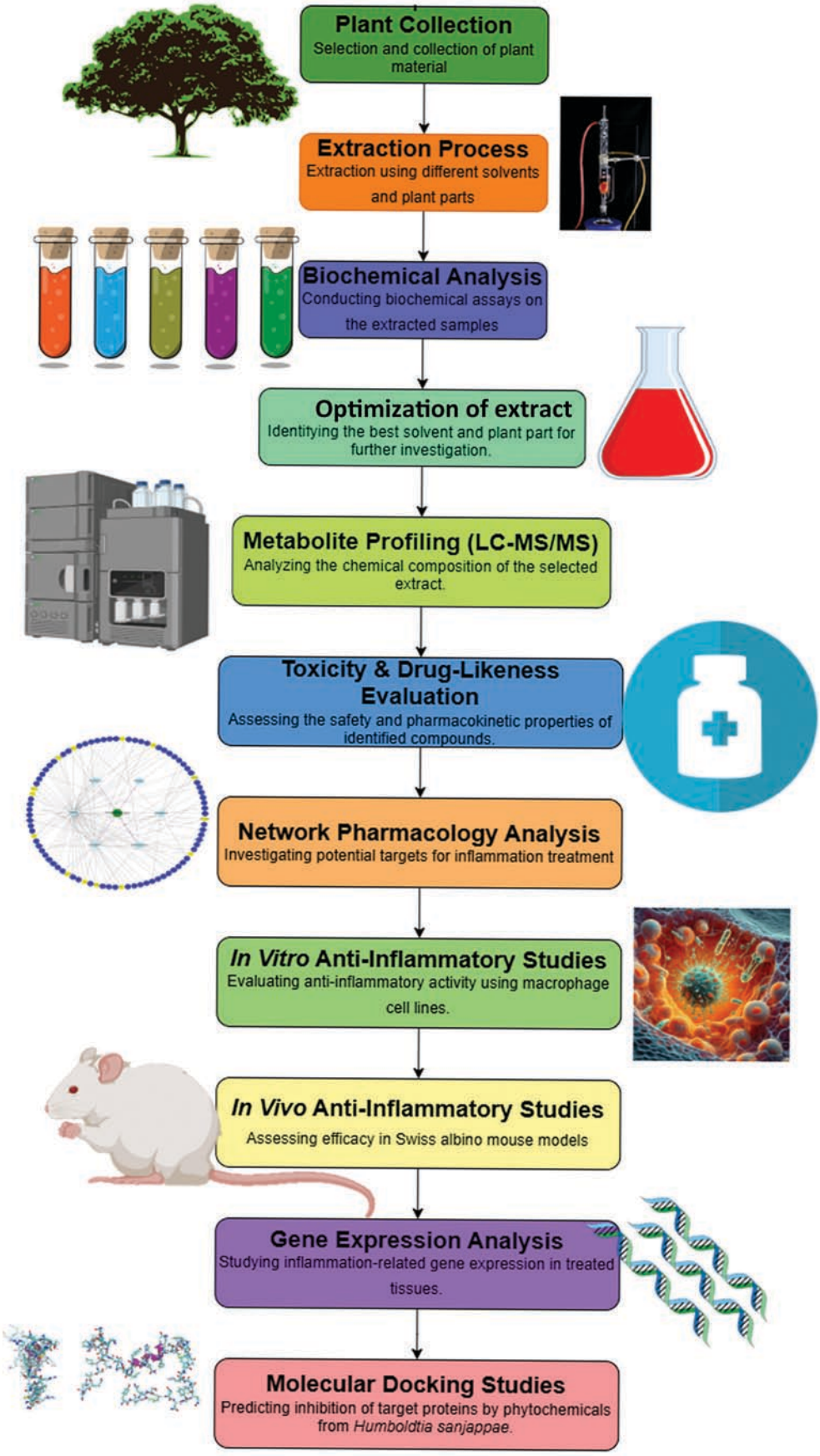

Figure 2: Methodological Flowchart

The study started by collecting leaves and bark from *Humboldtia sanjappae* tree. Then, ethanol, water, and their combinations were used to extract the samples. We used biochemical tests, LC-MS/MS, and *in silico* analyses to find the best extract and check its safety and pharmacokinetic profiles. Studies *in vitro*, *in vivo*, gene expression, and molecular docking confirmed the anti-inflammatory potential. The full workflow is shown in Figure 2.

3.1 Plant collection, Authentication and Extraction

Plant samples of *H. sanjappae* (Figure 3) were collected on 12 December 2022 from Ernakulam District, Western Ghats of Kerala (10.04829˚ N, 76.8399˚ E). Authenticated specimens were deposited in the 'DEV' herbarium, with accession number 9208.The mature leaves and the bark of the plants collected were carefully cleaned of all kinds of dust via washing. These materials were dried under shade for 21 days and powdered using a mixer grinder; the powder was extracted with different solvents, such as water, ethanol, and aqueous ethanol, in various ratios. Samples derived from *H. sanjappae* bark are denoted as "HB," followed by an alphabet (A to F) indicating the type and ratio of solvents used for extraction. Similarly, samples from *H. sanjappae* leaves are labelled as "HL," with the subsequent alphabet (A to F) representing the extraction solvent (Figure 4). 10 g of each material was subjected to 6 h of extraction.

3.2 Extract yield

All the extracts were evaporated to dryness using a rotary evaporator, and extract yield was calculated for the same.

$$\%\ of\ extract\ yield = \frac{Weight\ of\ the\ extract}{Weight\ of\ the\ dried\ plant\ material\ used\ for\ extarction} \times 100$$

3.3 Preliminary phytochemical analysis

The identification of phytoconstituents in plant material aids in forecasting the possible pharmacological activity of the plant (Emran *et al.*, 2015). Twelve different extracts were subjected to preliminary screening of phytochemicals. The standard analysis protocols were adapted from Harborne and Harborne (1973) along with several modified methods from various publications (Table 4).

Table 4: Procedure for preliminary phytochemical analysis

Alkaloids		
Mayer's test	1 mL of extract was supplied with two drops of Myers's reagent. Appearance of white creamy precipitate denotes the presence of alkaloids.	(Jha *et al.*, 2012)
Wagner's test	Along the side of the test tubes small quantity of Wagner's reagent was added. Presence of alkaloids can be confirmed by reddish- brown precipitate.	(Nandedkar & Mulani, 2016)
Dragendroff's test	1 mL of extract was mixed with Dargendroff's reagent. the heating of the mixture will indicate the formation of reddish orange colour precipitate	(Nobakht *et al.*, 2010)
Hager's test	2 mL of Hager's reagent mixed with extract. Creamy white precipitate indicates the presence of alkaloids.	(Prasad & Sharma, 2019)
Carbohydrate		
Molisch's test	2 mL of extract was mixed with few drops of α-naphthol reagent. The mixture was shaken well and to that concentrated Sulphuric acid was added slowly along the sides of test tube.	(Sarkar *et al.*, 2024)
Fehling's tets	The extract was boiled with 1 mL of Fehling's solution. Formation of a brick red precipitate indicates the presence of carbohydrate.	
Bendict's test	Equal quantity of extract and Benedict's reagent was mixed and the same was heated on a boiling water bath. The formation of characteristic coloured precipitate indicated the presence of sugar.	

Proteins		
Biuret test	1 drop of 2 % Copper sulphate solution was mixed with extract. To that mixture 1 mL of absolute alcohol and excess potassium hydroxide pellets were added. Pink colour ethanolic layer indicated the presence of protein.	(Lanjwani *et al.*, 2015)
Ninhydrin test	2 mL of extract was treated with two drops of ninhydrin solution. The formation of purple colour indicated the presence of amino acids.	
Phenolics and Tannins		
Ferric Chloride	1 mL of extract was dissolved in 5 mL of distilled water and 5% ferric chloride solution was added. Formation of a dark green colour indicated the presence of phenolic/tannin compounds.	(Panchal & Parvez, 2019)
Lead Acetate	1 mL of extract was dissolved in 1 mL of distilled water and 3 mL of 10 % lead acetate solution was added. Presence of phenolic/tannin was indicated by the formation of a white precipitate.	
Flavonoids		
Sodium hydroxide test	Subsequently, 10% aqueous sodium hydroxide was added to 2 mL of the ethanolic extract, resulting in a yellow colouration. The transition from yellow to colourless upon the addition of weak hydrochloric acid indicated the existence of flavonoids.	(Krvavych *et al.*, 2014)
Sulphuric acid test	1 mL of concentrated sulphuric acid was added to the extract. A yellow colouration that vanishes when standing shows the presence of flavonoids.	(Ayoola *et al.*, 2008)
Schinoda test	Extract was mixed with 5 mL of alcohol and a small piece of magnesium ribbon was added to it. Then, concentrated hydrochloric acid was added as drops. Pink to crimson colour indicated the presence of flavonoid glucosides.	(Ibrahim & Ghareeb, 2020)

Sterols		
Libermann's	When the extract solution is heated with acetic anhydride, followed by cooling and the addition of concentrated sulfuric acid, a blue colour develops. indicates the presence of sterols	
Libermann buchard's	Extract solution was mixed with Chloroform and concentrated Sulphuric acid was added to it and followed by the addition of Acetic anhydride. A colour change was observed from Red to blue finally green colour in the presence of steroidal moiety.	(Das & Gezici, 2018)
Triterpenoid		
Salkowski's test	When the extract is mixed with chloroform and concentrated sulfuric acid, a reddish-brown color appears on the inner face. Indicates the presence of terpenoids	(Das & Gezici, 2018)
Saponins		
Foam test	When the extract sample is shaken with water, frothing is formed in the presence of saponins	(Das & Gezici, 2018)

3.4 High Performance Thin Layer Chromatography

The study was conducted utilising the revised methodology of Annegowda *et al.* (2013). The HPTLC analysis was done on an aluminium-backed, precoated silica gel 60F254 HPTLC plate (Merck India) using a CAMAG HPTLC device with an automatic sample applicator (CAMAG ATS-IV).

The Camag automatic TLC sampler 4 connected to the Camag HPTLC system was used to apply samples on the plate. Using a Hamilton syringe (100µl), samples were applied on a Silica gel 60 F254 TLC plate (Merck India) that was already covered. The plate had a uniform thickness of 0.2 mm and was shaped into bands 8 mm wide.



Figure 3: A) *Humboldtia sanjappae*.Western Ghats of Kerala (10.04829 N, 76.8399˚E). B) Fruit of *H. sanjappae.* C) Inflorescence of *H. sanjappae.*

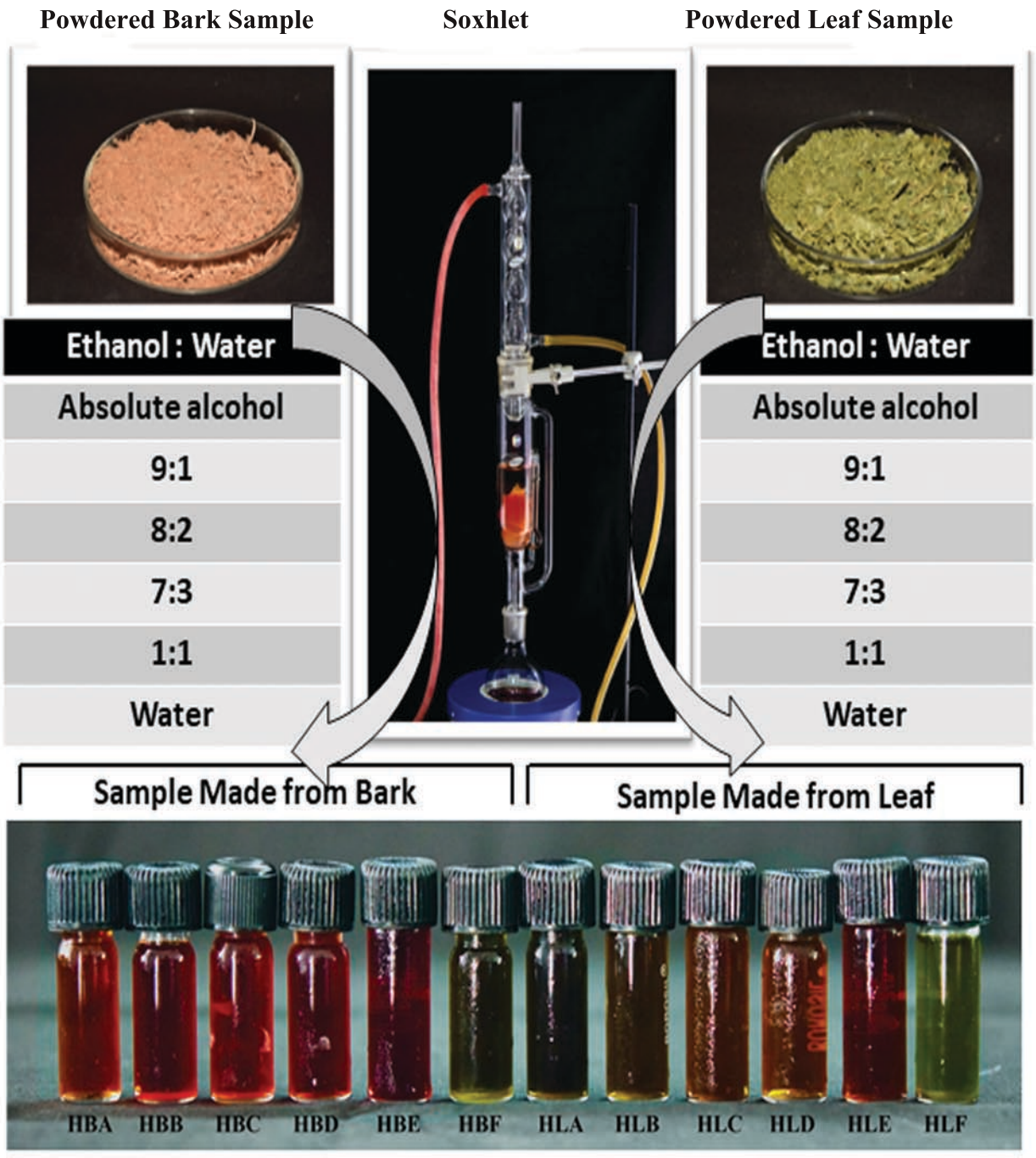

Figure 4: Soxhlet extraction of *H. sanjappae* bark and leaf using ethanol-water mixtures in different ratios, ranging from absolute alcohol to water.

A spot of 1 mg of extract was put on the plate for each sample. The plate was developed in a twin-trough chamber with a liquid system up to a distance of 9 cm.

A mobile phase consisting of toluene, ethyl acetate, and methanol in the ratio 7:3:1 was found optimal for separation. The plates were developed, then dried, and scanned with a CAMAG HPTLC Scanner-III at UV-254, UV-366, and visible light after being treated with an p-anisaldehydesulphuric acid solution to visualize more bands. The CAMAG HPTLC photo-documentation system took pictures of all the developed plates and made notes on them.

3.5 Quantitative phytochemical analysis

Total Phenolic Content: The total phenolic content was assessed using the Folin-Ciocalteu method. Aliquot of Gallic acid standard solutions (100, 200, 300, 400, and 500 µg/mL) were introduced into a 25 mL volumetric flask containing 9 mL of distilled water. A reagent blank was created with distilled water. One millilitre of Folin-Ciocalteu phenol reagent was introduced into the mixture and agitated. After 5 minutes, 10 millilitres of a 7% Na_2CO_3 solution was incorporated into the mixture. The volume was adjusted to the specified mark. Following a 90-minute incubation at ambient temperature, the absorbance relative to the reagent blank was measured at 550 nm using a U V visible spectrophotometer. The total phenolic content was quantified as Gallic acid equivalents (GAE) using the caliberation curve () (Singleton & Rossi, 1965).

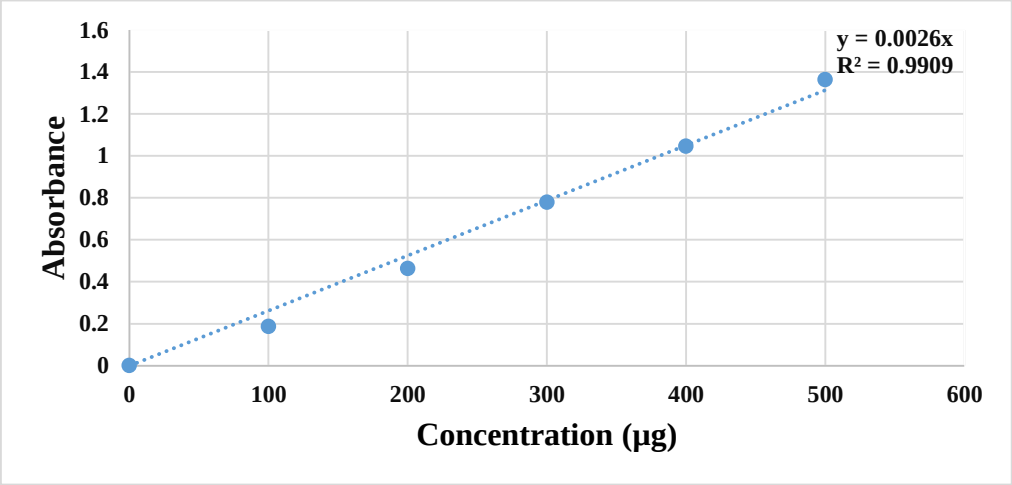

Figure 5: Calibration curve of gallic acid showing a linear relationship between concentration (µg) and absorbance at 550 nm.

Total Flavonoid Content: The total flavonoid concentration was quantified using the aluminium chloride colorimetric test established by Zhishen *et al.* (1999), with minor modifications. An aliquot of quercetin extracts or standard solutions (20, 40, 60, 80, 100, 120, and 140 µg/mL) was introduced into a 10 mL volumetric flask containing 4 mL of distilled water. 0.30 mL of 5% $NaNO_2$ was added to the flask, followed by the addition of 0.3 mL of 10% AlCl3 after 5 minutes. After 5 minutes, 2 mL of 1M NaOH was introduced, and the total volume was adjusted to 10 mL with distilled water. The solution was combined, and absorbance was assessed relative to the blank at 520 nm. The total flavonoid content was quantified in of quercetin equivalents (QE) using a calibration curve (Figure 6) (Zhishen *et al.*, 1999).

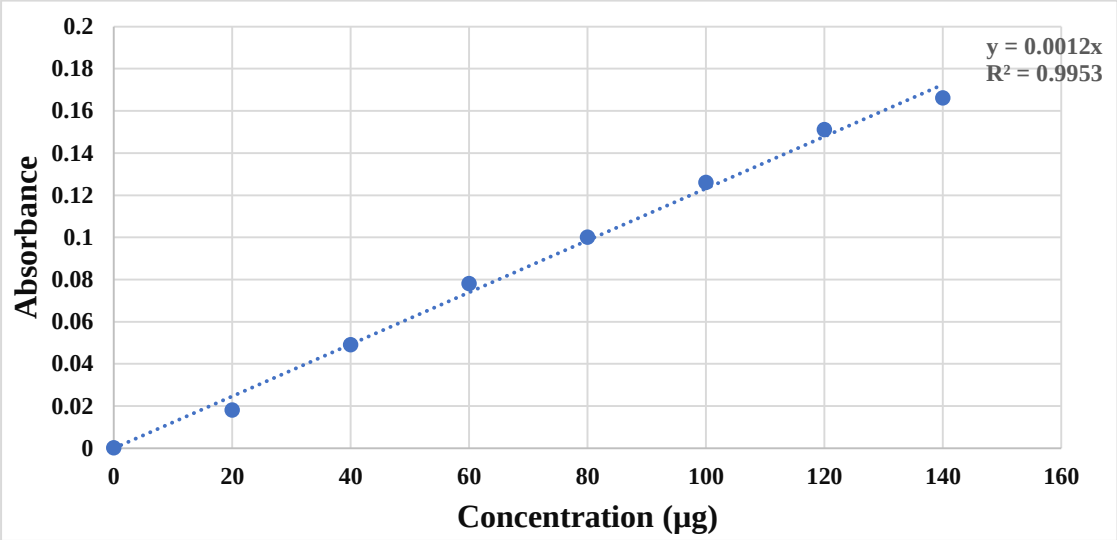

Figure 6: Calibration curve of quercetin showing a linear relationship between concentration (µg) and absorbance at 520 nm.

3.6 Antioxidant Activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay: A solution of DPPH was prepared by dissolving it in methanol at a concentration of 0.1 mM. Varying concentrations of the extract and ascorbic acid were mixed with the DPPH solution. The resulting mixture was then incubated in a dark environment at a temperature of 30 degrees Celsius for 20 min. The change in absorbance of the solution was measured and used to estimate the percentage inhibition. Percentage inhibition was calculated by plotting the

calibration curve for each sample (Sidhic *et al.*, 2023). The radical scavenging activity was calculated using the following formula;

$$\%\ of\ Radical\ scavenging\ activity = \frac{Absorbance\ of\ DPPH - Absorbance\ of\ Sample}{(Absorbance\ of\ DPPH)} \times 100$$

Ferric Reducing Antioxidant Power (FRAP) Assay: The stock solutions consisted of a 300 mM acetate buffer (prepared by dissolving 3.1 g of sodium acetate and 16 mL of acetic acid) with a pH of 3.6, a 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution (3.12 mg/mL) in 40 mM HCl, and a 20 mM $FeCl_3$ solution (3.25 mg/mL). To prepare the fresh working solution, 25 mL of acetate buffer, 2.5 mL of TPTZ solution, and 2.5 mL of FeCl3 solution were mixed together. The resulting solution was warmed at 37 degrees Celsius before use. For the FRAP assay, a test sample and ascorbic acid of varying concentrations was mixed with 2.80 mL of the prepared FRAP solution. The mixture was allowed to react for 30 min under dark conditions. After the incubation the absorbance of the coloured product, known as the ferrous tripyridyl triazine complex, was measured at a wavelength of 593 nm. Colour enhancement was calculated by plotting the calibration curve for each sample and expressed as percentage enhancement (EC50) (Sidhic *et al.*, 2023).

3.7 Liquid Chromatography–Mass Spectrometry (LC-MS/MS) profiling

Before performing the LC-MS/MS analysis, 10 mg of the plant extract was dissolved in 10 mL of HPLC-grade methanol to make sure it would dissolve as much as possible. The compounds were evenly mixed throughout the solution. It was then put through a 0.2-micron membrane filter to get rid of any particles and possible impurities, which made sure that the analysis was very accurate. We used the Agilent 6530C Q-TOF LC/MS system with the HPLC 1260 Infinity II Prime LC series to do the LC-MS/MS analysis. The separation was done with a C-18 reverse phased column (Agilent poroshell 120 EC-C8 µm, 3.0×150 mm). In a gradient elution, the mobile phase was made up of 0.1% acetonitrile (A) and water (B). As time proceeded with, the amount of solvent A slowly increased while the amount of solvent B decreased.

At two minutes, the mobile phase is made up of 10% percent A and 90% B. At 6 minutes, the amount of A goes up to 20% and the amount of B goes down to 80%. At 10 minutes, the composition changes to 30% A and 70% B. At 14 minutes, this pattern keeps going, with 40% A and 60% B. The gradient transition is finished at 20 minutes when the mobile phase reaches a 50% A and 50% B ratio.

40 psi, 325 °C source temperature, 10 L/min nitrogen flow, and 345 °C jet stream sheath temperature were the optimised MS parameters for the nebuliser. 2.9 L/min for nebulizing gas flow, 10 L/min for heating gas flow, 300°C for interface temperature, 250°C for DL temperature, 400°C for heat-block temperature, and 10 L/min for drying gas flow. Agilent Mass Hunter software (version 10) was used to extract mass spectral data, which was acquired in both positive and negative electrospray ionisation (ESI) modes.

3.8 Drug likeness and *in-silico* ADMET prediction

The pkCSM web server (https://biosig.lab.uq.edu.au/pkcsm/prediction), which was accessed on 12 October 2024, was used to do *in silico* ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) prediction on the compounds found in HBA by LC-MS/MS. Additionally, Lipinski's Rule of Five was applied to assess drug-likeness, ensuring that the identified compounds met criteria for oral bioavailability based on molecular weight, lipophilicity, hydrogen bond donors, and acceptors.

Absorption properties were tested by predicting how well the substance dissolves in water, how easily it passes through Caco-2 cells, how much of it is absorbed by the human intestine, and how easily it passes through the skin (log Kp). The interaction of the compounds with P-glycoprotein (P-gp) including their classification as P-gp substrates and inhibitors (both isoforms I and II), which influence drug retention and absorption was also assessed. Distribution parameters were analyzed by estimating the volume of distribution (VDs) to understand tissue penetration, the fraction of the compound unbound in human plasma to assess bioavailability, and the ability of the compounds to cross the blood-brain barrier (BBB) and penetrate the central nervous system (CNS).

Metabolism was studied by evaluating interactions with cytochrome P450 (CYP) enzymes. Predictions included whether the compounds were substrates of CYP2D6 and CYP3A4, as well as their potential to inhibit key metabolic enzymes, such as CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4. Excretion properties were assessed by analyzing interactions with the organic cation transporter 2 (OCT2), which contribute to the process of renal clearance, along with the overall total clearance rate of the compounds.

Toxicity predictions included hepatotoxicity assessment to determine the risk of liver damage, AMES toxicity testing to evaluate potential mutagenicity, and the maximum tolerated dose (MTD) for humans. Additionally, the potential for cardiotoxicity was determined through hERG I and II inhibition analysis, which assesses the likelihood of QT prolongation and arrhythmias. Skin sensitization potential was also predicted to evaluate dermal toxicity risks.

3.9 Acute Oral Toxicity evaluation using animal models

The acute oral toxicity of the *H. sanjappae* extract was assessed according to the OECD (Organization for Economic Cooperation and Development) (Bedi & Krishan, 2020) Guideline No. 423. The animal experimental procedures were carried out with the approval of IAEC, JSS College of Pharmacy, Ooty, India (IAEC (118/PO/Re BiRc/S/1999/CPCSEA)) under permission number JSSCP/OT/IAEC/24/2024-2025.

3.9.1 Number of animals and Dosage level

Eight-week-old female Swiss albino mice were randomly selected and housed under the following laboratory conditions. The temperature was maintained between 23.4 and 28.8°C, with relative humidity ranging from 54% to 78% and a 12-hour light/dark cycle. The animals were housed individually in standard polypropylene cages, measuring approximately 410 mm (length) × 282 mm (width) × 150 mm (height). Each cage was equipped with stainless steel top grills and included provisions for pelleted food and bottled drinking water. Paddy husk served as bedding material, which was replaced at least twice a week. All animal procedures were conducted in compliance with ethical guidelines and approved protocols.

Three animals were used at each step of the process. The first dose was set at 2000 mg/kg body weight because it could potentially induce mortality in some of the test animals. The animals were given the chosen dose, and then they were watched for signs of toxicity, like changes in behaviour and death, over a set period of time. if no fatalities occurred, the subsequent lower dose level was evaluated. Nonetheless, if mortality occurred in one or more subjects, the experiment was conducted again with additional subjects at the same or a reduced dosage. The technique persisted until the suitable dose-response relationship was determined.

3.9.2 Preparation of test item

The test substance HBA was made fresh right before it was given to the animals. the test item HBA was mixed with portable water to get two different concentrations: 200 mg/ml and 30 mg/ml. Homogeneity of the test item HBA in the vehicle was maintained during treatment by continuous stirring and shaking. The test solutions were given right after they were made, at a dose of 10 ml/kg body weight, to get the desired dose of 2000 mg/kg and 300 mg/kg.

3.9.3 Treatment and observations

The test solution was given to mice orally. mice had been fasting for 4 to 5 hours. A dose of 10 ml/kg of body weight was given to each animal. Three to four hours after the dose, food was given back, while access to water remained unrestricted. The treated mice were checked five times on the first day of treatment. The first check was at 30 minutes after the treatment started, and the next four checks were conducted hourly for the next four hours. After that, observations were conducted until day 14 and clinical signs were noted. Body weights were measured on day 1 (prior to administration), day 8 (seven days post-administration), and day 15 (fourteen days post-administration). At the end of the study, the mice were euthanized using diethyl ether and then their bodies were examined.

3.10 Potential target screening of active compounds and inflammation

Information on possible targets for HBA compounds from Swiss Target Prediction (http://www.swisstargetprediction.ch/ accessed on 14th September 2024) and STITCH (http://stitch.embl.de/ accessed on 14th September 2024) through entering

the canonical SMILES and setting the species to "*Homo sapiens*". The inflammatory targets from GeneCard (http://www.genecards.org/, accessed on September 18, 2024), the OMIM database (https://omim.org/, viewed on September 18, 2024), and Des-Genet (http://www.disgenet.org/, accessed on September 18, 2024) were obtained. A Venn diagram made with the Gene Venn tool (https://www.bioinformatics.org/gvenn/ Venn accessed on 24th September 2024) indicated the common targets of phytochemicals and inflammation.

3.10.1 Network Construction and Visualization

A phytochemical-target interaction network was constructed to show how the plant *H. sanjappae*, and its phytochemicals, and their biological targets are related to each other. The network had three kinds of nodes: (1) the plant (*H. sanjappae*), (2) its phytochemicals, and (3) the biological targets. Edges showed how the plant and its phytochemicals interacted with each other or how phytochemicals interacted with targets. the plant node was represented in green, the phytochemicals were blue, and the target nodes were red. The most shared target, based on node degree, was highlighted in yellow to show how important it is in the network. a circular layout algorithm was used to find the ideal locations for nodes so that they could be seen better.

Matplotlib was used to make the final network visualisation as a high-resolution image with custom Figure size and resolution settings.

3.10.2 Prediction of protein–protein-interaction network

The protein–protein interactions of common genes were evaluated using the STRING database (https://stringdb.org/, accessed on 1 January 2025). In that the organism designated as "*Homo sapiens*." The protein-protein interaction network was visualised using Cytoscape V3.10.2 (accessed on 1 January 2025).

The analysis was conducted using the full STRING network with functional associations. The edges show both functional and physical interactions between proteins. We used a medium confidence threshold of 0.4 to filter the interactions. To get an entire view of the network, all active interaction sources were chosen. These included text mining, experiments, databases, co-expression, neighbourhood, gene

fusion, and co-occurrence. In order to make the interactions look more appealing, 3D bubble visualisation was used.

3.10.3 Gene ontology and KEGG pathway analysis

***Gene Ontology (GO) analysis*:** The Database for Annotation, Visualisation, and Integrated Discovery (DAVID) (http://david.ncifcrf.gov/, accessed on January 3, 2025) was used to do the GO analysis on the organism "*Homo sapiens*." The GO classification split gene functions into three main groups: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). All the terms were sorted by using their p values and picked the 15 most important ones to show in a graph. The graph was made with SRplot (https://www.bioinformatics.com.cn/en), a free online tool for making graphs and visualising data.

***Pathway Enrichment Assessment*:** Pathway enrichment analysis was conducted with ShinyGO v0.81, an online tool for gene ontology and pathway analysis, accessible at https://bioinformatics.sdstate.edu/go/. The input comprised 117 significant genes recognised as common targets between phytochemicals and inflammation-associated pathways. The analysis was performed with the subsequent parameters:

i. Pathway Database: Kyoto Encyclopaedia of Genes and Genomes (KEGG).
ii. False Discovery Rate (FDR) Threshold: A significance criterion of 0.05 was implemented to prevent false positives.
iii. Number of Pathways to Exhibit: The twenty most enriched pathways were documented, ranked by FDR-adjusted p-values.
iv. Pathway Dimension Filter: 2-200 genes.

In addition to ShinyGO for KEGG pathway analysis**,** DAVID was also utilized to interpret and identify significant pathways. Enriched pathways were illustrated, and their biological significance was analyzed for inflammation and phytochemical interactions.

3.11 Effect of HBA on Lipopolysaccharide-Induced Anti-Inflammatory Activity in Macrophages

3.11.1 Cell Culture and Treatment: Murine RAW 264.7 macrophage cells were grown in RPMI-1640 media supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were sustained at 37°C in a humidified incubator with 5% CO_2. For the experiment, RAW 264.7 cells were inoculated at a density of 1 × 10^7 cells/mL in 24-well plates with full growth medium. Following 24 hours of adhesion, cells were pre-treated with varying doses (5, 10, and 20 µg/mL) of HBA extract, formulated in serum-free RPMI-1640 medium. Aspirin (1 mM), a conventional anti-inflammatory agent, served as the positive control. Following 2 hours of pre-treatment, cells were stimulated with lipopolysaccharide (LPS, 1 µg/mL) and incubated for 24 hours. Untreated cells functioned as the negative control. Details are given in the Table 5 and Figure 7.

3.11.2 Cytokine Quantification by ELISA*:* The concentrations of Interleukin-1β (IL-1β), Interleukin-6 (IL-6), and Tumour Necrosis Factor-α (TNF-α) were measured utilising PeproTech ELISA kits in accordance with the manufacturer's guidelines. The absorbance was quantified at 450 nm via a microplate reader (Sidhic *et al.*, 2023).

Table 5: Treatment Groups and Conditions

Treatment Group	Pre-treatment (2h)	LPS stimulation (24 h)
Untreated control	None	None
LPS control	None	1 µg/mL of LPS
Aspirin	1 mM	1 µg/mL of LPS
HBA	5 µg/mL	1 µg/mL of LPS
HBA	10 µg/mL	1 µg/mL of LPS
HBA	20 µg/mL	1 µg/mL of LPS

3.11.3 Quantification of Nitric Oxide (NO): The Griess reaction was used to check how much nitric oxide (NO) was made in the culture supernatant. In brief 100 µL of cell culture supernatant was mixed with the same amount of Griess reagent (which

contains 1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 2.5% phosphoric acid) and left to sit at room temperature for 10 minutes. We used a sodium nitrite standard curve to find out how much NO there was by measuring absorbance at 540 nm (Ghosh *et al.*, 2024; Sidhic *et al.*, 2023).

3.11.4 Gene Expression Analysis by qRT-PCR: Total RNA was extracted from RAW 264.7 cells using TRIzol reagent, and complementary DNA (cDNA) was synthesized using the Takara cDNA synthesis kit (Takara, Bangalore, India) following the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed using Applied Biosystems 7300 Real-Time PCR System, with specific primers for NF-κB and Cyclooxygenase-2 (COX-2) genes.

The PCR cycling conditions were as follows:

- Initial denaturation: 95°C for 15 s
- Annealing: 60°C for 45 s
- Extension: 73°C for 30 s
- Cycle number: 40 cycles

Relative gene expression was analyzed using the ΔΔCT method, with GAPDH as the internal control. The expression levels were calculated as fold changes compared to untreated control cells (Livak & Schmittgen, 2001).

3.12 *In vivo* evaluation of anti-inflammatory potential using animal model

3.12.1 Animal Selection and Housing

For the study, eight-week-old male Swiss albino mice were chosen at random. The animals were kept in a controlled laboratory setting with a temperature range of 23.4 to 28.8°C and a relative humidity of 54 to 78%. Throughout the study, a 12-hour cycle of light and dark was maintained. Standard polypropylene cages measuring L 410 × B 282 × H 150 mm were used to house the mice. The cages had stainless steel top grills with space for pelleted food and bottles of drinking water. The bedding was made of paddy husk, which was replaced at least twice a week.

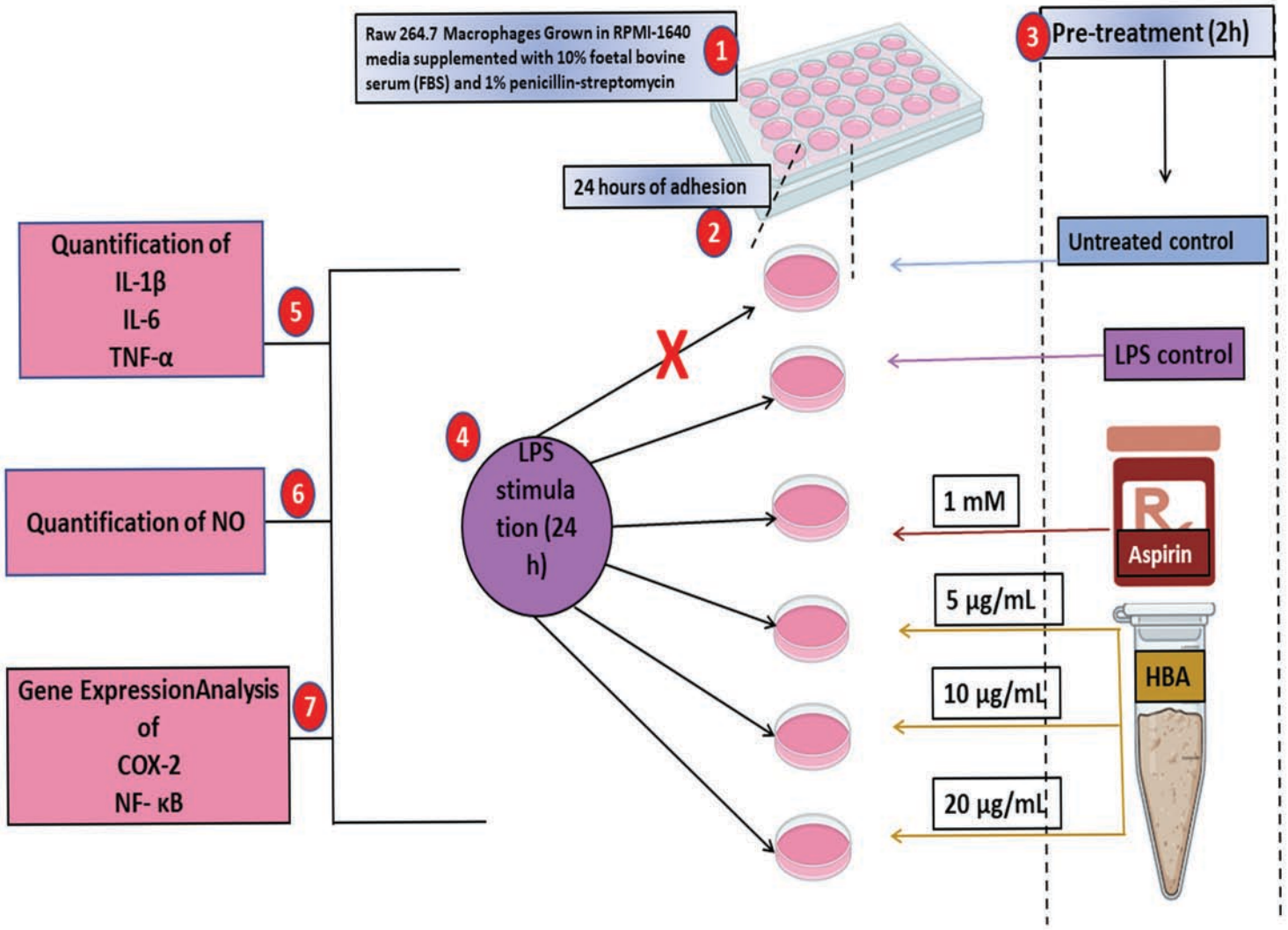

Figure 7: The illustration representing the workflow for evaluating the anti-inflammatory effects.

3.12.2 Acclimatization and Ethical Compliance

Animals were kept in the lab for seven days before the experiments started to allow them to get used to the conditions and reduce stress. All animal experiments followed the ethical rules set by the institution and the country, and the right ethics committee approved the protocols (IAEC (118/PO/ReBiRc/S/1999/CPCSEA) under permission number JSSCP/OT/IAEC/24/2024-2025

3.12.3 Experimental Design

Two separate studies were conducted to evaluate the acute and chronic anti-inflammatory effects of HBA. Animals were divided into five groups (n=6 per group) for each study:

1. Normal Control – received vehicle treatment.
2. Disease Control – received vehicle treatment.
3. Standard Treatment – treated with indomethacin (5 mg/kg, *i.p.*).
4. HBA 100 mg/kg – treated with HBA (100 mg/kg, *p.o.*).
5. HBA 200 mg/kg – treated with HBA (200 mg/kg, *p.o.*).

Each study was performed separately, with specific inflammation induction methods and treatment protocols (Figure 8a and Figure 8b).

The carrageenan-induced paw edema model was used to evaluate acute inflammation. Baseline paw thickness (0 hour) was measured using a digital Vernier callipers before inflammation induction and animals were treated with their respective treatments. Acute inflammation was induced by subplantar injection of 100 µl of 1% freshly prepared carrageenan into the right hind paw. Respective treatments were administered 30 mins prior to carrageenan induction. Paw thickness was recorded at 0, 0.5, 1, 2, 4, and 6 hours post-treatment.

The formalin-induced inflammation model was used to assess chronic inflammation. On day 0, animals were injected with 100µl of 5% formalin in the sub plantar region of the right hind paw to induce persistent inflammation. Starting from day 1, animals received their respective treatments daily. The paw thickness was measured using a digital Vernier calliper on day 0, and then daily from day 1 to day 5**.** The reduction in

paw thickness over time was used to evaluate the chronic anti-inflammatory effect of HBA.

To see how well HBA worked as an anti-inflammatory agent, the percentage change in paw thickness and the average increase in paw volume were measured using a plethysmometer.

3.12.4 Histopathology

At the end of the study, paw tissues were collected from respective groups fixed in 10% formalin. After removing lipid debris by soaking in alcohol, and then the sections were dehydrated in increasing ethanol concentrations. The tissue samples were embedded in paraffin wax. 5-10µm thin coronal section of wound tissue with thickness of 2mm was taken using microtome. Slides were stained with Haematoxylin and Eosin (H&E). The stained tissue sections were observed under a motic microscope (40x) and the paw region was photographed to assess the changes observed by different treatments.

3.12.5 Gene Expression

Total RNA was extracted from paw tissue using TRIzol reagent, and complementary DNA (cDNA) was synthesised using the Takara cDNA synthesis kit (Takara, Bangalore, India) in accordance with the manufacturer's protocol. The Applied Biosystems 7300 Real-Time PCR System was employed to conduct quantitative real-time PCR (qRT-PCR) with specific primers for the NF-κB and Cyclooxygenase-2 (COX-2) genes. The PCR cycling conditions were; initial denaturation: 95°c for 15 seconds, annealing: 60°c for 45 seconds, extension: 73°c for 30 seconds, the number of cycles is 40. The ΔΔCT method was employed to analyse relative gene expression, with GAPDH serving as the internal control. The expression levels were determined as fold changes in comparison to the untreated control cells (Livak & Schmittgen, 2001).

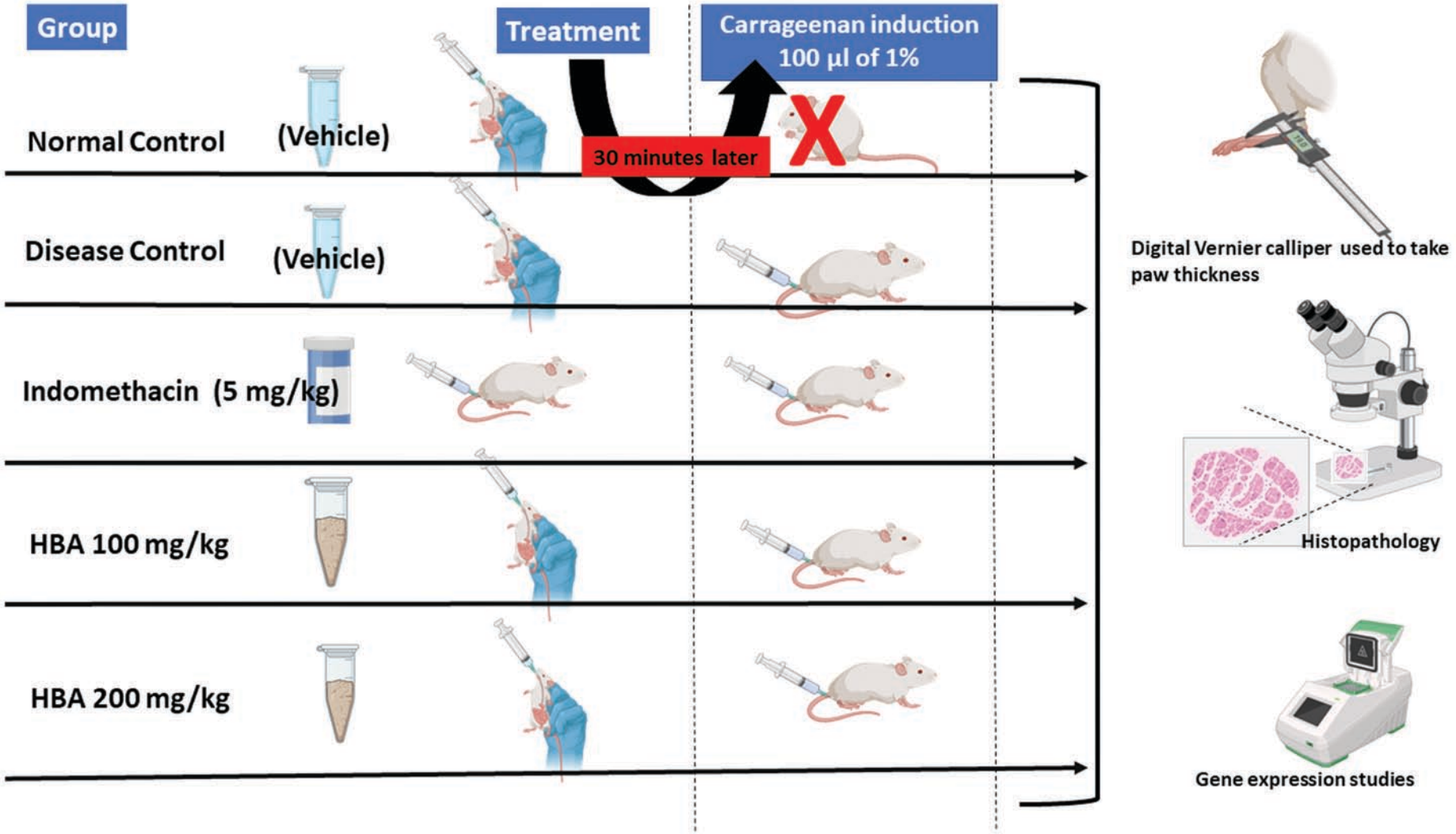

Figure 8a: The figure illustrates the experimental design for testing the effects of different treatments in a carrageenan induced acute inflammatory model in mice.

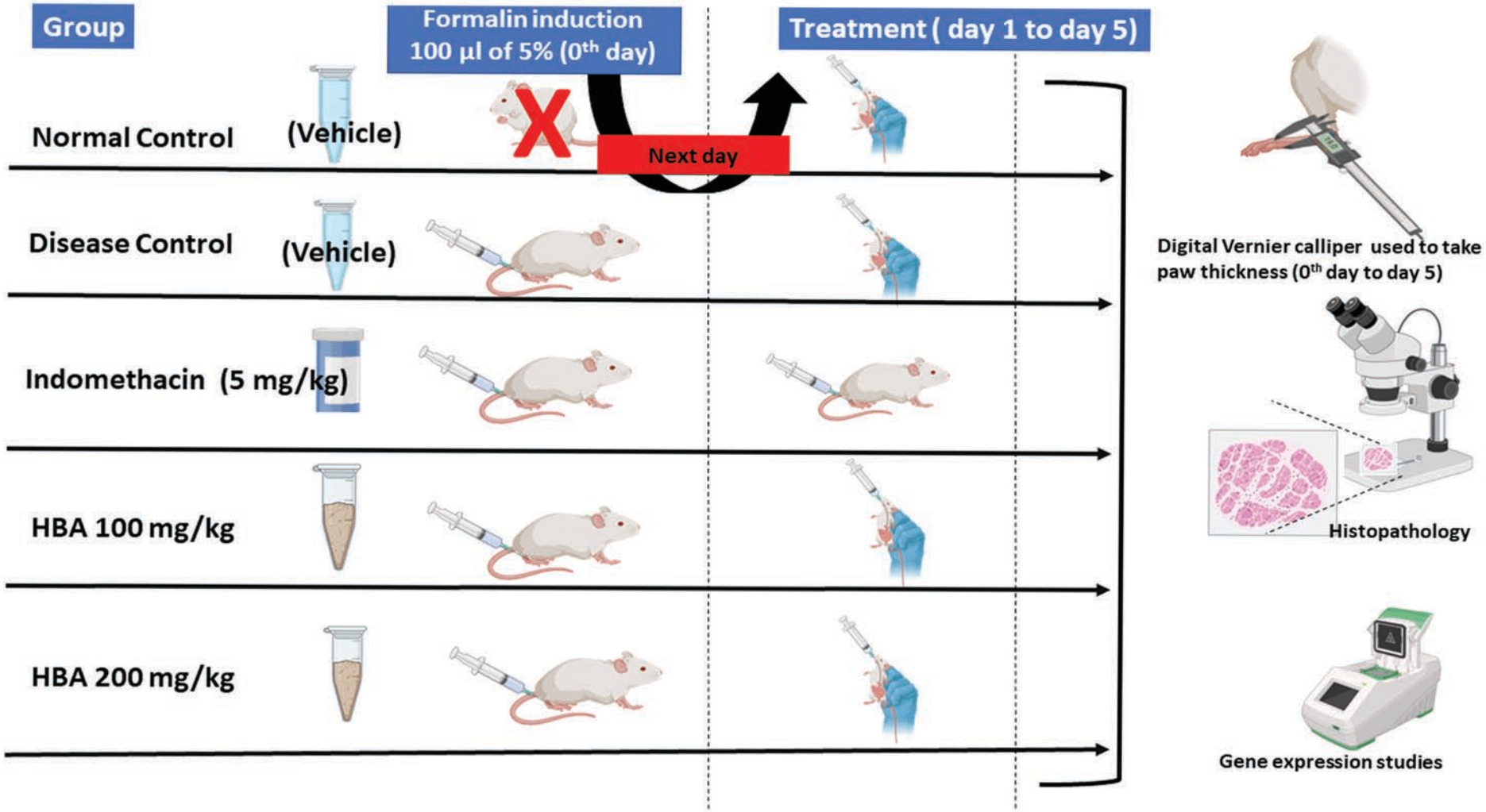

Figure 8b: The figure illustrates the experimental design for testing the effects of different treatments in a formalin induced chronic inflammatory model in mice.

3.13 Molecular Docking and MM-GBSA Analysis of Identified Phytochemicals from *HBA* Against Inflammatory Targets

3.13.1 Ligand Preparation

The 3D structures of the phytochemicals from *H. sanjapape* that have been discovered using LC-MS/MS were downloaded from PubChem and processed using Schrödinger Maestro software. Ligand optimisation was done with the LigPrep module to make sure that the energy was as low as possible and the protonation states were correct.

3.13.2 Protein Target Preparation

Crystal structures of COX-1, COX-2, IL-6, IL-1β, and TNF-α were obtained from the Protein Data Bank (PDB). Schrödinger's Protein Preparation Wizard was used to prepare the proteins. This included pre-processing, assigning bond order, getting rid of water molecules, and minimising energy.

3.13.3 Molecular Docking

The Glide module in Schrödinger was employed to conduct molecular docking in order to assess the binding interactions between phytochemicals and target proteins. The receptor grid was generated in the vicinity of the active site, and docking was performed in both standard precision (SP) and extra precision (XP) modes. Binding interactions and docking scores were examined.

3.13.4 Molecular Mechanics-Generalized Born Surface Area

Molecular Mechanics-Generalized Born Surface Area (MM-GBSA) method was added to Schrödinger's Prime module, to figure out how much free energy the docked complexes had. then the ΔG binding energy was calculated to see how well the ligand binds and how stable it is.

3.14 Statistical Analysis

The statistical analysis of all the data was performed using IBM SPSS Statistics version 25.

Chapter 4

RESULTS AND DISCUSSIONS

4.1	Effect of Extraction Solvents and Plant Part on Phytochemical Composition and Antioxidant Potential
4.2	Comprehensive Analysis of Bioactive Compounds from Ethanol Extract of *H. sanjappae* Bark Using LC-MS/MS, ADMET-Based Pharmacokinetics, and *In Vivo* Acute Oral Toxicity
4.3	Network Pharmacology and *In Vitro* Analysis of Ethanol Extract of *H. sanjappae* Bark in Treating Inflammation
4.4	*In Vivo* Evaluation of Anti-inflammatory Potential and Molecular Docking Analysis of Key Phytochemicals

4.1 Effect of extraction solvents and plant part on phytochemical composition and antioxidant potential

The therapeutic properties of plants are associated with their phytochemical constituents and secondary metabolites, such as phenolic compounds, flavonoids, alkaloids, tannins, and other substances (Ghasemzadeh *et al.*, 2011; Mohammedi & Atik, 2011). Several parameters, including time, temperature, solvent concentration, and solvent polarity influence the extraction and purification of phytochemicals from plant material. Diverse phytochemicals are extracted using solvents of differing polarity, as no one solvent can effectively extract all phytochemical found in plant material (Iloki-Assanga *et al.*, 2015; Lapornik *et al.*, 2005).

This study incorporates preliminary phytochemical screening and HPTLC analysis to compare phytoconstituents and see how their chemical constitutes differ. Also, the study wants to find the best extraction solvent and the best part of the *H. sanjappae* plant to use for extracting based on yield, TPC, TFC, and antioxidant activity. Understanding these factors is essential for maximizing phytochemical yield, optimizing extraction efficiency, and enhancing the potential therapeutic applications of the plant.

4.1.1 Results

4.1.1.1 Extract Yield

Sample HBE, which was extracted using a 1:1 ethanol-to-water ratio, had the highest yield (18.2%) (Figure 9). After that, HBF (16.2%) extracted using water and HBA (16%) extracted using ethanol showed good extract yields. The samples HBD (14.2%), HBB (14%), and HBC (14%) had lower yields because the ratios of ethanol to water were 7:3, 9:1, and 8:2, respectively. The results suggest that a balanced ethanol-to-water mixture (1:1) improves extraction efficiency, possibly because it lets a wider range of phytochemicals be more soluble. On the other hand, extreme solvent compositions, like only ethanol or water, made extraction less efficient.

The extraction yield of the leaf changed according to the ethanol-to-water solvent ratio used. The maximum yield (17.8%) was recorded in sample HLF, extracted alone with water, demonstrating that water was the most efficacious solvent for extracting phytoconstituents from the leaf. This was followed up by HLD (15.4%), extracted using a 7:3 ethanol-to-water ratio, and HLC (13.4%), extracted with an 8:2 ratio. Samples HLE (12.6%), HLB (12.2%), and HLA (12%) exhibited diminished yields, associated with ethanol-to-water ratios of 1:1, 9:1, and pure ethanol, respectively. Higher water content improved extraction efficiency of leaf phytochemicals whereas ethanol alone was less effective indicating poorer solubility of leaf bioactives in ethanol.

These findings show that, a balanced ethanol-to-water ratio (1:1) optimises bark extraction efficiency, water alone serves as the most efficacious solvent for leaf extraction, and the selection of solvent clearly influences yield depending on the composition of the plant part.

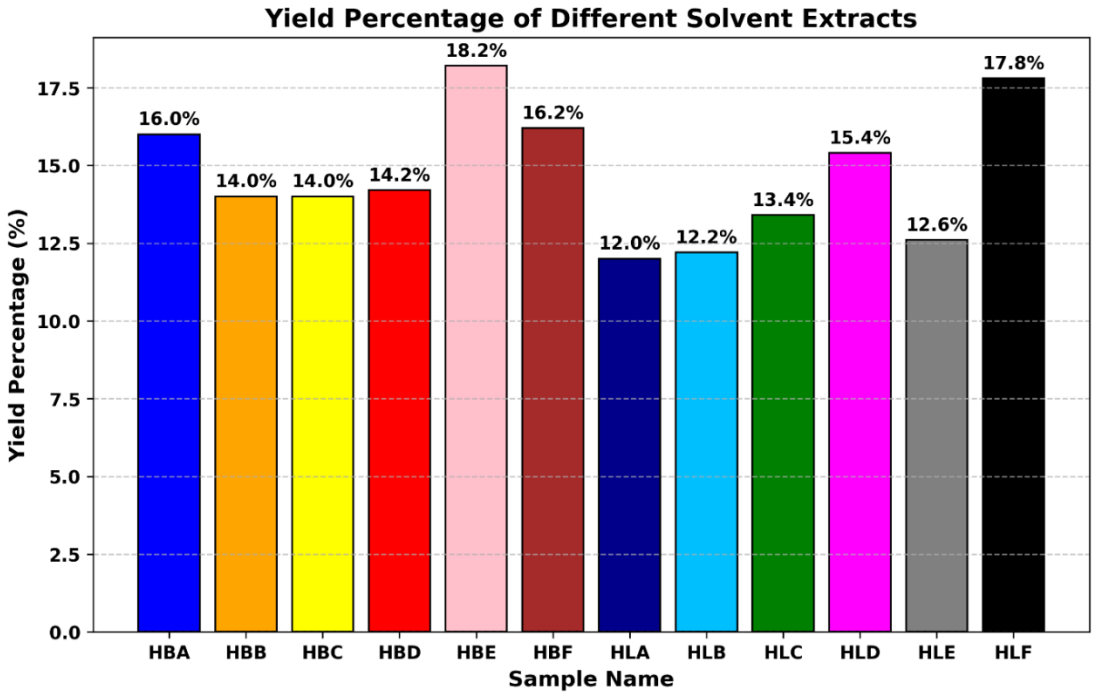

Figure 9: The bar chart represents the yield percentage of different extracts.

4.1.1.2 Preliminary Phytochemical analysis

The preliminary phytochemical study of the extracts indicated the presence of several bioactive molecules, represented in Table 6. Alkaloid tests revealed positive findings in some extracts; HBF, HLA, HLB, and HLC tested consistently positive using several methods. It was confirmed by Molisch's, Fehling's, and Benedict's assays that carbohydrates were abundant in the majority of extracts. Biuret and Ninhydrin tests demonstrated that protein was consistent among all the samples. All extract revealed the presence of phenolics, tannin and flavonoids. A few extracts such as HBA, HBB, HA, HLA, HLB, and HLC has shown the presence of sterol groups. Saponins were found in all the extracts and the foam formation during the test indirectly indicated that the amount of them varying each of the samples. Figure 10 shows the different levels of phytochemicals in the samples on the heat map.

These data show how important it is to choose the right plant parts and the right solvents when extracting phytochemicals. They also show how these factors affect the overall phytochemical profile and possible medical uses.

4.1.1.3 High Performance Thin Layer Chromatography

HPTLC demonstrated a total of 46 different Rf values under three detection wavelengths: 366 nm (UV), 254 nm (UV), and 550 nm (visible light). HPTLC plate and chromatograms are given in the Figure 11 and 12 respectively. The Venn diagram (Figure 13) illustrates the distribution of total number of Retention Factor (Rf) values detected at three distinct wavelengths. Each circle represents a wavelength, with the numbers denoting the number of peaks observed solely or in common among the various wavelengths. The Table 7 presents the relationship between Rf values and the corresponding wavelengths of light under which they are detected. The distribution of peaks and their area percentages are represented in the scatter plot (Figure 14).

A total of 20, 21 and 27 Rf values were found at 254, 366 and 550 nm respectively. Each band represents a different metabolite suggests that secondary metabolites differ based on the solvent and plant parts used.

Table 6: Preliminary phytochemical analysis of different extracts

Test	HBA	HBB	HBC	HBD	HBE	HBF	HLA	HLB	HLC	HLD	HLE	HLF
Alkaloid												
Mayer's test	–	+	–	–	+	+	+	+	+	–	–	–
Wagner's test	–	+	+	+	–	–	++	+	+	+	–	–
Dragendroff's test	–	–	+	–	–	–	++	++	+	+	–	–
Hager's test	–	–	–	–	–	–	++	+	+	+	–	–
Carbohydrate												
Molisch's test	++	++	++	++	+	+	+++	++	++	++	+	+
Fehling's tets	+++	++	++	+	+	+	+++	++	++	++	+	+
Bendict's test	+++	++	++	++	+	+	++	++	+	+	+	+
Proteins												
Biuret test	+	+	+	+	+	+	+	+	+	+	+	+
Ninhydrin test	+	+	+	+	+	+	+	+	+	+	+	+
Phenolics and Tannins												
Ferric Chloride	+++	+	+	+	+	+	+	+	+	+	+	+
Lead Acetate	+++	+	+	+	+	+	+	+	+	+	+	+
Flavones												
Sodium hydrochloride test	+++	++	++	++	++	++	++	++	+	+	+	+
Sulphuric acid test	++	+	+	+	+	+	+	+	+	+	+	+

Schinoda test	++	+	+	+	+	+	+	+	+	+	+	+
Sterols												
Libermann's	+	+	–	–	–	–	+	+	+	–	–	–
Liber buchards	+	–	–	–	–	–	+	+	+	–	–	–
Triterpenoid												
Salkowski’s test	+++	+	+	–	–	–	+++	+	+	–	–	–
Saponins												
Foam test	+++	++	++	+	+	+	+++	++	++	++	+	+
"+" indicates presence, while "–" indicates absence. A greater number of "+" or "–" symbols signifies the intensity of the reaction and the strength of the result.												

Table 7: Rf Values and their detection wavelengths

Observed Wavelength (nm)						
254	**366**	**550**	**254 and 366**	**254 and 550**	**366 and 550**	**254, 366 and 550**
0.14	0.12	0.01	0.54	0.13	0.1	0.04
0.46	0.25	0.17	0.84	0.15	0.22	0.06
0.47	0.32	0.24		0.7	0.26	0.19
0.74	0.34	0.33		0.72	0.29	0.2
0.78	0.45	0.36		0.97	0.48	
0.88	0.62	0.39			0.76	
0.9	0.96	0.42			0.95	
0.91	0.98	0.58				
0.94		0.6				
		0.67				
		0.83				

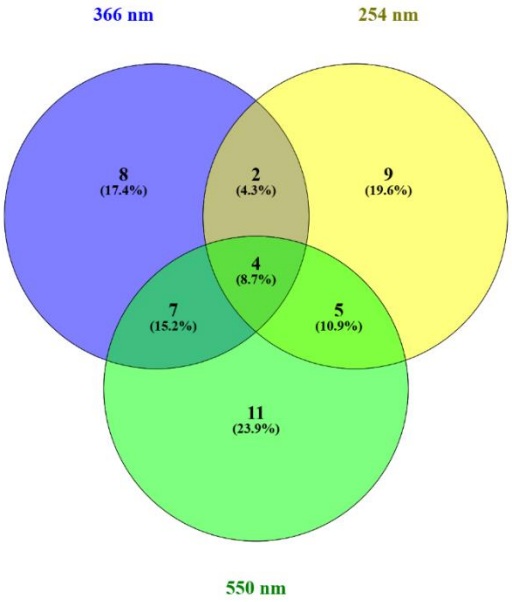

Figure 13: Distribution of total number of Rf values detected at three distinct wavelengths.



Figure 10 : The heat map represents the varying intensity of phytochemicals between samples. Yellow represents a lower concentration of phytochemicals, while green represents a medium level of concentration, and red indicates the highest co-ncentration of phytochemicals.

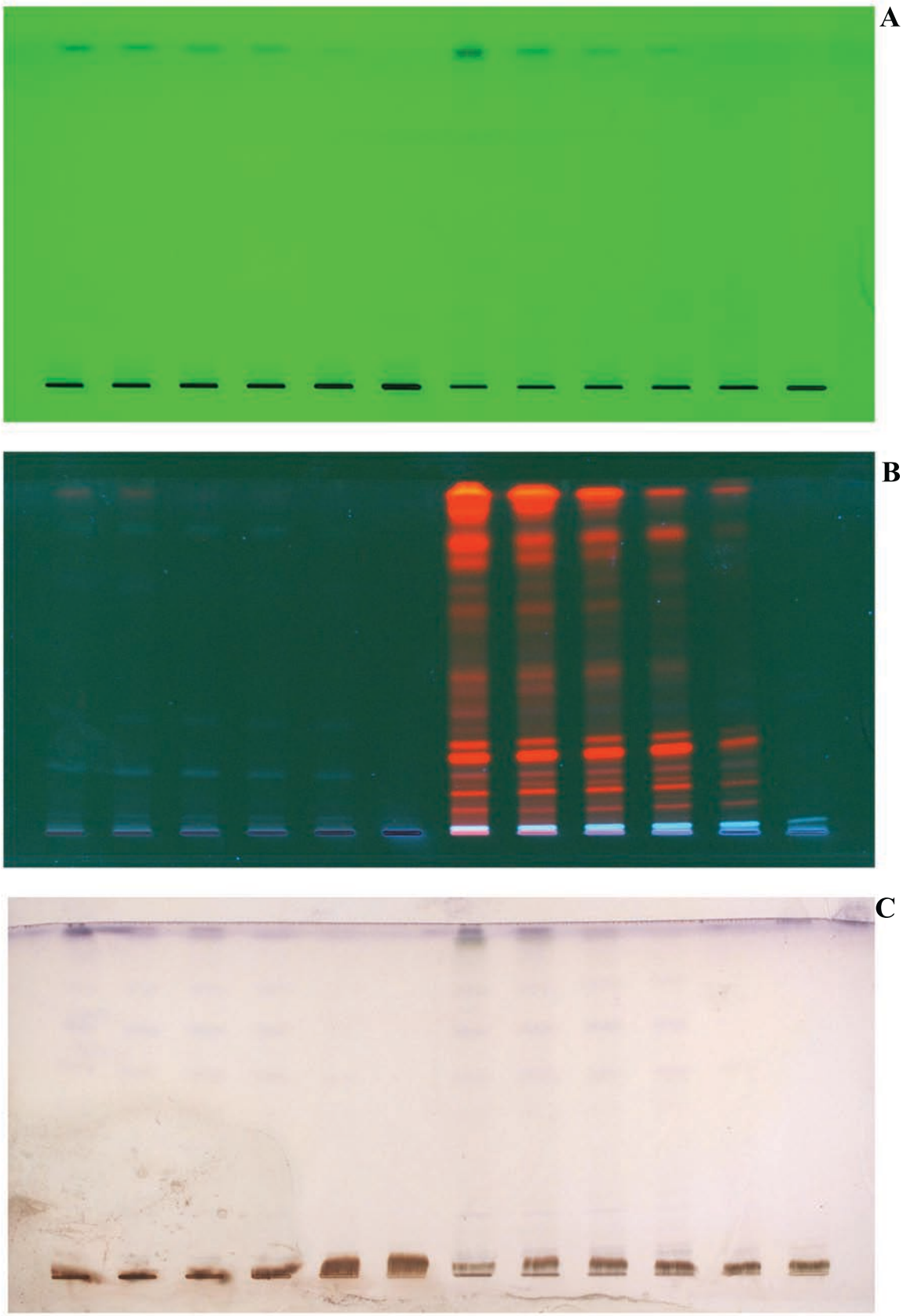

Figure 11: HPTLC plates of different extract of *H. sanjapape*. Track 1 to 12 indicates, HBA, HBB, HBC, HBD, HBE, HBF, HLA, HLB, HLC, HLD, HLE and HLF respectively. HPTLC-Plate under A) 254 nm, B) 366 nm and C) 550 nm.

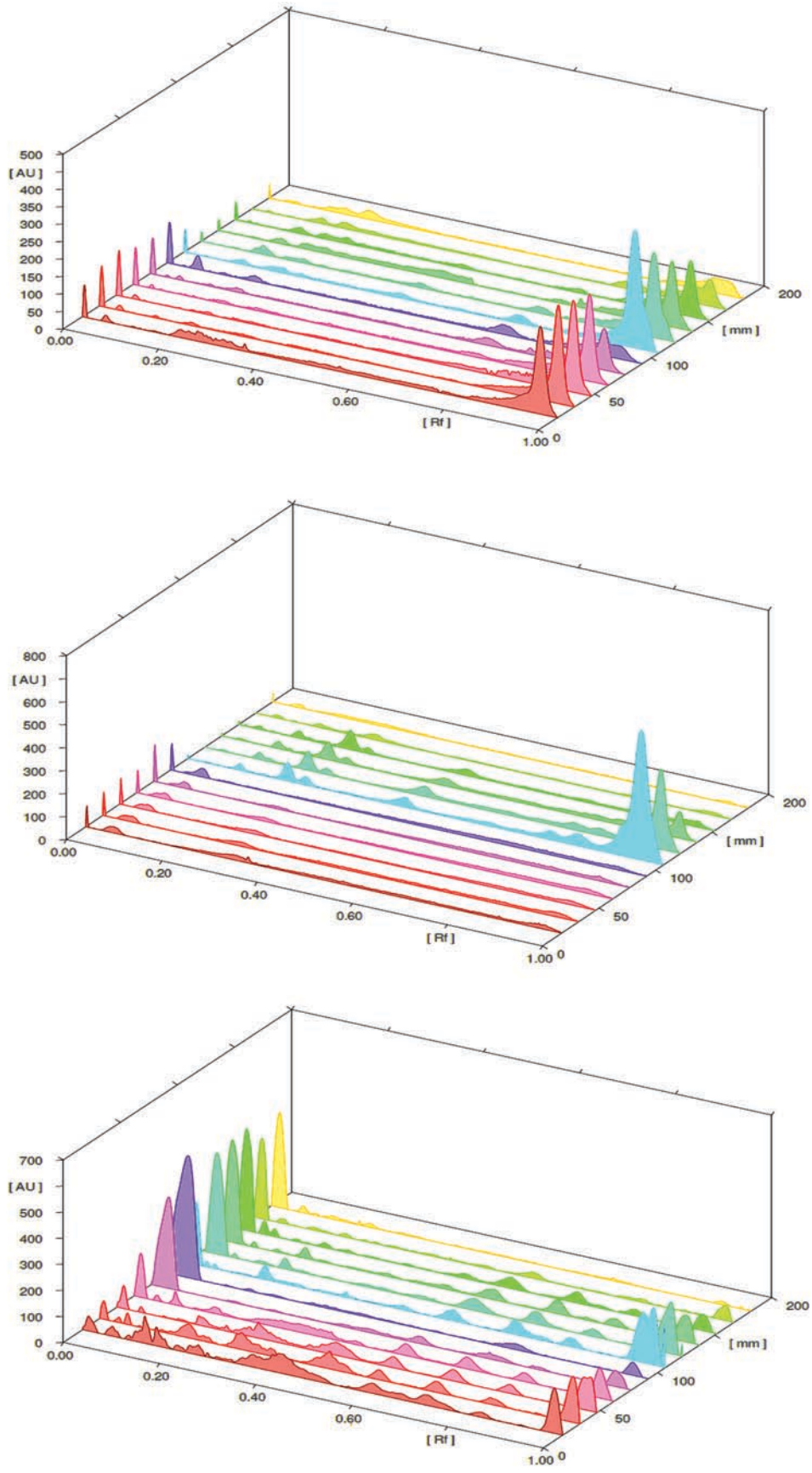

Figure 12: HPTLC chromatogram of different extract of *H. sanjappae.*Track 1 to 12 indicates, HBA, HBB, HBC, HBD, HBE, HBF, HLA, HLB, HLC, HLD, HLE & HLF respectively. Chromatogram under A) 254 nm, B) 366 nm, C) 550 nm.

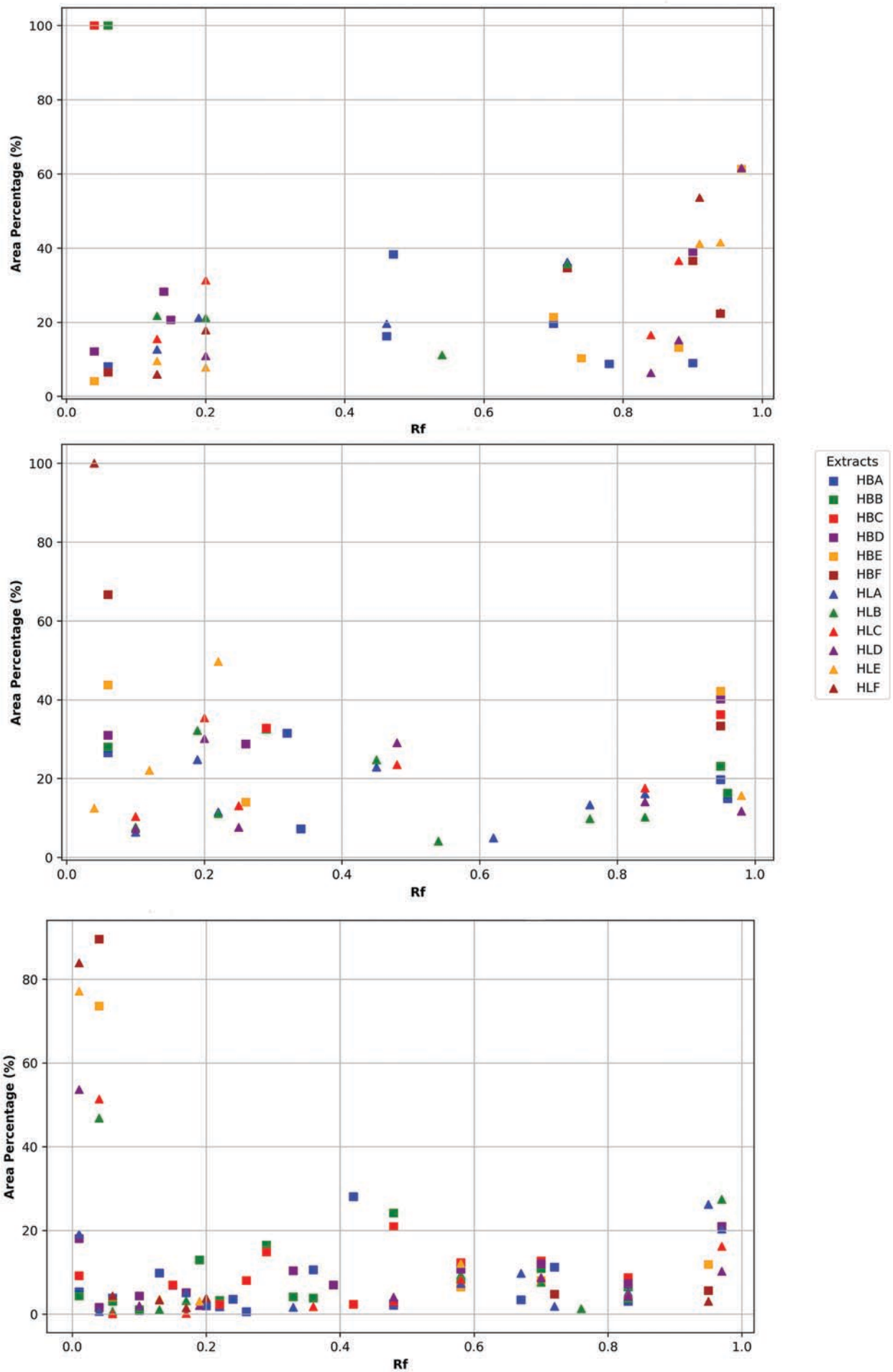

Figure 14: Comparison of Rf and area percentages at diferent detection wavelengths. A) at 254 nm, B) at 366 nm and C) at 550 nm.

The HPTLC evaluation showed that the extracts had substantially distinct phytochemical profiles. Certain extracts have shown different peaks with the same Rf values, while some extracts had a distinct peak itself. Under 254 nm UV light, Rf 0.54 was exclusively seen in HLB, but Rf 0.74 and 0.91 were alone detected in HBF. HBA exhibited a higher number of distinct peaks at different Rf values, specifically 0.47, 0.78, and 0.875. However, Rf 0.14 and 0.15 were identified just in HBD. Furthermore, the same Rf values were observed at varying area percentages in different extracts, indicating differences in compound abundance. For example, Rf 0.97 was observed in HBE, HBF, HLD, HLE, and HLF; however, its area percentage varied significantly across the extracts, registering 61.36, 22.33, 61.59, 41.53, and 22.61, respectively (Table 8-10).

Under visible light at 550 nm, HBD and HBA displayed separate bands at Rf 0.24 and 0.39, respectively. The common peaks in different extracts showed differences in area percentage. The total number of Rf values varies amongst the extracts, as seen in Figure 14. HBA and HLA extracts exhibit the highest total Rf values, indicating a higher level of chemical complexity. Conversely, the extracts of HBE and HBF have the lowest values, suggesting that there are fewer of the identified chemicals.

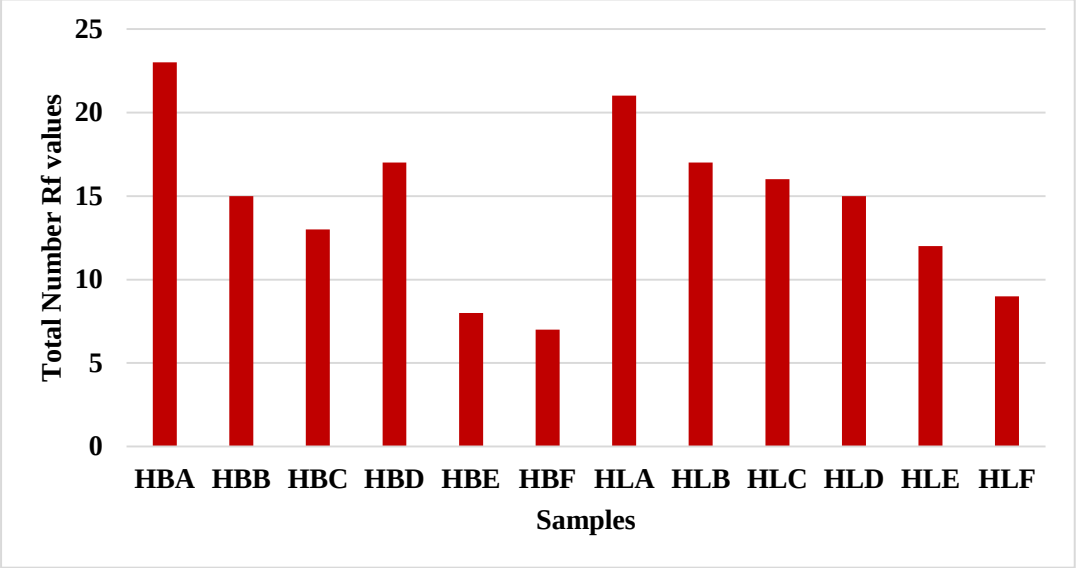

Figure 15: The graph illustrates the variation in the total number of Rf values among different extracts.

Table 8: Comparison of Maximum Rf and Area Percentage of different solvent extract of bark at 254 nm

Rf	Area Percentage (%)											
	HBA	**HBB**	**HBC**	**HBD**	**HBE**	**HBF**	**HLA**	**HLB**	**HLC**	**HLD**	**HLE**	**HLF**
0.04	-	-	100	12.13	4.13	-						
0.06	8.08	100	-	-	-	6.50						
0.13							12.64	21.72	15.51	5.95	9.53	5.93
0.14	-	-	-	28.26	-	-						
0.15	-	-	-	20.62	-	-						
0.19							21.20	-	-	-	-	-
0.20							-	21.2	31.32	10.93	7.76	17.84
0.46	16.23	-	-	-	-	-	19.57	-	-	-	-	-
0.47	38.31	-	-	-	-	-						
0.54							-	11.12	-	-	-	-
0.70	19.61	-	-	-	21.38	-						
0.72	-	-	-	-	-	34.59	36.27	35.73	-	-	-	-
0.74						10.33	-	-	-	-	-	
0.78	8.75	-	-	-	-	-						
0.84						-	-	16.58	6.32	-	-	
0.88	-	-	-	-	13.13	-	-	-	36.59	15.21	-	-
0.90	9.02	-	-	38.99	-	-	-	-	-	-	-	-
0.91	-	-	-	-	-	36.57	-	-	-	-	-	
0.94	-	-	-	-	-	-	-	-	-	-	41.18	53.62
0.97	-	-	-	-	61.36	22.33	-	-	-	61.59	41.53	22.61

Table 9: Comparison of Maximum Rf and Area Percentage of different solvent extract of bark at 366 nm

Rf	Area Percentage (%)											
	HBA	**HBB**	**HBC**	**HBD**	**HBE**	**HBF**	**HLA**	**HLB**	**HLC**	**HLD**	**HLE**	**HLF**
0.04	-	-	-	-	-	-	-	-	-	-	12.53	100
0.06	26.56	28.04	30.95	31.00	43.79	66.66	-	-	-	-	-	-
0.1	-	-	-	-	-	-	6.37	7.73	10.37	7.33	-	-
0.12	-	-	-	-	-	-	-	-	-	-	22.06	-
0.19	-	-	-	-	-	-	24.80	32.21	-	-	-	-
0.20	-	-	-	-	-	-	-	-	35.41	30.13	-	-
0.22	-	-	-	-	-	-	11.49	11.14	-	-	49.68	-
0.25	-	-	-	-	-	-	-	-	13.10	7.61	-	-
0.26	-	-	-	28.77	14.02	-	-	-	-	-	-	-
0.29	-	32.54	32.78	-	-	-	-	-	-	-	-	-
0.32	31.51	-	-	-	-	-	-	-	-	-	-	-
0.34	7.23	-	-	-	-	-	-	-	-	-	-	-
0.45	-	-	-	-	-	-	22.92	24.77	-	-	-	-
0.48	-	-	-	-	-	-	-	-	23.53	29.08	-	-
0.54	-	-	-	-	-	-	-	4.11	-	-	-	-
0.62	-	-	-	-	-	-	4.95	-	-	-	-	-
0.76	-	-	-	-	-	-	13.31	9.83	-	-	-	-
0.84	-	-	-	-	-	-	16.15	10.21	17.58	14.13	-	-
0.95	19.75	23.14	36.27	40.23	42.19	33.34	-	-	-	-	-	-
0.96	14.94	16.27	-	-	-	-	-	-	-	-	-	-
0.98	-	-	-	-	-	-	-	-	-	11.72	15.72	-

Table 10: Comparison of Maximum Rf and Area Percentage of different solvent extract of leaf and Bark at 550 nm

Rf	Area Percentage (%)											
	HBA	**HBB**	**HBC**	**HBD**	**HBE**	**HBF**	**HLA**	**HLB**	**HLC**	**HLD**	**HLE**	**HLF**
0.01	5.34	4.37	9.15	18.06	-	-	19.15	-	-	53.65	77.19	83.92
0.04	-	1.48	-	1.63	73.61	89.58	0.68	46.8	51.38	-	-	-
0.06	3.83	3.06	-	.	-	-	0.67	0.65	0.07	4.32	4.17	4.36
0.1	0.86	1.1	-	4.32	-	-	-	-	-	1.99	-	-
0.13	9.83	-	-	-	-	-	-	1.05	-	-	3.53	3.37
0.15	6.87	-	6.99	-	-	-	-	-	-	-	-	-
0.17	-	-	-	5.13	-	-	5.1	3.2	0.18	-	-	1.48
0.19	-	12.93	-	-	-	-	-	-	-	2.06	3.04	
0.2	2	-	-	-	-	-	-	-	-	-	-	3.82
0.22	1.79	3.29	2.36	-	-	-	-	-	-	-	-	-
0.24	3.58	-	-	-	-	-	-	-	-	-	-	-
0.26	0.56	-	8.04	-	-	-	-	-	-	-	-	-
0.29	-	16.51	14.87	-	-	-	-	-	-	-	-	-
0.33	-	4.13	-	10.41	-	-	1.62	-	-	-	-	-
0.36	10.57	3.87	-	.	-	-	-	-	1.76	-	-	-

0.39	-	-	-	6.98	-	-	-	-	-	-	-	-
0.42	28.1	-	2.39	.	-	-	-	-	-	-	-	-
0.48	-	24.16	20.88	2.48	-	-	2.15	-	3.08	4.06	-	-
0.58	-	7.78	12.28	10.74	6.49	-	7.32	9.49	8.34	10.77	12.07	-
0.6	-	-	-	-	-	-	-	-	-	-	-	-
0.67	3.39	-	-	-	-	-	9.76	-	-	-	-	-
0.7	.	10.89	12.71	12.02	8.02			7.59	8.74	8.65		
0.72	11.27	-	-	-	-	4.77	1.85	-	-	-	-	-
0.76		-	-	-	-	-	-	-	1.32	-	-	-
0.83	3.1	6.45	8.76	7.24	-	-	5.17	3.81	4.57	4.29		
0.95	-	-	-	-	-	-	26.21	-	-	-	-	3.06
0.97	-	-	-	21	11.87	5.65	20.33	27.41	16.21	10.22	-	-

4.1.1.4 Total Phenolic and Total Flavonoid Contents

The results show that the solvent makeup has a big effect on the total phenolic and flavonoid content of the different samples (Table 11, Figure 16).

Table 11: Total Phenolics and flavonoid content of different extracts

Samples	Total Phenolic Content (µg GAE/mg of Extract)	Total Flavonoid Content (µg QE/mg of Extract)
HBA	418.35± 3.64^{a}	355.76 ±6.85^{a}
HBB	378.77 ± 6.62^{c}	204.76 ± 6.10^{c}
HBC	411.51 ± 6.41^{ab}	278.14 ± 4.57^{b}
HBD	399.14 ± 4.63^{b}	361.25 ± 6.88^{a}
HBE	342.98 ± 5.69^{d}	191.13 ± 5.27^{c}
HBF	296.82 ± 5.95^{e}	201.57 ± 4.96^{c}
HLA	165.54 ± 5.53^{h}	130.96 ± 8.56^{e}
HLB	217.57 ± 6.46^{g}	149.77 ± 8.00^{d}
HLC	216.48 ± 6.99^{g}	157.83 ± 6.00^{d}
HLD	215.57± 1.68^{g}	113.81 ± 5.49^{e}
HLE	235.35 ± 5.42^{f}	154.32 ± 7.61^{d}
HLF	123.17 ± 5.28^{j}	78.11 ± 2.74^{f}

Data are represented as mean ± SD, Differences between the mean values. Values are statistically significant at $p < 0.05$. The values with different superscript letters represent significantly different from each other.

The highest overall phenolic content was found in HBA and HBC. The values were not very different from each other, but they were higher than those found in other samples. For TFC the highest values were found in HBA and HBD, which were not significantly different from each other

The results suggest that solvent composition has a substantial effect on TPC and TFC in *H. sanjappae* extracts, with bark (HBA, HBB, HBC, HBD, HBE, HBF) having greater values than leaves (HLA, HLB, HLC, HLD, HLE, HLF). Ethanol-rich solvents produced the most phenolic and flavonoid content, with ethanol alone (HBA) having the highest value. As the water ratio increased, phenolic and flavonoid content

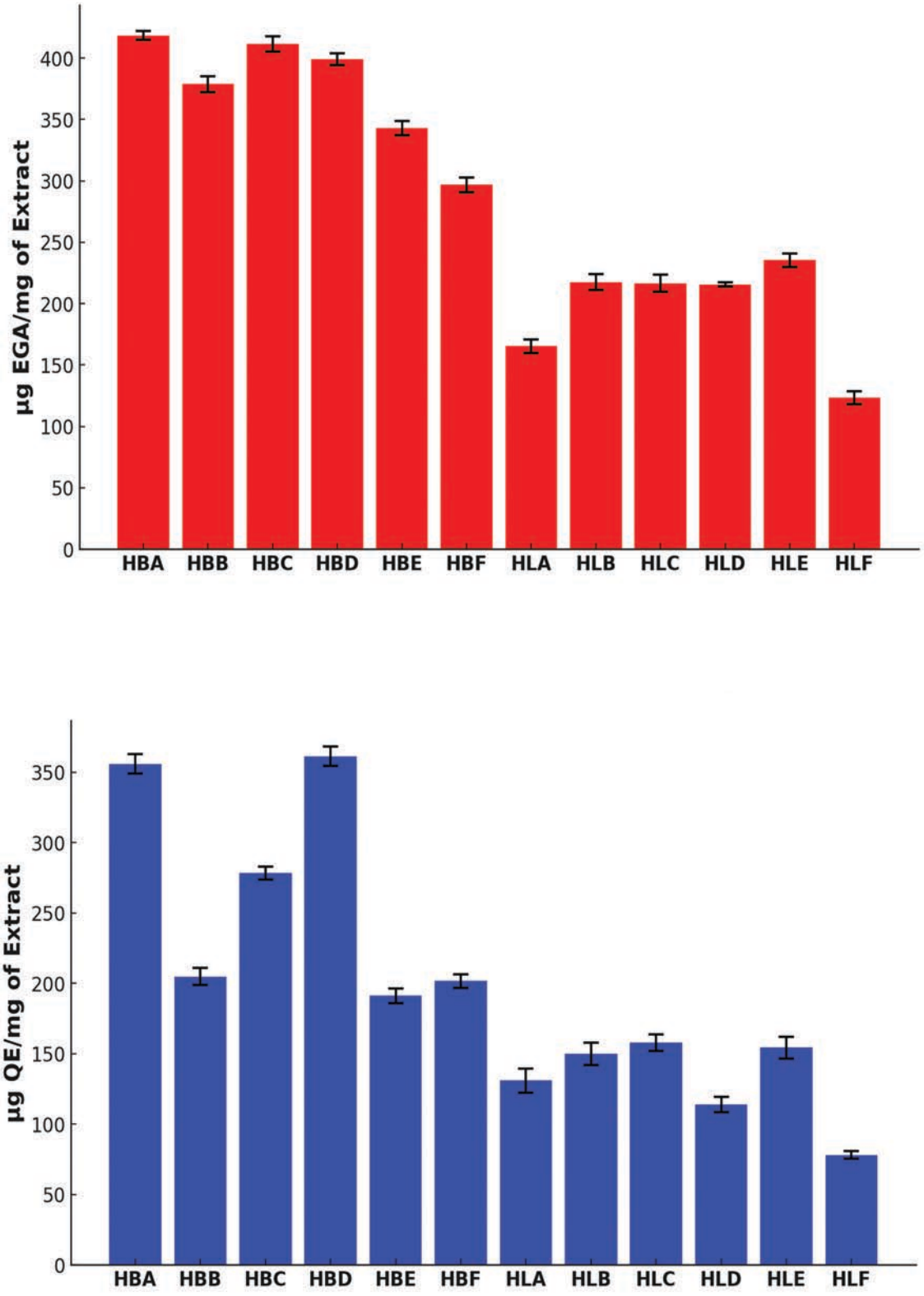

Figure 16: The bar graph indicate variations in phenolic and flavonoid concentrations across different samples. A) Total Phenolic Contents, B) Total Flavonoid Contents.

declined in most of the cases, with the lowest amounts seen in water-alone extracts (HLF). This shows that ethanol is more efficient at extracting phenolic and flavonoid contents from bark and leaves.

4.1.1.5 Antioxidant Potential of plant extracts

The DPPH and FRAP assay results demonstrate the antioxidant ability of various extracts. Lower IC_{50} and EC_{50} values indicate stronger antioxidant activity (Table 12, Figure 17). The antioxidant potential varies across all samples and bark extract consistently exhibited higher antioxidant levels than leaf extract. HBA and HBB showed the highest antioxidant activity, reflected by lowest IC_{50} values (6.53 ± 1.49 and 8.46 ± 1.38 µg/mL) and EC_{50} values (4.33 ± 1.65 and 3.85 ± 0.11 µg/mL). These results suggest that a higher ethanol content in the bark extracts enhances the extraction of antioxidant rich phytocompounds.

Table 12: Antioxidant potential of different extracts

Sample	DPPH activity IC_{50} (µg/mL)	FRAP activity EC_{50} (µg/mL)
HBA	6.53 ± 1.49^{h}	4.33 ± 1.65^{f}
HBB	8.46 ± 1.38^{h}	3.85 ± 0.11^{f}
HBC	35.48 ± 1.93^{ef}	4.40 ± 0.27^{f}
HBD	30.3 ± 3.06^{fg}	4.12 ± 0.06^{f}
HBE	14.44 ± 1.69^{gh}	5.93 ± 0.30^{f}
HBF	184.43 ± 19.40^{b}	19.41 ± 1.02^{b}
HLA	40.88 ± 1.30^{de}	16.41 ± 0.80^{c}
HLB	50.6 ± 3.47^{de}	10.98 ± 0.91^{de}
HLC	46.03 ± 2.60^{def}	13.66 ± 0.58^{d}
HLD	58.88 ± 2.08^{cd}	11.57 ± 0.56^{de}
HLE	71.65 ± 6.93^{c}	10.23 ± 0.42^{e}
HLF	252.25 ± 2.63^{a}	26.53 ± 2.00^{a}

Data are represented as mean ± SD, Differences between the mean values. Values are statistically significant at $p < 0.05$. The values with different superscript letters represent significantly different from each other.

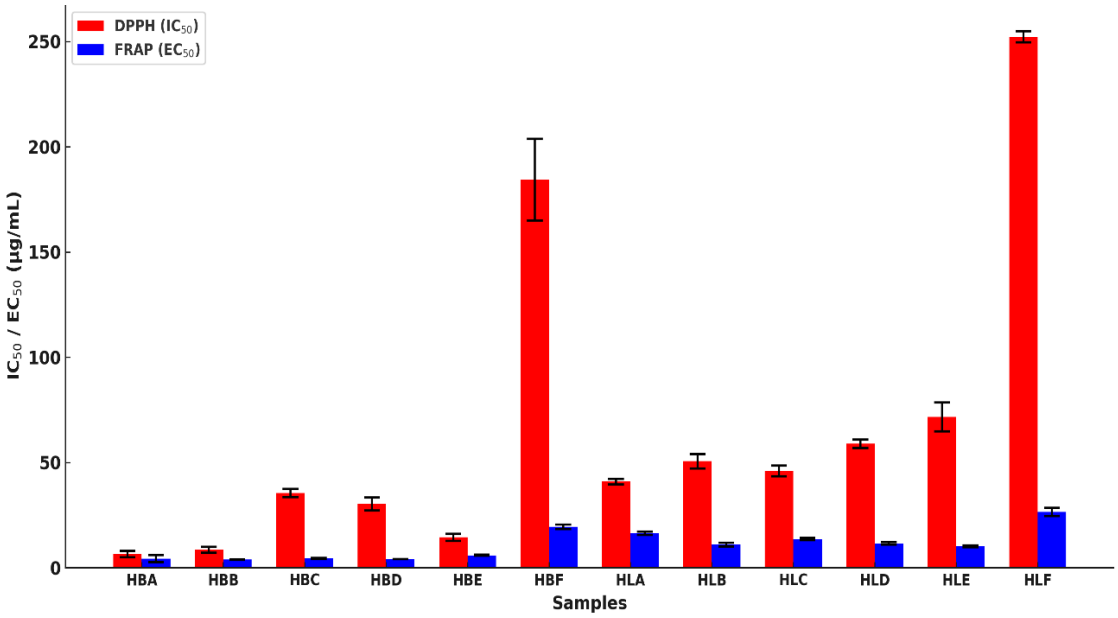

Figure 17: Antioxidant potential of different samples expressed in µg/mL.

4.1.2 Discussion

In metabolomics, it's important to choose the right extraction solvent and prepare the samples correctly to get a wide range of metabolites. The field mostly uses two methods: targeted metabolomics, which quantifies specific metabolites with optimized sample preparation, and untargeted metabolomics, which tries to find a wide range of metabolites without identifying exactly what the compounds are (Patti, 2011; Sheridan *et al.*, 2012).

This study analyzed the plant extracts in both ways. The untargeted strategy employed HPTLC, which discovered distinct bands representing various metabolites without prior knowledge of their compound characteristics. HPTLC is frequently used to create fingerprint profiles of plant extracts (Annegowda *et al.*, 2012). It is often preferred over other chromatographic techniques due to its ease of use, ability to process multiple samples simultaneously on a single plate, high throughput, and cost-effectiveness, making it a fast and efficient analytical method This approach was specifically used to analyze various solvent extracts of *H. sanjappae* for a comprehensive metabolic profile.

Specifically, this method was applied to examine different solvent extracts of *H. sanjappae* in order to obtain a thorough metabolic profile. Untargeted HPTLC

analysis has been used in a number of studies in the past to distinguish between different extracts. The study by Shawky and Selim (2017) used HPTLC to examine the effects of different extraction solvents and plant parts on the chemical composition of *Astragalus spinosus.* The study found that the metabolite composition of plant extracts is significantly influenced by the solvent's characteristics. Additionally, Nigella sativa oils from Egypt, Ethiopia, and Syria were Analyzed by Shawky *et al.* (2018) using HPTLC fingerprinting, showing variations in chemical composition and bioactivities according to geographic origin. From an untargeted strategy perspective, HPTLC served as an effective tool, which explored the chemical diversity of 12 *H. sanjappae* extracts. It allowed for analysis without prior knowledge of about any specific target compounds. This method enabled the identification of a wide range of metabolites.

In contrast, the targeted strategy was used for *H. sanjappae* extracts by assessing total TPC, TFC, and antioxidant activity using DPPH and FRAP tests. This allowed for the accurate characterisation of bioactive substances and their contribution to antioxidant activity. The selection of solvent is crucial when extracting polyphenols from botanical sources. Polar solvents, including water and methanol, are frequently utilised due to their superior ability to dissolve polar polyphenolic substances. Polyphenols often possess hydroxyl groups (-OH) linked to aromatic rings, rendering them hydrophilic (water-soluble) and appropriate for extracting using polar solvents (Belščak-Cvitanović *et al.*, 2018; Vuolo *et al.*, 2019).

Aqueous-alcohol has been proposed as a suitable solvent for the extraction of polyphenols from *Phoradendron californicum* oak extracts. Numerous studies indicate that water is effective in extracting polyphenolic chemicals from plants (Iloki-Assanga *et al.*, 2015; Reis *et al.*, 2012). But in another study conducted by Wang and Dai (2012) examined the total phenolic and total flavonoid content of the water and alcohol extracts of ten herbs and found that phenolic components were extracted more strongly in the ethanol extract than in the water extract. The results align well with our study, where the TPC and total TFC were higher in ethanol extracts compared to water extracts.

The findings seem positive and consistent with our research, which showed that ethanol extracts had higher levels of total TFC and TPC than water extracts. Studies have shown that the polarity of the solvent has a significant effect on the extract yield and antioxidant activity of phenolic compounds in plant material

(Ghaffar & Perveen, 2024; Muhamad *et al.*, 2014). Plant-based antioxidants, especially polyphenolic compounds, have gained importance due to potential health benefits. Antioxidants are essential compounds that help shield living organisms from oxidative stress caused by free radicals (Rudrapal *et al.*, 2022).

A correlation analysis was undertaken to determine the relationship between the plant's TPC, TFC, and antioxidant capacity. The results indicate that the concentration of phenolics and flavonoids are contributing to the antioxidant capacity of different extract of *H. sanjappae* (Figure 1). Previous research has also confirmed that phenolic compounds in medicinal plants play a significant role in their antioxidant capacity (Wang & Dai, 2012).

4.1.3 Conclusion

The impact of extraction solvents and plant components on the phytochemical profile and antioxidant potency of *H. sanjappae* were evaluated in this study. The findings clearly demonstrate that the selection of solvent and the choice of plant organ greatly impact the bioactive chemicals. Of the various ethanol-water solvent systems evaluated, ethanol had the best efficacy, resulting in the greatest quantities of phenolics content and flavonoids. Furthermore, the ethanol extract made up of bark (HBA) demonstrated a more superior phytochemical composition and enhanced antioxidant activity compared to all other leaf extract, indicating that the bark serves as a more abundant source of bioactive compounds.

The untargeted HPTLC fingerprinting method corroborated these findings by demonstrating significant chemical differences among the extracts, with unique HPTLC bands in HBA as well as highest number of Rf values were represented by the same extract compared to all other extrcats, hence supporting its greater metabolite diversity. The good positive connection among TPC, TFC, and antioxidant activity underscores the significance of polyphenols and flavonoids in the antioxidant capacity of *H. sanjappae* extracts.

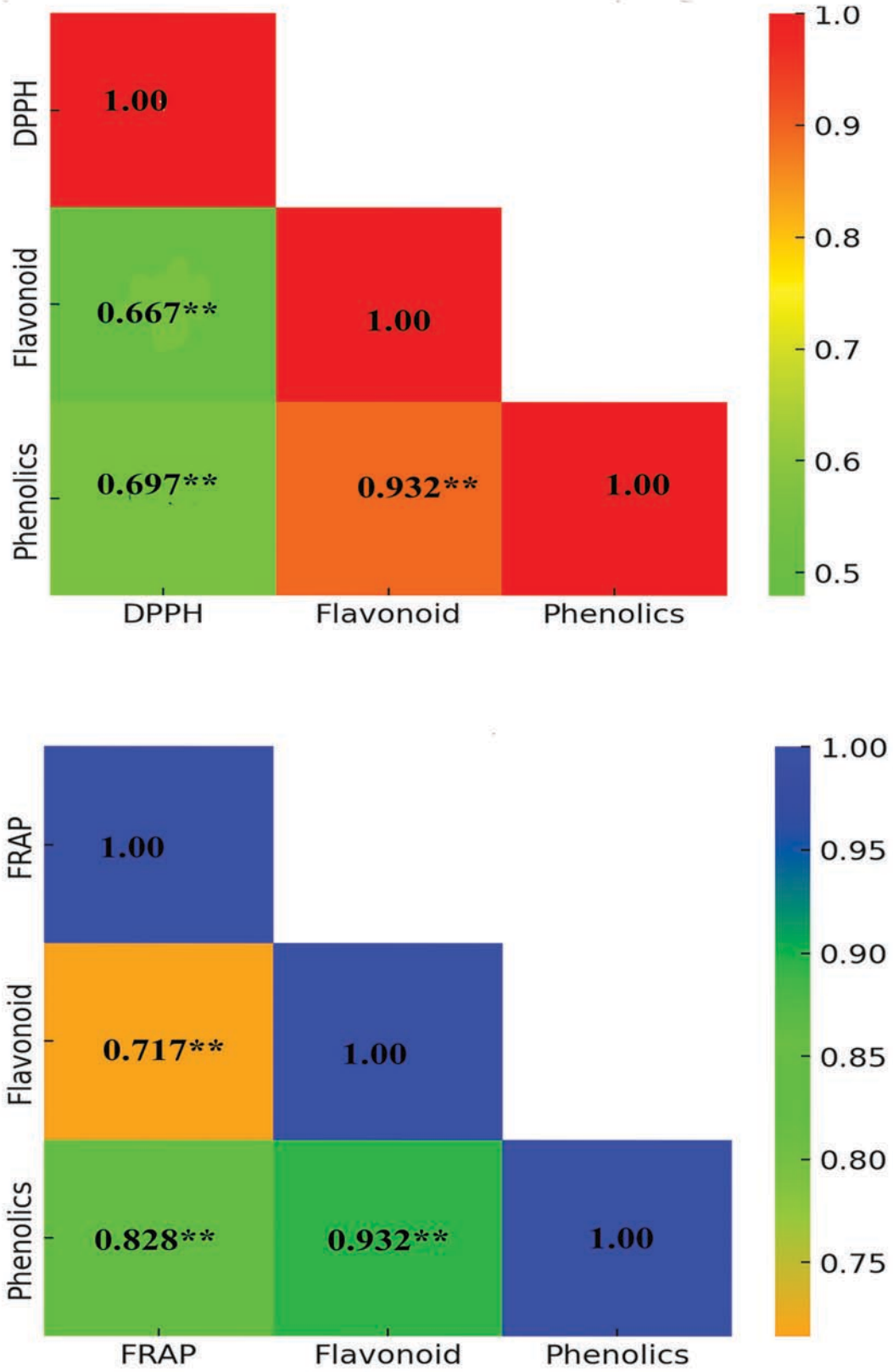

Figure 18: Correlation heatmaps. A) Correlation between DPPH radical scavenging activity, Total Flavonoid Content, and Total Phenolic Content. B) Correlation between FRAP antioxidant activity, total flavonoid content, and total phenolic content.

4.2 Comprehensive Analysis of Bioactive Compounds from ethanol extract of *Humboldta sanjappae* bark Using LC-MS/MS Analysis, ADMET Based Pharmacokinetics Prediction and *In vivo* Acute Oral Toxicity Assessment.

Many plant extracts are widely used in traditional medicine despite the lack of comprehensive safety evaluations. Many supporters of herbal remedies assert that products with a long history of widespread use are generally safe when used correctly at standard therapeutic doses (Fong, 2002). Still, the use of the plant or plant product as a medicine carries potential concerns about hepatotoxicity, nephrotoxicity, allergic responses, and interactions with other medications (Moreira *et al.*, 2014). Consequently, merging traditional knowledge with present pharmacological research is essential to guarantee the efficacy and safety of plant medicines.

Modern analytical techniques such as Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) allow for the precise identification of compounds from plant extracts. It is an efficient method for the precise and accurate identification of various metabolites in plants. Its broad utilisation in metabolomic research is due to its high-throughput capacity, diverse ionisation techniques, and capacity for comprehensive metabolite profiling (Sarkar *et al.*, 2025). Conventional methods for screening plant extract to understand their safety profile are time-consuming, expensive and necessitate more advanced analytical laboratories and the findings do not always correlate well between animal models and humans. Computational techniques such as network pharmacology, QSAR modeling, machine learning, and molecular docking are valuable in this context because they can identify potential targets of natural compounds, facilitating experimental validation efforts (Agu *et al.*, 2023). The integration of *in silico* ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) analysis using tools like pkCSM alongside *in vivo* acute oral toxicity studies provides a robust approach for assessing compound potential as a drug. The interplay between pharmacokinetics, toxicity, and potency is essential for the development of effective drugs. ADMET and toxicity studies provides scientific validation for the safer use of natural products, bridging the gap between ethnobotanical claims and modern pharmacological evidence (Chandrasekaran *et al.*, 2018; Patil, 2016). Hence, *in silico* approaches help to reduce the need of expensive and time-consuming laboratory experiments.

The current study focuses on the characterization of the phytochemical components of HBA through LC-MS/MS analysis, and it employs an *in silico* approach to evaluate the pharmacokinetic properties and toxicity of the identified plant metabolite. Figure 19 explains the work flow of the study. This study presents the most extensive LC-MS/MS-based metabolite profiling of *H. sanjappae* to date. To the best of our knowledge, no prior study has reported *in silico* methods to explore the pharmacokinetic characteristics and toxicity profile of its metabolites. Furthermore, *in vivo* acute oral toxicity studies were conducted to provide a critical evaluation of the safety and therapeutic window, effectively bridging the gap between computational predictions and practical applications.

4.2.1 Results

4.2.1.1 LC-MS/MS analysis and identified compounds

The LC-MS/MS analysis identified 57 phytochemicals belonging to 14 primary categories (Table 13, Figure 20 & 21). Flavones constituted the primary class, comprising 21.05% of the total compounds, followed by phenols and catechins, each with 14.035% (Figure 22). Our study aligns with previous research from this family; however, no prior studies on this genus have reported such a comprehensive metabolite analysis. *Cassia glauca*, a member of the Fabaceae family, is a well-documented medicinal plant known for its diverse phytochemical constituents. Various bioactive compounds have been identified in this species, including Torososide B, different forms of Cinnamtannin, kaempferol derivatives, and epicatechin-derived compounds (Dwivedi *et al.*, 2021). These findings align with our study, which indicates that *H. sanjappae* also contains Torososide B, along with various forms of Cinnamtannins (A1, A2, A3, and D1). Kaempferol 3-O-feruloyl-caffeoyl-sophoroside 7-O-glucoside is also present in *H. sanjappae*. Additionally, it has been shown to contain (-)-Epicatechin and its derivatives, such as (-)-Epicatechin-3'-O-glucuronide and 4'-O-methyl-(-)-Epicatechin-3'-O-β-glucuronide. UHPLC-HRMS/MS analysis of five *Copaifera* species from Fabaceae revealed flavonoids like luteolin, 3′,4′,7-trihydroxyflavanone, apigenin, and catechins. Similarly, our study confirms *H. sanjappae* also contains apigenin, hydroxyflavanones, and catechins,

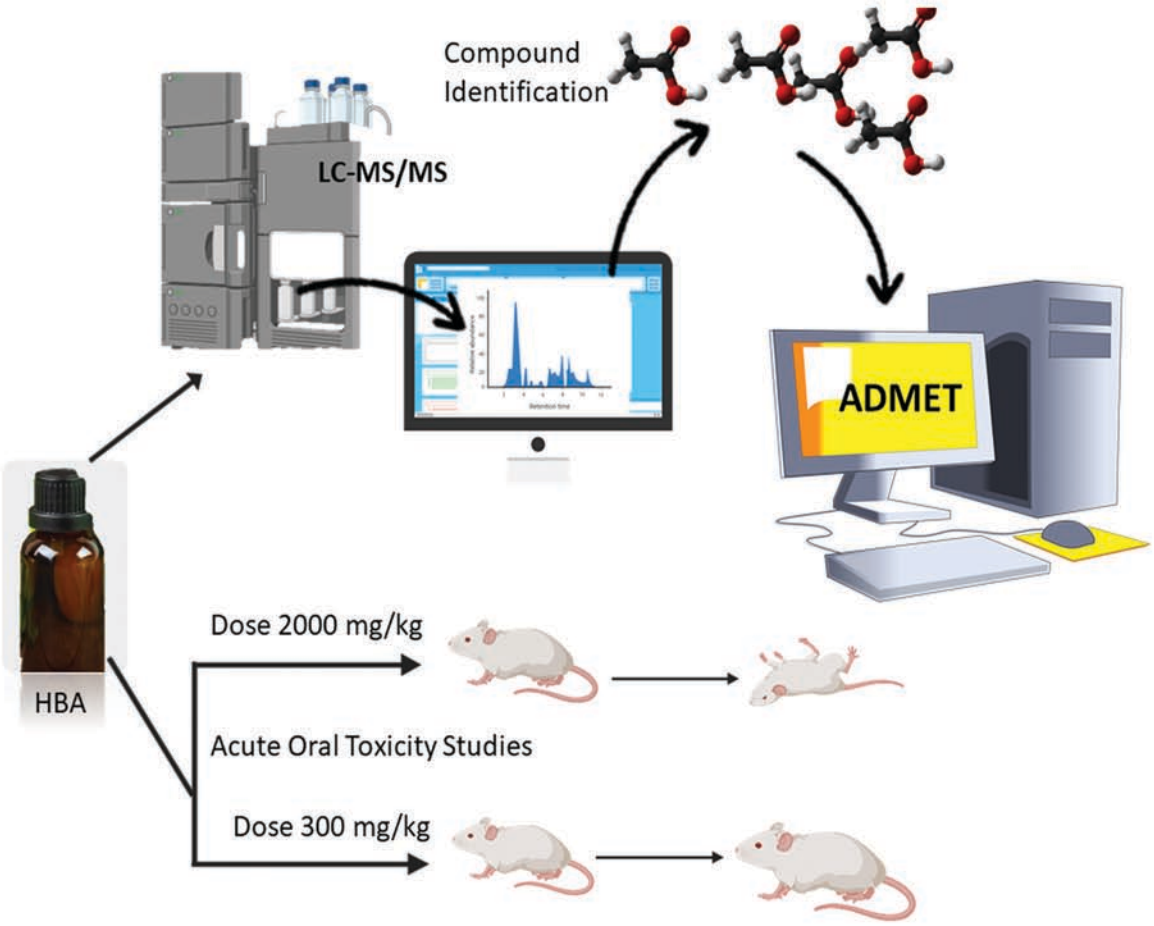

Figure 19: Illustartion represents the work flow of LC-MS/MS analysis, ADMET evaluation and Acute oral toxicity studies.

reinforcing phytochemical similarities within Fabaceae and their pharmacological potential (da Silva *et al.*, 2021).

Based on previous reports, the identified chemicals exhibited a diverse range of biological activities, highlighting the medicinal potential of the plant extract. Morusin, Procyanidin B2, and Neoeriocitrin demonstrated substantial anticancer, anti-inflammatory, and antidiabetic properties (Aldemir *et al.*, 2022; Gupta *et al.*, 2014; Li *et al.*, 2023; Wang *et al.*, 2017). Other phytocompounds, including Herniarin and Eudesmin, exhibited neuroprotective characteristics, positioning them as possible options for therapeutics targeting neurological disorders (Castillo *et al.*, 2023; Mirzavi *et al.*, 2024). Other compounds detected through LC-MS/MS analysis shown promising biological activities are listed in Table 14.

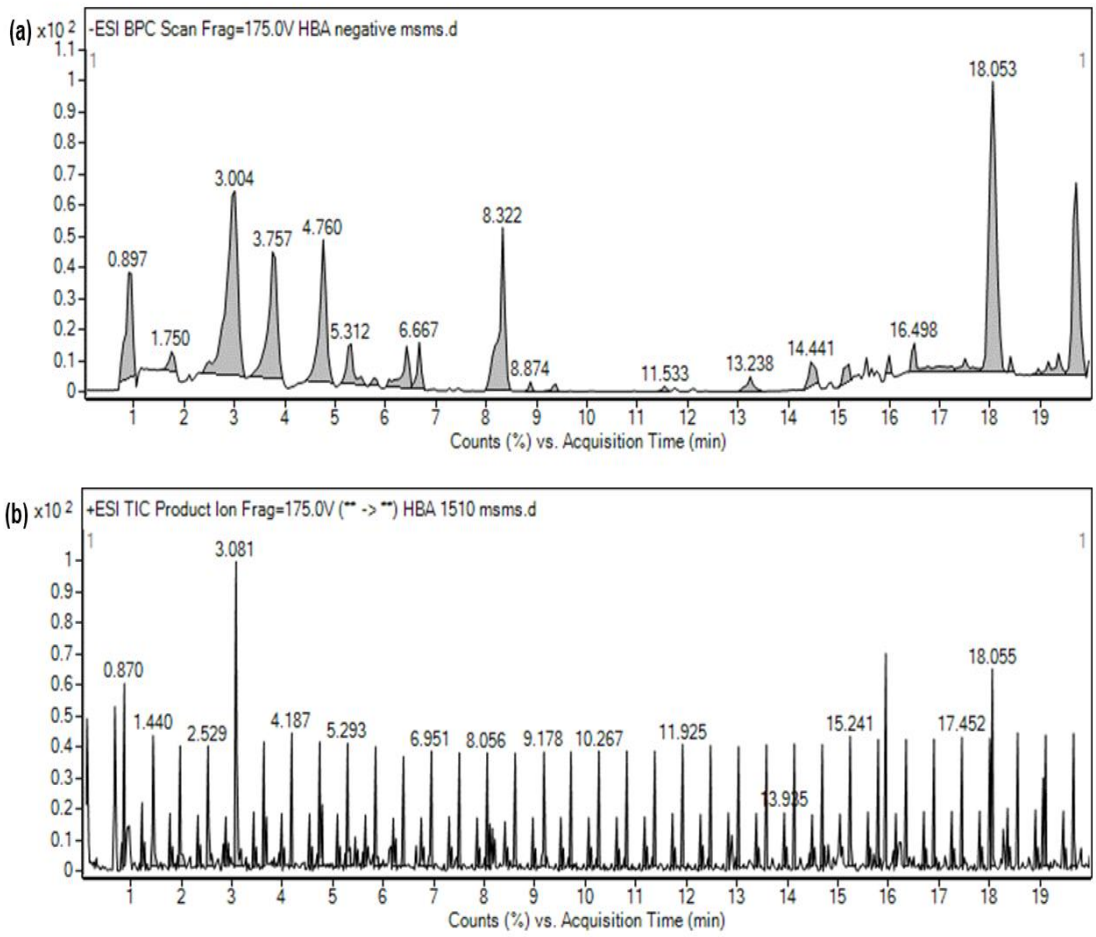

Figure 20: Total ion chromatogram by LC-MS/MS of the ethanolic bark extract of *H. sanjappae* a) negative ion mode and b) positive ion mode.

Table 13: Identified Phytochemicals in ethanolic extract of *H. sanjappae* bark detected by LC-MS/MS.

Sl.No	Tentative identification	Class of compound	Ionization mode	m/z	Mass	MS/MS	Molecular formula	RT
1.	**Cinnamtannin A1**	Tannin	Negative (M-H)-	865.2025	866.2096	696.1439 525.0919 289.0745 125.0251	C_{45} H_{38} O_{18}	0.930
2.	**Cinnamtannin A1**	Tannin	Negative (M-H)-	865.2025	866.2096	696.1439 525.0919 289.0745 125.0251	C_{45} H_{38} O_{18}	0.930
3.	**Trichotomine**	Alkaloid	Negative (M+HCOO)-	577.1378	532.1397	125.0246 289.0730	C_{30} $H2_0$ N_4 O_6	2.970
4.	**Kaempferol 3-O-feruloylcaffeoyl-sophoroside 7-O-glucoside**	Flavonoid glycoside	Negative (M+HCOO)-	1155.2806	1110.2828	577.1394 407.0782 125.0247	C_{52} H_{54} O_{27}	3.062
5.	**Cinnamtannin A2**	Tannin	Negative (M-H)-3	1153.2648	1154.2723	832.1789 577.1335 407.0783 125.0251	C_{60} H_{50} O_{24}	3.664
6.	**(-)-Epicatechin**	Catechins	Negative (M-H)-	289.0718	290.0805	271.0633 245.0828 221.0826 203.0720	C_{15} H_{14} O_6	3.874

						179.0353 151.0403 137.0247 125.0243 109.0296		
7.	**Neoeriocitrin**	Flavanone glycoside	Negative (M-H)-	595.1685	596.1754	476.1302 269.0419 125.0251	C_{27} H_{32} O_{15}	4.141
8.	**Cinnamtannin A3**	Tannin	Negative (M-H)-	1441.3301	1442.3374	865.1966 575.1190 407.0789 287.0578 125.0244	C_{75} H_{62} O_{30}	5.622
9.	**Morusin**	Flavones	Negative (M+HCOO)-	465.1560	420.1556	334.8392 151.0371	C_{25} H_{24} O_{6}	6.090
10.	**Physagulin F**	Steroid lactone	Negative (M-H)-	543.2608	544.2647	420.4758 328.5773 123.0841	C_{30} H_{40} O_{9}	7.352
11.	**Cinnamtannin D1**	Tannin	Negative (M-H)-	863.1872	864.1945	737.1504 540.0944 423.0721 289.0737 125.0217	C_{45} H_{36} O_{18}	7.754
12.	**Gambiriin B3**	Catechins	Negative (M-H)-	561.1409	562.1499	289.0674 125.0218	C_{30} H_{26} O_{11}	7.837
13.	**trans-delta-Viniferin 3''-glucoside**	Stilbenes	Negative (M-H)-	615.1887	616.1936	435.0798 271.0986 125.0246	C_{34} H_{32} O_{11}	8.891
14.	**Lonchocarpenin**	Chalcones	Negative (M+HC-	493.1870	448.1878	428.2492 227.1000	C_{27} H_{28} O_{6}	9.794

						143.0328		
15.	**Luteolin**	Flavones	Negative (M-H)-	285.0405	286.0499	241.0461 217.0461 193.8618 176.0419 162.8408 150.0365 133.0276 107.0124	$C_{15}H_{10}O_6$	10.170
16.	**Apigenin**	Flavones	Negative (M-H)-	269.0455	270.0555	225.0561 201.0576 181.0647 151.0049 117.0350 107.0142	$C_{15}H_{10}O_5$	11.549
17.	**Genkwanin**	Flavones	Negative (M-H)-	283.0612	284.0709	283.0622 268.0396 250.8749 240.0467 224.0490 212.0469 200.0484 191.8598 160.8412 135.0090 125.0009	$C_{16}H_{12}O_5$	11.758
18.	**Diosmetin**	Flavones	Negative (M-H)-	299.0561	300.0662	284.0346 256.0392	$C_{16}H_{12}O_6$	12.084
19.	**Formononetin**	Flavones	Negative (M-H)-	267.0663	268.0757	252.0440 223.0383 195.0468	$C_{16}H_{12}O_4$	13.221

						170.0091 132.0201		
20.	**Biochanin A**	Flavones	Negative (M-H)-	283.0612	284.0710	268.0394 250.8721 239.0351 211.0426 191.8612 132.0206	C_{16} H_{12} O_5	15.854
21.	**Dihydrocaffeic acid 3-Oglucuronide**	Phenol	Positive $(M^+Na)^+$	381.0788	358.0898	307.1287 175.1132	C_{15} H_{18} O_{10}	0.837
22.	**(-)-epicatechin-3'-Oglucuronide**	Catechins	Positive $(M^+H)^+$	453.1382	452.1304	273.0731 139.0392	C_{21} H_{24} O_{11}	1.540
23.	**Verbasoside**	Glycoside	Positive $(M^+Na)^+$	485.1615	462.1722	339.1049	C_{20} H_{30} O_{12}	2.244
24.	**4'-O-methyl-(-)-epicatechin-3'-O-betaglucuronide**	Catechins	Positive $(M^+Na)^+$	517.1301	494.1407	387.1436 185.0768	C_{23} H_{26} O_{12}	2.780
25.	**Gravolenic acid**	Glycoside	Positive $(M^+H)^+$	281.1022	280.0947	221.0808 135.0430	C_{14} H_{16} O_6	2.796
26.	**Vanilloloside**	Phenol	Positive $(M^+H)^+$	317.1227	316.1157	263.0914 194.0568	C_{14} H_{20} O_8	2.847
27.	**Procyanidin B5**	Catechins	Positive $(M^+H)^+$	579.1475	578.1403	409.0905 287.0542 127.0395	C_{30} H_{26} O_{12}	3.081
28.	**Gambiriin A3**	Catechins	Positive $(M^+Na)^+$	603.1450	580.1556	451.0889 313.0677 245.0404 139.0414	C_{30} H_{28} O_{12}	3.852
29.	**2''-o-p-Coumaroylaloesin**	Glycoside	Positive $(M^+Na)^+$	563.1517	540.1627	476.8778 393.0904	C_{28} H_{28} O_{11}	4.806

						287.0536 231.0638 139.0402		
30.	**Procyanidin B2**	Catechins	Positive $(M^+H)^+$	579.1497	578.1395	409.0900 287.0539 127.0390	$C_{30}H_{26}O_{12}$	5.309
31.	**3',4',5,7-Tetrahydroxyisoflavanone**	Flavones	Positive $(M^+H)^+$	289.0703	288.0630	215.0704 139.0389	$C_{15}H_{12}O_6$	6.063
32.	**Phenethyl rutinoside**	Glycoside	Positive $(M^+NH4)^+$	448.2162	430.1823	320.0514 220.1677 129.0553	$C_{20}H_{30}O_{10}$	6.264
33.	**Fustin**	Flavones	Positive $(M^+H)^+$	289.0705	288.0634	261.0749 139.0390	$C_{15}H_{12}O_6$	6.766
34.	**Eupatoriochromene**	Benzopyran	Positive $(M^+H)^+$	219.1017	218.0944	161.0950	$C_{13}H_{14}O_3$	7.420
35.	**Glaucarubol 15-O-beta-D-glucopyranoside**	Quassinoid	Positive $(M^+Na)^+$	581.2211	558.2315	339.1218 273.1112 215.0682 129.0549	$C_{26}H_{38}O_{13}$	8.877
36.	**(-)-Citronellol**	Terpenoid	Positive $(M^+NH4)^+$	174.1853	156.1515	117.0706	$C_{10}H_{20}O$	8.927
37.	**Deoxyshikonin**	Terpenoid	Positive $(M^+H)^+$	273.1129	272.1059	213.0937 121.0642	$C_{16}H_{16}O_4$	8.977
38.	**Proanthocyanidin A2**	Catechins	Positive $(M^+H)^+$	577.1313	576.1266	425.0845 379.0845 163.0383	$C_{30}H_{24}O_{12}$	9.279
39.	**Graminiliatrin**	Terpenoid	Positive $(M^+H)^+$	435.1639	434.1567	325.0644 273.1119 149.0582	$C_{22}H_{26}O_9$	9.430
40.	**Tectorigenin**	Flavones	Positive	301.0706	300.0633	229.0511	$C_{16}H_{12}O_6$	11.389

			$(M^+H)^+$					
41.	**Tecostanine**	Alkaloid	Positive $(M^+H)^+$	184.1697	183.1624	102.0923	$C_{11}H_{21}NO$	12.528
42.	**Cajanin**	Flavones	Positive $(M^+H)^+$	301.0703	300.0631	153.0191	$C_{16}H_{12}O$	13.232
43.	**Eudesobovatol A**		Positive $(M^+H)^+$	505.3325	504.3250	505.3319	$C_{33}H_{44}O_4$	13.449
44.	**Sinapoylspermine**	Phenol	Positive $(M^+Na)^+$	431.2610	408.2720	328.1713 127.0389	$C_{21}H_{36}N_4O_4$	13.801
45.	**5,7-Dihydroxy-8-prenylflavone**	Flavones	Positive $(M^+H)^+$	323.1271	322.1201	135.0442	$C_{20}H_{18}O_4$	14.739
46.	**Sophoraflavanone B**	Flavanone glycoside	Positive $(M^+H)^+$	341.1381	340.1310	263.1047 135.0444	$C_{20}H_{20}O_5$	14.806
47.	**Ursiniolide A**	Terpenoid	Positive $(M^+H)^+$	405.1896	404.1824	201.0907	$C_{22}H_{28}O_7$	14.990
48.	**Eudesmin**		Positive $(M^+H)^+$	387.1788	386.1717	181.0859 107.0496	$C_{22}H_{26}O_6$	15.057
49.	**Mexicanin E**	Terpenoid	Positive $(M^+H)^+$	233.1173	232.1100	173.0953	$C_{14}H_{16}O_3$	15.107
50.	**Kadsurenone**	Phenol	Positive $(M^+H)^+$	357.1692	356.1629	123.0445	$C_{21}H_{24}O_5$	15.191
51.	**Herniarin**	Glycoside	Positive $(M^+H)^+$	177.0549	176.0478	149.0233	$C_{10}H_8O_3$	15.208
52.	**Indicaxanthin**	Betalains	Positive $(M^+NH_4)^+$	327.1410	309.1075	281.1354 133.0835	$C_{14}H_{17}N_2O_6$	15.292
53.	**Torososide B**	Terpenoid	Positive $(M^+H)^+$	933.2894	932.2821	763.2422 644.2233 533.1763 371.1435 271.0601	$C_{40}H_{52}O_{25}$	15.560

						135.0462		
54.	**Picrasin B**	Terpenoid	Positive $(M^+H)^+$	377.1953	376.1884	153.0545	C_{21} H_{28} O_6	16.464
55.	**Arbutin**	Glycoside	Positive $(M^+H)^+$	273.0967	272.0897	273.1666 129.0185	C_{12} H_{16} O_7	18.424
56.	**Sesamin**	Phenol	Positive $(M^+H)^+$	355.1187	354.1117	135.0445	C_{20} H_{18} O_6	18.507
57.	**Valerianine**	Pyridine derivative	Positive $(M^+H)^+$	178.1228	177.1155	119.0500	C_{11} H_{15} N O	19.227
58.	**Kanzonol P**	Phenol	Positive $(M^+H)^+$	369.1684	368.1615	135.0443	C_{22} H_{24} O_5	19.680

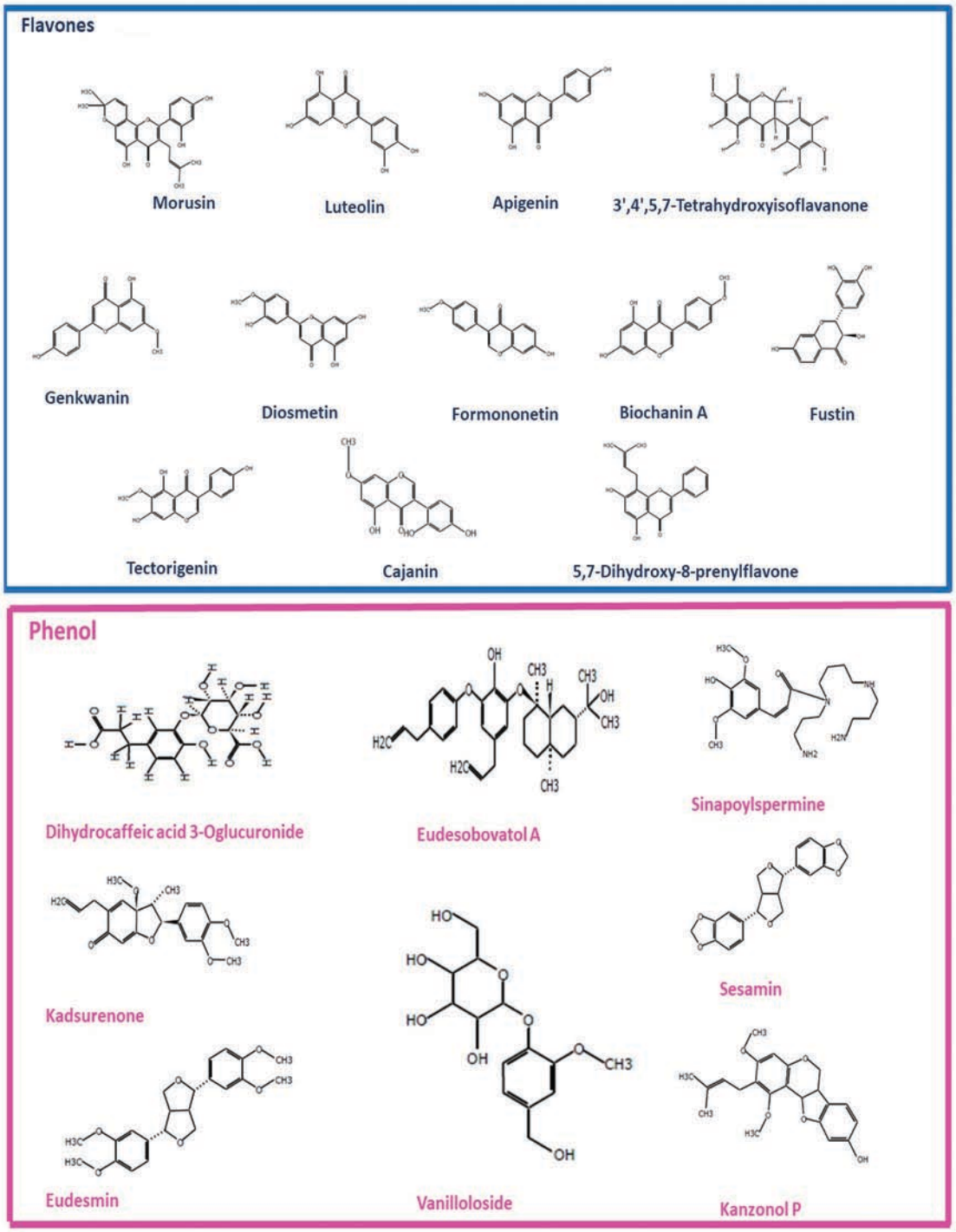

Figure 21a: Phytochemicals belonging Flavones and Phenols.

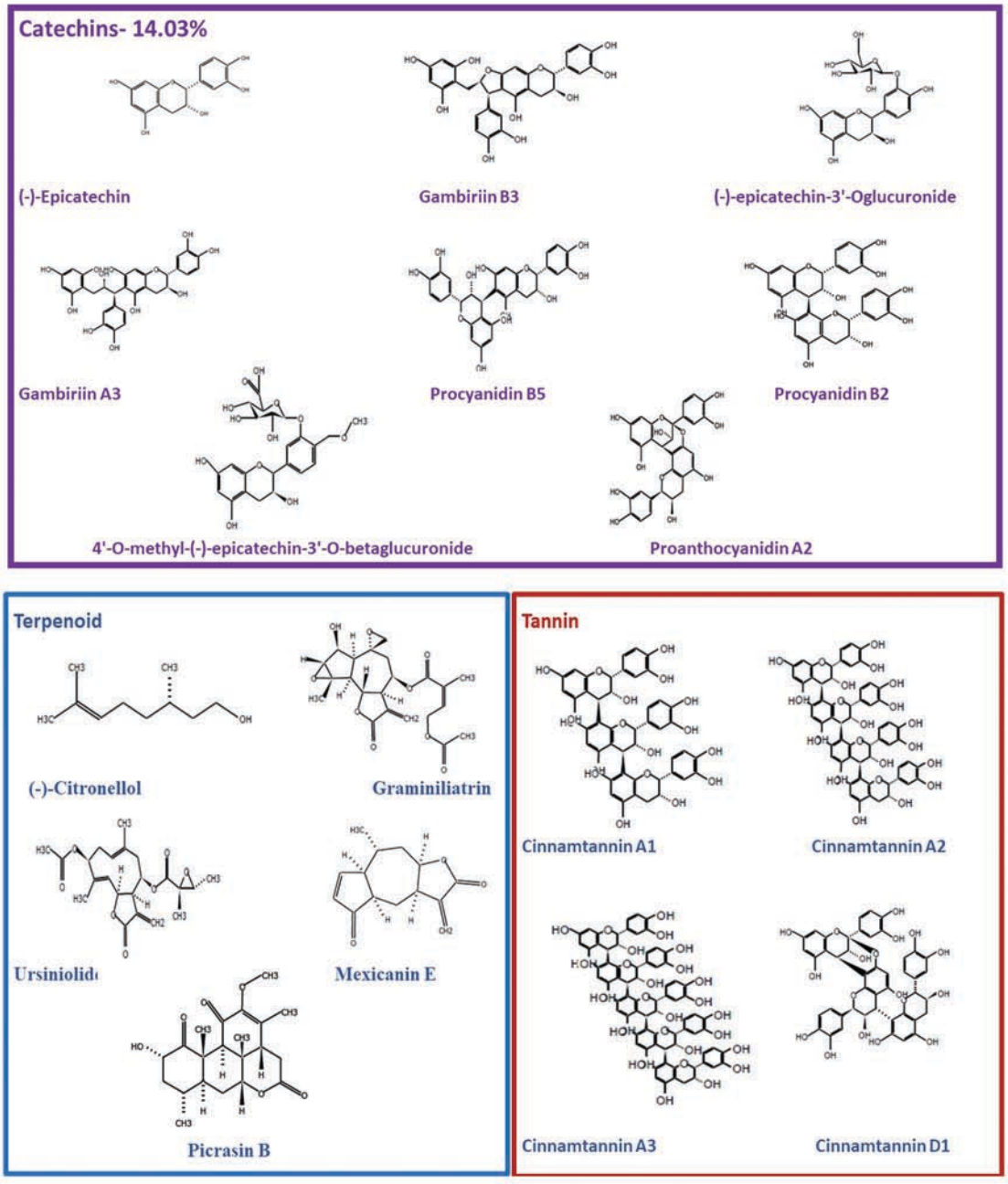

Figure 21b: Phytochemicals belonging Catechins, Terpenoid and Tannin.

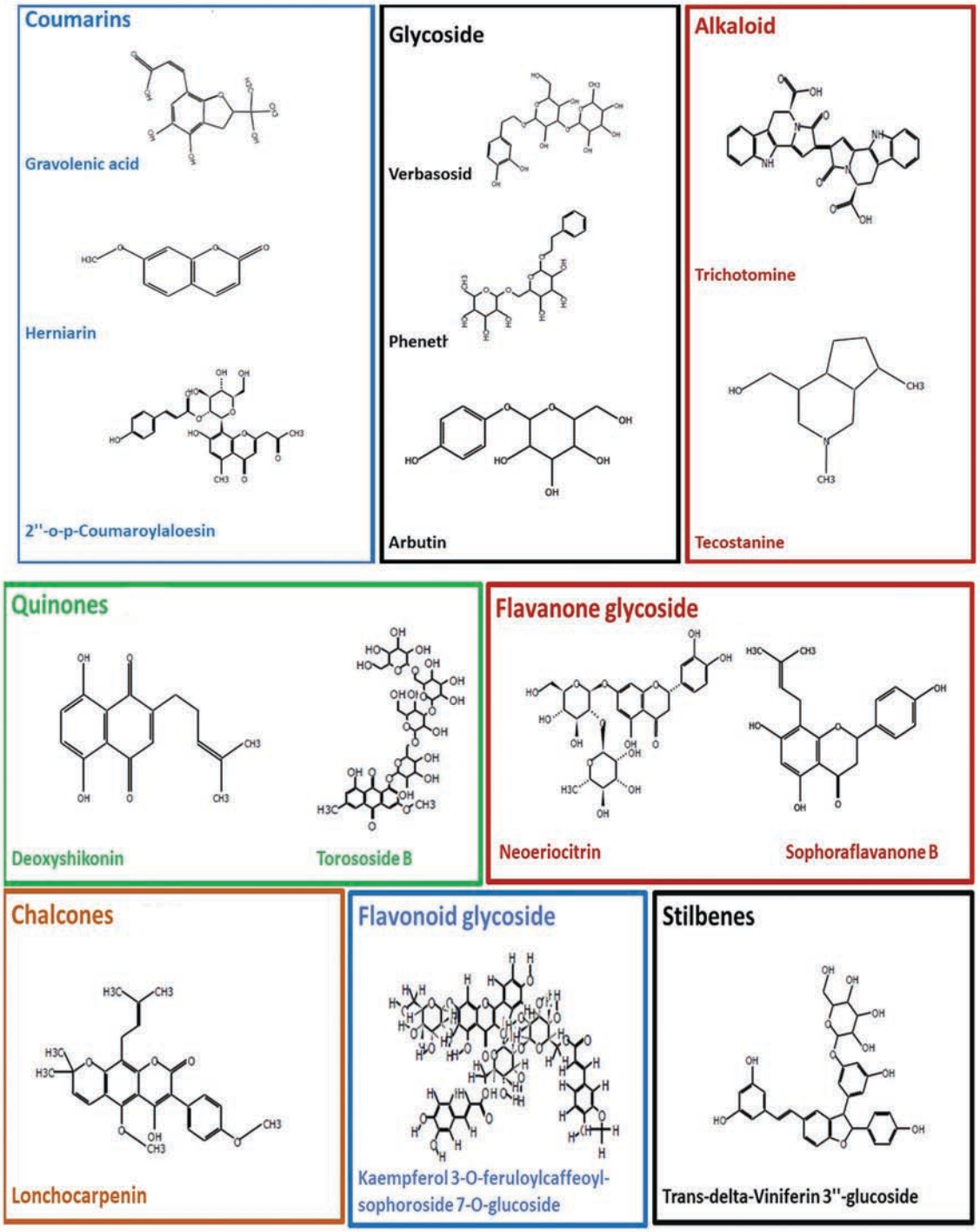

Figure 21c: Phytochemicals belonging Betalains, Benzopyran, Pyridine derivative Coumarins, Glycoside, Alkaloid, Quinones, Flavone glycoside, Chalcones, Flavonoid glycoide and Stilbenes.

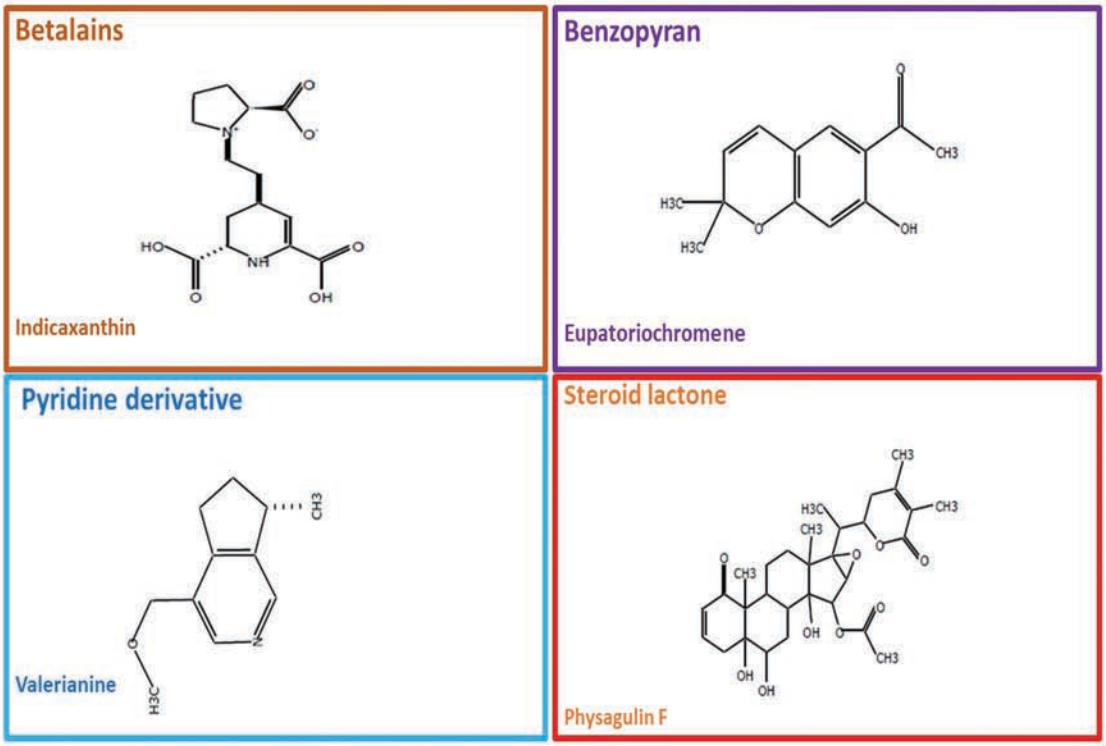

Figure 21d: Phytochemicals belonging Betalains, Benzopyran, Pyridine derivativeand Steroid lactone.

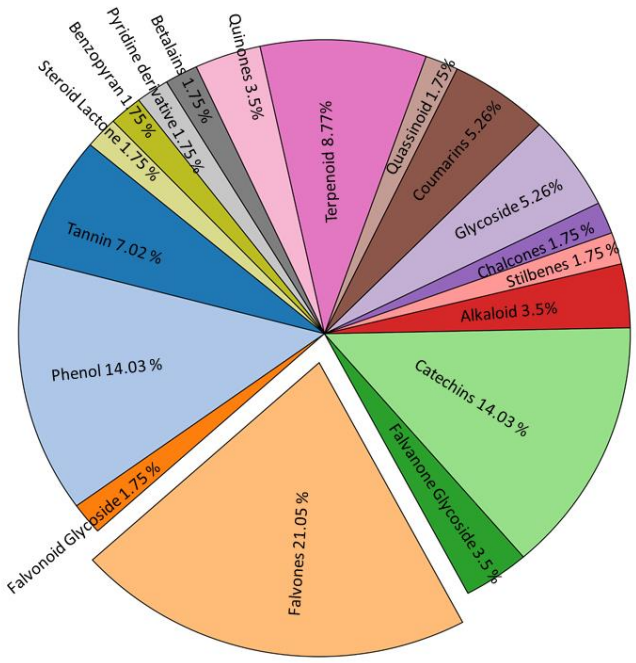

Figure 22: Percentage distribution of different phytochemical classes.

Table 14: Pharmacological properties of compounds identified from HBA

Compound	Biological activity	Reference
Cinnamtannin A1	Vasorelaxation, anticancer	(Byun *et al.*, 2014; Kin *et al.*, 2013)
Kadsurenone	Anticancer	(Hou *et al.*, 2018)
Eudesmin	Antitumour, neuroprotective	(Castillo *et al.*, 2023; Jiang *et al.*, 2017)
Eudesobovatol A	Cytotoxic effects	(Matsuda *et al.*, 2001)
Sesamin	Immunomodulatory and anti-inflammatory, Chondroprotective, Antinociceptive	(Henriques Monteiro *et al.*, 2014; Majdalawieh *et al.*, 2022; Phitak *et al.*, 2012)
Morusin	Anticancer, anticonvulsant	(Gupta *et al.*, 2014; Li *et al.*, 2023)
Luteolin	Anticonvulsant, antidepressant	(Shaikh *et al.*, 2013; Zhu *et al.*, 2019)
Apigenin	Antidepressant, cardioprotective, anti-diabetic	(Hossain *et al.*, 2014; Liu *et al.*, 2017; Zhu *et al.*, 2019)
Genkwanin	Anticancer, Antioxidant, Hepatoprotective, anti-arthritic	(Bao *et al.*, 2019; Ijaz *et al.*, 2022; Pandey *et al.*, 2024)
Diosmetin	Antioxidant, anti-inflammatory	(Q. Liu *et al.*, 2017;

		Wójciak *et al.*, 2022)
Formononetin	Antidiabetic, anti-inflammatory, Antinociceptive	(Cavendish *et al.*, 2015; Oza & Kulkarni, 2018; Qiu *et al.*, 2017)
Biochanin A	Anti-inflammatory	(Sithisarn *et al.*, 2013; Zhang & Chen, 2015)
Fustin	Anticancer	(Gospodinova *et al.*, 2023)
Tectorigenin	Anti-inflammatory, Antioxidant Antidiabetic and hypolipidemic	(Han *et al.*, 2012; Lee *et al.*, 2000)
Cajanin	Anti-inflammatory	(Hong *et al.*, 2022)
Neoeriocitrin	Antidiabetic	(Aldemir *et al.*, 2022)
Sophoraflavanone	Anticancer	(Zhu *et al.*, 2023)
(-)-Epicatechin	Anticancer	(Almaguer *et al.*, 2021; Dueñas *et al.*, 2010)
Procyanidin B2	Antiinflammatory, Antidiabetic, Anti arthritic	(Cai *et al.*, 2022; Wang *et al.*, 2017; Yin *et al.*, 2015)
Proanthocyanidin A2	Antioxidant, antiviral activities	(Barreiros *et al.*, 2000; Xu *et al.*, 2010)
Arbutin	Antimelanogenic and antioxidant	(Boo, 2021)
Herniarin	Ameliorates neuro-inflammation	(Mirzavi *et al.*, 2024)
(-)-Citronellol	Antioxidant	(Jagdale *et al.*, 2015)
Indicaxanthin	Prevent obesity-related dysmetabolic conditions, inhibit human melanoma cell proliferation and anticancer activity	(Allegra *et al.*, 2018)
Physagulin F	Anti-diabetic	(Pujari & Mamidala, 2015)

4.2.1.2 Assessment of Drug-likeness, Pharmacokinetic and Toxicity (ADMET) Profiles of the Identified Phytoconstituents from HBA

The term "drug-likeness" describes the qualitative evaluation of a compound's ability to have the structural and physicochemical characteristics required to be a promising drug candidate. It helps predict the compound's oral bioavailability, pharmacological activity, and safety in humans (Lipinski, 2004). Drug-likeness is assessed using various computational techniques and guidelines. The most common ones include Lipinski's Rule of Five (RO5), an established criterion to guide the development of orally deliverable compounds. According to the criteria, at least three of the four

stipulations must be fulfilled by the compound to classify it as a potential drug candidate, including a molecular weight of ≤ 500 Da, lipophilicity (log P) value ≤ 5, a maximum of 5 hydrogen bond donors, and no more than 10 hydrogen bond acceptors (Abchir *et al.*, 2024). The computed drug-likeness and the pharmacokinetic parameters based on ADMET of the 57 identified phytocompounds are summarized in Table 15 .

Most compounds exhibited advantageous drug-likeness characteristics, suggesting potential for substantial absorption and low toxicity. Some of the compounds like Cinnamtannin A1, trans-delta-Viniferin 3''-glucoside, (-)-epicatechin-3'-Oglucuronide etc. were failed to comply with Lipinski's rule of five. The findings highlight appropriateness of some molecules for advanced drug development.

In ADMET studies, absorption measures how well a compound enters the bloodstream after being given, which affects its bioavailability and therapeutic potential. Checking things like permeability, water solubility, and intestinal absorption can help figure out how well an oral drug will work and make the formulas better so the body can absorb them. The heatmap (Figure 23) shows the absorption profile of different compounds from HBA in detail, showing their pharmacokinetic properties and bioavailability potential.

The water solubility of a compound is significant because it affects how efficiently the body absorbs it (Pires *et al.*, 2015). All the compounds detected in the extract are water-soluble. In addition to water solubility, understanding the gastro intestinal permeability and intestinal absorption are critical in the development of a drug (Awortwe *et al.*, 2014; Radchenko *et al.*, 2016). In preclinical studies, the human epithelial colorectal adenocarcinoma cell line Caco-2 is commonly used as an *in vitro* model to predict the gastrointestinal permeability of drug candidates (Awortwe *et al.*, 2014). The apparent permeability coefficient (Papp) is a key criterion used in Caco-2 cell permeability assays to estimate the intestinal absorption potential of drug candidates. It is estimated from the rate of permeation and concentration of compound at different time point (Artursson *et al.*, 2001).

In silico methods offer a fast and inexpensive approach to predict the Papp of drug candidates, eliminating the need for labor-intensive *in vitro* assays. pkCSM is an *in silico* tool that predicts Caco-2 permeability based on the molecular structure of compounds, using graph-based signatures to estimate their intestinal absorption properties. The *in silico* Caco-2 permeability predictions can be interpreted as

compounds with a Papp > 10×10^{-6} cm/s are likely to be effectively absorbed, a Papp between 1×10^{-6} and 10×10^{-6} cm/s exhibit variable absorption, while a Papp < 1×10^{-6} cm/s show poor absorption (Pires *et al.*, 2015).

The identified phytocompounds like Apigenin, Genkwanin, Formononetin, Eupatoriochromene, (-)-Citronellol, Deoxyshikonin, Tecostanine, Eudesobovatol A, Sophoraflavanone B, Ursiniolide A, Eudesmin, Mexicanin E, Kadsurenone, Herniarin, Picrasin B, Sesamin, Valerianine, Kanzonol P showed high Caco-2 permeability with log Papp in 10^{-6} cm/s value > 0.9 (Radchenko *et al.*, 2016). Majority of the compounds demonstrated the capacity for absorption via the human intestine, with Graminiliatrin and Ursiniolide A exhibiting 100% absorption. Predictions indicate that Kaempferol 3-O-feruloyl-caffeoyl-sophoroside 7-O-glucoside, Dihydrocaffeic acid 3-O-glucuronide, 4'-O-methyl-(-)-epicatechin-3'-O-beta-glucuronide, and Torososide B demonstrate little intestinal absorption. Moreover, all of these compounds break Lipinski's rule of five, which projects inadequate oral bioavailability for drug-like entities.

Skin permeability coefficient (Kp) is a crucial parameter in drug delivery and transdermal pharmacology. It refers to the rate at which a substance passes through the skin, typically measured in cm/h. The Kp value depends on the compound's chemical structure, molecular weight, skin condition, and formulation as well as the membrane's physicochemical and biological characteristics (Alves *et al.*, 2015). Generally, for effective transdermal delivery, a log Kp between -3 and -1 is considered optimal, allowing for moderate permeability and sufficient drug absorption without systemic side effects (Brown & Williams, 2019). log Kp value computed for all the compounds except (-)-Citronellol, Tecostanine, and Valerianine were > -2.5, suggests moderate to good skin permeability (Pires *et al.*, 2015).

P-glycoprotein is the most recognised ATP binding cassette (ABC) transporter that eliminates toxins and xenobiotics from cells to create a biological barrier. As per *in vitro* and *in vivo* research, it is important for drug absorption and disposition. If a compound is a P glycoprotein substrate, it means that the compound is recognised and

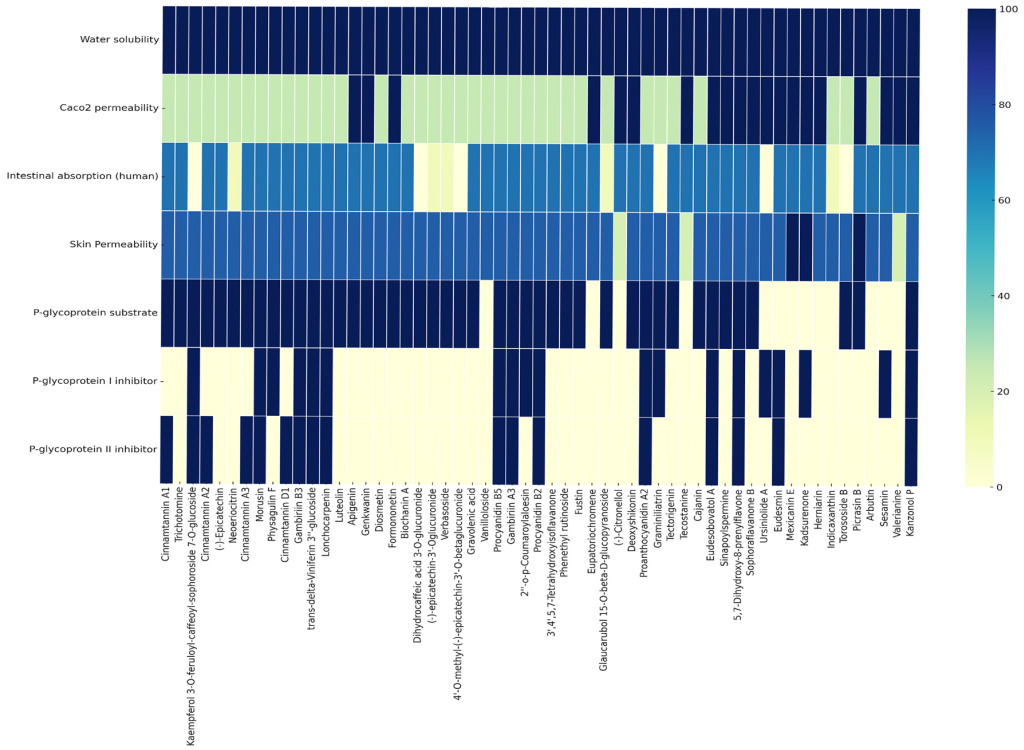

Figure 23: Heatmap illustrating the pharmacokinetic properties of various compounds on a 0–100 scale, with darker shades indicating higher values. Key parameters analyzed include water solubility, Caco-2 permeability, intestinal absorption, skin permeability, and interactions with P-glycoprotein (substrate and inhibitors).The variability in colour gradients across different compounds highlights differences in their absorption related properties, which are crucial for evaluating their bioavailability and potential as drug candidates.

Table 15: ADMET properties of the identified phytochemical compounds of HBA

			Absorption							Distribution				Metabolism							Excretion		Toxicity						
Sl.No	Phytochemical	LIPINSKY	Water solubility(log mol/L)	Caco2 permeability (log Papp in 10–6 cm/s)	Intestinal absorption (human) %	Skin Permeability (log Kp)	P-glycoprotein substrate	P-glycoprotein I inhibitor	P-glycoprotein II inhibitor	VDss (human)	Fraction unbound (human)	BBB permeability	CNS permeability	CYP2D6 substrate	CYP3A4 substrate	CYP1A2 inhibitior	CYP2C19 inhibitior	CYP2C9 inhibitior	CYP2D6 inhibitior	CYP3A4 inhibitior	Total Clearance	Renal OCT2 substrate	AMES toxicity	hERG I inhibitor	hERG II inhibitor	Hepatotoxicity	Skin Sensitisation	Max. tolerated dose (human)
1	Cinnamtannin A1	NO	-2.892	-1.826	60.469	-2.735	Yes	No	Yes	-0.086	0.378	-2.843	-4.855	No	No	No	No	No	No	No	-3.326	No	No	No	Yes	No	No	0.438
2	Trichotomine	YES	-2.892	-0.898	40.994	-2.735	Yes	No	No	-1.193	0.108	-0.978	-3.346	No	No	No	No	No	No	No	0.68	No	No	No	No	No	No	0.505
3	Kaempferol 3-O-feruloyl-caffeoyl-sophoroside 7-O-glucoside	NO	-2.892	-1.418	0	-2.735	Yes	Yes	Yes	-0.05	0.28	-3.373	-6.587	No	No	No	No	No	No	No	-1.74	No	No	No	No	No	No	0.438
4	Cinnamtannin A2	NO	-2.892	-2.62	53.166	-2.735	Yes	No	Yes	-0.023	0.381	-3.715	-5.56	No	Yes	No	No	No	No	No	-5.823	Yes	No	No	Yes	No	No	0.438
5	(-)-Epicatechin	YES	-3.117	-0.283	68.829	-2.735	Yes	No	No	1.027	0.235	-1.054	-3.298	No	No	No	No	No	No	No	0.183	No	No	No	No	No	No	0.438
6	Neoeriocitrin	NO	-2.9	-0.855	16.143	-2.735	Yes	No	No	0.827	0.178	-1.836	-4.925	No	No	No	No	No	No	No	0.125	No	No	No	Yes	No	No	0.475
7	Cinnamtannin A3	NO	-2.892	-3.221	46.968	-2.735	Yes	No	Yes	0.011	0.381	-4.634	-6.402	No	Yes	No	No	No	No	No	-8.245	No	No	No	Yes	No	No	0.438
8	Morusin	YES	-4.056	0.048	93.485	-2.736	Yes	Yes	Yes	0.079	0.002	-1.16	-1.838	No	Yes	Yes	Yes	Yes	No	No	0.224	No	No	No	Yes	No	No	0.231
9	Physagulin F	YES	-5.213	0.431	98.023	-2.883	Yes	Yes	No	-0.059	0.312	-1.036	-3.143	No	Yes	No	No	No	No	No	0.353	No	No	No	No	No	No	-0.69
10	Cinnamtannin D1	NO	-2.892	-1.879	62.158	-2.735	Yes	No	Yes	-0.112	0.375	-2.637	-4.791	No	No	No	No	No	No	No	-3.503	Yes	No	No	Yes	No	No	0.438
11	Gambiriin B3	NO	-2.892	-1.001	58.869	-2.735	Yes	Yes	Yes	-0.472	0.293	-1.844	-3.849	No	Yes	No	No	No	No	No	0.112	No	No	No	Yes	No	No	0.438
12	trans-delta-Viniferin 3''-glucoside	NO	-2.978	-1.118	55.972	-2.735	Yes	Yes	Yes	-1.564	0.112	-1.63	-3.797	No	Yes	No	No	No	No	No	-0.156	No	No	No	Yes	No	No	0.397
13	Lonchocarpenin	YES	-6.167	0.496	93.948	-2.703	Yes	Yes	Yes	0.271	0.04	-0.669	-1.85	No	Yes	No	Yes	Yes	No	Yes	0.599	No	Yes	No	Yes	No	No	0.024
14	Luteolin	YES	-3.094	0.096	81.13	-2.735	Yes	No	No	1.153	0.168	-0.907	-2.251	No	No	Yes	No	Yes	No	No	0.495	No	No	No	No	No	No	0.499
15	Apigenin	YES	-3.329	1.007	93.25	-2.735	Yes	No	No	0.822	0.147	-0.734	-2.061	No	No	Yes	Yes	No	No	No	0.566	No	No	No	No	No	No	0.328
16	Genkwanin	YES	-3.497	0.917	93.982	-2.746	Yes	No	No	0.011	0.096	-0.337	-2.171	No	Yes	Yes	Yes	Yes	No	No	0.617	No	No	No	No	No	No	0.032
17	Diosmetin	YES	-3.238	0.326	79.898	-2.735	Yes	No	No	0.709	0.068	-0.954	-2.316	No	No	Yes	Yes	Yes	No	No	0.598	No	No	No	No	No	No	0.42
18	Formononetin	YES	-3.691	1.253	96.112	-2.752	No	No	No	-0.121	0.096	0.157	-1.993	No	Yes	Yes	Yes	Yes	No	No	0.258	No	No	No	No	No	No	0.008
19	Biochanin A	YES	-3.735	0.897	93.028	-2.737	Yes	No	No	-0.341	0.03	-0.221	-2.115	No	Yes	Yes	Yes	Yes	No	No	0.247	No	No	No	No	No	No	0.4
20	Dihydrocaffeic acid 3-O-glucuronide	YES	-2.741	-0.757	0	-2.735	Yes	No	No	-0.149	0.643	-1.223	-4.106	No	No	No	No	No	No	No	0.753	No	No	No	No	No	Yes	No	0.614
21	(-)-epicatechin-3'-Oglucuronide	NO	-2.875	-0.878	13.865	-2.735	Yes	No	No	0.975	0.306	-1.415	-4.384	No	No	No	No	No	No	No	-0.388	No	No	No	No	No	No	0.439
22	Verbasoside	NO	-2.294	-0.628	25.383	-2.735	Yes	No	No	0.747	0.618	-1.294	-4.613	No	No	No	No	No	No	No	0.75	No	No	No	No	No	No	0.393
23	4'-O-methyl-(-)-epicatechin-3'-O-betaglucuronide	NO	-2.833	-0.921	0	-2.735	Yes	No	No	1.244	0.384	-1.308	-4.162	No	No	No	No	No	No	No	-0.307	No	No	No	No	No	No	0.41
24	Gravolenic acid	YES	-2.7	0.513	46.824	-2.735	Yes	No	No	-0.562	0.514	-0.97	-3.31	No	No	No	No	No	No	No	0.591	No	No	No	No	No	No	0.89
25	Vanilloloside	YES	-1.445	0.237	41.011	-2.778	No	No	No	-0.151	0.647	-1.129	-4.016	No	No	No	No	No	No	No	0.184	No	No	No	No	No	Yes	No	0.493
26	Procyanidin B5	NO	-2.892	-1.049	55.588	-2.735	Yes	Yes	Yes	-0.17	0.329	-1.947	-3.941	No	No	No	No	No	No	No	-0.077	No	No	No	Yes	No	No	0.438
27	Gambiriin A3	NO	-2.892	-1.371	39.171	-2.735	Yes	Yes	Yes	-0.16	0.342	-2.279	-4.031	No	Yes	No	No	No	No	No	0.104	No	No	No	Yes	No	No	0.438
28	2''-o-p-Coumaroylaloesin	NO	-3.165	0.111	51.438	-2.735	Yes	Yes	No	0.392	0.058	-1.669	-3.555	No	Yes	No	No	No	No	Yes	0.366	No	No	No	Yes	Yes	No	0.006
29	Procyanidin B2	NO	-2.892	-1.225	66.749	-2.735	Yes	Yes	Yes	-0.158	0.309	-1.94	-3.983	No	No	No	No	No	No	No	-0.085	Yes	No	No	Yes	No	No	0.438
30	3',4',5,7-Tetrahydroxyisoflavanone	YES	-3.314	0.116	72.72	-2.735	Yes	No	No	0.501	0.101	-0.796	-3.2	No	No	Yes	No	No	No	No	0.017	No	No	No	No	No	No	0.047
31	Phenethyl rutinoside	YES	-2.356	0.32	35.262	-2.736	Yes	No	No	0.408	0.649	-0.961	-4.246	No	No	No	No	No	No	No	1.11	No	No	No	No	No	No	0.323
32	Fustin	YES	-2.913	-0.174	73.643	-2.735	Yes	No	No	0.982	0.27	-0.739	-3.111	No	No	No	No	No	No	No	-0.067	No	Yes	No	No	No	No	0.104
33	Eupatoriochromene	YES	-3.083	1.297	93.134	-2.828	No	No	No	0.053	0.319	0.135	-2.539	No	No	Yes	No	No	No	No	0.187	No	No	No	No	No	No	0.684
34	Glaucarubol 15-O-beta-D-glucopyranoside	NO	-2.783	-0.203	27.227	-2.735	Yes	No	No	-0.114	0.492	-1.397	-4.641	No	No	No	No	No	No	No	0.725	No	No	No	Yes	No	No	-1.335
35	(-)-Citronellol	YES	-2.957	1.489	92.83	-1.525	No	No	No	0.195	0.447	0.627	-2.222	No	No	No	No	No	No	No	0.482	No	No	No	No	No	Yes	0.7
36	Deoxyshikonin	YES	-3.786	0.928	91.339	-3.084	Yes	No	No	0.247	0.218	0.021	-2.154	No	No	No	Yes	No	No	No	0.077	No	No	No	No	No	No	0.63
37	Proanthocyanidin A2	NO	-2.892	-1.085	69.461	-2.735	Yes	Yes	Yes	-0.328	0.305	-1.769	-4.086	No	No	No	No	No	No	No	0.11	Yes	No	No	Yes	No	No	0.438
38	Graminiliatrin	YES	-4.322	0.834	100	-2.879	Yes	Yes	No	-0.063	0.376	-1.063	-3.152	No	Yes	No	No	No	No	No	0.723	No	Yes	No	No	No	No	-0.554

39	Tectorigenin	YES	-3.435	-0.132	84.511	-2.735	Yes	No	No	0.166	0.051	-1.06	-2.404	No	Yes	Yes	Yes	Yes	No	No	0.132	No	No	No	No	No	No	0.334
40	Tecostanine	YES	-1.398	1.476	83.382	-2.416	No	No	No	0.666	0.703	0.126	-2.425	No	No	No	No	No	No	No	0.431	No	No	No	No	No	Yes	0.394
41	Cajanin	YES	-3.401	-0.283	82.752	-2.735	Yes	No	No	0.208	0.042	-1.202	-2.344	No	No	Yes	Yes	Yes	No	No	0.594	No	No	No	No	No	No	0.502
42	Eudesobovatol A	NO	-6.047	1.165	88.914	-2.733	Yes	Yes	Yes	0.27	0	-0.585	-1.605	No	Yes	No	Yes	Yes	No	Yes	0.374	No	No	No	Yes	No	No	-0.189
43	Sinapoylspermine	YES	-2.623	-0.053	50.961	-2.742	Yes	No	No	1.81	0.663	-0.86	-3.784	No	Yes	No	No	No	No	No	1.033	No	No	No	Yes	Yes	No	0.203
44	5,7-Dihydroxy-8-prenylflavone	YES	-4.192	0.685	93.243	-2.746	Yes	Yes	Yes	-0.042	0	0.071	-1.979	No	Yes	Yes	Yes	Yes	No	Yes	0.482	No	No	No	Yes	Yes	No	-0.113
45	Sophoraflavanone B	YES	-3.567	1.304	91	-2.739	Yes	No	No	0.272	0.017	-0.925	-2.13	No	Yes	Yes	Yes	No	No	No	0.119	No	No	No	Yes	No	No	-0.246
46	Ursiniolide A	YES	-4.352	1.045	100	-2.905	No	Yes	No	-0.049	0.309	-0.761	-2.915	No	Yes	No	No	No	No	No	1.274	No	No	No	No	No	No	-0.375
47	Eudesmin	YES	-5.288	1.081	96.702	-2.716	No	Yes	Yes	-0.19	0	-0.744	-2.897	No	Yes	Yes	Yes	Yes	No	Yes	0.15	No	No	No	No	No	No	0.324
48	Mexicanin E	YES	-2.731	1.268	99.98	-3.115	No	No	No	0.17	0.433	0.078	-2.674	No	No	Yes	No	No	No	No	0.518	No	No	No	No	No	No	0.314
49	Kadsurenone	YES	-4.409	1.432	96.963	-3.139	No	Yes	No	0.07	0.088	-0.199	-2.803	No	Yes	No	No	No	No	No	0.418	No	No	No	No	No	No	0.635
50	Herniarin	YES	-1.761	1.62	98.02	-2.21	No	No	No	-0.235	0.319	0.263	-2.16	No	No	Yes	No	No	No	No	0.79	No	No	No	No	No	No	0.366
51	Indicaxanthin	YES	-1.716	-0.026	7.378	-2.735	No	No	No	-1.202	0.692	-0.801	-3.669	No	No	No	No	No	No	No	-0.126	No	No	No	No	Yes	No	0.954
52	Torososide B	NO	-2.868	-1.037	0	-2.735	Yes	No	No	-0.272	0.299	-2.69	-6.747	No	No	No	No	No	No	No	0.324	No	No	No	Yes	No	No	-0.043
53	Picrasin B	YES	-4.062	1.36	99.526	-4.289	Yes	No	No	0.017	0.338	-0.335	-2.941	No	Yes	No	No	No	No	No	0.586	No	Yes	No	No	No	No	-0.225
54	Arbutin	YES	-1.607	0.009	38.027	-2.801	No	No	No	0.026	0.784	-0.962	-3.557	No	No	No	No	No	No	No	0.524	No	No	No	No	No	No	0.485
55	Sesamin	YES	-4.223	1.399	97.81	-2.772	No	Yes	No	-0.17	0	-0.862	-2.939	No	Yes	Yes	Yes	Yes	No	Yes	-0.126	No	Yes	No	No	No	No	0.089
56	Valerianine	YES	-2.244	1.712	97.064	-1.985	No	No	No	0.187	0.356	0.207	-2.181	No	No	Yes	No	No	No	No	0.32	No	No	No	No	No	Yes	0.674
57	Kanzonol P	YES	-4.97	1.675	94.574	-2.79	Yes	Yes	Yes	0.009	0	0.014	-1.982	No	Yes	Yes	Yes	Yes	No	Yes	0.364	No	No	No	No	No	No	0.011

transported by P glycoprotein, thus decreasing its oral bioavailability. If it is a P glycoprotein inhibitor, the compound can block the function of the pump, which leads to increased intracellular concentration of substrates, causing toxic effects (Dewanjee *et al.*, 2017; Seelig, 2020). According to the results, the compounds Formononetin, Vanilloloside, Eupatoriochromene, (-)-Citronellol, Tecostanine, Mexicanin E, Herniarin, Indicaxanthin, Arbutin and Valerianine are neither an inhibitor nor a substrate of P glycoprotein balancing their availability in the cells. Because arbutin and valerianine are neither an inhibitor nor a substrate of P glycoprotein, their availability in cells is balanced.

Another important pharmacokinetic parameter is volume of distribution at steady state (VDss) which is a measure of drug distribution throughout the body, particularly in tissues relative to plasma. A compound is said to have a high distribution volume if its log VDss value > 0.45, which indicates that it is more widely distributed in tissues as rather than being restricted to plasma (Belal, 2018). The phytoconstituents (-)-Epicatechin, Neoeriocitrin, Luteolin, Apigenin, Diosmetin, (-)-epicatechin-3'-Oglucuronide, Verbasoside, 4'-O-methyl-(-)-epicatechin-3'-O-betaglucuronide, 3',4',5,7-Tetrahydroxyisoflavanone, Fustin, Tecostanine and Sinapoylspermine had distribution volumes (logVDss) of > 0.45 , making them well distributed ones in the tissues (Pires *et al.*, 2015). The unbound (free) drug is the active form, as it can cross cell membranes, enter tissues, and bind to its intended targets. This can be represented by the fraction unbound (Fu) which denotes to the proportion of a drug that is remains unbound to plasma proteins and is free to exert its pharmacological effects, including interactions with target proteins such as receptors, enzymes, or ion channels. When the drug's Fu value is 1, it indicates that nearly all of it is unbound and ready for action and tissue distribution (Bohnert & Gan, 2013). In this study, most of the phytocompounds showed Fu value ~1, showing availability of unbound form of the compounds suggesting their potential for pharmacologically activity.

The permeability of compounds through the blood-brain barrier (BBB) and their central nervous system (CNS) permeability are necessary determinants of their capacity to induce therapeutic effects on the brain (Nagpal *et al.*, 2013; Zhao *et al.*, 2022). Molecules that target the central nervous system must be able to cross the blood-brain barrier to be effective as therapeutic agents. However, in order to prevent

CNS-related adverse effects, peripherally active drugs should not cross the blood-brain barrier. Therefore, understanding a compound's BBB permeability is crucial in both cases to ensure proper efficacy and safety profiles (Garg *et al.*, 2008). The majority of the compounds exhibit negative BBB permeability values, indicating that they face significant barriers to crossing into the brain. Compounds like Cinnamtannin A1, Cinnamtannin A3 and Torososide B have extremely poor permeability, making them unlikely candidates for direct neurological applications. The compounds Eudesobovatol A, 5,7-Dihydroxy-8-prenylflavone, Formononetin, Kanzonol P, Lonchocarpenin, and Morusin exhibit less negative CNS permeability values**,** suggesting they have a relatively higher capacity to distribute within the central nervous system (CNS)**.** BBB permeability influences how easily a compound enters the brain. These six compounds exhibit moderate BBB permeability values, making them more likely to cross the BBB efficiently.

The human cytochrome P450 isoforms (CYP) that are responsible for metabolising and detoxifying drugs inside the liver, help make xenobiotics more excretable by oxidizing them. Thus, the identified phytochemicals were assessed for their potential to inhibit certain important forms of CYP (Yeni & Rachmania, 2022; Zhao *et al.*, 2021). The CYP profile of various compounds from HBA is comprehensively represented in the Figure 24. It was found that none of the compounds acted as substrates or inhibitors for CYP2D6. One of the prominent CYP expressed in the liver, CYP3A4, is responsible for the metabolic clearance of many anticancer medications. So, drug toxicity, drug–drug interactions and other adverse effects could result from their (Awadelkareem *et al.*, 2022; Kacevska *et al.*, 2008). Seven compounds act as CYP3A4 substrate, which is not ideal. According to predictions, 90% of the tested compounds are not substrate for this cytochrome. However, Lonchocarpenin, 2"-O-pCoumaroylaloesin, Eudesobovatol A, Eudesmin, 5,7-Dihydroxy-8-prenylflavone, Sesamin, and Kanzonol P act as substrate effects on CYP3A4.

CYP1A2 is a principal hepatic cytochrome P450 enzyme in the human liver, essential for the-bioactivation of numerous procarcinogens and the metabolism of around 15% of therapeutic pharmaceuticals, such as clozapine, theophylline, tacrine, and zolmitriptan 14 (Zhou *et al.*, 2009a; Zhou *et al.*, 2009b). 18 compounds were found to

be inhibitors of CYP1A2. 3',4',5,7-Tetrahydroxyisoflavanone, Eupatoriochromene, Herniarin, Mexicanin E, and Valerianine were recognised as inhibitors of CYP1A2, exhibiting no inhibitory effects on CYP2C19, CYP2C9, or and not a substrate for CYP3A4. This selective suppression indicates a possible impact on CYP1A2-mediated drug metabolism while preserving the function of other principal CYP enzymes, thereby reducing extensive herb-drug interactions.

CYP2C9 and CYP2C19 are crucial drug-metabolizing enzymes that collectively process approximately 20% of therapeutically vital drugs (Hirota *et al.*, 2013). As per our prediction, 16 compounds inhibit either or both CYP2C9 and CYP2C19, with 13 compounds inhibiting both enzymes. Specifically, CYP2C19 is inhibited by two compounds, Lonchocarpenin and Deoxyshikonin, which do not inhibit CYP2C9. Conversely, CYP2C9 is inhibited by Herniarin, which does not inhibit CYP2C19. Numerous polyphenolic substances have been recognised as significant CYP2C9 inhibitors, including Chalcones like Xanthohumol and phloretin (Kimura *et al.*, 2010; Yuan *et al.*, 2014), along with rhapontigenin, a stilbene noted for its strong inhibitory effect on CYP2C9 (Cieniak *et al.*, 2013). Numerous studies indicate that different components of medicinal plants, especially those utilised in herbal therapy, can influence CYP2C9 activity, potentially resulting in herb-drug interactions (Wang *et al.*, 2020). Even while these inhibitory effects raise questions about potential negative interactions and changed drug metabolism, it is necessary to consider that natural products have a lot of therapeutic potential. Applying FDA guidelines for drug-drug interactions to herb-drug interactions has limitations. Herbal extracts contain many compounds, some of which may not be active, unlike pure drugs. Also, the concentrations used in studies might not match what's absorbed in the body, which could exaggerate the inhibitory effects of herbal medicines. The data related to OCT substarte, total clearance values are given in Figure 25 and 27.

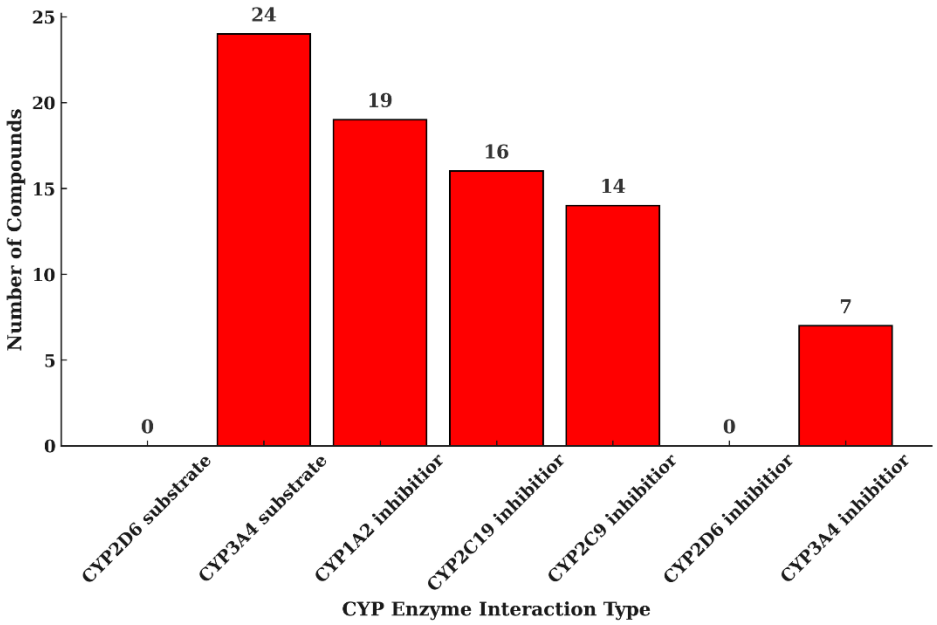

Figure 24: The bar chart illustrates the distribution of CYP substrates and inhibitors among the identified compounds, highlighting their potential metabolic interactions.

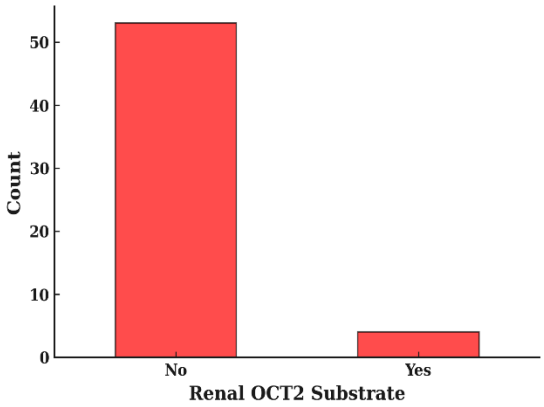

Figure 25: Illustrates the distribution of renal OCT2 substrates and the number of compounds in HBA.

A total of 5 toxicity prediction parameters such as the AMES toxicity, maximum tolerated dose (MRTD), human ether-go-go gene (hERG I) inhibition, hepatotoxicity and skin sensitisation were used to predict the safety profile of the identified compounds (Olaokun & Zubair, 2023). The toxicity parameters and number of compounds of HBA that represents the toxicity are given in the Figure 26. The AMES test is a widely used assay to evaluate the mutagenicity of chemicals. A compound is

said to be mutagenic if it shows positive result in AMES test. Since many mutagens are also carcinogenic, the test is a crucial initial screening method in toxicology and drug research. Majority of the identified compounds are AMES negative and hence non-mutagenic (Pires *et al.*, 2015). Out of the compounds tested, five were found to be AMES positive. These compounds included Sesamin, Lonchocarpenin, Fustin, Graminiliatrin, and Picrasin B. However, despite their AMES-positive results, some studies have indicated that Sesamin and Fustin do not exhibit genotoxic effects at specific doses (Hori *et al.*, 2011; Park *et al.*, 2004). This suggests that their potential toxicity may be dose-dependent and requires further investigation. Such findings highlight the importance of considering both AMES test results and in-depth toxicological assessments when evaluating compound safety.

The MRTD of a drug is the highest dosage of a drug that can be given without causing severe toxicity in animals or humans (Pires *et al.*, 2015). It is used to predict a range of safe and efficient dosages for clinical studies. MRTD is expressed in terms of log (mg/kg/day). All the compounds except a few of them shows low (≤ 0.477 log (mg/kg/day) MRTD value indicating their high potency and are effective at small doses (Didigwu & Nnadi, 2024; Ferrari, 2023). High MRTD value of 16 compounds suggests those have a wide safety margin between its therapeutic dose and toxicity dose making them safer for clinical use. Among that tested compounds Gravolenicacid and Indicaxanthin were shown highest MRTD values, suggesting the highest safety and lower risk of toxicity at therapeutic doses. Previous reports on other compounds indicate that kaempferol and quercetin exhibited the highest MRTD values, with 0.531 and 0.499 log(mg/kg/day) respectively (Aini *et al.*, 2023). If these values fall within the safe range, our findings further highlight the potential use of Gravolenic acid and Indicaxanthin as promising candidates with favourable safety profiles.

Most of the compounds are non-hepatotoxic and found to be having no skin sensitisation. The liver is integral to the metabolism and excretion of xenobiotics, giving it particularly vulnerable to their unfavourable and toxic effects. Synthetic drugs are identified as a primary cause of liver toxicity, however many herbs can contribute similarly. Numerous herbal remedies with a proven history of effectiveness

are utilised by humans (Jamkhandea *et al.*, 2013). Dihydrocaffeic acid 3-O-glucuronide, Vanilloloside, Sinapoylspermine, 5,7-Dihydroxy-8-prenylflavone, and Indicaxanthin are anticipated to demonstrate hepatotoxicity, indicating possible liver toxicity hazards. This suggests that these chemicals may adversely affect liver function, either via metabolic stress, enzyme inhibition, or oxidative damage. Additional experimental validation and mechanistic investigations are required to evaluate their effects on liver health and confirm safe therapeutic application.

Hagvall *et al.* (2020) reported that oxidized citronellol triggers higher contact allergy cases than purified citronellol. Similarly, PKCM analysis found that only three of the compounds (-)-Citronellol, Tecostanine, and Valerianine can cause skin sensitization.

Likewise, hERG I, encodes a voltage-gated potassium channel and is responsible for regulating cardiac action potential. Inhibition of hERG I can lead to QT prolongation and elevate the issues of risk of arrhythmias (Harinarayanamoorthy *et al.*, 2013; Pearlstein *et al.*, 2003). Thus, it is essential to assess the hERG channel blocking potential of drug candidates early in the process of drug discovery to evaluate their cardiac safety (Polak *et al.*, 2011; Rainsford, 2007). None of the identified phytocompounds are found to be hERG I inhibitors suggesting that these compounds do not present a risk of cardiotoxicity making them safer candidates for further clinical evaluation.

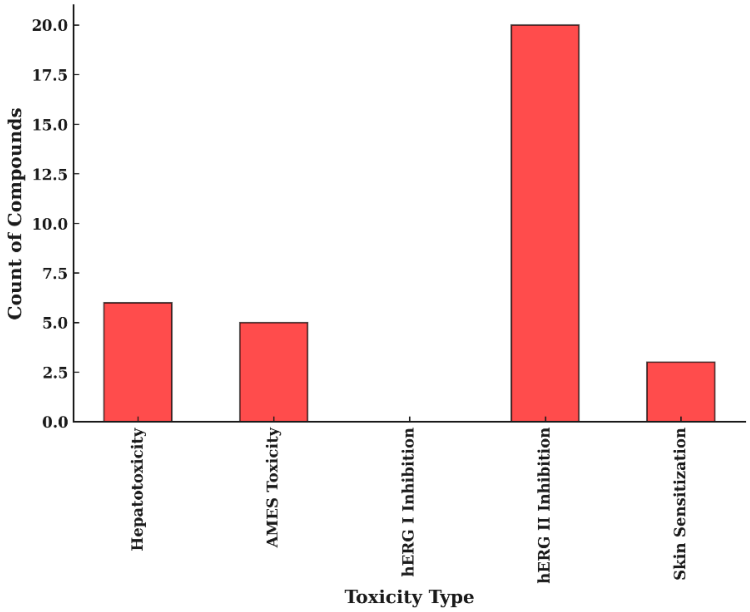

Figure 26: Frequency of phytochemicals, associated with specific toxicity types.

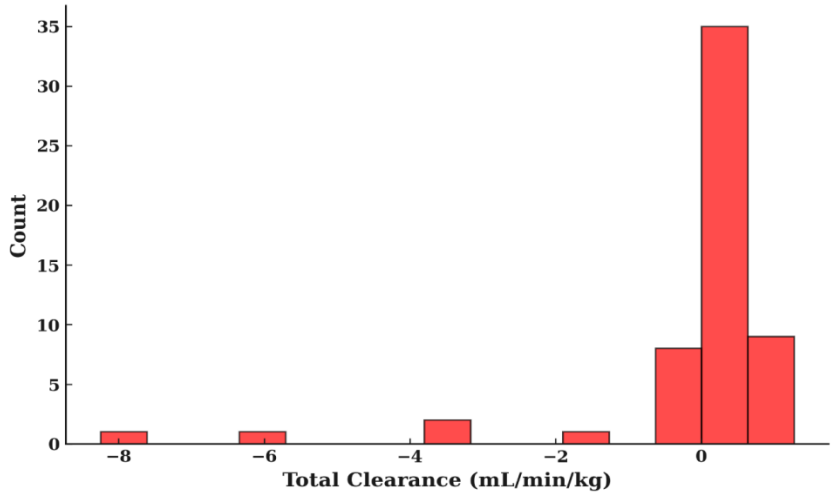

Figure 27: Distribution of total clearance values (mL/min/kg) and the number of compounds in HBA.

4.2.1.3 Acute Oral toxicity assessment

Physiological parameters: Body weights, Toxic signs and Pre-terminal deaths: A significant reduction in body weight was observed in mice treated with 2000 mg/kg. in contrast all mice treated with 300 mg/kg gained weight during the observation period (Table 16 and Figure 28). Pre-terminal deaths occurred in 2 animals treated with 2000 mg/kg. No toxic signs and pre-terminal deaths were observed when treated with 300 mg/kg. The effects administering HBA at high dose and low dose are represented in the Figure 28.

Table 16: Body weights of marked animals over the toxicity studies

Animals marked	Body Weights (g)			
	Day (-1)	Day 1	Day 8	Day 15
Head	27.1	27.0	27.4	27.9
Body	26.3	26.3	26.5	26.7
Tail	26.7	26.6	26.7	26.9
Head and Body	24.7	24.7	24.9	25.1
Body and Tail	25.3	25.4	25.7	25.9
Head and Tail	26.1	26.2	26.4	26.7

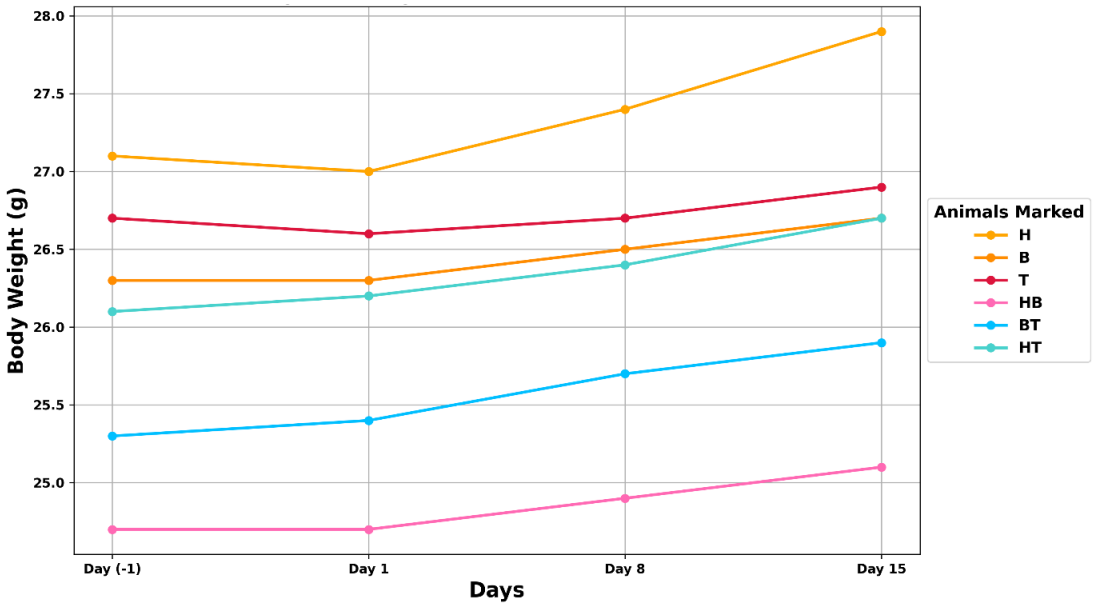

Figure 29: Graph showing the body weights of marked animals over the specified days. Each line representing an animal group, with weights tracked over time from Day (-1) to Day 15 (**H: Head, B: Body, T: Tail, HB: Head Body, BT: Body Tail, HT: Head Tail).*

Statistical Analysis of Body Weight Changes: Statistical analysis was performed to evaluate the connection between body weights at various days (Table 17). The results indicating steady weight progression without substantial variations. The correlation between Day 8 and Day 15 was the strongest (r = 0.996, p = 0.000), signifying near-perfect stability in weight increase. The connection between Day 1 and Day 8 (r = 0.988, p = 0.000) and between Day (-1) and Day 15 (r = 0.976, p = 0.001) was statistically significant. The results indicate that the test substance did not cause notable weight loss or fluctuation, hence reinforcing its possible safety profile. The lack of significant weight fluctuations further substantiates the validity of the toxicity study findings.

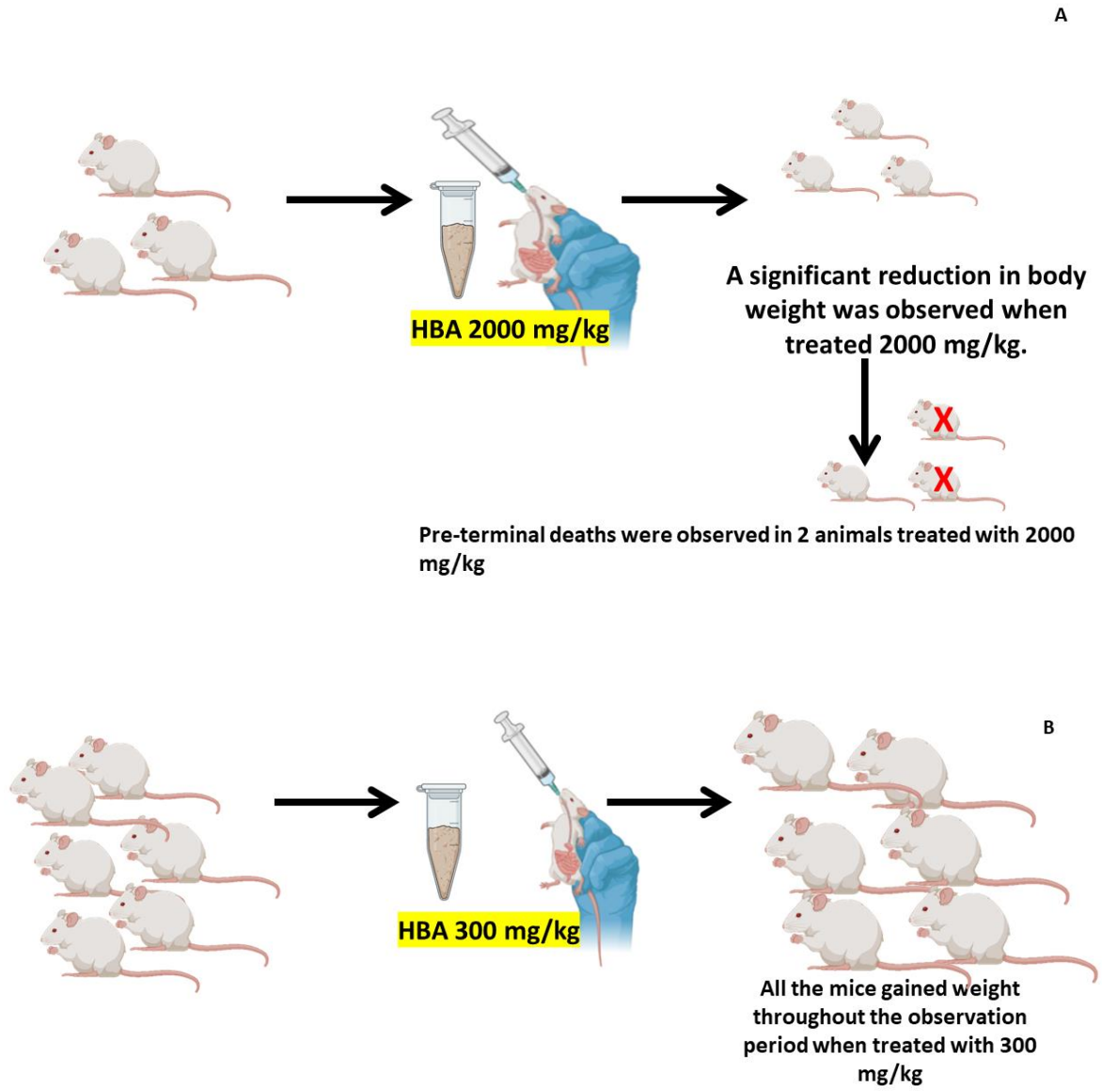

Figure 28: A) The effects of administering HBA at a high dose (2000 mg/kg) in mice, showing a significant reduction in body weight and pre-terminal deaths in two animals. B) depicts the response to a lower dose (300 mg/kg), where all treated mice exhibited weight gain throughout the observation period, suggesting a better tolerance at this dose.

Table 17: Correlation Analysis of Body Weight Changes during the Study.

Pair	Correlation (r)	Significance (p-value)
Day (-1) & Day 8	0.988	0.00
Day (-1) & Day 15	0.976	0.001
Day (-1) & Day 15	0.996	0.00

Necropsy: After the observation period, the mice were euthanized under diethyl ether anesthesia and underwent a thorough necropsy; no abnormalities were observed in the mice treated with 300 mg/kg.

The findings from the acute oral toxicity test of the test item HBA, using the Acute Toxic Class Method on Swiss albino mice, indicate that the test item's LD50 can be classified as GHS Category 4, which corresponds to an LD50 range of greater than 300 mg/kg and up to 2000 mg/kg. This classification is in accordance with OECD Guideline No. 423, dated December 17, 2001.

4.2.2 Discussion

Herbal treatments are increasingly acknowledged for their potential to address diverse health issues and are receiving significant global interest. Examining the chemical composition of medicinal plants is essential for both ethnomedicinal populations that depend on them and the scientific community, as it facilitates the assurance of their quality and understanding of their therapeutic efficacy. This study introduces an innovative approach employing tandem mass spectrometry to identify the principal constituents of *H. sanjappae.* Through LC-MS/MS it is possible to analyse the complete metabolome of a plant (Sawada & Hirai, 2013). The correct identification of plant metabolites helps to determine the biological function of each metabolite, which in turn helps to identify a plant's overall bioactivity (Cocan *et al.*, 2018; Vitale *et al.*, 2022). Certain components of medicinal plants despite their drug-like properties, can be toxic and may cause adverse effects if not used appropriately. So, toxicity testing is an important part of finding biologically active chemicals (Pour *et al.,* 2011; Zhuo *et al.*, 2020). Ensuring the safety and efficacy of therapeutic drug is essential for the pharmaceutical industry and patient health (Alshammari, 2016).

The acute oral toxicity of many Fabaceae plant extracts has been assessed to ascertain their safety profiles. The aqueous bark extract of *Tetrapleura tetraptera* demonstrated good tolerance, with no notable toxic effects detected at a therapeutic dosage of 200 mg/kg or greater acute dosages (Dongmo *et al.*, 2019). An acute toxicity investigation of *Erythrina senegalensis* extract in mice further validated its safety, as none of the administered doses resulted in mortality or notable behavioural alterations. In a sub-chronic toxicity investigation conducted on rats, daily oral administration of ethyl acetate extract of *E. senegalensis* at a dosage of 600 mg/kg led to a notable rise in relative body weight during the final week of therapy(Atsamo *et al.*, 2011). Nonetheless, the comparative weights of the organs remained unchanged. The bark extract of *H. sanjappae* demonstrated a wide safety margin, with no observable toxicity noted between 300 mg/kg and 2000 mg/kg. The results suggest that these plant extracts have no immediate toxicological risks within the examined dosage ranges.

Various methods are now available to assess the toxicity of drugs, with *in silico* tools emerging as a cost-effective option. Through Drug-likeness and ADMET evaluations, it was observed that the phytochemicals of *H. sanjappae* have favourable properties for becoming a drug candidate; however, the results regarding toxicity were inconclusive, as some compounds exhibited toxic effects. 3',4',5,7-Tetrahydroxyisoflavanone, Eupatoriochromene, Mexicanin E, Herniarin, and Valerianine are some of the most promising phytochemicals for drug development. These compounds show no inhibition of CYP2C19, CYP2C9, and are not substrates for CYP3A4. Most importantly, they adhere to the Lipinski's Rule of Five. Additionally, they do not exhibit hepatotoxicity or skin sensitization, and their intestinal absorption rates are relatively high, ranging from 70% to 98%. Their total clearance rates are moderate as well.

Despite these promising initial findings, the study highlights the significance of conducting further experimental validation. Subsequent *in vivo* toxicity analysis confirmed the safety of the extract. This study's acute oral toxicity evaluation reinforces the notion that the extract, although including chemicals known for toxicity, was classed as GHS Category 4 with an LD50 above 300–2000 mg/kg,

indicating a safe dosage range. The toxicity of a singular detrimental molecule in a crude extract containing numerous useful chemicals can frequently be alleviated by the synergistic effects of other elements in the extract (Ma *et al.*, 2009; Yarnell, 2015). This phenomenon arises when different substances combine, amplifying therapeutic effects while mitigating potential unfavourable consequences. The findings underscore the safety profile of the extract, indicating that the synergistic effects of its components enhance its acceptability and therapeutic potential, thus endorsing its application as a natural remedy (Yarnell, 2015).

4.2.3 Conclusion

In conclusion, the study provides comprehensive insights into the pharmacokinetic and toxicological evaluation of the 57 identified compounds from the plant *H. sanjappae* which is ethnomedicinally significant. The ADMET analysis revealed the potential of these compounds as promising candidates for therapeutic applications, warranting further investigation in preclinical settings. Interestingly, while some individual constituents of the extract exhibited toxicity, acute oral toxicity study of the crude extract's synergistic composition appeared to neutralize these effects, highlighting its potential safety as a therapeutic agent. However, extensive pharmacological investigations, including pre-clinical studies, are needed to fully realize its therapeutic potential. The findings provide a strong foundation for future research to develop safe and effective herbal drugs from this plant.

These findings not only provide scientific use but also validate scientific foundation for developing standardized formulations.

4.3 Network pharmacology and *In vitro* analysis of Ethanol extract of *Humboldtia sanjappae* bark in treating inflammation

Network pharmacology is a new field that studies how drugs interact with multiple targets (usually protein) in the body (Hopkins, 2007). Network pharmacology use computational methods to investigate the characteristics of pharmaceuticals (Xin *et al.*, 2021; Zhou *et al.*, 2020). Currently, there is an increasing interest in natural therapies (Cheng *et al.*, 2009). Numerous studies concentrate on reverse pharmacology and employ plant-derived chemicals to formulate novel pharmaceuticals (Patwardhan & Vaidya, 2010; Willcox *et al.*, 2011). However, scientifically proving traditional knowledge is challenging and requires laboratory testing and clinical trials to ensure safety and effectiveness. To avoid rigorous laboratory experiments, the network pharmacology approach can be employed (Barkat *et al.*, 2021; Saggar *et al.*, 2022). This technique can examine the polypharmacological effects of crude plant extracts to elucidate the role of phytochemicals in the treatment of various diseases (Tang & Aittokallio, 2014). Polypharmacology can provide more effective therapeutic options for managing chronic inflammatory medical conditions by targeting numerous elements linked to inflammation.

Inflammation is a crucial response provided by the immune system; previously, it was thought that inflammation was associated with immune system diseases, but nowadays, it is understood that inflammation is indicative of several diseases (Ahmed, 2011; Schmid-Schönbein, 2006). Persistent unresolved inflammation can cause several related issues and can lead to even cancer (Allavena *et al.*, 2008). Several medications are on the market to treat inflammation. Since, inflammation is a complex mechanism, prolonged use of the medications is not advisable because of their side effects (Gooch *et al.*, 2007; Nikolaou *et al.*, 2013). Plant-based medicines are now becoming more beneficial in treating various inflammations. Because, plants consist of different chemical compounds, they help regulate multiple signalling pathways related to inflammation, and they are reported to have the ability to reduce inflammatory markers such as TNF-α, IL-6, and IL-1β etc (Aravilli *et al.*, 2017; Bernhoft, 2010; Merecz-Sadowska *et al.*, 2020; Zhu *et al.*, 2018). World Health Organization recommend using plant-based diets to maintain a healthy lifestyle and

also traditional medicine is relied upon by approximately 65% of the global population. The, use of plants remains significant during this period, as shown by the data. Hence, plants have emerged as a significant source of novel drug discovery (Kharaty *et al.*, 2023; Owona *et al.*, 2020). Our crude extract contains various phytochemicals that can be tested for their effects on inflammation using the network pharmacology approach. This chapter evaluates its potential against inflammation *in silico* and *in vitro.*

1) To predict potential molecular targets of the phytochemicals using network pharmacology and identify their role in inflammation.
2) To analyze gene-pathway interactions related to inflammation and determine the influence of phytochemicals on these pathways.
3) To validate the *in silico* findings through *in vitro* assays**,** evaluating the anti-inflammatory effects of the crude extract and its active compounds.

4.3.1 Results

4.3.1.1 Network pharmacology analysis of HBA

Network pharmacology analysis of HBA has provided valuable insights into the anti-inflammatory potential of its various phytochemicals. Among the 57 identified phytochemicals, 21 were selected for analysis based on data availability.

Potential targets of Phytochemicals from HBA and Inflammation: A total of 123 targets were predicted for phytochemicals of HBA. Out of the 57 compounds identified through LC-MS/MS analysis, 21 phytochemicals were only used to construct the network due to the lack of available data. Targets with a predicted probability score lower than 0.4 were excluded from further analysis, as they were considered less likely to exhibit significant interactions with the compound of interest. A total of 16365 targets were represented by inflammation. A total of 117 common targets were found (Table 18), suggesting potential similarities between phytochemical targets and those associated with inflammation (Figure 30).

Table 18: Common targets associated with phytochemical and inflammation

Sl. No	Gene	Elaborated name
1.	ABCB1	ATP-dependent translocase
2.	ABCC1	Multidrug resistance-associated protein 1
3.	ABCC5	Multidrug resistance-associated protein 5
4.	ABCG2	Multidrug resistance-associated protein 5
5.	ACHE	Acetylcholinesterase
6.	ADORA1	Adenosine receptor A1
7.	ADORA2A	Adenosine receptor A2a
8.	AKR1B1	Aldo-keto reductase family 1 member B1
9.	AKR1B10	Aldo-keto reductase family 1 member B10
10.	AKT1	RAC-alpha serine/threonine-protein kinase
11.	ALOX12	Arachidonate 12-lipoxygenase
12.	ALOX15	Arachidonate 15-lipoxygenase
13.	ALOX5	Arachidonate 5-lipoxygenase
14.	ALPL	Alkaline phosphatase, tissue-nonspecific isozyme
15.	APOA1	Truncated apolipoprotein A-I
16.	APP	Gamma-secretase C-terminal fragment 50
17.	ARG1	Arginase-1
18.	BCHE	Cholinesterase
19.	CA1	Carbonic anhydrase 1
20.	CA12	Carbonic anhydrase 12
21.	CA13	Carbonic anhydrase 13
22.	CA14	Carbonic anhydrase 14

23.	CA2	Carbonic anhydrase 2
24.	CA4	Carbonic anhydrase 4
25.	CA5A	Carbonic anhydrase 5A
26.	CA6	Carbonic anhydrase 6
27.	CA7	Carbonic anhydrase 7
28.	CA9	Carbonic anhydrase 9
29.	CASP3	Caspase-3 subunit p12
30.	CASP8	Caspase-8 subunit p10
31.	CASP9	Caspase-9 subunit p10
32.	CBR1	Carbonyl reductase NADPH 1
33.	CCNA2	Cyclin-A2
34.	CCNB1	G2/mitotic-specific cyclin-B1
35.	CCNB2	G2/mitotic-specific cyclin-B2
36.	CCND1	G1/S-specific cyclin-D1
37.	CD38	ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1
38.	CDK1	Cyclin-dependent kinase 1
39.	CDK2	Cyclin-dependent kinase 2
40.	CDK5	Cyclin-dependent-like kinase 5
41.	CDK5R1	Cyclin-dependent kinase 5 activator 1
42.	CDK6	Cyclin-dependent kinase 6
43.	CFTR	Cystic fibrosis transmembrane conductance regulator
44.	COL18A1	Collagen alpha-1(XVIII) chain
45.	CSNK2A1	Casein kinase II subunit alpha
46.	CYP19A1	Aromatase
47.	CYP1A1	Cytochrome P450 1A1
48.	CYP1A2	Cytochrome P450 1A2
49.	CYP1B1	Cytochrome P450 1B1
50.	CYP2C8	Cytochrome P450 2C8
51.	CYP3A4	Cytochrome P450 3A4
52.	DUSP1	Dual specificity protein phosphatase 1

53.	EDF1	Endothelial differentiation-related factor 1
54.	EGFR	Epidermal growth factor recepto
55.	ESR1	Estrogen receptor
56.	ESR2	Estrogen receptor beta
57.	FLT3	Receptor-type tyrosine-protein kinase FLT3
58.	FOS	Proto-oncogene c-Fos
59.	GLO1	Lactoylglutathione lyase
60.	GNB1	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1
61.	GSK3B	Glycogen synthase kinase-3 beta
62.	HMOX1	Heme oxygenase 1
63.	HSD17B1	Estradiol 17-beta-dehydrogenase 1
64.	HSD17B2	Estradiol 17-beta-dehydrogenase 2
65.	IL2	Interleukin-2
66.	IL6	Interleukin-6
67.	JUN	Transcription factor AP-1
68.	LPAR1	Lysophosphatidic acid receptor 1
69.	MAOA	Amine oxidase (flavin-containing) A
70.	MAPK1	Mitogen-activated protein kinase 1
71.	MAPK3	Mitogen-activated protein kinase 3
72.	MAPK8	Mitogen-activated protein kinase 8
73.	MMP12	Macrophage metalloelastase
74.	MMP2	72 kDa type IV collagenase
75.	MMP9	67 kDa matrix metalloproteinase-9
76.	MTRNR2L12	MT-RNR2 like 12
77.	MTRR	Methionine synthase reductase
78.	NDOR1	NADPH-dependent diflavin oxidoreductase 1
79.	NLRP3	NACHT, LRR and PYD domains-containing protein 3

80.	NOS1	Nitric oxide synthase
81.	NOS2	Nitric oxide synthase
82.	NOS3	Nitric oxide synthase
83.	NOX4	NADPH oxidase 4
84.	NQO1	NAD(P)H dehydrogenase (quinone) 1
85.	PARP1	Poly (ADP-ribose) polymerase 1
86.	PDE4A	AMP-specific 3',5'-cyclic phosphodiesterase 4A
87.	PDE5A	cGMP-specific 3',5'-cyclic phosphodiesterase
88.	PGF	Placenta growth factor
89.	PKM	Pyruvate kinase PKM
90.	POR	NADPH--cytochrome P450 reductase
91.	PPARA	Peroxisome proliferator-activated receptor alpha
92.	PTAFR	Platelet-activating factor receptor
93.	PTGS2	Prostaglandin G/H synthase 2
94.	PTPN1	Tyrosine-protein phosphatase non-receptor type 1
95.	PTPRS	Receptor-type tyrosine-protein phosphatase S
96.	RGS6	Regulator of G-protein signaling 6
97.	S1PR2	Sphingosine 1-phosphate receptor 2
98.	SKP1	S-phase kinase-associated protein 1
99.	SMAD2	Mothers against decapentaplegic homolog 2
100.	STAT3	Signal transducer and activator of transcription 3
101.	SYK	Tyrosine-protein kinase SYK
102.	TAS2R38	Taste receptor type 2 member 38
103.	TNKS	Poly (ADP-ribose) polymerase tankyrase-1

104.	TNKS2	Poly (ADP-ribose) polymerase tankyrase-2
105.	TOP1	DNA topoisomerase 1
106.	TOP2A	DNA topoisomerase 2-alpha
107.	TP53	Cellular tumor antigen p53
108.	TTR	Transthyretin
109.	TYR	Tyrosinase
110.	UGT1A1	UDP-glucuronosyltransferase 1-1
111.	UGT1A10	UDP-glucuronosyltransferase 1-10
112.	UGT1A6	UDP-glucuronosyltransferase 1-6
113.	UGT1A7	UDP-glucuronosyltransferase 1-7
114.	UGT1A8	UDP-glucuronosyltransferase 1-8
115.	UGT1A9	UDP-glucuronosyltransferase 1-9
116.	VEGFC	Vascular endothelial growth factor C
117.	XDH	Xanthine dehydrogenase/oxidase

These common targets were regarded as possible candidates for the anti-inflammatory effects of HBA.

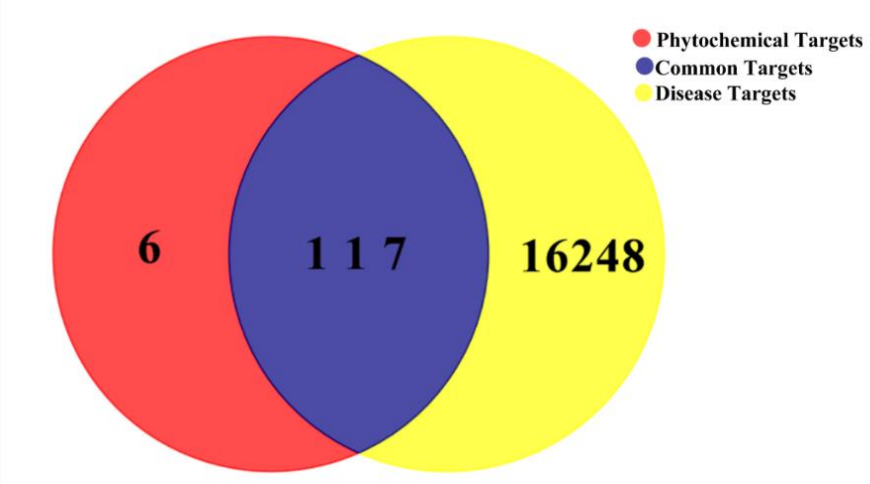

Figure 30: Venn diagram of disease and phytochemical target genes

Compound-target network: Figure 31 illustrates the different compounds present in the crude extract that can influence various molecular targets in the biological system. The network represented a total of 145 nodes and 226 edges. Luteolin was found to

have a good number of interactions with targets, and the target CYP19A1 was found to have multiple interactions with various phytochemicals. This highlights the multi-target and multi-component effects of the compounds in the treatment of inflammation. Overall, the compound -target network explains HBA's polypharmacological action.

Protein–protein-Interaction of disease and phytochemical targets: A protein-protein interaction network comprising 117 common genes has been created via the STRING database. Upon visualising the PPI network, 117 nodes and 1230 edges were identified (Figure 32a & b). The mean node degree of the protein-protein interaction network is 21, signifying that, on average, each protein interacts with 21 other proteins. The mean local clustering coefficient was 0.657, indicating a considerable tendency for proteins to establish strong associations within the network. The network's expected number of interactions (edges) was 467, while the actual observed interactions far exceeded this at 1230. This disparity suggests that the network is far more interconnected than would be anticipated by random occurrence. The PPI enrichment study produced a p-value below 1.0e-16, indicating that the observed network is statistically significant and not attributable to random chance.

4.3.1.4 Gene ontology and pathway enrichment analysis

Gene Ontology is a systematic and standardised framework utilised to categorise and elucidate the roles of genes and their products. It offers a comprehensive depiction of genes and their products across three classifications: molecular function, biological process, and cellular component (Shahzadi *et al.*, 2024; Thomas, 2017).

117 targets were involved in 395 different biological processes in which xenobiotic metabolic process, cellular response to hypoxia, response to lipopolysaccharide, Cellular response to reactive oxygen species, Nitric oxide biosynthetic process, etc were noted. The target proteins were distributed in 64 different cellular components, including, Cytosol, Cytoplasm, Nucleus, and Plasma membrane contained most targets. 148 types of molecular functions were noted in which Enzyme binding, identical protein binding, Protein homodimerization activity, and Zinc ion binding were noted. igure 33 & 34 illustrates top 15 most significant Gene Ontology (GO) terms for all three categories.

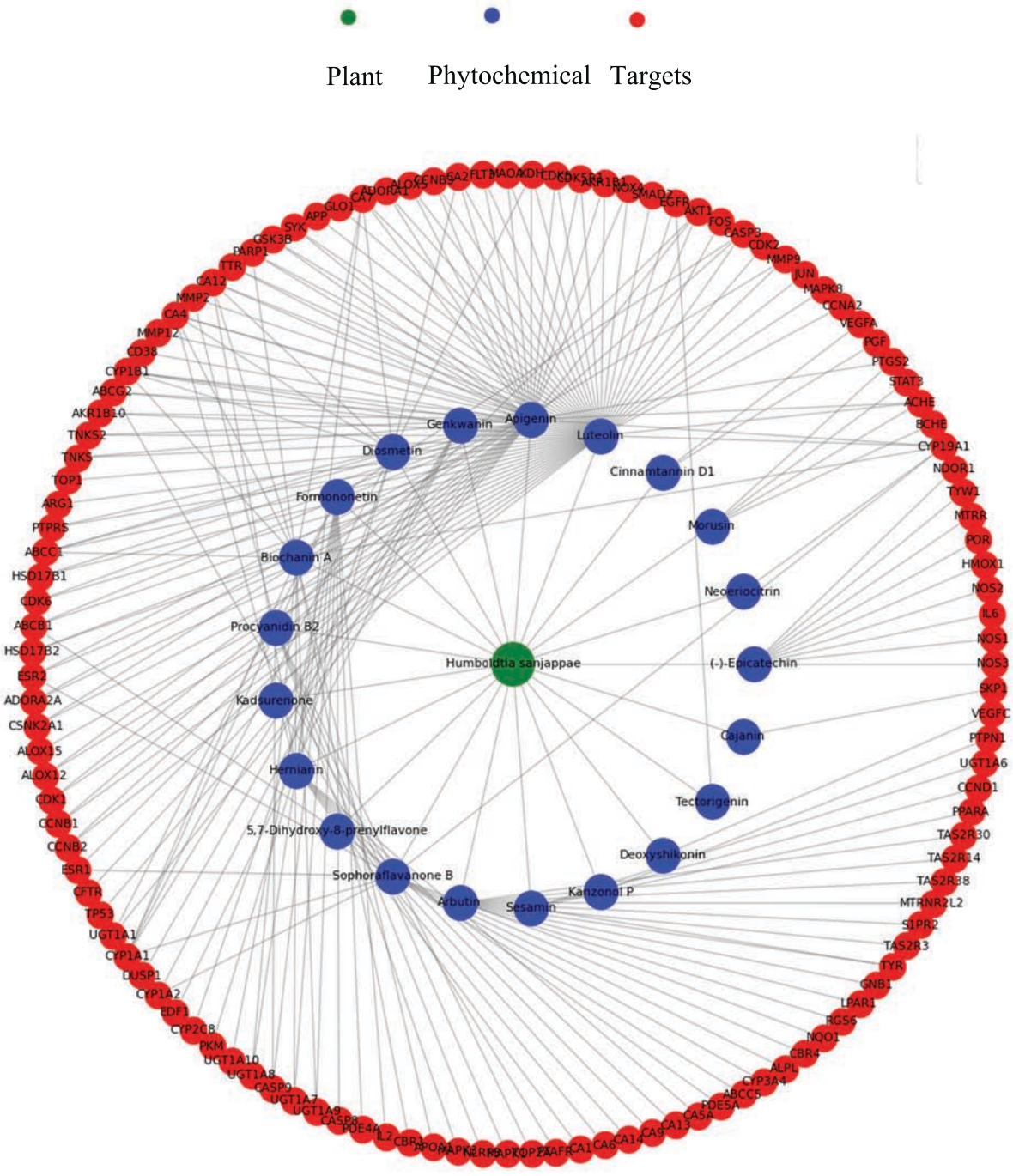

Figure 31: Compound target network. The plant node is represented by green, phytochemicals are blue, and target nodes are red.

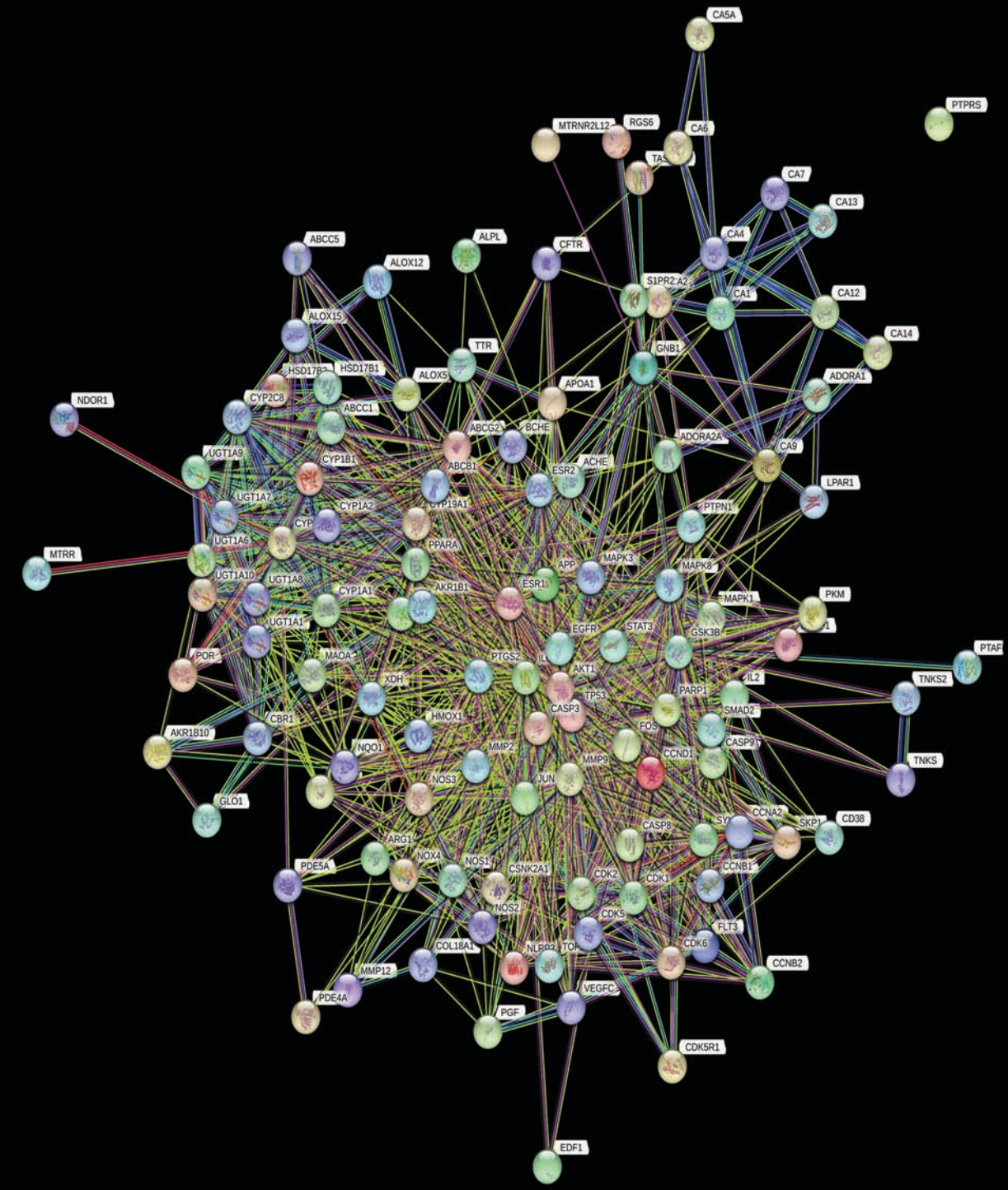

Figure 32a: Protein-protein interactions of the target genes. Line colors representing different types of interaction evidence. Nodes correspond to proteins, and edges indicate interactions, with the Colour of the edge reflecting the specific type of evidence supporting each interaction, such as experimental data, co-expression, or text mining.

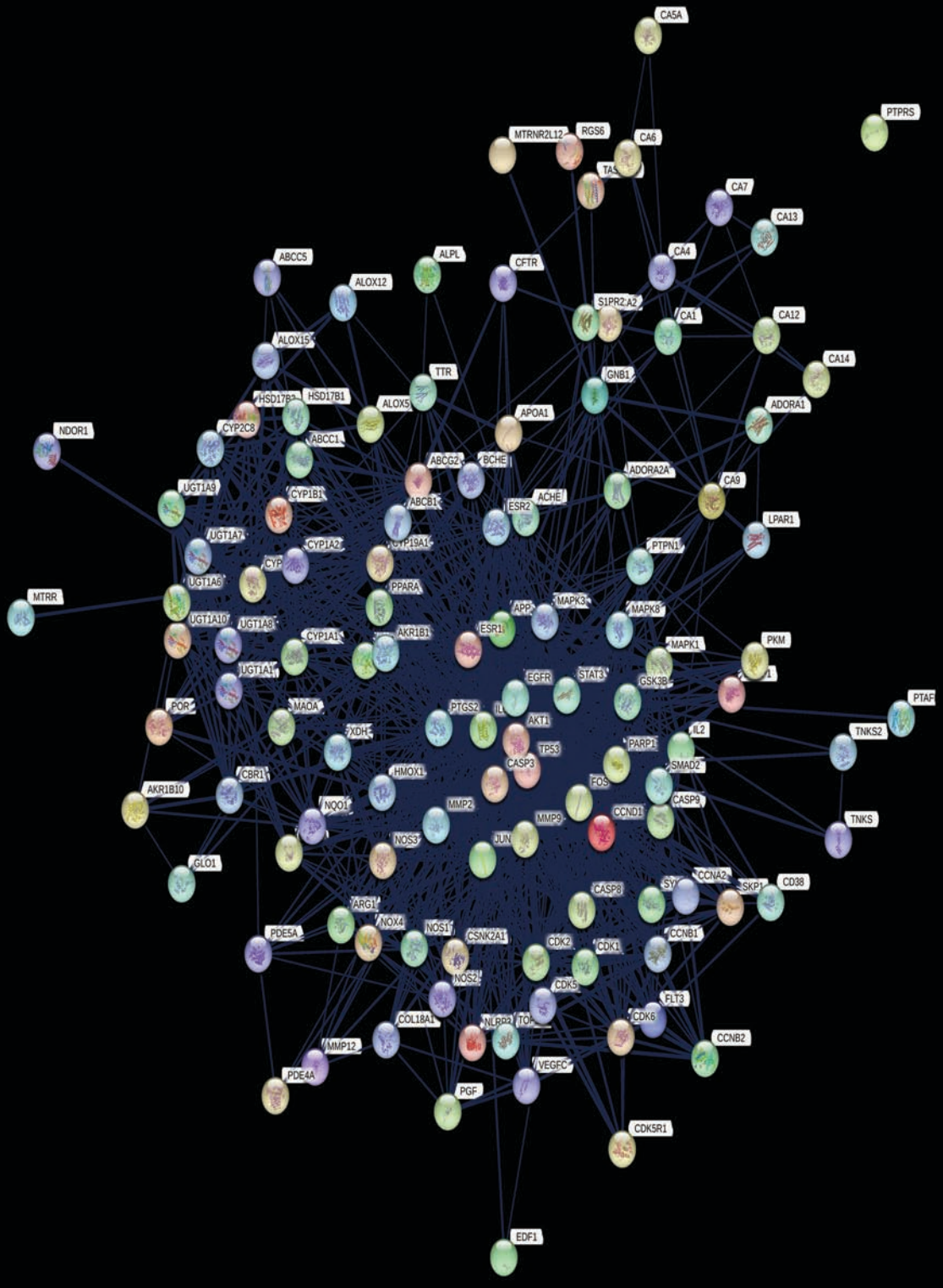

Figure 32b: Protein-protein interactions (PPIs) of the target genes. The nodes represent proteins, and the edges indicate interactions, with thicker lines representing higher confidence in the interaction based on experimental evidence and computational predictions.

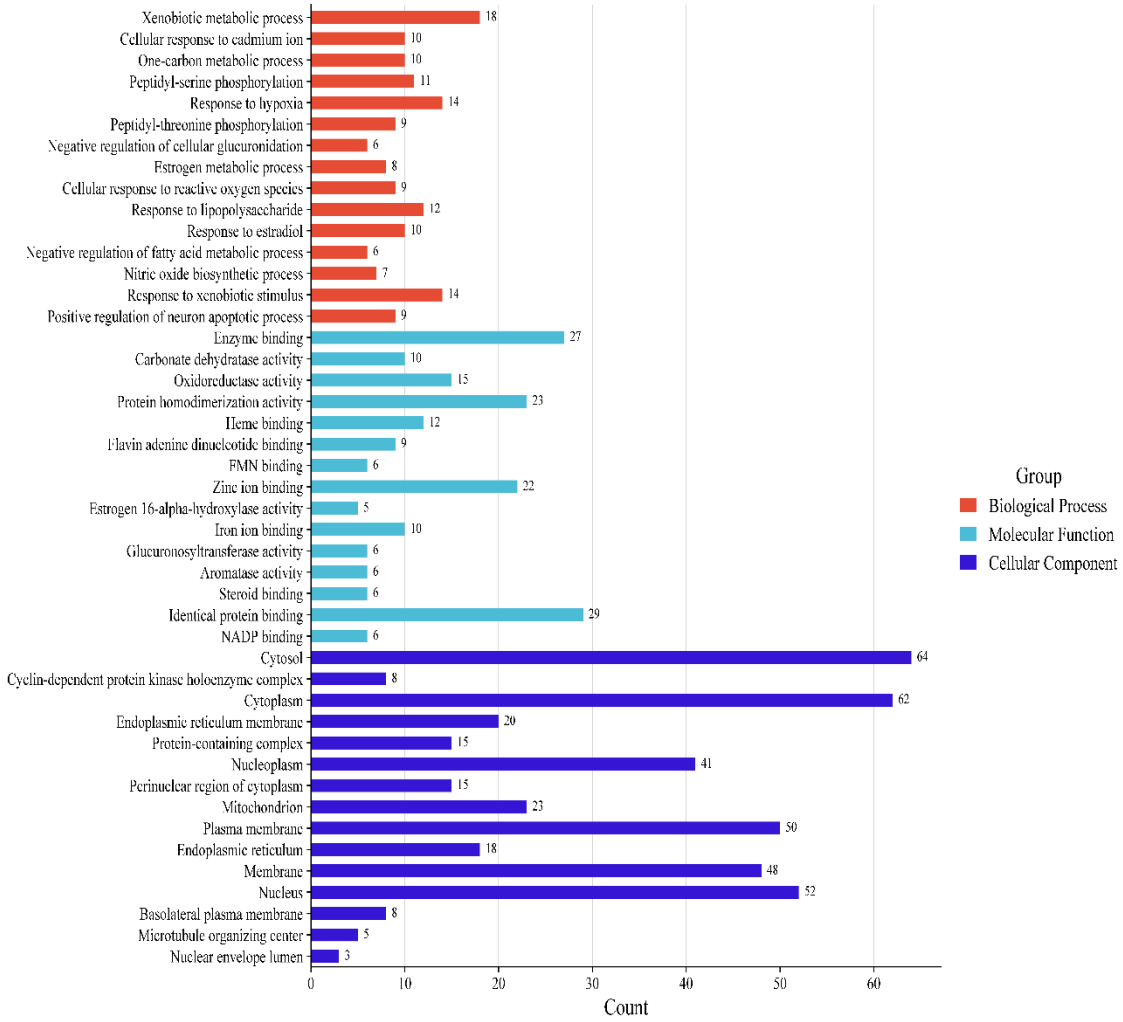

Figure 33: This plot presents the top 15 most significant Gene Ontology (GO). Displays the number of genes associated with each category.

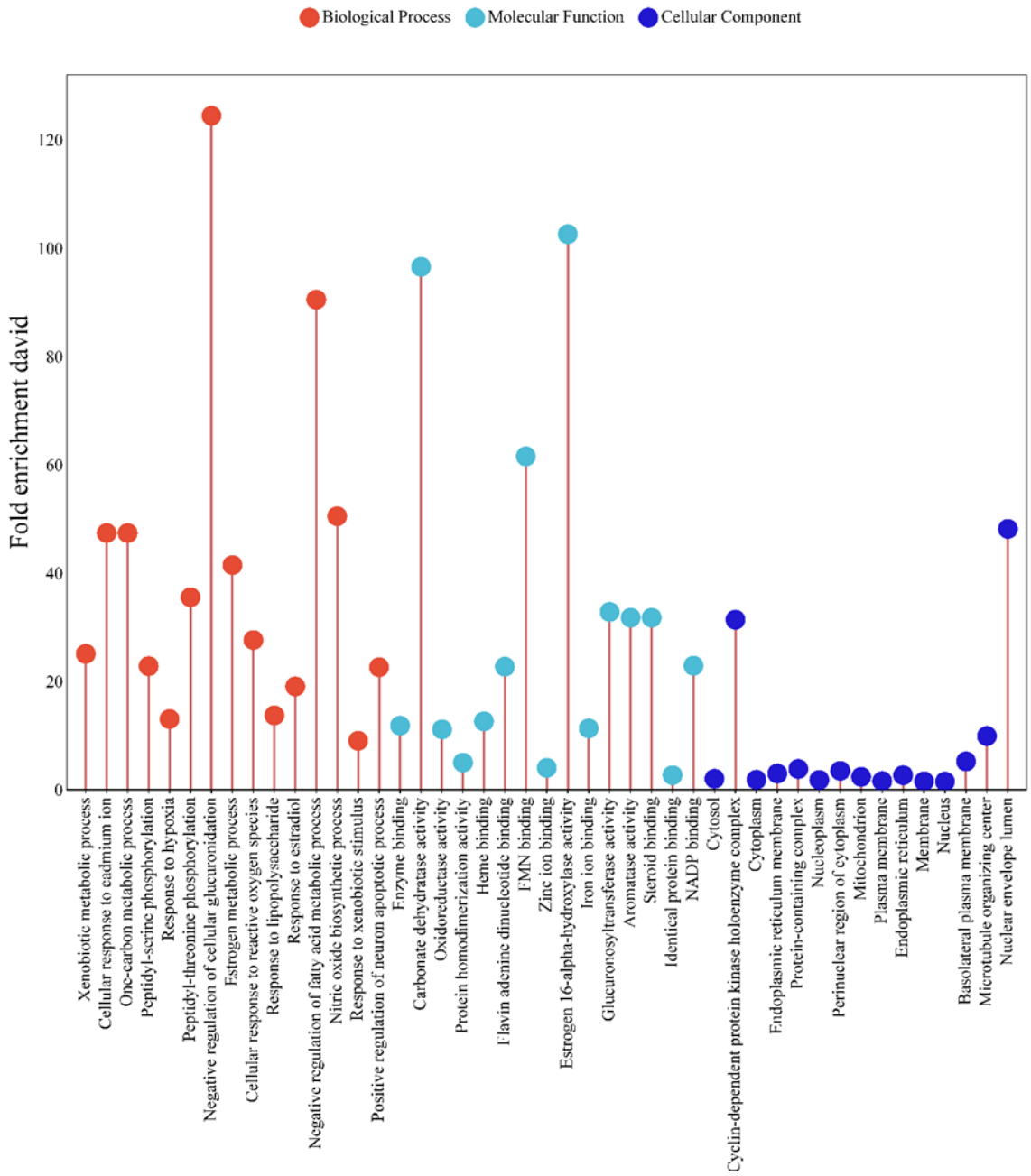

Figure 34: This plot presents the top 15 most significant Gene Ontology (GO). Displays the number of genes associated with each category. Represents the fold enrichment of each category.

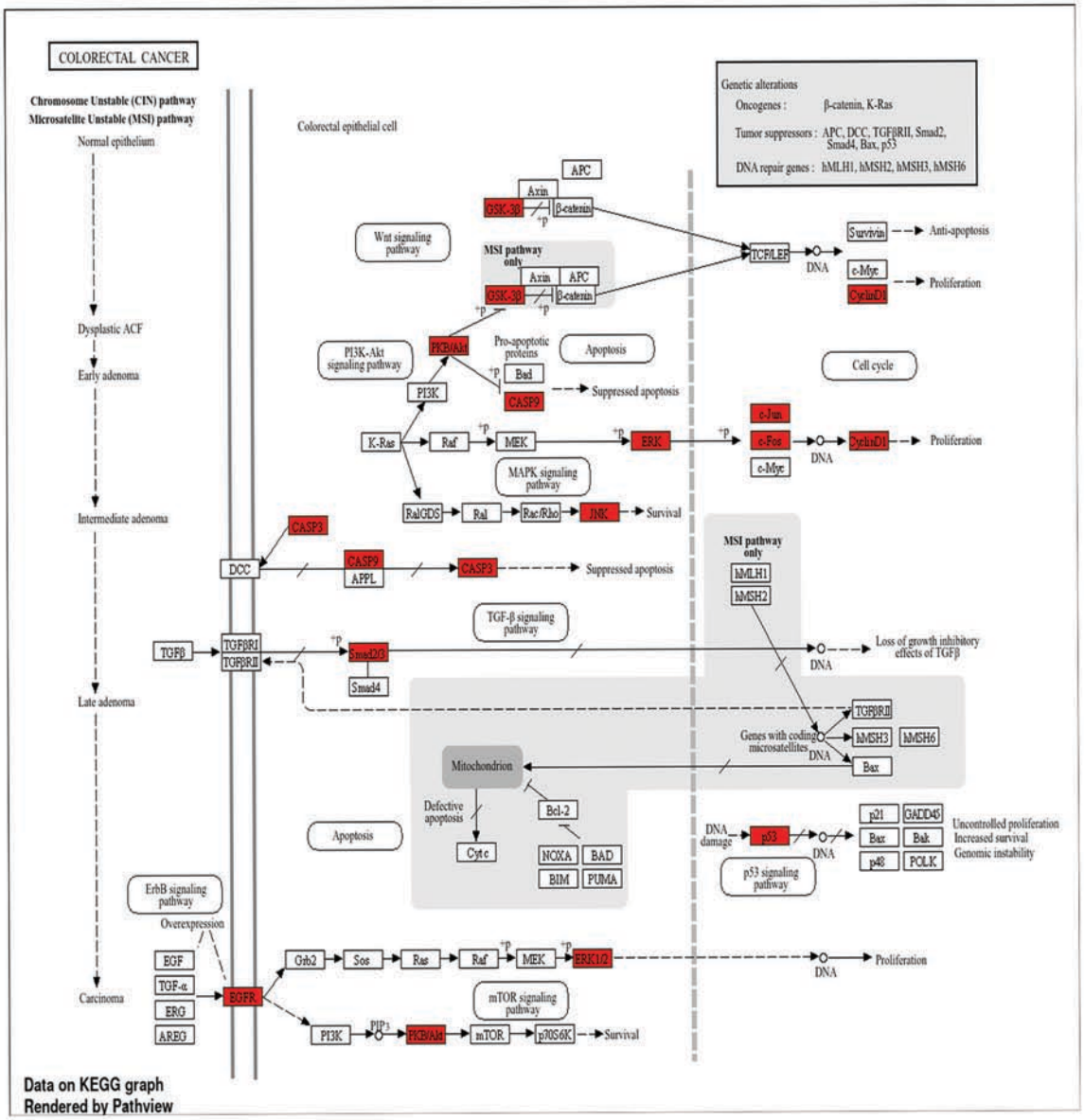

Figure 35a: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes), representing Colorectal Cancer.

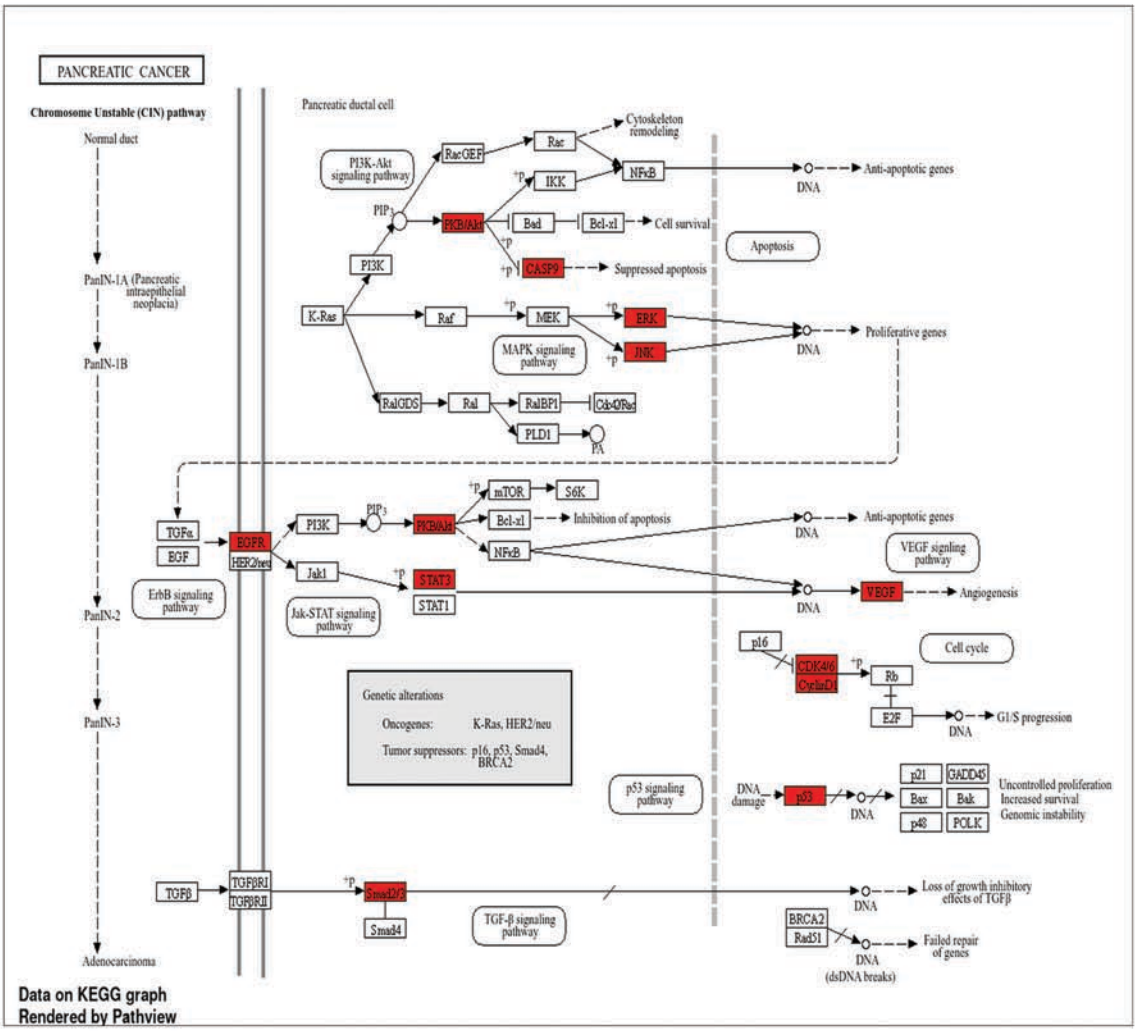

Figure 35b: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes), representing Pancreatic Cancer.

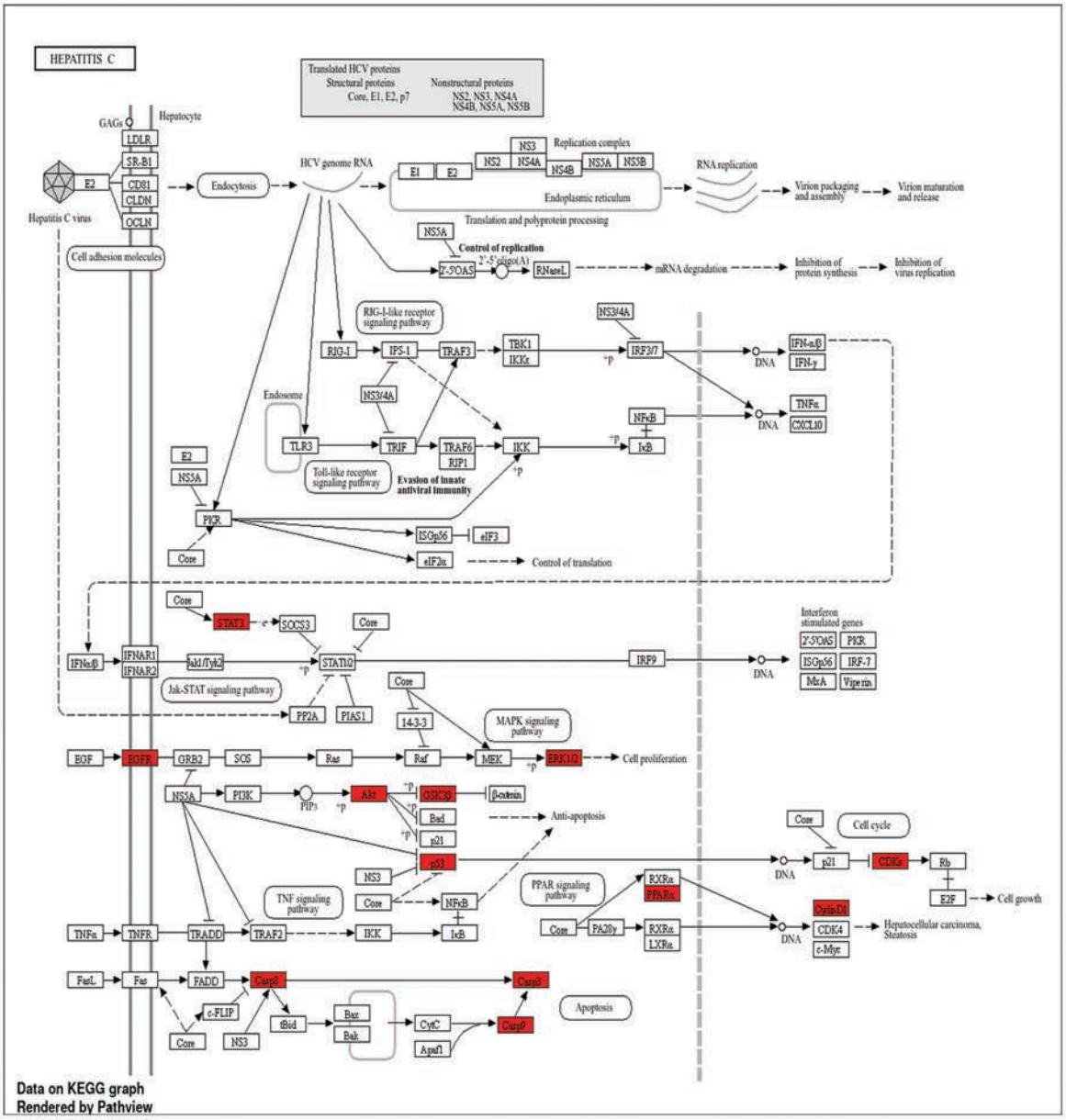

Figure 35c: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes), representing Hepatitis C.

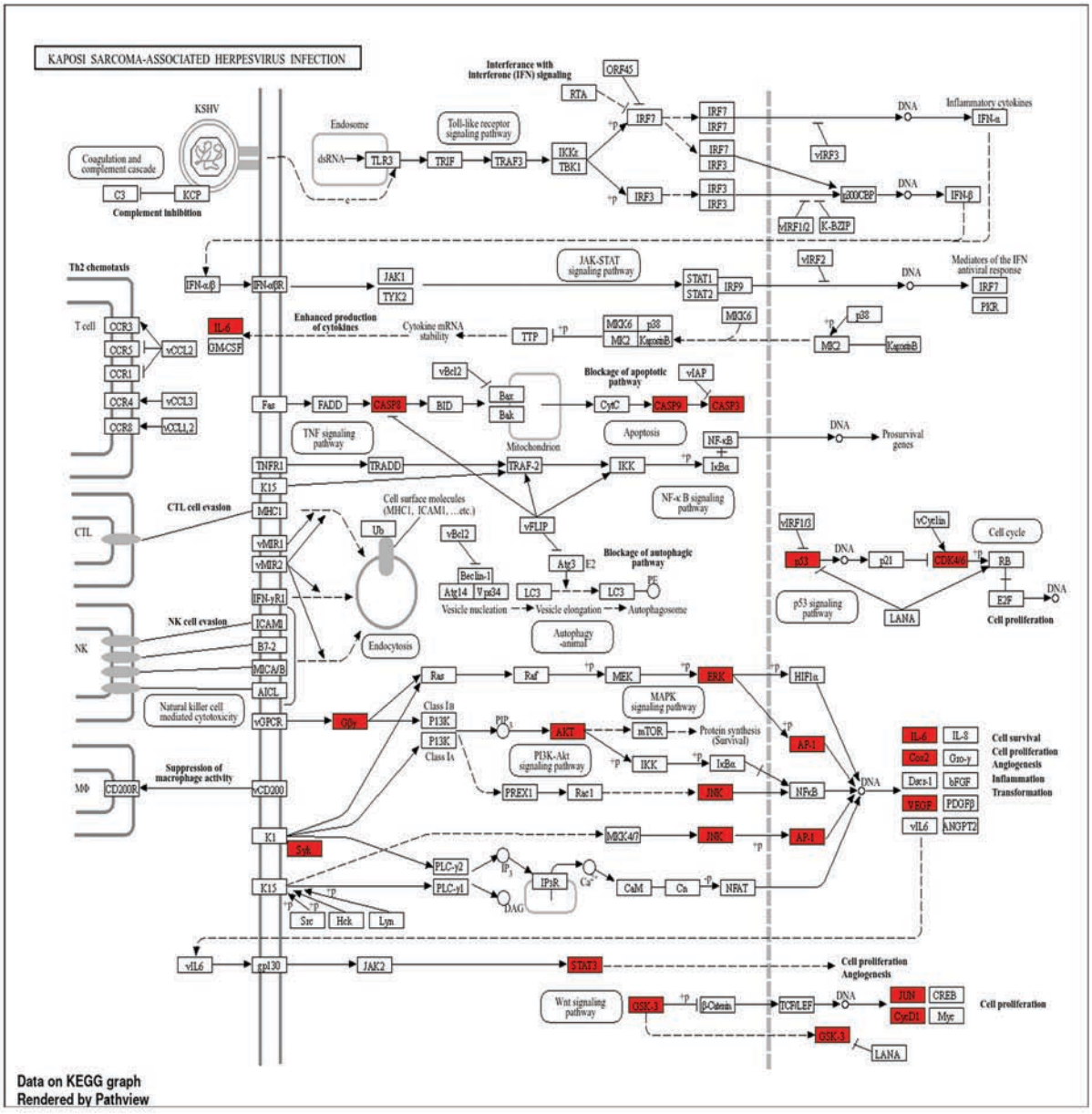

Figure 35d: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes), representing Kaposi sarcoma associated Herpes Virus infection

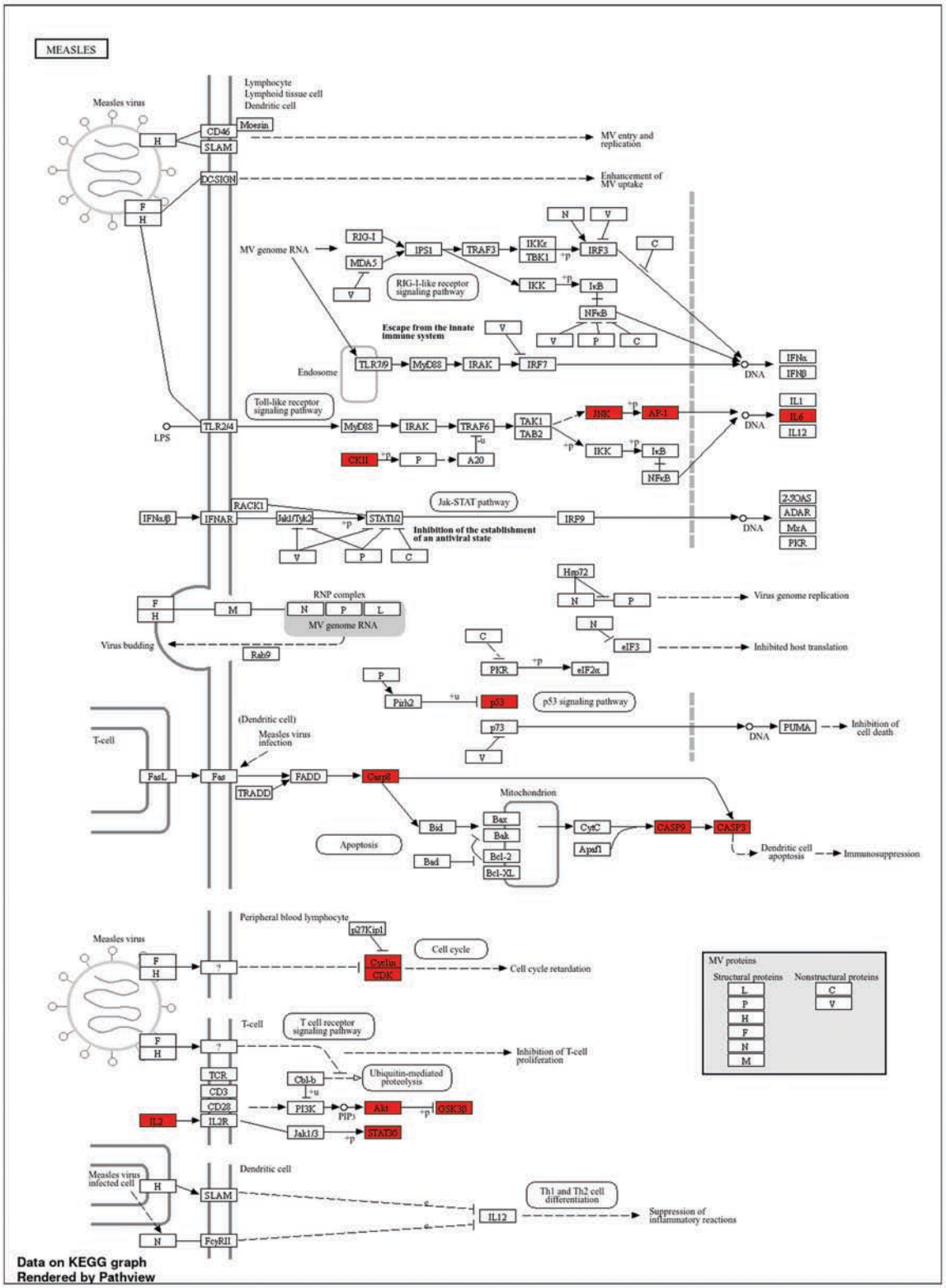

Figure 35e: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes), representing Measles.

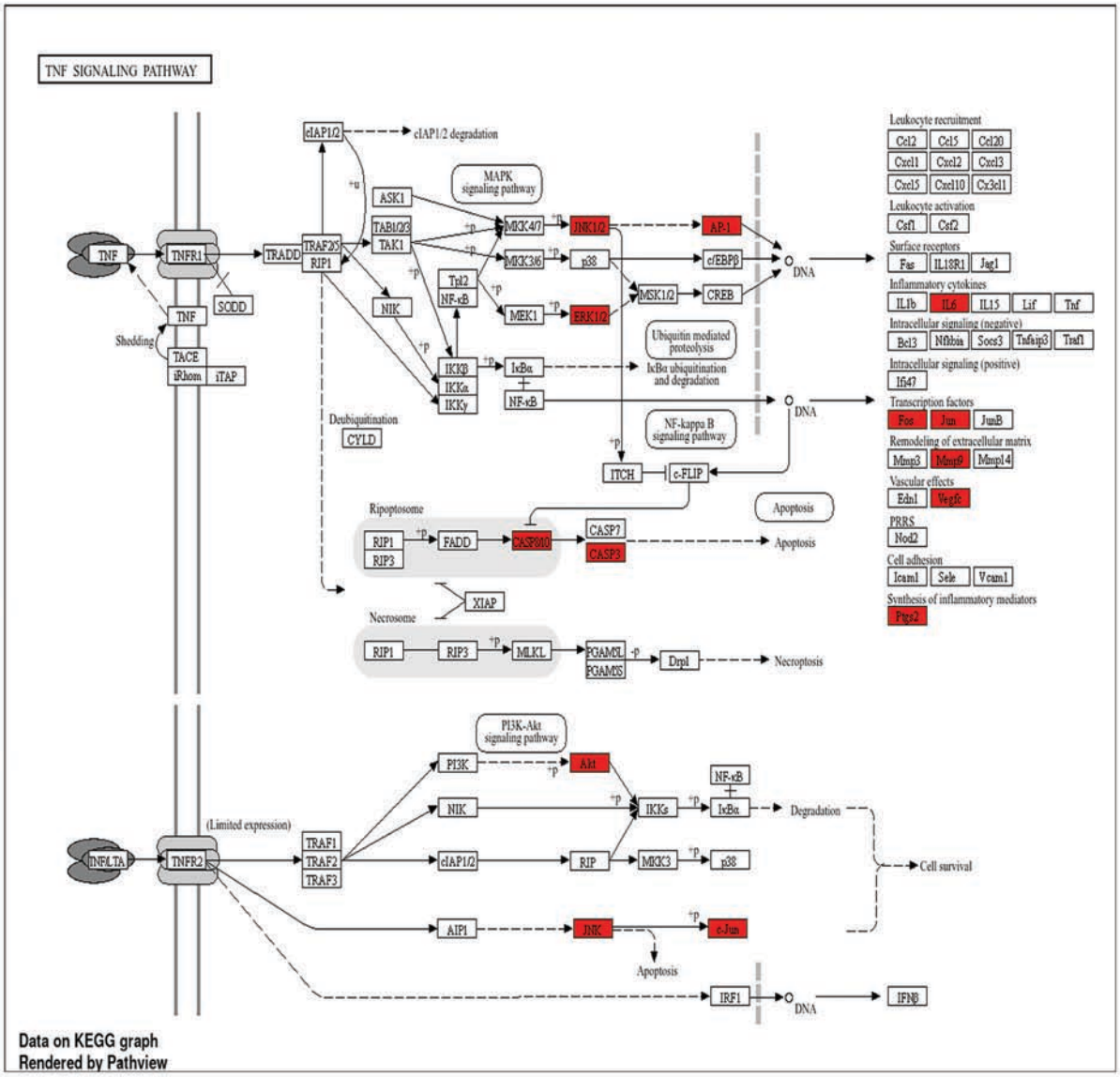

Figure 35f: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes),representing TNF Signaling pathway.

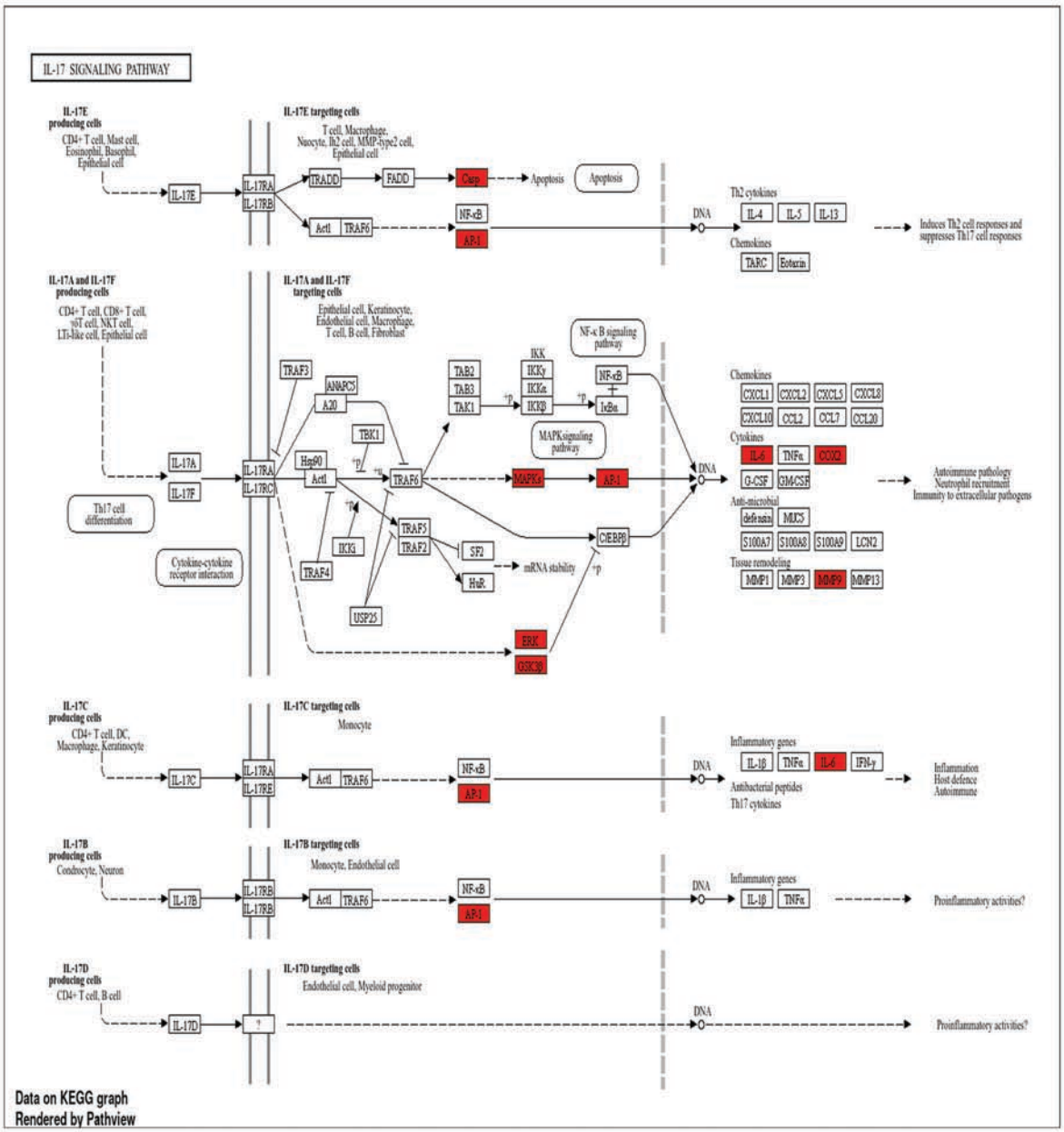

Figure 35g: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes),representing IL-17 Signaling pathway.

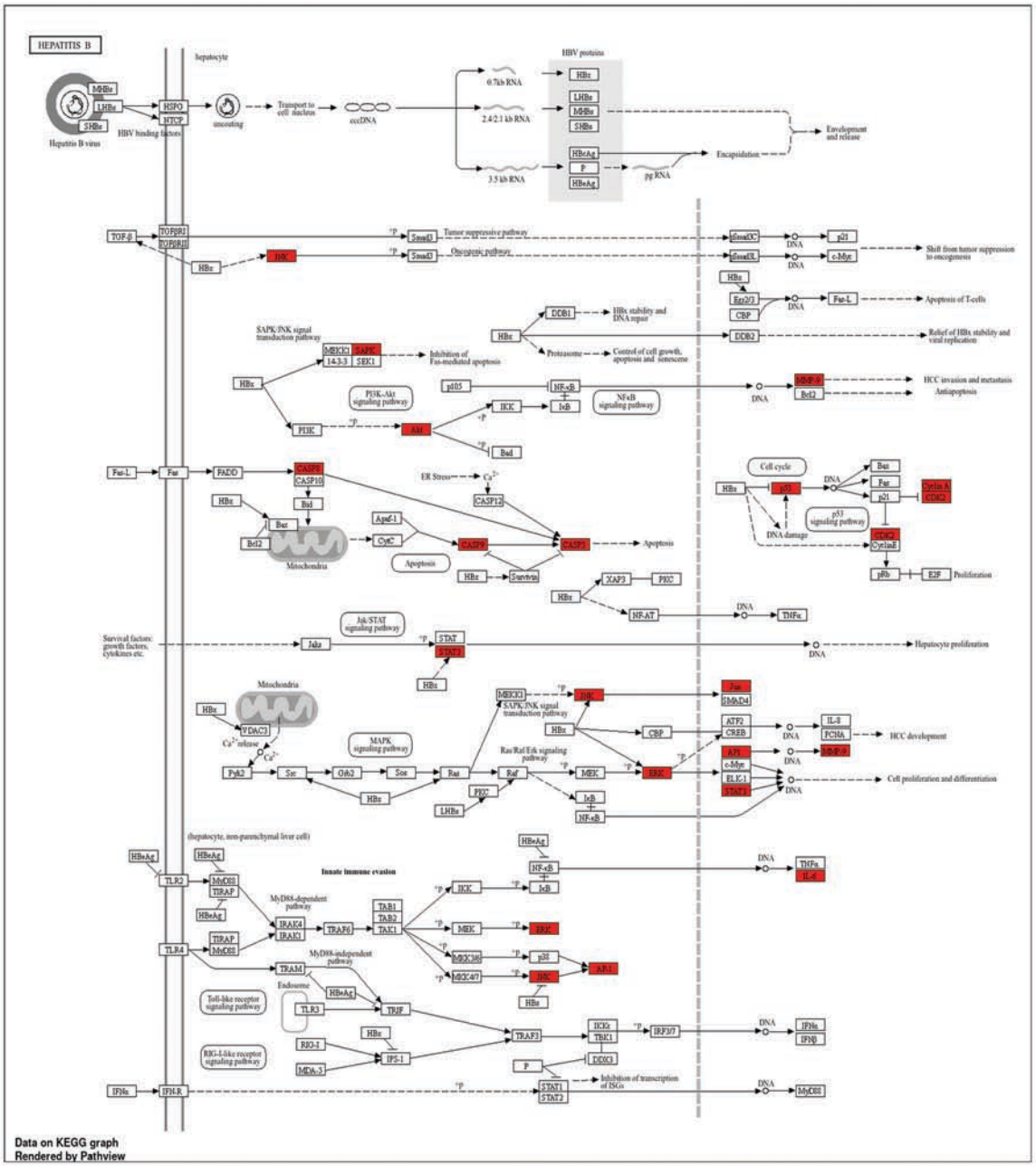

Figure 35h: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes),representing Hepatitis B pathway.

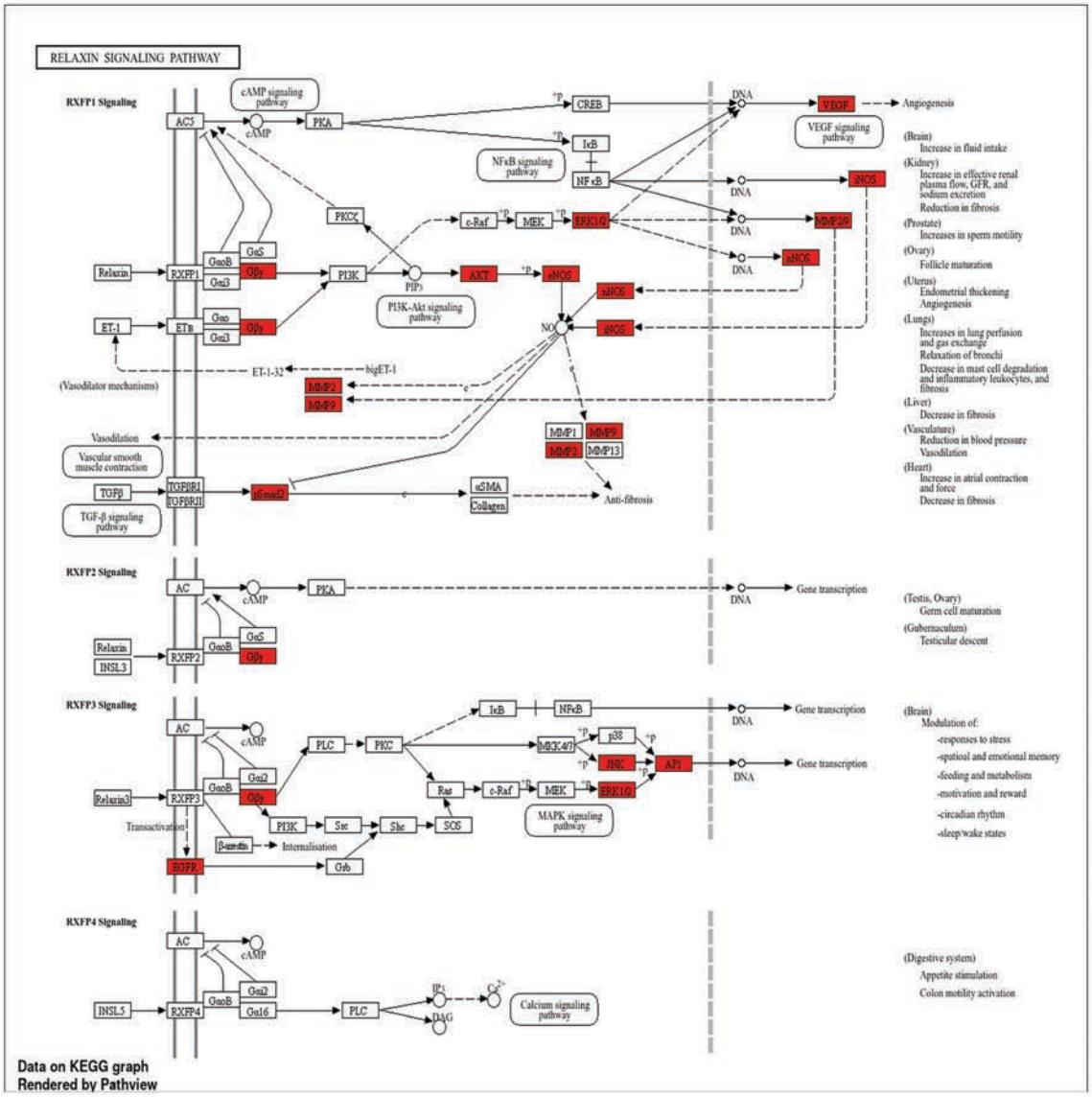

Figure 35i: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes), representing Relaxin signaling pathway.

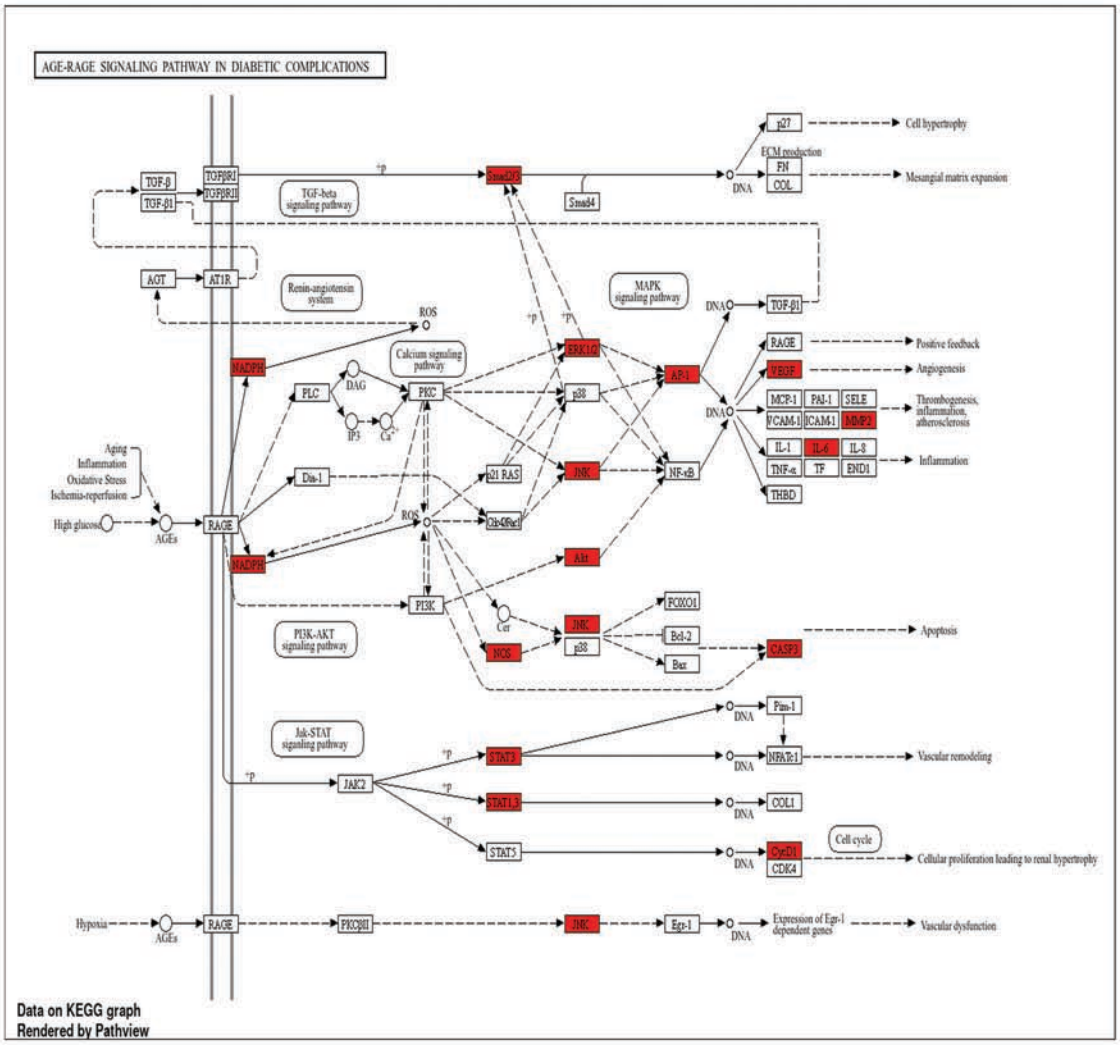

Figure 35j: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes), representing AGE-RAGE Signaling pathway in Diabetic complications.

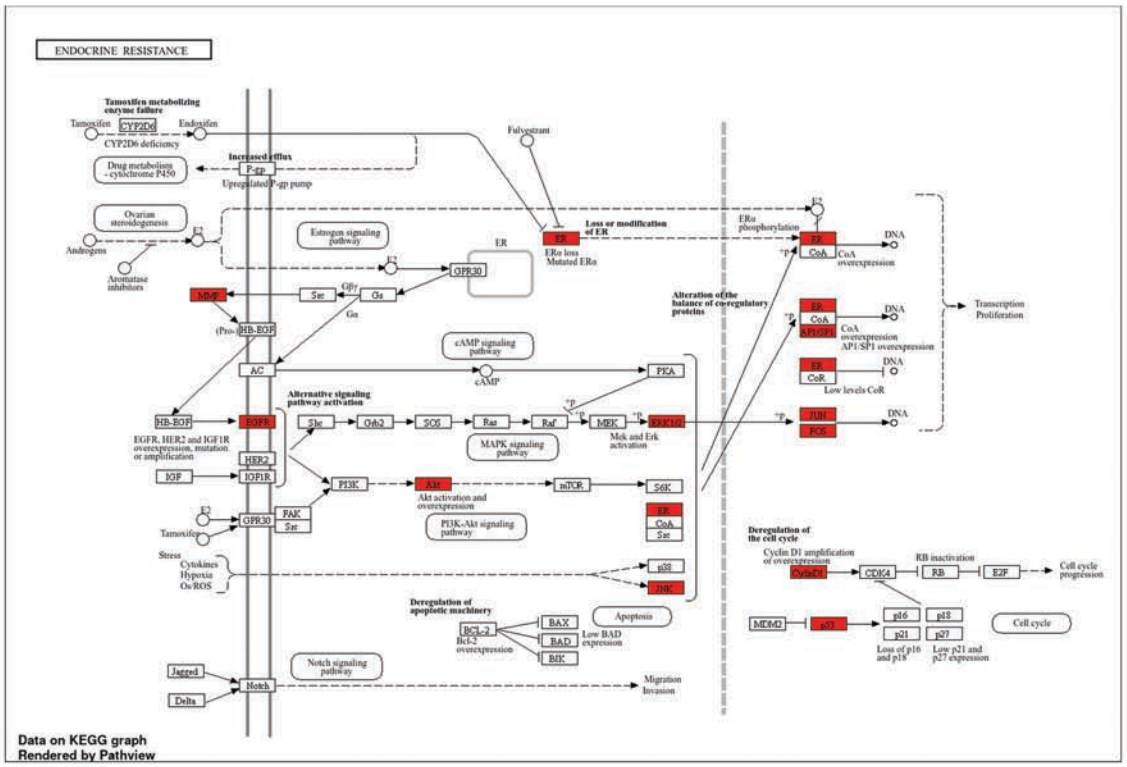

Figure 35k: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes), representing Endocrine resistance pathway in Diabetic complications.

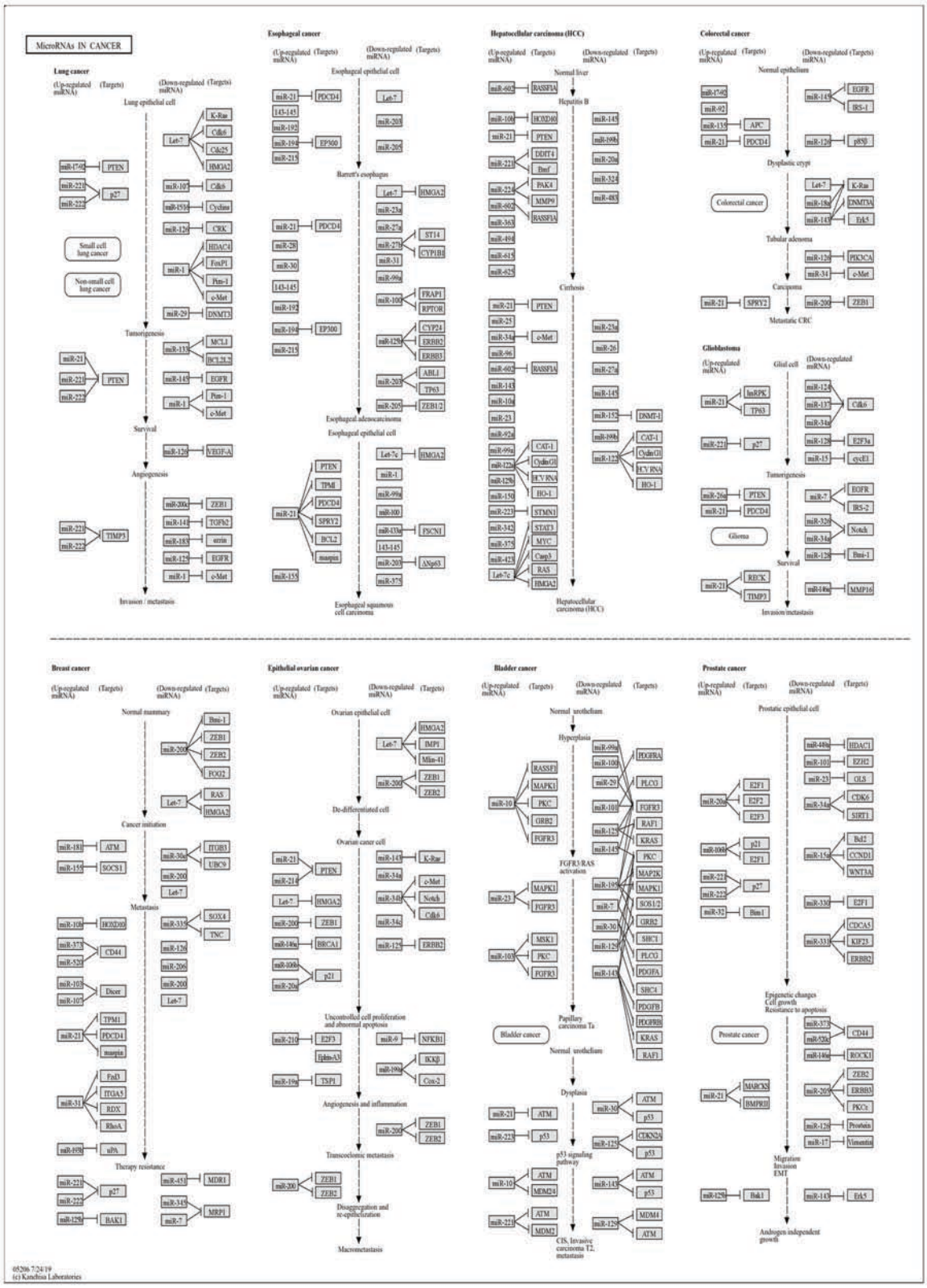

Figure 35l: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes), representing Micro RNAs in various Cancer related Signaling pathways.

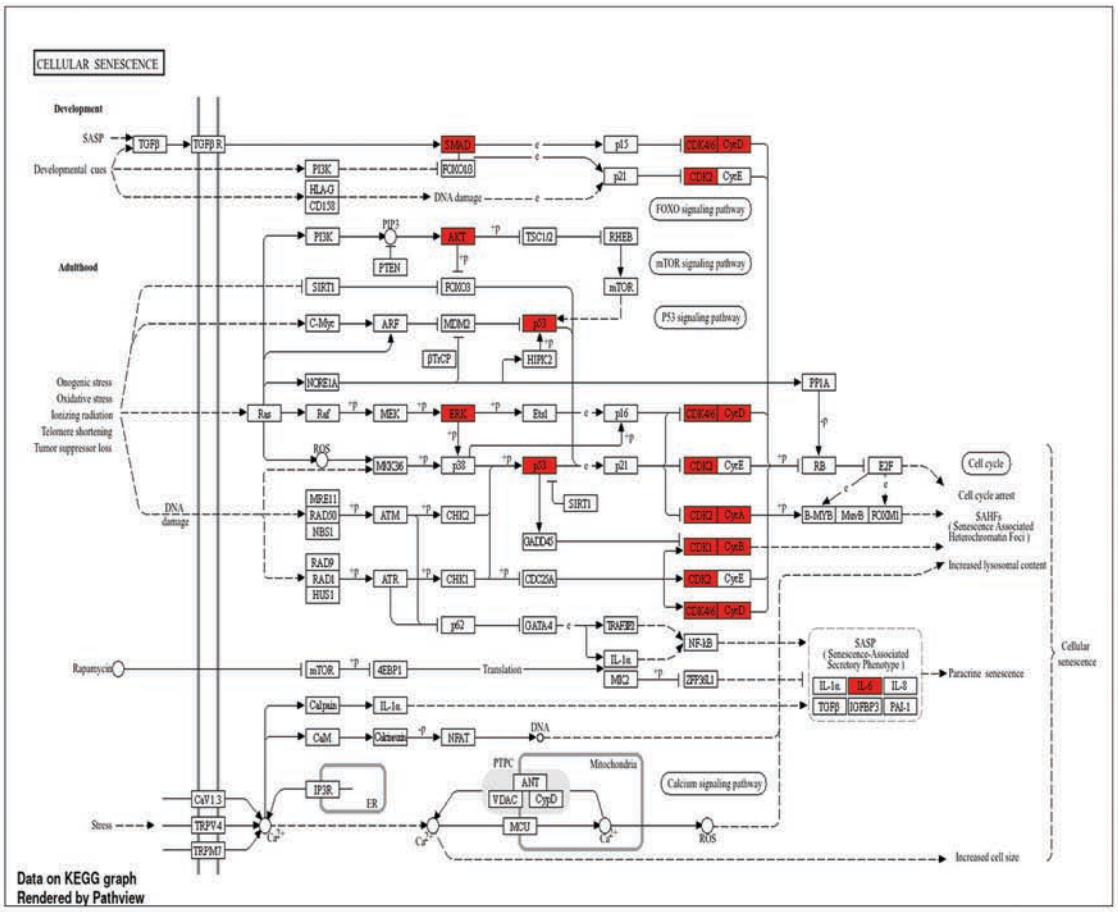

Figure 35m: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes), representing Cellular senescence pathways.

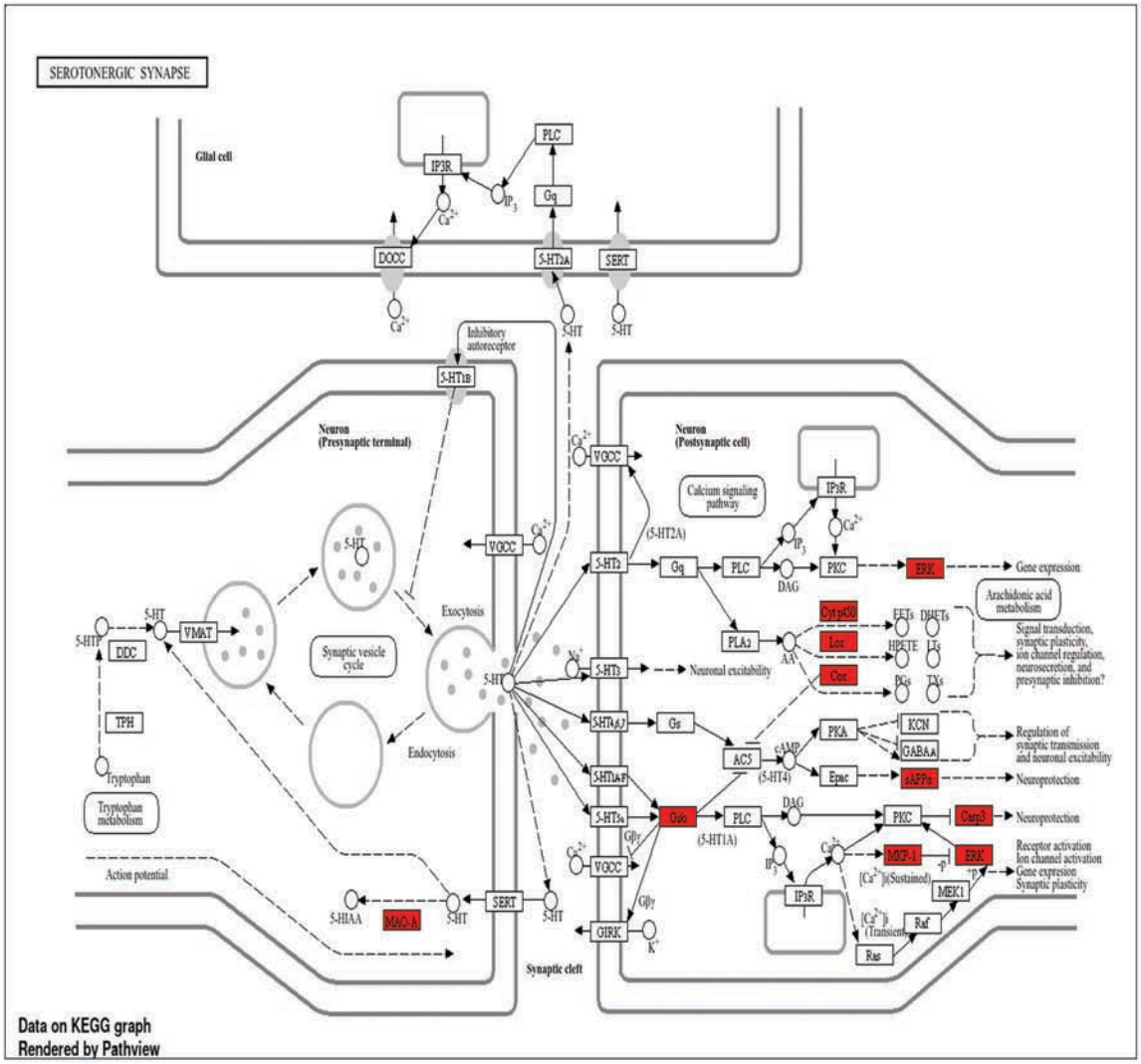

Figure 35n: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes),representing Serotonergic synapse pathways.

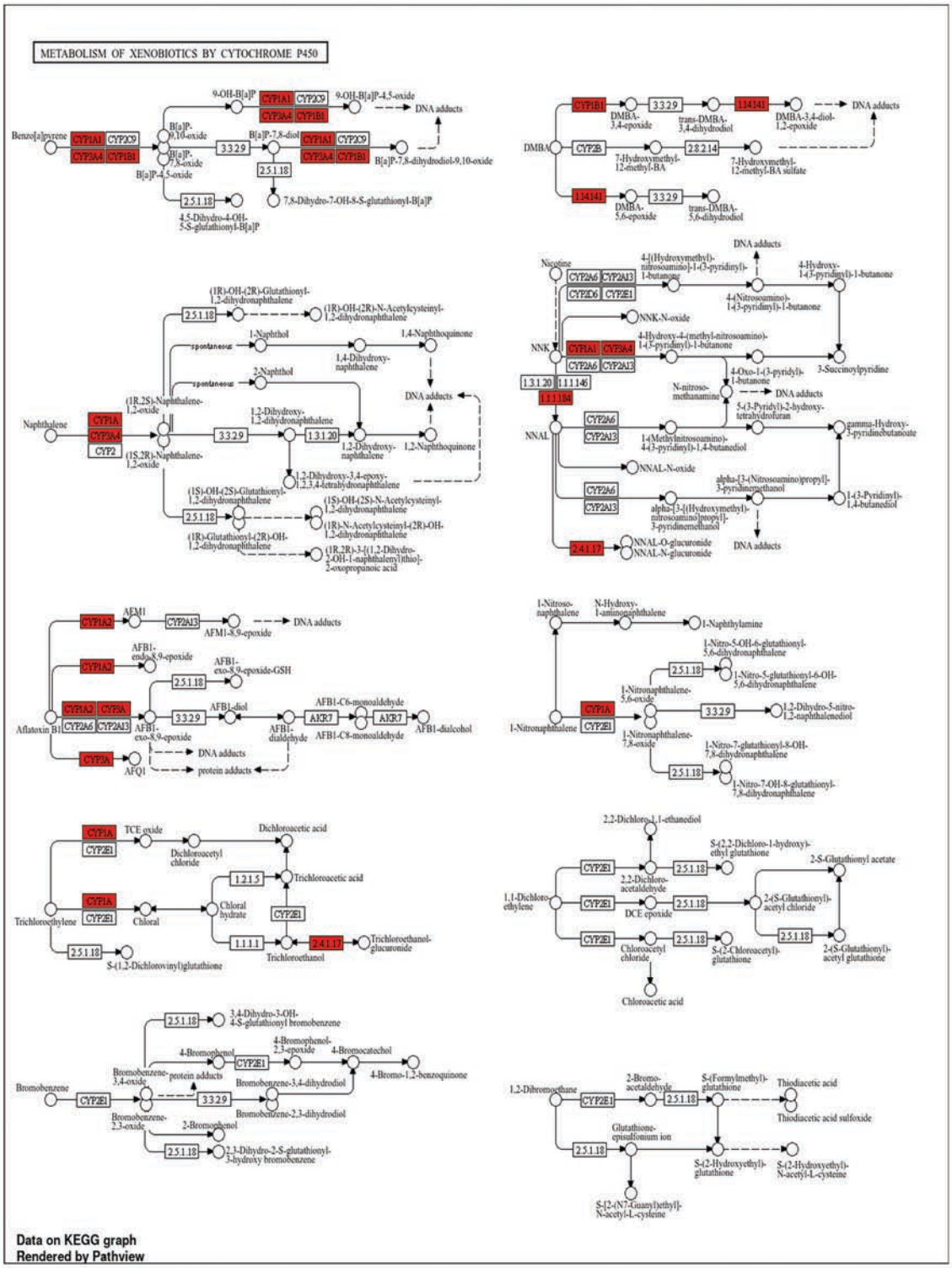

Figure 35o: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes), representing pathways related to Metabolism of xenobiotics by Cytochrome P450.

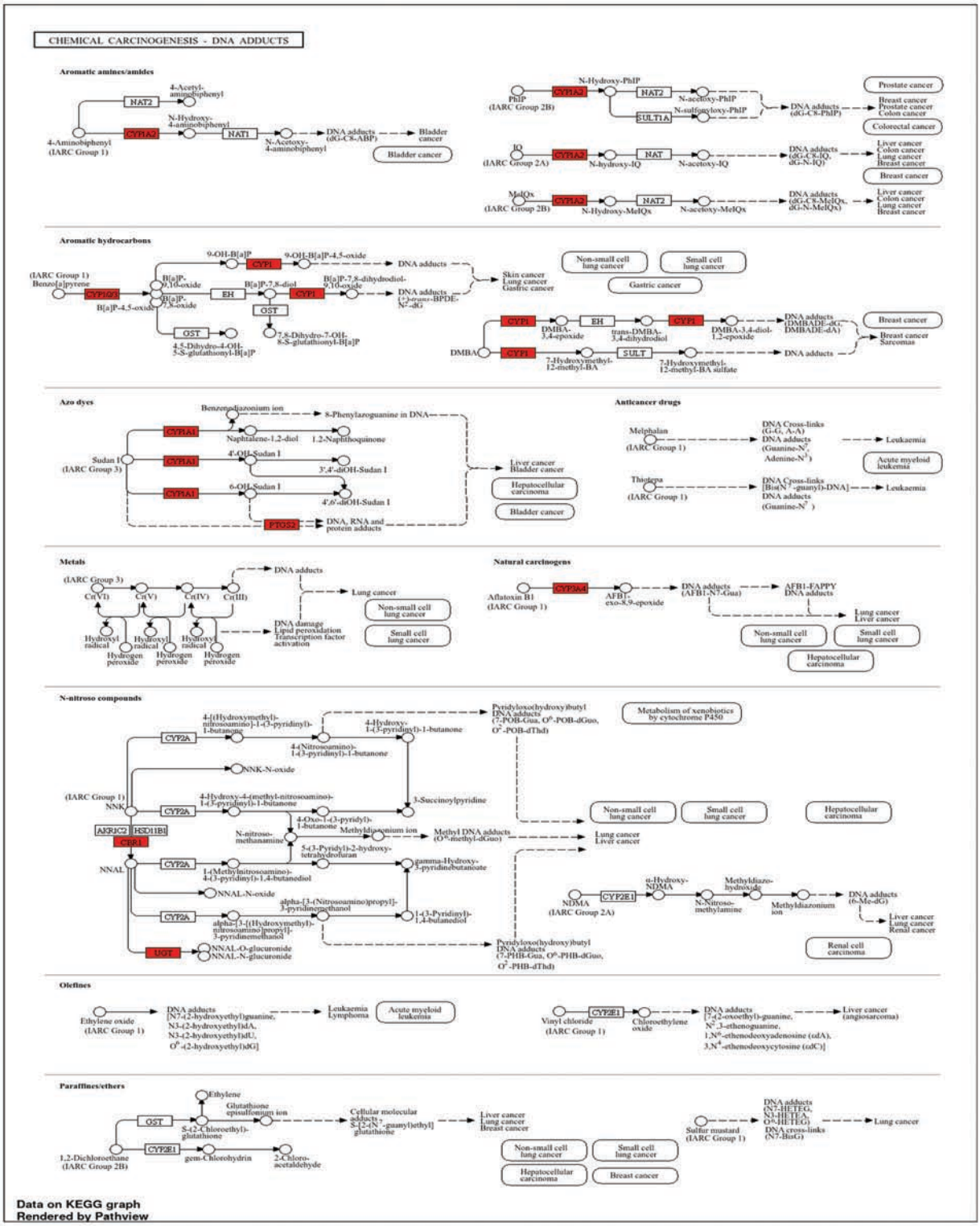

Figure 35p: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes), representing illustrates how various chemical carcinogens are metabolized by enzymes leading to the formation of DNA adducts that can cause mutations and ultimately result in different types of cancer.

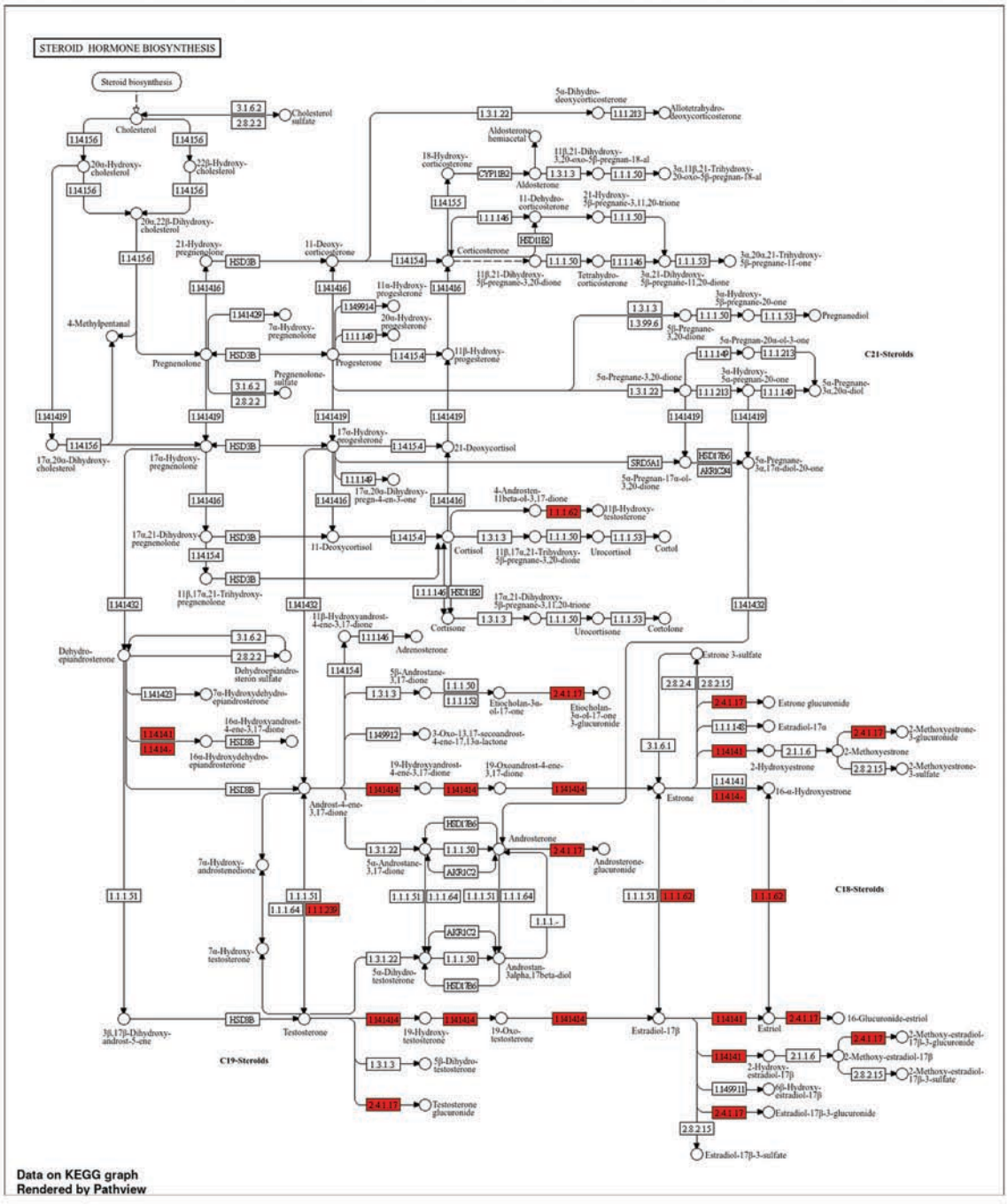

Figure 35q: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes), representing Steroid Hormone biosynthesis pathway.

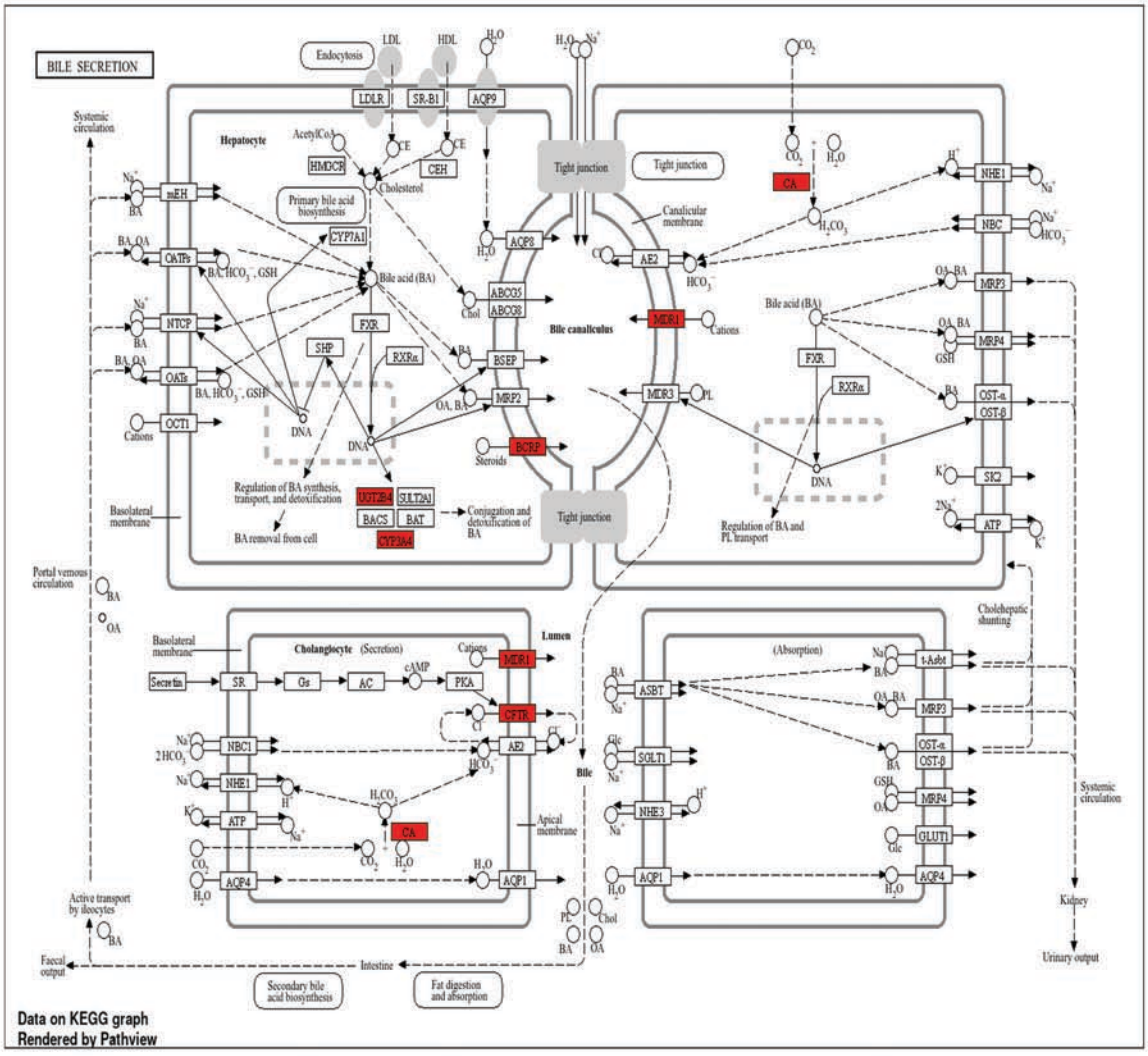

Figure 35r: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes), representing Bile secretion pathway.

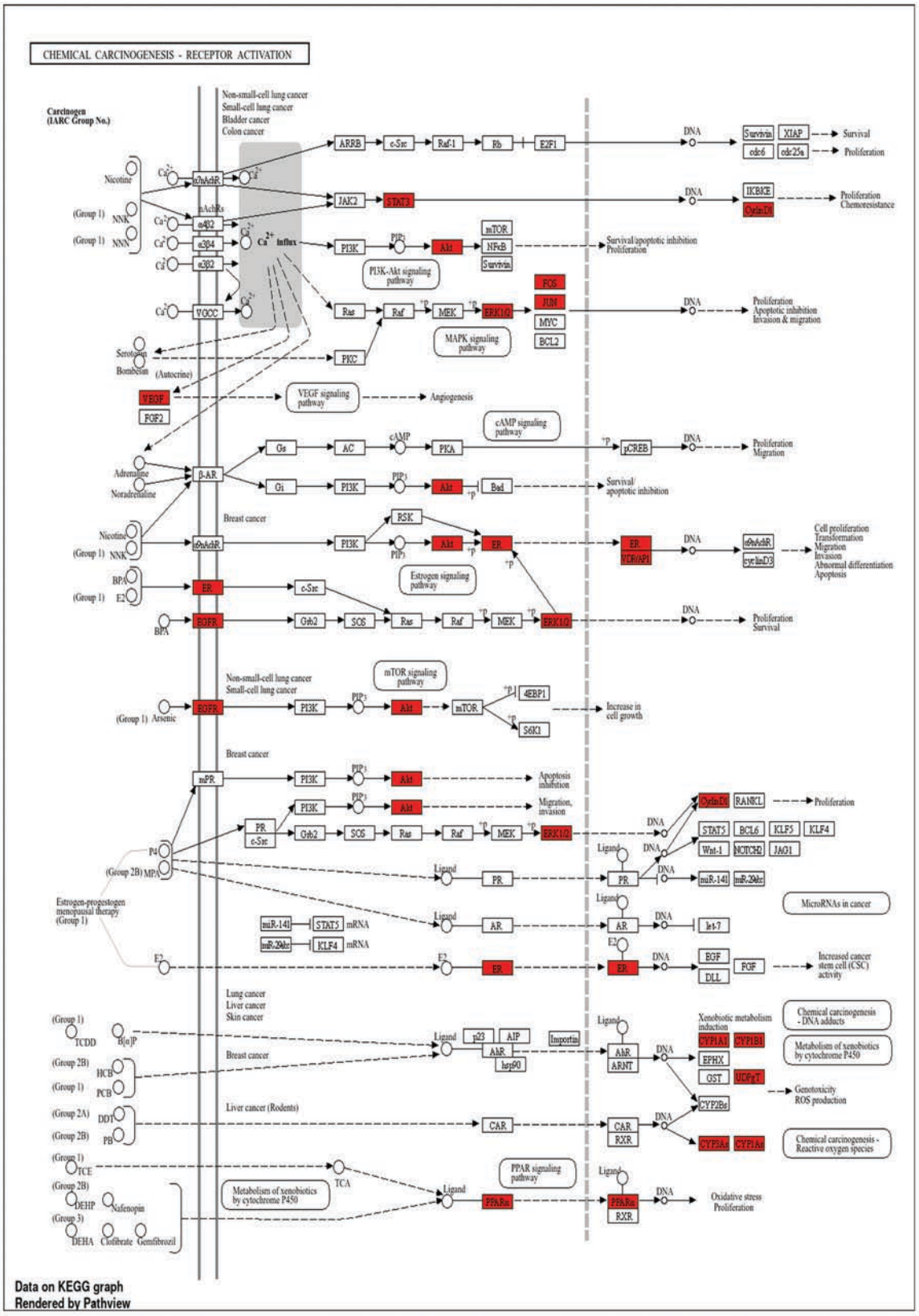

Figure 35s: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes), representing Chemical carcinogenesis during receptor activation pathways.

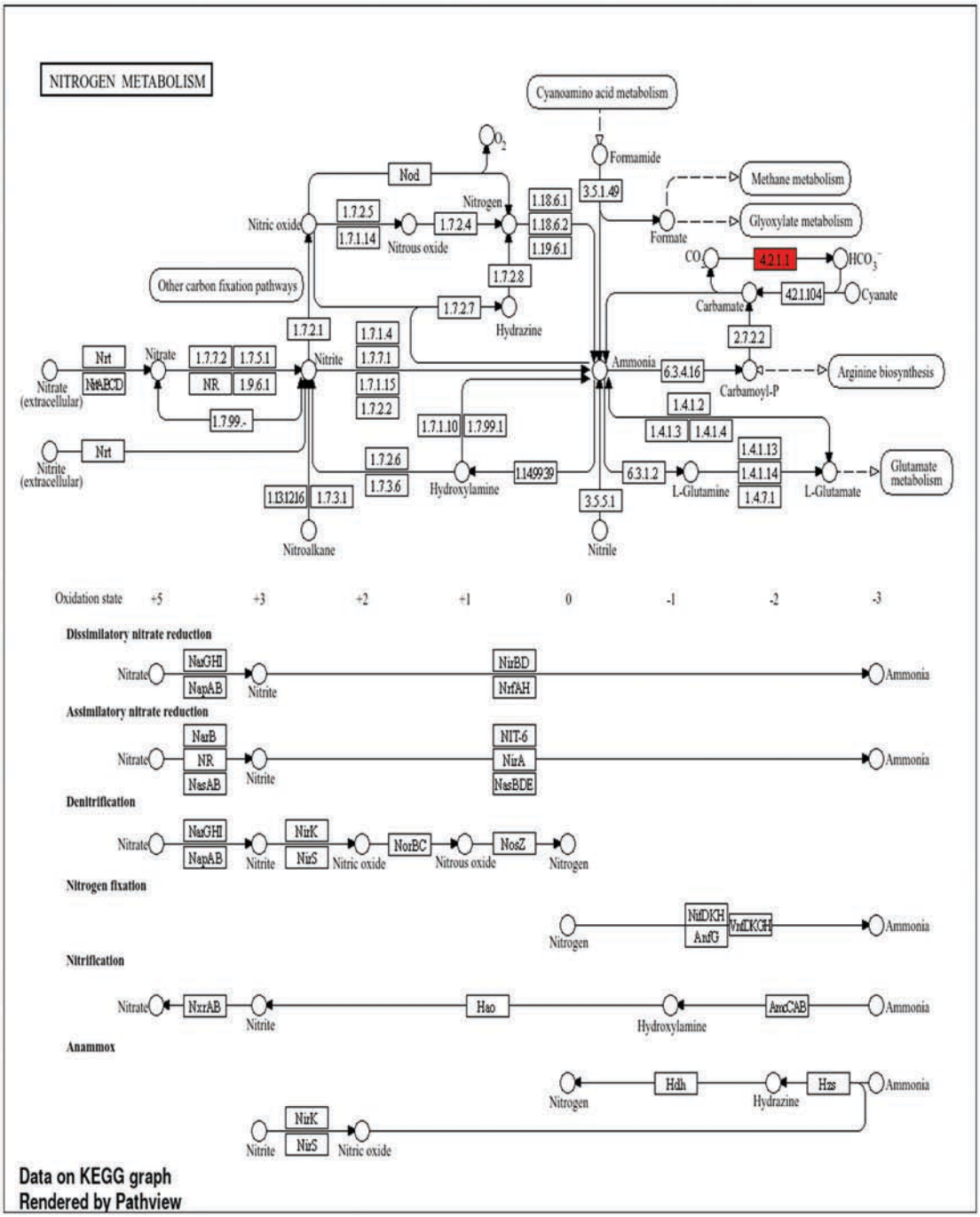

Figure 35t: KEGG Pathway Map (Kyoto Encyclopedia of Genes and Genomes), representing Nitrogen metabolismsignaling pathways.

The KEGG pathway denotes the Kyoto Encyclopaedia of Genes and Genomes. DAVID incorporates the KEGG database to examine the input genes of interest across several biological pathways (Kanehisa & Goto, 2000; Kanehisa *et al.*, 2012). This allows the mapping of genes or gene sets across several biological pathways (Aoki-Kinoshita & Kanehisa, 2007). It was identified that 149 different biological pathways were linked with the gene list that we have provided. From which the most significant 20 pathways (Figure 35a-t) were identified based on low FDR (False Discovery Rate) and sorted by fold enrichment. A hierarchical clustering tree was created describing the relationships among notable pathways (Figure 36: tree cluster). Numerous genes that are shared by several pathways are grouped together. The shorter branches are more similar and pathways such as colorectal and pancreatic cancer are more related in terms of genes. within the same.

Furthermore, to explore the functional relationships between pathways, an interaction network diagram was constructed (Figure 37). In this network two pathways connects only when they share at least 20% similar genes. The thicker lines are direct indication of the gene overlap between connected pathways.

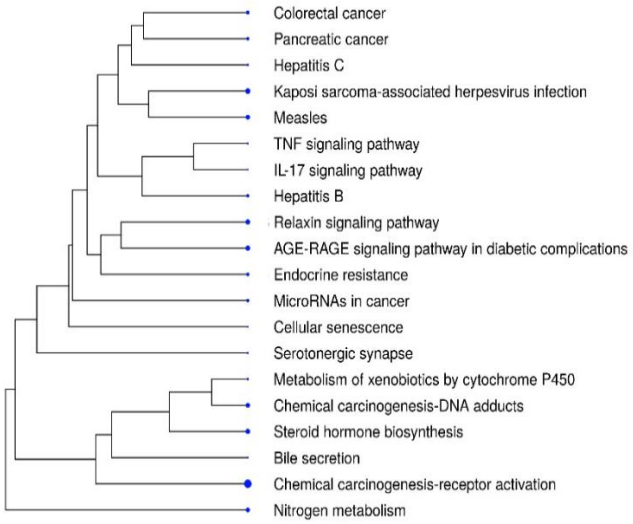

Figure 36: Clustering tree show how similar the significant pathways are based on the genes they share. Pathways with many overlapping genes are grouped closer together in the tree. Larger dots in the tree represent pathways with more significant results (smaller p-values).

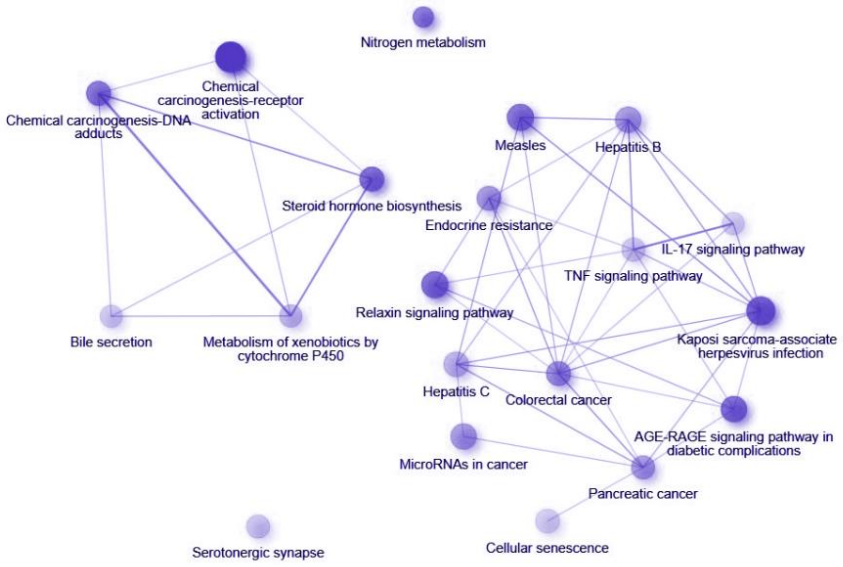

Figure 37: Pathway enrichment analysis, displaying interconnected biological pathways. The node size and edge thickness indicate the significance and degree of interaction between pathways

4.3.1.2 Effect of HBA Extract on Lipopolysaccharide-Induced Anti-Inflammatory Activity in Macrophages

The anti-inflammatory activity of the extract was evaluated using Raw 264.7macrophages as the model. Raw 264.7 cells stimulated with lipopolysaccharide (LPS) are a widely utilized cellular model of inflammation (Facchin *et al.*, 2022). The LPS is the cell wall component of most of the Gram-negative bacteria; the LPS stimulates the macrophage in a toll-like-receptor dependent manner, causes inflammatory responses (Hoppstädter *et al.*, 2019).

Effect of HBA on cytokines: Cytokines are signalling molecules during inflammation. The extent of inflammation responses can be understood by studying the cytokines (O'Shea & Murray, 2008; Schmitz *et al.*, 2011). The expression of cytokines under various treatments are represented in the Table 19 and Figure 38. In the present study, the normal macrophage was estimated for the level of IL-1β, and it was estimated to be 64.6 ±1.9 pg/mg protein. However, there was observed a significant elevation in the IL-1β levels upon stimulation with the LPS to 573.4 ± 4.5 pg/mg protein. The increased level of IL-1β is an indicator of inflammation in the cellular

conditions (Bent *et al.*, 2018; Kaneko *et al.*, 2019; Pyrillou *et al.*, 2020). However, the pre-treatment of macrophages with the different doses of HBA resulted in a significant reduction in IL-1β levels. The pre-treatment of Raw 264.7 cells with 5 µg/mL of HBA resulted in cellular IL-1β levels of 403.7 ± 6.2 pg/mg protein ($p < 0.01$). Similarly, the pre-treatment with 10 µg/mL (298.5 ± 8.4 pg/mg protein) and 20 µg/mL of HBA (156.2 ± 3.4 pg/mg protein) resulted in lower IL-1β levels ($p < 0.001$). The reduction in IL-1β levels is indicative of the anti-inflammatory potential of the HBA at the respective treatment doses.

Together with IL-1β, IL-6 was also found to significantly influence the inflammation in macrophages. IL-6 has a major role in the innate immune defense systems (Chen *et al.*, 2022; Hirani *et al.*, 2022; Rose-John *et al.*, 2023). The level of IL-6 in the untreated macrophages without LPS stimulation was estimated to be 133.4 ± 5.8 pg/mg protein; however, the exposure of LPS elevated the cellular IL-6 levels to 628.5 ± 8.2 pg/mg protein. On the contrary, the level was brought down by the treatment with 5 and 10 µg/mL of HBA, which reduced the cellular IL-6 levels to 507.1 ± 8.1 ($p < 0.05$) and 388.4 ± 2.8 pg/mg protein ($p < 0.001$). In the highest concentration of HBA treatment, the IL-6 was estimated to be 291.3 ± 6.6 pg/mg protein ($p < 0.001$).

TNF-α is a cytokine that plays a role in inflammatory responses and is also involved in pathogenesis (Bradley, 2008). The level of TNF-α in the untreated and unstimulated Raw 264.7 macrophages was estimated to be 115.2 ± 3.1 pg/mg protein. However, the level was elevated to 856.0 ± 11.2 pg/mg protein upon stimulation by the LPS. This clearly indicated the induction of acute inflammation in the experimental condition. In 5 µg/mL HBA treated macrophages, the level of TNF-α was reduced to 715.2 ± 8.8 pg/mg protein. A similar decrease in the TNF-α level was also noted in the 10 and 20 µg/mL HBA treatment, which brought down the TNF-α level to 602.8 ± 5.2 and 493.7 ± 6.4 pg/mg protein.

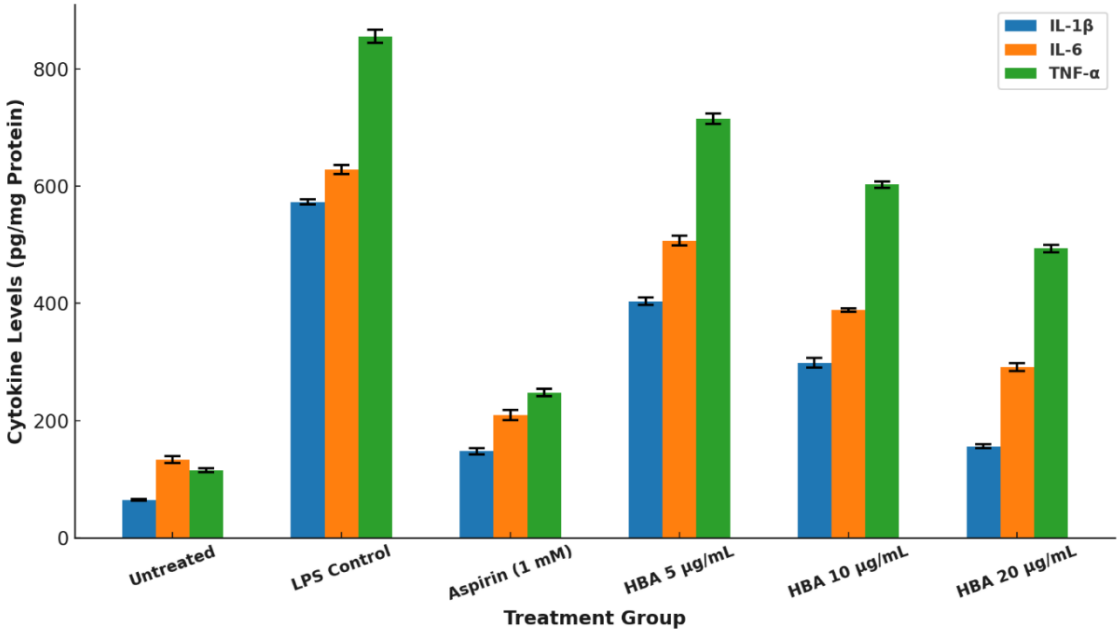

Figure 38: Anti-inflammatory activity of HBA against LPS induced cytokine production.

Effect of HBA on Nitric Oxide levels: The nitric oxide level is also an important inflammatory indicator in cells; the inducible nitric oxide synthase is an enzyme responsible for the overwhelming load of NO in the body (Król & Kepinska, 2020). Despite its physiological and immunological importance, nitric oxide is often associated with chronic inflammation and is thereby involved in many of the degenerative diseases (Iwata *et al.*, 2020). The level of nitric oxide in the untreated macrophage cells was estimated to be 10.7 ± 0.64 µM/mg protein. The level was increased to 75.2 ± 2.1 µM/mg protein in the macrophages exposed to LPS. Interestingly, the level was brought down by the pre-treatment with the 5 µg/mL of HBA (58.8 ± 3.4 µM/mg protein). In the 10 µg/mL of HBA treatment, the NO level was estimated to be 42.3 ± 1.9 µM/mg protein, and, in the 20 µg/mL HBA treatment, it was further reduced to 30.7 ± 2.5 µM/mg protein (Table 19, Figure 39). Hence, it is clearly indicated that the pre-treatment with different doses of HBA dose-dependently reduced the inflammatory insults in cultured macrophages.

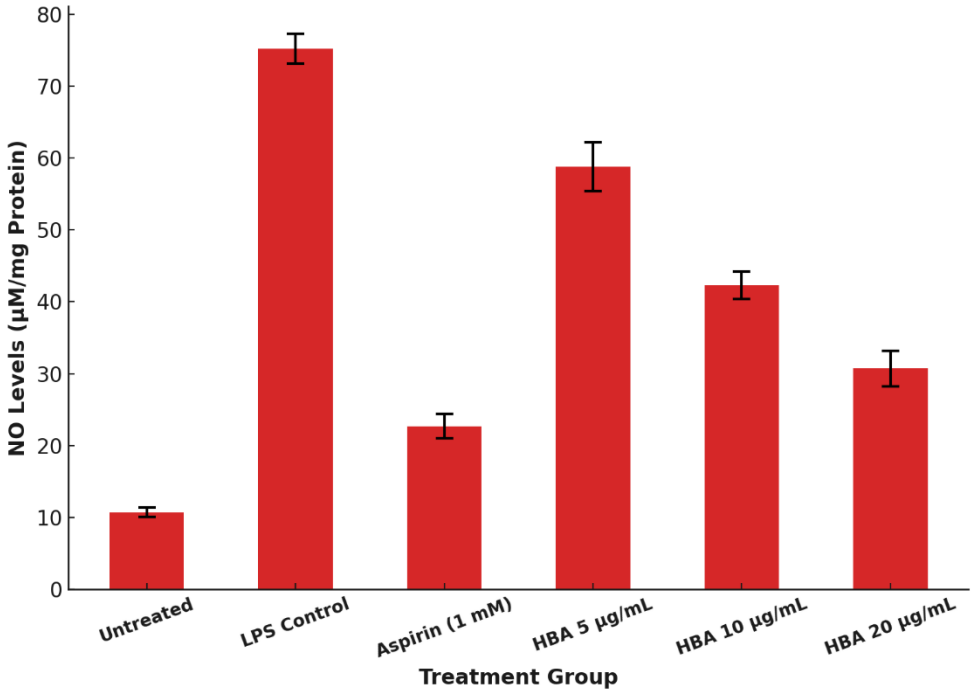

Figure 39: Effect of HBA on NO levels in LPS -induced RAW 264.7 cells.

Table 19: Anti-inflammatory activity of HBA against lipopolysaccharide-induced activation of Raw 264.7 cells and comparison with standard aspirin (1 mM).

Treatment Group	IL-1β (pg/mg Protein)	IL-6 (pg/mg Protein)	TNF-α (pg/mg Protein)	NO (µM/mg Protein)
Untreated	64.6 ± 1.9	133.4 ± 5.8	115.2 ± 3.1	10.7 ± 0.64
LPS Control	573.4 ± 4.5	628.5 ± 8.2	856.0 ± 11.2	75.2 ± 2.1
Aspirin (1 mM)	147.5 ± 5.1 ***	209.5 ± 9.1 ***	247.5 ± 6.3 ***	22.7 ± 1.7 ***
HBA 5 µg/mL	403.7 ± 6.2 **	507.1 ± 8.1 *	715.2 ± 8.8 *	58.8 ± 3.4 *
HBA 10 µg/mL	298.5 ± 8.4 ***	388.4 ± 2.8 ***	602.8 ± 5.2 ***	42.3 ± 1.9 ***
HBA 20 µg/mL	156.2 ± 3.4 ***	291.3 ± 6.6 ***	493.7 ± 6.4 ***	30.7 ± 2.5 ***

*indicates significant variation with respect to LPS control ($p < 0.05$); ** indicates higher significant variation withrespect to that of LPS control ($p < 0.01$), and *** indicates highest significant variation with respect to that of LPS control ($p < 0.001$). All the results are indicated as mean ± standard deviation of six independent experiments.

Effect of HBA on NF-κB and COX2 expression: To further explain the mechanism of anti-inflammatory activity NF-κB and COX2 expression was assessed HBA significantly brought down the expression of NF-κB and COX2 (Figure 40).

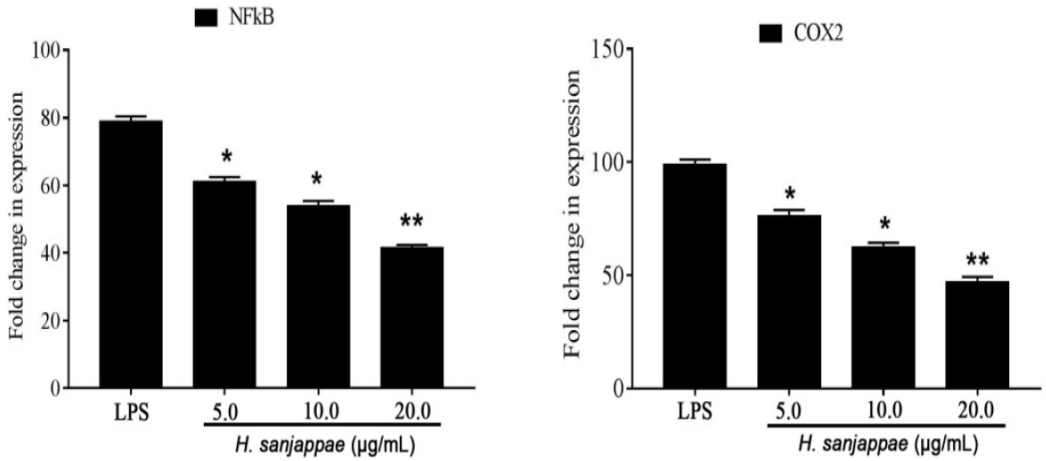

Figure 40: The expression of NF-κB and COX2 genes in the Raw 264.7 macrophages treated with LPS and different doses of HBA. * indicates significant variation with respect to LPS control ($p < 0.05$); ** indicates higher significant variation with respect to that of LPS control ($p < 0.01$).

4.3.2 Discussion

The network pharmacology analysis of HBA revealed 117 common targets. This means that in the inflammatory process, out of a greater number of targets, 117 targets will definitely be modulated by the phytochemicals of HBA.

LPS-stimulated macrophages, is a widely accepted inflammation model (Daniels *et al.*, 2000; Hu *et al.*, 2008) Quantitative evaluation of IL-6, IL-1β, TNF-α, and NO revealed a reduction in their levels in HBA-pre-treated, inflammation-induced macrophages. Notably, IL-6 levels dropped significantly after HBA treatment, aligning with previous research suggesting that plant-derived compounds can inhibit the production of inflammatory cytokines (Hwang *et al.*, 2019). Our results is in agreement with the study by Yoon *et al.* (2009) on *Scutellaria baicalensis*, as both investigations demonstrate significant inhibition of key pro-inflammatory mediators, including NO and cytokines such as IL-6.

Similar to how *Scutellaria baicalensis* reduces inflammation, our study revealed that HBA also affects NO production, it might have lowering the activity of the inducible nitric oxide synthase (iNOS) enzyme. This suggests that HBA plays a role in regulating NO levels. Furthermore, our findings align with *in silico* pathway enrichment analysis, which linked HBA activity to nitrogen metabolism. The observed decrease in NO levels provides strong experimental validation of this connection. Since excessive iNOS activity leads to increase NO production, oxidative stress, and inflammatory tissue damage, its inhibition is a crucial aspect of anti-inflammatory therapeutics (Sakurai *et al.*, 1995; Zamora *et al.*, 2000).

COX-2 inhibition is a vital strategy for mitigating inflammatory reactions (Ju *et al.*, 2022). It has been previously documented that phytochemicals play significant role in decreasing COX-2 expression (Orabi *et al.*, 2022). Hence, gene expression of COX-2 was done, due to their established roles in inflammation (Mitchell *et al.*, 1995; Neurath & Finotto, 2011; Zelová & Hošek, 2013). COX-2 expression was downregulated in macrophages, highlighting the anti-inflammatory potential of HBA. Since phytochemicals influence COX-2 expression, these same compounds may also help prevent certain cancers, as chronic inflammation is a known risk factor for cancer development. In several cancers, including colorectal and pancreatic cancer, COX-2 is often upregulated (Doiphode *et al.*, 2023). Pathway enrichment analysis further revealed that more than 10 targets of HBA play a role in these cancer-related pathways, reinforcing its potential for cancer prevention and treatment.

Like COX-2, TNF-α is another gene involved in inflammation and cancer (Qu *et al.*, 2022). While TNF-α was not explicitly identified as a target of the phytochemicals of HBA, pathway enrichment analysis indicated a substantial association between the TNF signalling pathway and HBA. This is consistent with our *in vitro* findings, where TNF-α secretion was significantly reduced following HBA treatment in LPS-induced macrophages. The suppression of TNF-α aligns with previous studies on plant-derived anti-inflammatory compounds, which have been shown to inhibit TNF-α production. For example, *Turnera subulata* extracts effectively reduced TNF-α and IL-1β levels in LPS-stimulated RAW 264.7 macrophages. Reducing these important pro-inflammatory cytokines represented that the extracts controlled inflammatory

reactions mediated by macrophages (Luz *et al.*, 2022). Interestingly, as like the *Turnera subulata* extract, HBA treatments lowered IL-1β levels even though *in silico* analysis did not specifically identify IL-1β as a target. This result indicates that the HBA can be used to treat various inflammation-associated diseases.

Although network pharmacology gave important new perspectives, it did not draw attention to NF-κB and IL-1β as clear targets. This difference emphasises the need of experimental confirmation and the limits of computer predictions. The noted decrease in NF-κB and IL-1β points to HBA perhaps having deeper immunomodulating action than just what the network model demonstrates. This is because the *in silico* data captures already existing literature. Due to the availability of research, only 21 phytochemicals of HBA were possible to evaluate. Hence, the remaining molecules in the HBA must have unexplored actions on the genes.

4.3.3 Conclusion

This study evaluated network pharmacology with *in vitro* validation to clarify the anti-inflammatory efficacy of HBA. The computational analysis identified 117 common targets, of HBA, IL-6, TNF-α, and COX-2 were some of them. They were notably reduced in the LPS-stimulated macrophages during the treatment with HBA. Moreover, gene expression analysis validated the inhibition of NF-κB and COX-2. These findings strengthen the compound's regulatory activities at the transcriptional level. Despite IL-1β and NF-κB not being initially recognised as direct targets within the network model, their downregulation following treatment implies that HBA may have extensive anti-inflammatory effects through indirect mechanisms, potentially including NF-κB suppression or inflammasome modulation. The noted reduction in NO levels corresponds with the pathway enrichment analysis in the *in silico* study that associates the HBA's activity with nitrogen metabolism. The work validates HBA's capacity to modulate inflammatory mediators; however, more investigation is required to examine other regulatory mechanisms, other molecular targets, and their therapeutic efficacy *in vivo.*

4.4 *In vivo* evaluation of anti-inflammatory potential and molecular docking analysis of key phytochemicals.

Murine models are essential in preclinical research, offering significant insights into the pharmacokinetics, pharmacodynamics, toxicity, and efficacy of phytochemicals (Ruggeri *et al.*, 2014). These models enable researchers to evaluate physiological and biochemical responses in a regulated setting, facilitating the transition from *in vitro* investigations to human clinical trials, which has predictive utility (Olive & Tuveson, 2006; Wege, 2018). Researchers can explore the mechanisms of action, metabolism, and potential adverse effects of bioactive substances using mouse models, ultimately aiding in the development of innovative plant-based medicines (Morgan & Elangbam, 2016; Shanks *et al.*, 2009).

Herbal bioactive compounds have gained considerable attention due to their multi-targeted mechanisms of action (Efferth & Koch, 2011). Ethanol extract of *Humboldtia sanjapape* bark, has shown potential anti-inflammatory effects *in silico and in vitro* in our previous studies. However, its efficacy in inflammation models and underlying molecular mechanisms require further exploration. This study evaluates the anti-inflammatory potential of HBA in both acute and chronic inflammation models using Swiss albino mice. The therapeutic efficacy was assessed through paw thickness measurements, histopathological analysis, and gene expression studies. Additionally, molecular docking and MM-GBSA analysis were conducted to evaluate the binding affinity of key phytochemicals against inflammatory mediators such as COX-1, COX-2, IL-6, IL-1β, and TNF-α.

4.4.1 Results

4.4.1.1 Acute Inflammation Study

Acute inflammation in mice is typically characterized by a rapid immune response resulting in localized swelling at the site of chemical induction.

Changes paw thickness and paw volume

Measurements of Paw Thickness: The Disease Control group demonstrated a notable rise in paw thickness, reaching its peak at 2 hours, signifying the inflammatory response. Conversely, the Normal Control group had little variations, because of the

lack of inflammation. The Standard Indomethacin (5 mg/kg i.p.) group exhibited effective anti-inflammatory effects, with paw thickness stabilising after 2 hours and markedly diminishing by 6 hours.

In the HBA-treated groups, both the 100 mg/kg and 200 mg/kg dosages exhibited decrease in paw thickness. The 200 mg/kg dosage significantly reduced paw thickness, reaching up to the standard treatment. Statistical analysis indicated substantial differences between the Disease Control and treatment groups. HBA 200 mg/kg demonstrating a consistent reduction in inflammation after 2 hours (Table 20, Figure 41).

Table 20: Change in paw thickness over time (mm) in the acute inflammation model.

Group	Time interval					
	0 min	**30 mins**	**1 hr**	**2 hr**	**4 hr**	**6 hr**
Normal Control	0.12 ± 0.013	0.12 ± 0.013	0.12 ± 0.006	0.12 ± 0.008	0.13 ± 0.008	0.12 ± 0.010
Disease Control	0.13 ± 0.010	0.16 ±0.012	0.18 ± 0.012	0.22 ± 0.009	0.19 ± 0.014	0.16 ± 0.010
Standard Treatment	0.12 ± 0.01	0.16 ± 0.01	0.17 ± 0.01	0.16 ± 0.01	0.13 ± 0.01	0.12 ± 0.01
HBA 100 mg/kg	0.13 ± 0.01	0.17 ± 0.01	0.18 ± 0.02	0.16 ± 0.02	0.15 ± 0.02	0.14 ± 0.02
HBA 200 mg/kg	0.12 ± 0.01	0.17 ± 0.02	0.16 ± 0.02	0.14 ± 0.01	0.13 ± 0.01	0.11 ± 0.01

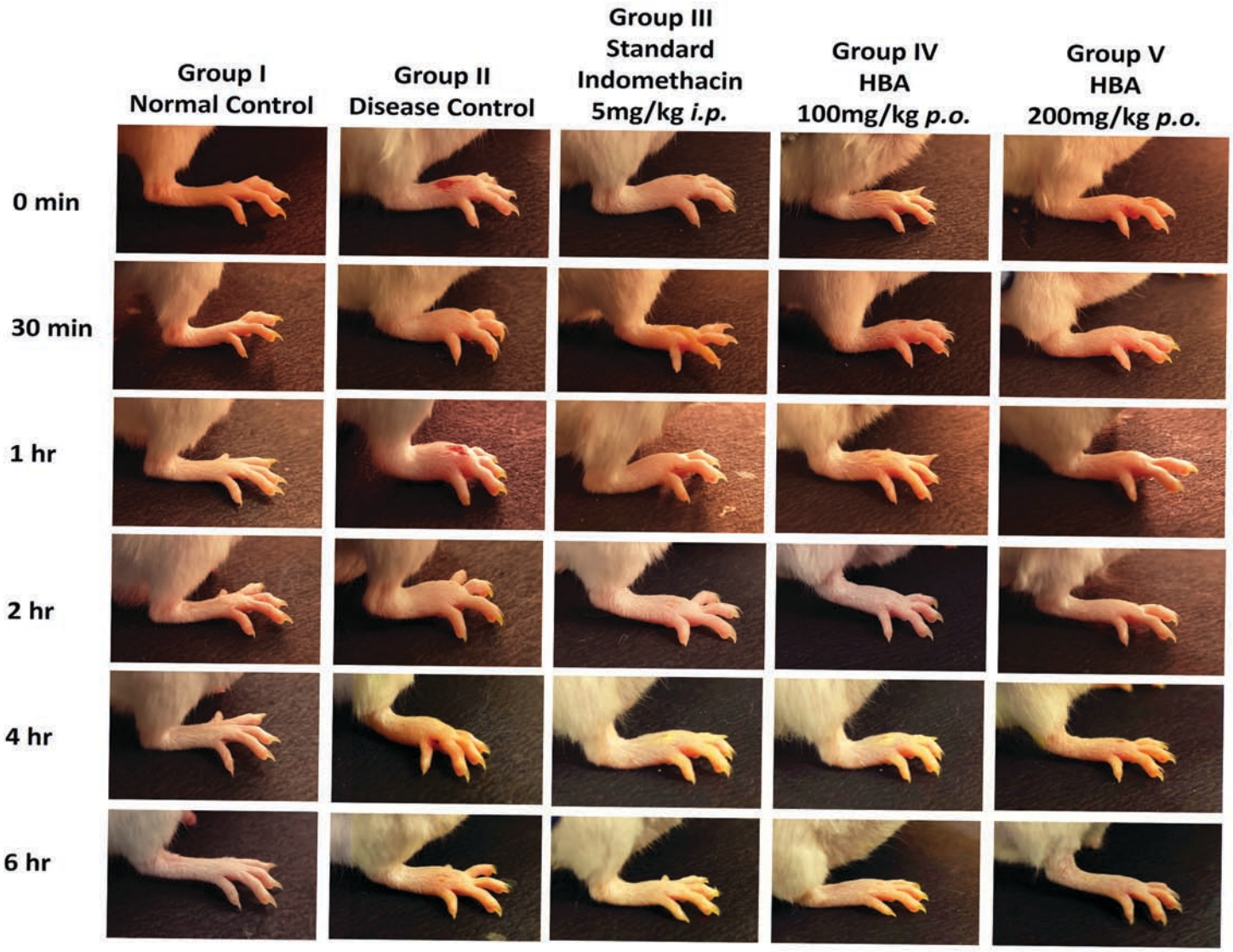

Figure 41: Changes in the paw thickness over time is represented by right hind paw of mice in acute inflammation model

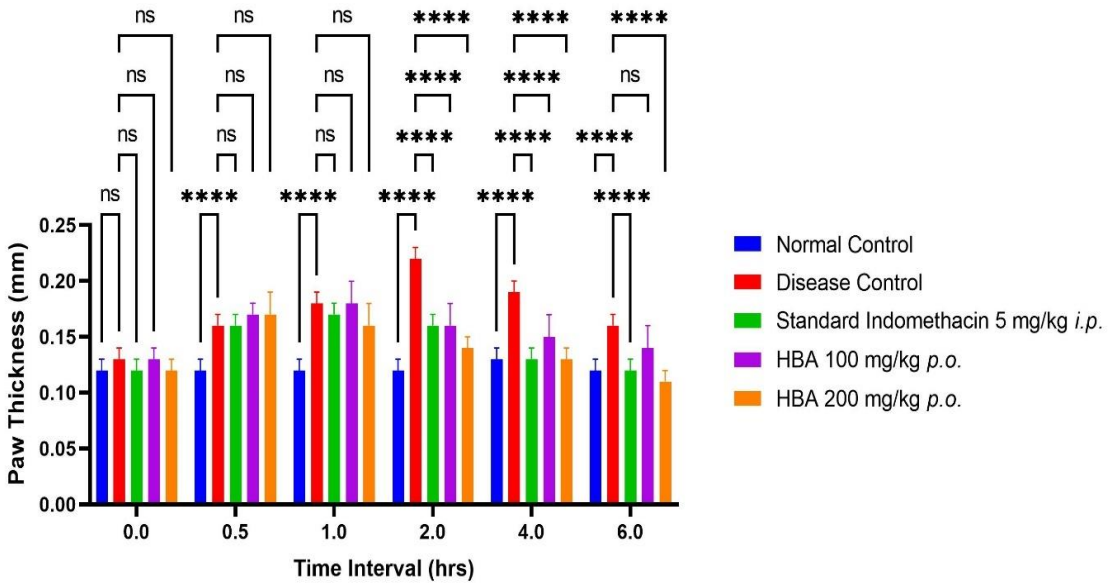

Figure 42: Changes in the paw thickness over time in the acute inflammation model.

Average Variation in Paw Volume: The Disease Control group had a continuous rise in paw volume, peaking at 6 hours (0.56 ± 0.02 mL), so indicating persistent inflammation. The Normal Control group exhibited no substantial alteration in paw volume. The HBA-treated groups demonstrated dose-dependent anti-inflammatory effects, with the 100 mg/kg dose resulting in a moderate reduction in paw volume (0.24 ± 0.04 mL at 6 hours), whereas the 200 mg/kg dose showed a more significant decrease, returning paw volume to baseline levels (0 ± 0.02 mL at 6 hours) (Table 21, Figure 43).

Table 21: Mean change in paw volume over time (ml) of acute inflammation model

Group	Time interval					
	0 hr	**0.5 hr**	**1 hr**	**2 hr**	**4 hr**	**6 hr**
Normal Control	0	-0.01 ± 0.01	-0.01 ± 0.02	0.00 ± 0.02	-0.01 ± 0.03	0.00 ± 0.02
Disease Control	0	0.11 ± 0.01	0.24 ± 0.02	0.36 ± 0.02	0.45 ± 0.03	0.56 ± 0.02
Standard Treatment	0	0.11 ± 0.03	0.14 ± 0.03	0.22 ± 0.02	0.12 ± 0.05	0.03 ± 0.05
HBA 100 mg/kg	0	0.12 ± 0.05	0.19 ± 0.05	0.3 ± 0.03	0.34 ± 0.06	0.24 ± 0.04
HBA 200 mg/kg	0	0.07 ± 0.03	0.12 ± 0.04	0.19 ± 0.06	0.09 ± 0.05	0 ± 0.02

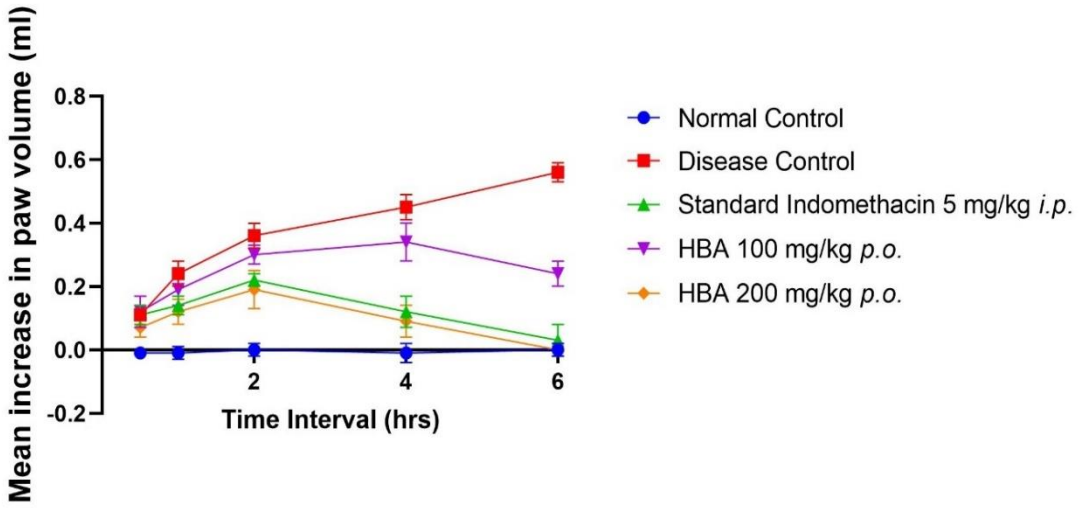

Figure 43: Changes in the paw volume over time in the acute inflammation model.

Histopathology observations of acute model

Group I (Normal control): The epidermal (E) and dermal (D) layers of the histopathological sections of Group I (control) animals were intact, indicating a well-preserved and normal skin architecture. However, there were no indications of irregular thickening or disruption in the epidermis, which maintained its typical stratified structure. The dermis is well-organised, with a normal distribution of collagen and the absence of inflammatory alterations. It is important to note that the animals preserved a healthy tissue morphology, as there was no indication of vascular congestion, oedema, or infiltration of inflammatory cells.

Group II (Carrageenan treated/ Disease control): the histopathological sections of Group II animals, exhibited substantial pathological changes. A significant amount of epidermal hyperplasia was observed, which was distinguished by an abnormal thickening of the epidermal layer. This thickening was likely the result of excessive keratinocyte proliferation. Furthermore, a significant infiltration of inflammatory cells was observed, particularly in the dermal layer, suggesting an active

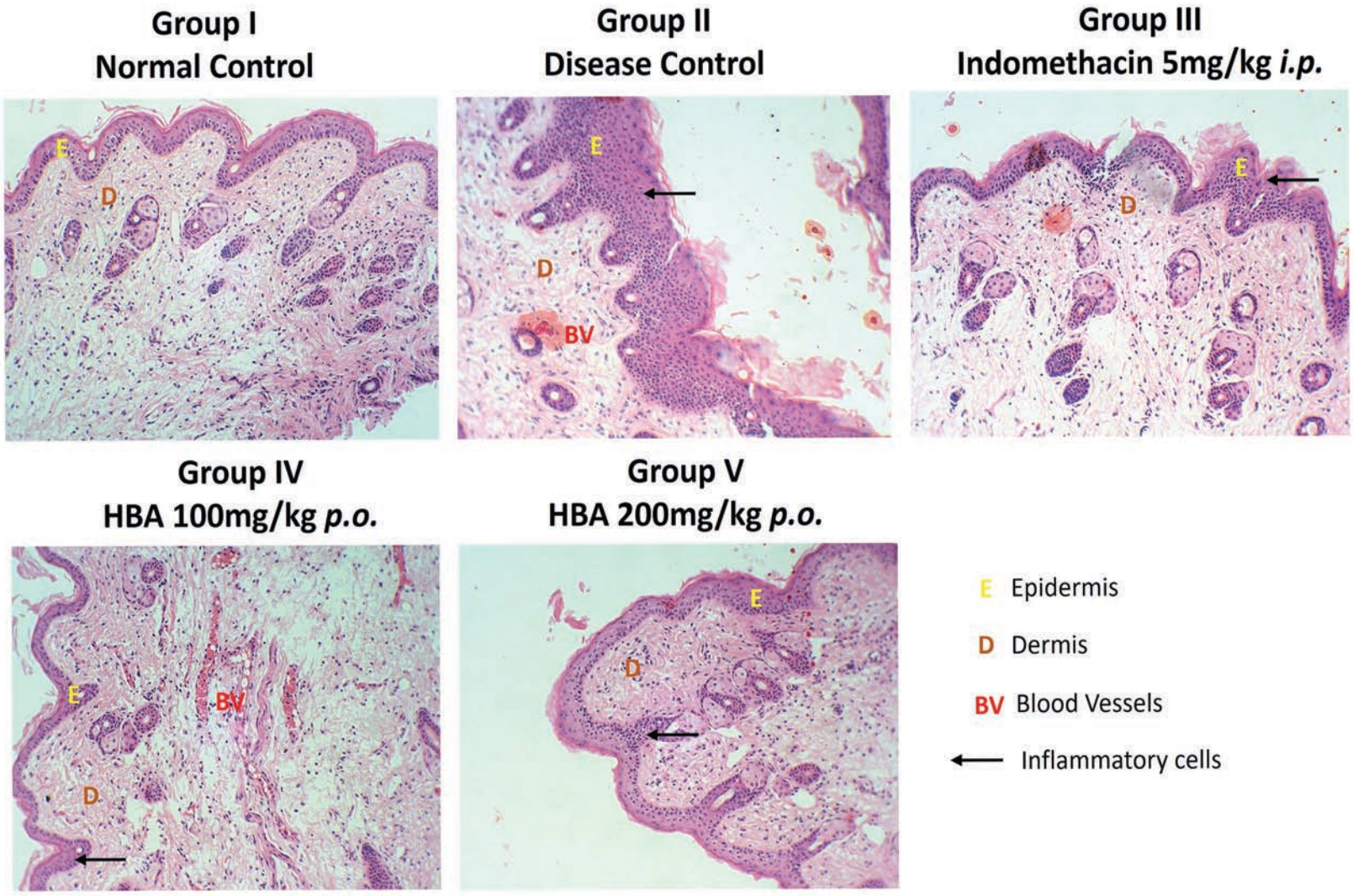

Figure 44: Histological examination of paw tissue sections after 6 hrs treatment of acute inflammation model.

inflammatory response. The disease model is associated with severe pathological changes, as indicated by the structural abnormalities and signs of tissue disruption.

Group III (Indomethacin): In comparison to Group II, animals in Group III that were administered Indomethacin exhibited substantial enhancements in histopathological characteristics. The degree of epidermal hyperplasia was diminished, suggesting a partial restoration of the normal skin structure. Additionally, the degree of inflammatory cell infiltration was lower in the Indomethacin-treated group than in the disease-induced group, indicating that the treatment effectively reduced inflammation. Nevertheless, there were still some residual indications of tissue remodelling and inflammation.

Group IV (HBA 100 mg/kg): The histopathological sections of Group IV animals that were treated with a low dose of HBA demonstrated decrease in epidermal hyperplasia. The severity of the condition was less than that of Group II, despite the fact that the epidermal layer remained somewhat thicker than that of normal controls. Mild structural enhancements were also observed in the dermal layer, and a few blood vessels were present, which may suggest that vascularization or tissue remodelling was enhanced.

Group V (HBA 200 mg/kg): The animals in Group V, which were administered a high dose of HBA, exhibited the most significant histopathological recovery. The epidermal and dermal layers exhibited architecture that was similar to that of normal controls, with minimal evidence of pathological changes. The ductal structures appeared nearly normal, and the epidermal and dermal hyperplasia were mild. Furthermore, the high-dose treatment demonstrated a robust anti-inflammatory effect, as evidenced by the substantial reduction in inflammatory cell infiltration. All the histological sections of acute inflammation study are represented in Figure 44.

Gene expression studies in acute model

The fold induction of selected inflammatory genes (COX-1, COX-2, IL-1β, IL-6, and TNF-α) across various experimental groups, including normal, diseased, Indomethacin-treated, and HBA-treated groups (100 mg/kg and 200 mg/kg), is

depicted in the bar graph (Figure 45). The diseased group demonstrates a substantial upregulation of all inflammatory genes, with TNF-α exhibiting the highest induction (~2.5-fold), suggesting an intense inflammatory response. On the other hand, Indomethacin treatment results in a substantial reduction in gene expression, particularly for IL-1β and TNF-α, which approaches near-normal levels. HBA exhibits a partial reduction in inflammatory gene expression at a dosage of 100 mg/kg; however, it is less efficacious than Indomethacin. Nevertheless, HBA at a dosage of 200 mg/kg exhibits a more potent anti-inflammatory effect, as it substantially reduces the levels of gene expression indicating a dose-dependent response. In general, the findings suggest that higher dosages of HBA have a more significant effect on the suppression of inflammation, which is comparable to the standard anti-inflammatory drug, Indomethacine.

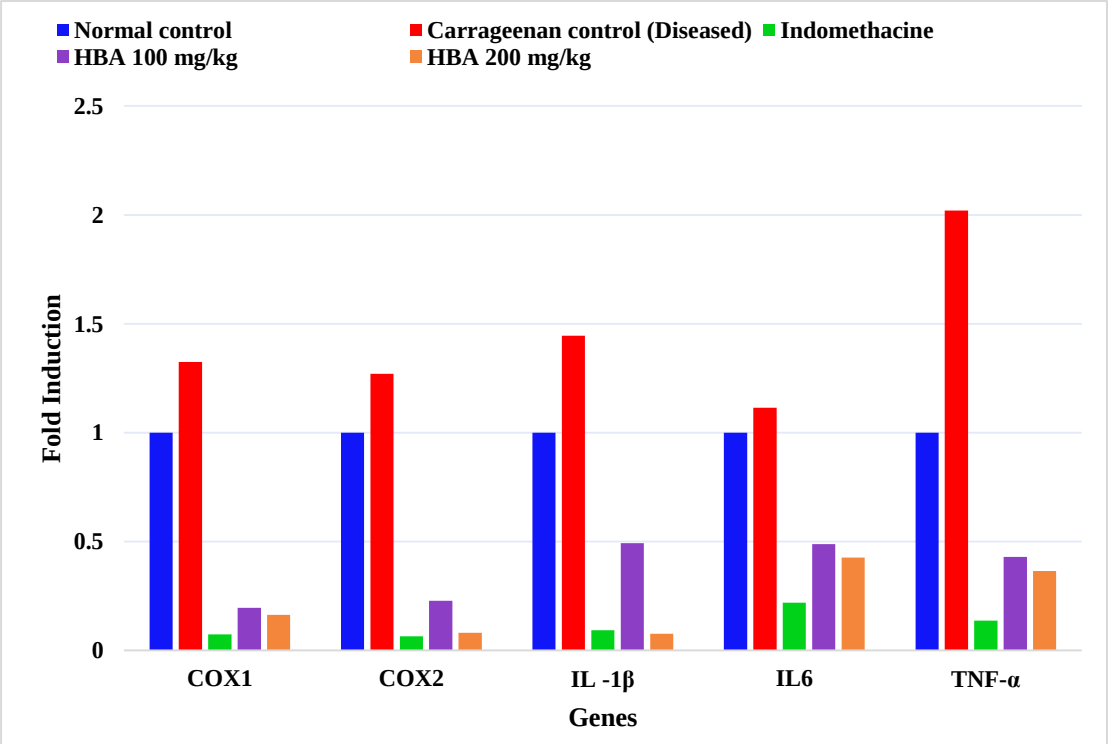

Figure 45: The expression of various inflammatory markers under acute inflammation.



Figure 46: Changes in the paw thickness over time is represented by right hind paw of mice in chronic inflammation model.

4.4.1.2 Chronic Inflammation Study

Chronic inflammation in mice is characterized by a prolonged and sustained immune response, typically lasting weeks to months.

Changes paw thickness and paw volume

Measurement of paw thickness: In the disease control group, inflammation increased over time, reaching a maximum of 0.23 ± 0.01 mm on Day 4, followed by a moderate decrease on Day 5 (0.21 ± 0.01 mm). This was demonstrated by the change in paw thickness. Indomethacin treatment was effective in reducing inflammation, as the paw thickness reached a maximum of 0.19 ± 0.01 mm on Day 3 and subsequently decreased to 0.14 ± 0.01 mm on Day 5, which is in close to the normal range. HBA 100 mg/kg moderately reduced inflammation in the HBA-treated groups, with a peak of 0.21 ± 0.01 mm on Day 4 and a modest decrease to 0.18 ± 0.01 mm on Day 5. The most effective treatment was HBA 200 mg/kg, which exhibited a lower peak of 0.17 ± 0.01 mm on Day 3 and a subsequent decrease to 0.12 ± 0.01 mm on Day 5, which was consistent with the normal control group. HBA 200 mg/kg is as effective as Indomethacin in reducing inflammation, according to these findings, while HBA 100 mg/kg has a moderate effect (Table 22, Figure 46 & 47).

Table 22: Mean change in paw thickness over time (mm) of chronic inflammation model

Group	Day					
	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Normal control	0.12 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.12 ± 0.02	0.13 ± 0.01	0.12 ± 0.01
Disease Control	0.12 ± 0.01	0.16 ± 0.01	0.19 ± 0.02	0.22 ± 0.01	0.23 ± 0.01	0.21 ± 0.01
Indomethacin	0.12 ± 0.02	0.14 ± 0.01	0.17 ± 0.01	0.19 ± 0.01	0.17 ± 0.01	0.14 ± 0.01
HBA 100 mg/kg	0.13 ± 0.02	0.15 ± 0.02	0.18 ± 0.02	0.2 ± 0.02	0.21 ± 0.01	0.18 ± 0.01
HBA 200 mg/kg	0.12 ± 0.02	0.14 ± 0.01	0.16 ± 0.01	0.17 ± 0.01	0.15 ± 0.01	0.12 ± 0.01

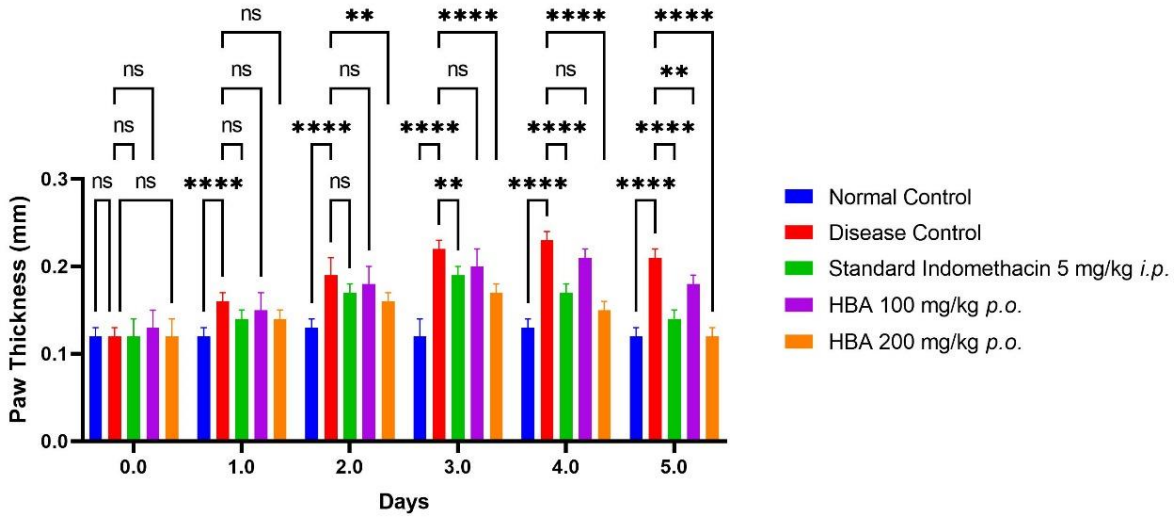

Figure 47: Changes in the paw thickness over time in the chronic inflammation model

Average Variation in Paw Volume: The paw volume of the disease control group steadily increased, reaching a maximum of 0.63 ± 0.06 ml on Day 5, which suggests that there was severe inflammation. The inflammation was effectively reduced by indomethacin and HBA 200 mg/kg, resulting in a decrease in paw volume to 0.08 ± 0.04 ml and 0.03 ± 0.04 ml, respectively, by Day 5. The paw volume was reduced to 0.13 ± 0.03 ml by HBA 100 mg/kg, resulting in a moderate effect. In terms of inflammation management, HBA 200 mg/kg was equally efficacious as Indomethacin (Table 23, Figure 48).

Table 23: Mean change in paw volume over time (ml) of chronic inflammation model

Group	Day				
	Day 1	Day 2	Day 3	Day 4	Day 5
Normal control	0.02 ± 0.02	0.02 ± 0.03	0.01 ± 0.02	0.03 ± 0.04	0.02 ± 0.02
Disease Contro	0.27 ± 0.1	0.34 ± 0.06	0.44 ± 0.07	0.53 ± 0.07	0.63 ± 0.06
Indomet hacine	0.19 ± 0.06	0.26 ± 0.05	0.21 ± 0.04	0.18 ± 0.05	0.08 ± 0.04
HBA 100 mg/kg	0.25 ± 0.05	0.32 ± 0.04	0.26 ± 0.04	0.2 ± 0.03	0.13 ± 0.03
HBA 200 mg/kg	0.15 ± 0.03	0.21 ± 0.04	0.18 ± 0.03	0.15 ± 0.02	0.03 ± 0.04

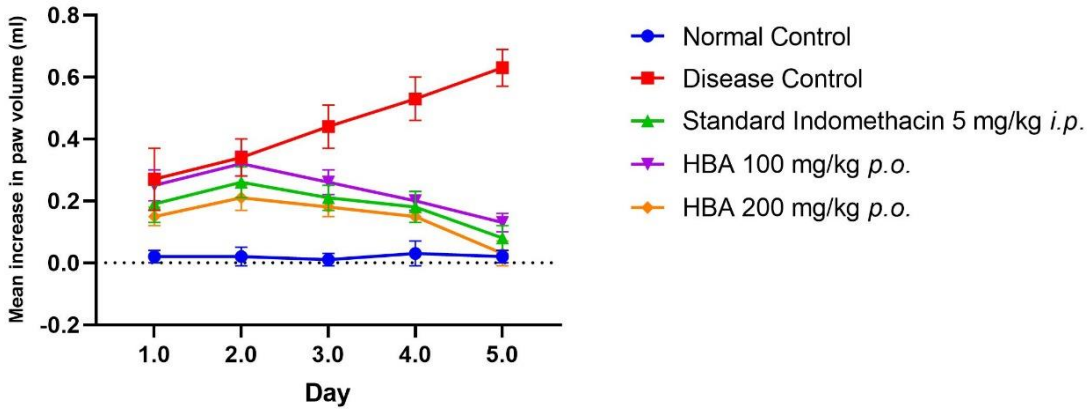

Figure 48: Changes in the paw volume over time in the chronic inflammation model.

Histopathology observations of chronic model

Group I (Normal control): The epidermal and dermal architecture of animals was well-preserved and normal in the histopathological sections. No evidence of thickening or irregularities were observed in the epidermis, which maintained its distinctive stratified structure. The connective tissues and blood vessels were evenly distributed throughout the dermal layer, which appeared structurally intact. It is crucial to note that the skin morphology was healthy, as there was no evidence of haemorrhage, oedema, or vascular abnormalities, and there was no infiltration of inflammatory cells.

Group II (Formalin treated/ Disease Control): The histological examination of Group II animals, which were subjected to formalin treatment to induce a chronic disease state, revealed severe epidermal hyperplasia. This condition is characterised by an excessive thickening of the epidermis as a result of sustained cellular proliferation. Furthermore, there was a significant infiltration of inflammatory cells into both the epidermal and dermal layers, suggesting a persistent inflammatory response. The number of blood vessels also increased significantly, with a significant number of them exhibiting symptoms of haemorrhage. This suggests that the vessels are fragile and prone to leakage, which is a characteristic of chronic tissue inflammation and damage.

Group III (Indomethacin): animals that were administered Indomethacin exhibited a decrease in epidermal hyperplasia in comparison to Group II in their histopathological sections. But, dermal hyperplasia persisted, indicating that Indomethacin had a partial impact on suppressing epidermal proliferation. The level of inflammatory cell infiltration was diminished, eventhough the inflammation had not been entirely resolved. This implies that Indomethacin had a moderate anti-inflammatory effect but was not entirely effective in restoring normal skin architecture in the chronic model.

Group IV (HBA 100 mg/kg): In contrast to the disease-induced group, animals in Group IV demonstrated a decrease in epidermal hyperplasia, despite the fact that the epidermis remained slightly thickened. Despite some structural improvements, inflammatory cell infiltration remained present, suggesting that inflammation was ongoing. These results indicate that the modest dose of HBA had a beneficial effect on reducing epidermal alterations; however, it was insufficient to completely suppress inflammation in the ch ronic model.

Group V (200 mg/kg): The animals which were administered a high dose of HBA, exhibited the most substantial histopathological recovery. The epidermal and dermal structures were strikingly similar to those of normal controls, with the exception of moderate epidermal hyperplasia. The ductal architecture appeared virtually normal, indicating a restoration of tissue integrity. Furthermore, the high-dose treatment exerted a good anti-inflammatory effect, as indicated by the low infiltration of inflammatory cells. The results of these studies indicate that a high dose of HBA was highly effective in restoring normal histological features and mitigating the chronic inflammatory response. All the histological sections of chronic inflammation study are represented in Figure 49.

Gene expression studies in chronic model

The fold induction of inflammatory genes (COX-1, COX-2, IL-1β, IL-6, and TNF-α) in chronic inflammation model is depicted in the bar graph (Figure 50). COX-2 and TNF-α demonstrate the most significant upregulation of all inflammatory markers in

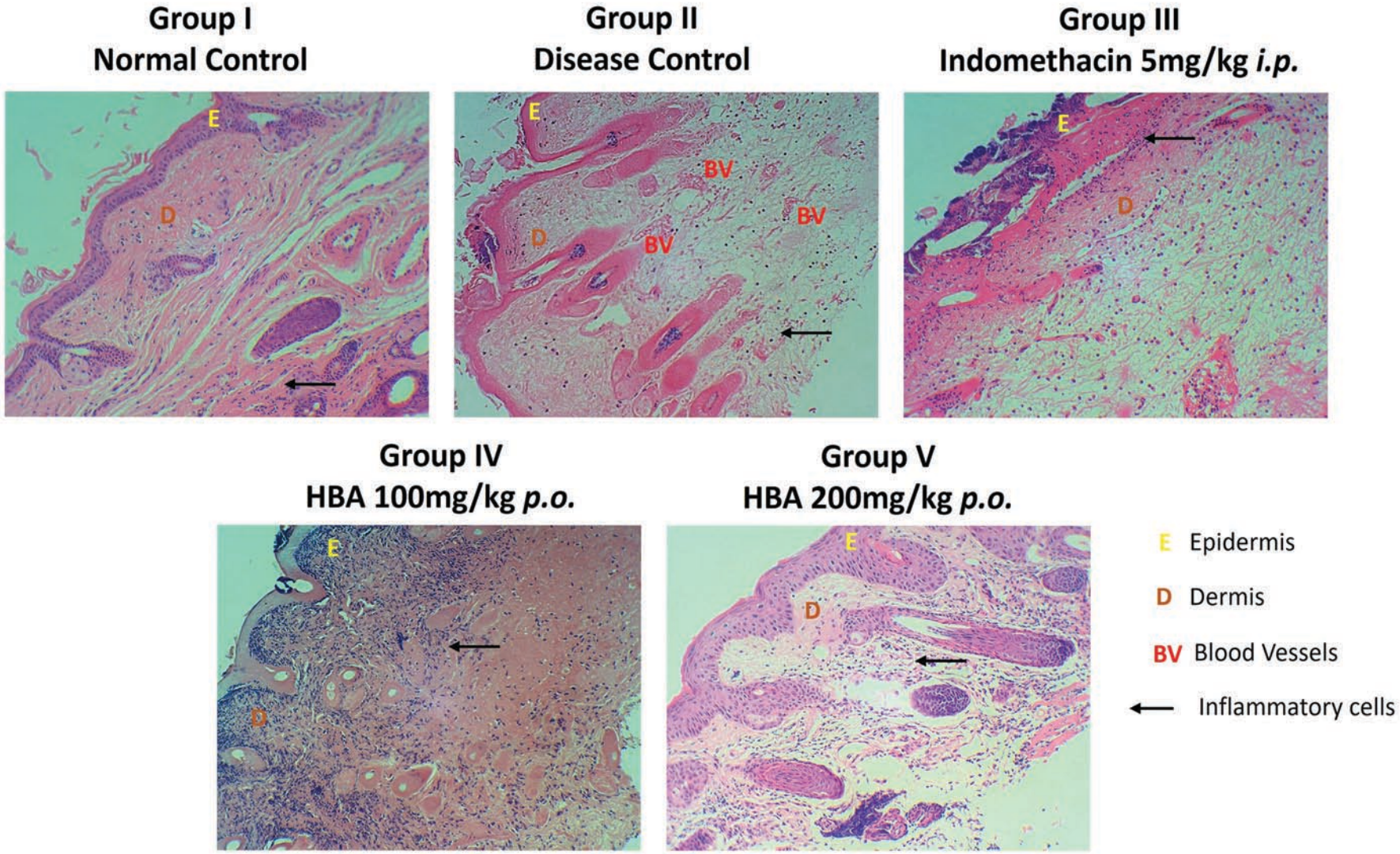

Figure 49: Histological examination of paw tissue sections after 5 days of treatment in chronic inflammation model.

the diseased group, with an induction of approximately 2.7–3.0 times. This confirms the presence of sustained chronic inflammation. The treatment with indomethacin results in a decrease in the expression of inflammatory genes, particularly in the case of cytokines. However, COX-1 and COX-2 remain elevated compared to cytokines. But the expression of COX-1 and COX-2 is reduced compared to disease control. HBA exhibits moderate anti-inflammatory effects at a dosage of 100 mg/kg, resulting in a decrease in inflammatory gene expression in comparison to the diseased group. However, it remains higher than the Indomethacin group. HBA at a dosage of 200 mg/kg demonstrates a more potent anti-inflammatory effect, as it substantially reduces IL-1β, IL-6, and TNF-α. This suggests a dose-dependent reduction in inflammation. These findings suggest that HBA, particularly at high dosages, has potential anti-inflammatory properties that are comparable to Indomethacin.

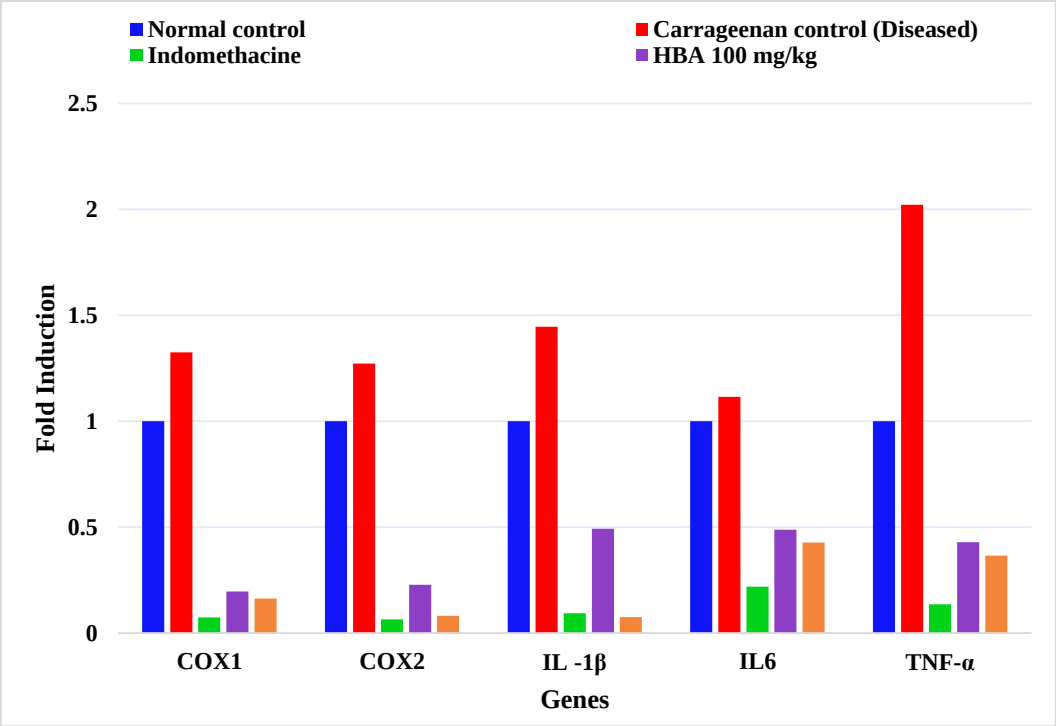

Figure 50: The expression of various inflammatory markers under chronic inflammation.

4.4.1.3 Molecular Docking and MM-GBSA Analysis

The molecular docking and MMGBSA results indicate that the selected phytochemicals exhibit strong binding affinities with inflammatory targets, supporting their potential anti-inflammatory activity (Table 24, Figure 51-55). (-)-Epicatechin showed the highest docking score (-11.9751) with COX-1, along with a MMGBSA dG bind of -49.13, suggesting a stable interaction. 5,7-Dihydroxy-8-prenylflavone interacted effectively with COX-2, with a docking score of -10.0586 and a binding energy of -46.30, indicating strong affinity.

Among cytokine targets, Cinnamtannin A2 exhibited moderate binding with IL-6 (docking score: -7.30883, MMGBSA: -30.41), while (-)-Epicatechin-3'-O-glucuronide showed higher binding stability with IL-1β (docking score: -7.9686, MMGBSA: -44.86). Neoeriocitrin demonstrated strong binding with TNF-α, with a docking score of -11.0504 and MMGBSA of -50.33, highlighting its potential to inhibit TNF-α.

Table 24: Molecular docking and binding energy analysis of phytochemicals targeting key inflammatory proteins

Protein	Compound	Pub chem ID	Docking Score	MMGBSA dG bind	glide_evdw	glide_ecoul	glide_emodel
COX-1	(-)-Epicatechin	72276	-11.9751	-49.13439533	-36.1786	-12.5258	-47.6789
COX-2	5,7-Dihydroxy-8-prenylflavone	5281903	-10.0586	-46.30508145	-33.5893	-5.27902	-39.9286
IL-6	Cinnamtannin A2	16130899	-7.30883	-30.41146598	-30.6387	-28.9789	-60.1444
1L-1β	(-)-epicatechin-3'-Oglucuronide	76969982	-7.9686	-44.86523958	-35.1701	-21.8335	-69.1814
TNF-α	Neoeriocitrin	114627	-11.0504	-50.33301878	-21.0455	-19.874	-69.0886

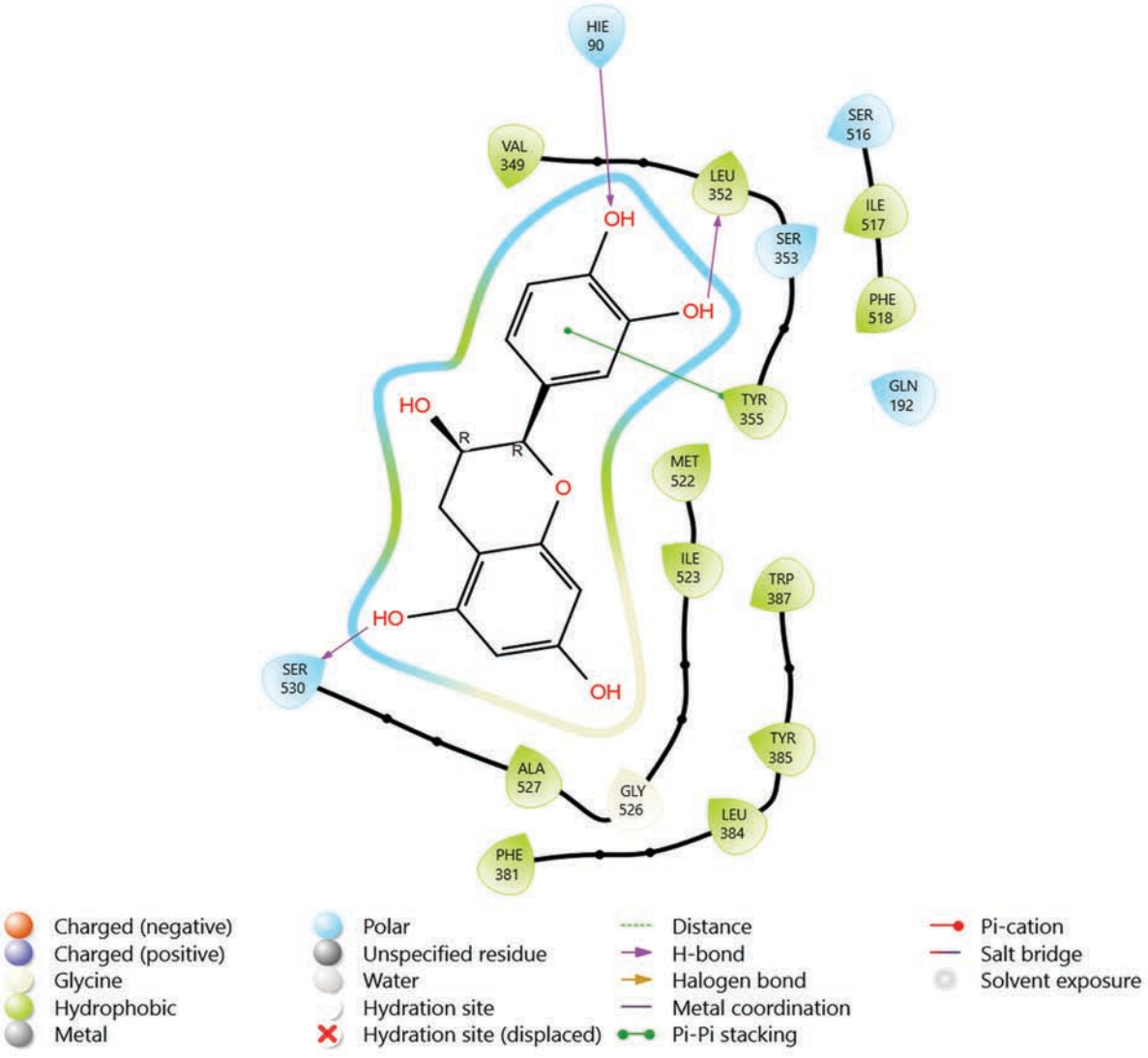

Figure 51: Ligand protein interaction diagram of COX-1 V/S (-)-Epicatechin.

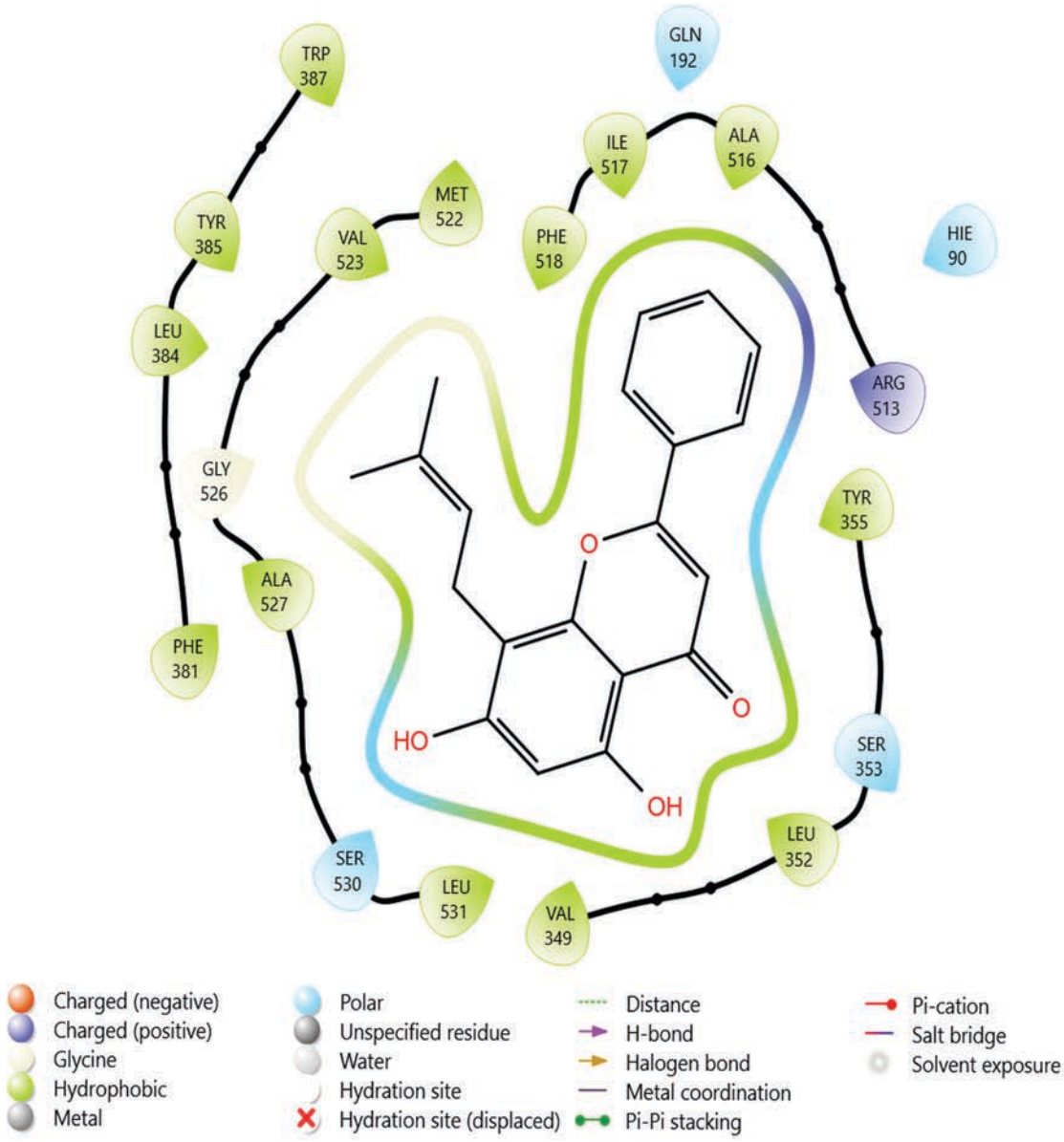

Figure 52: Ligand protein interaction diagram of COX-2 V/S 5,7-dihydroxy-8-prenyl flavone.

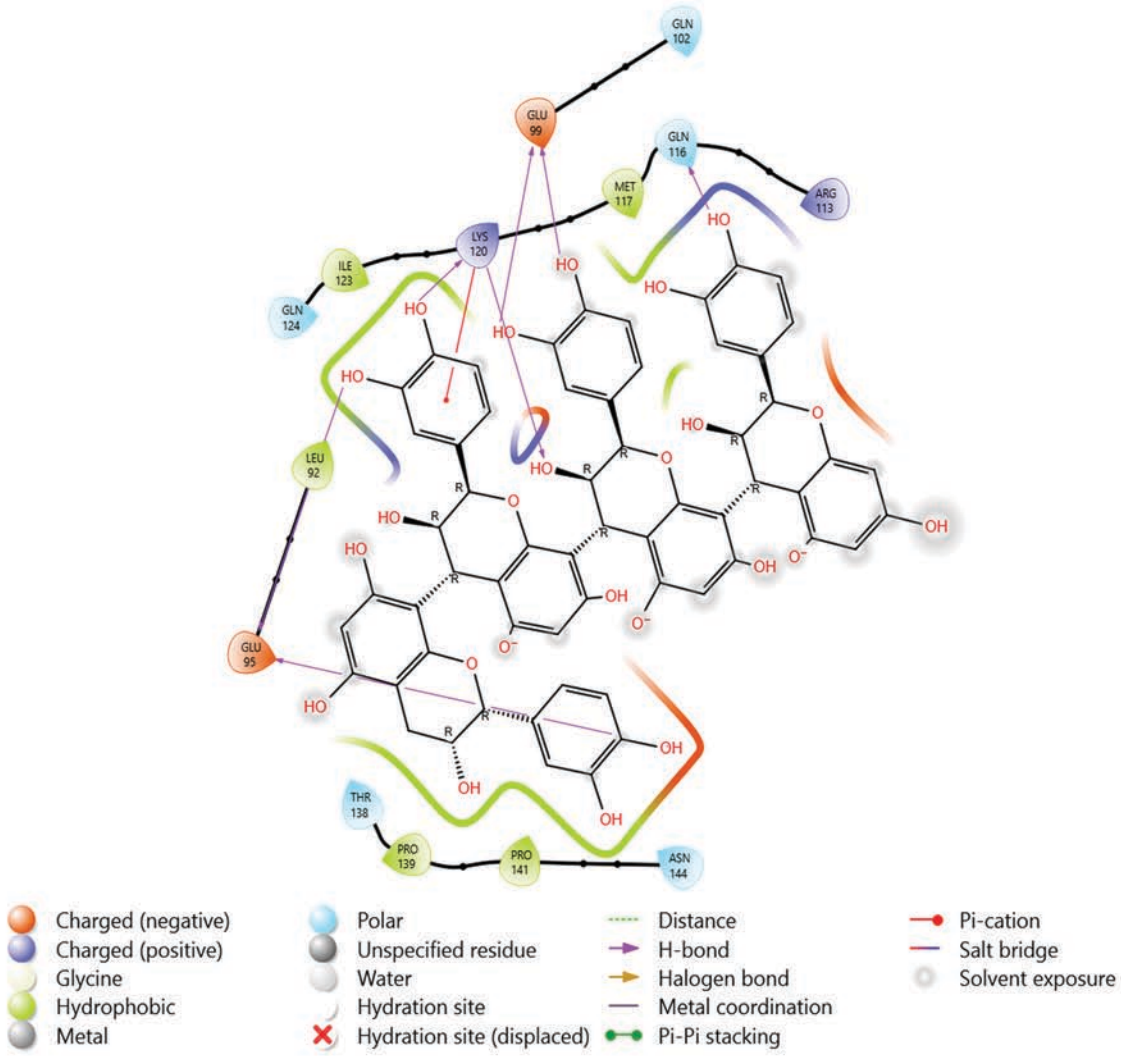

Figure 53: Ligand protein interaction diagram of IL-6 V/S Cinnamtannin A2

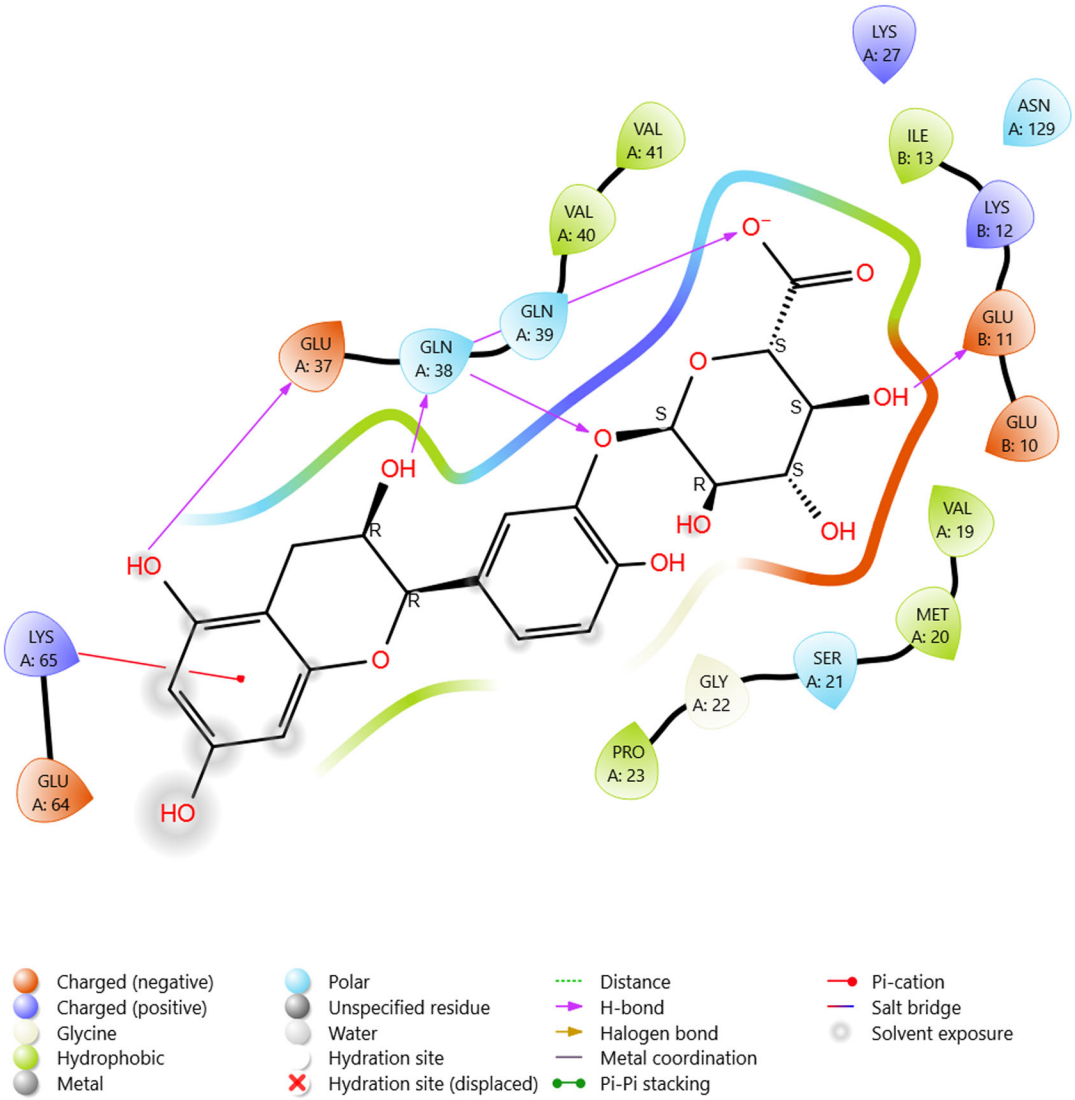

Figure 54: Ligand protein interaction diagram of IL-1β V/S (-)-epicatechin-3'-O-glucuronide

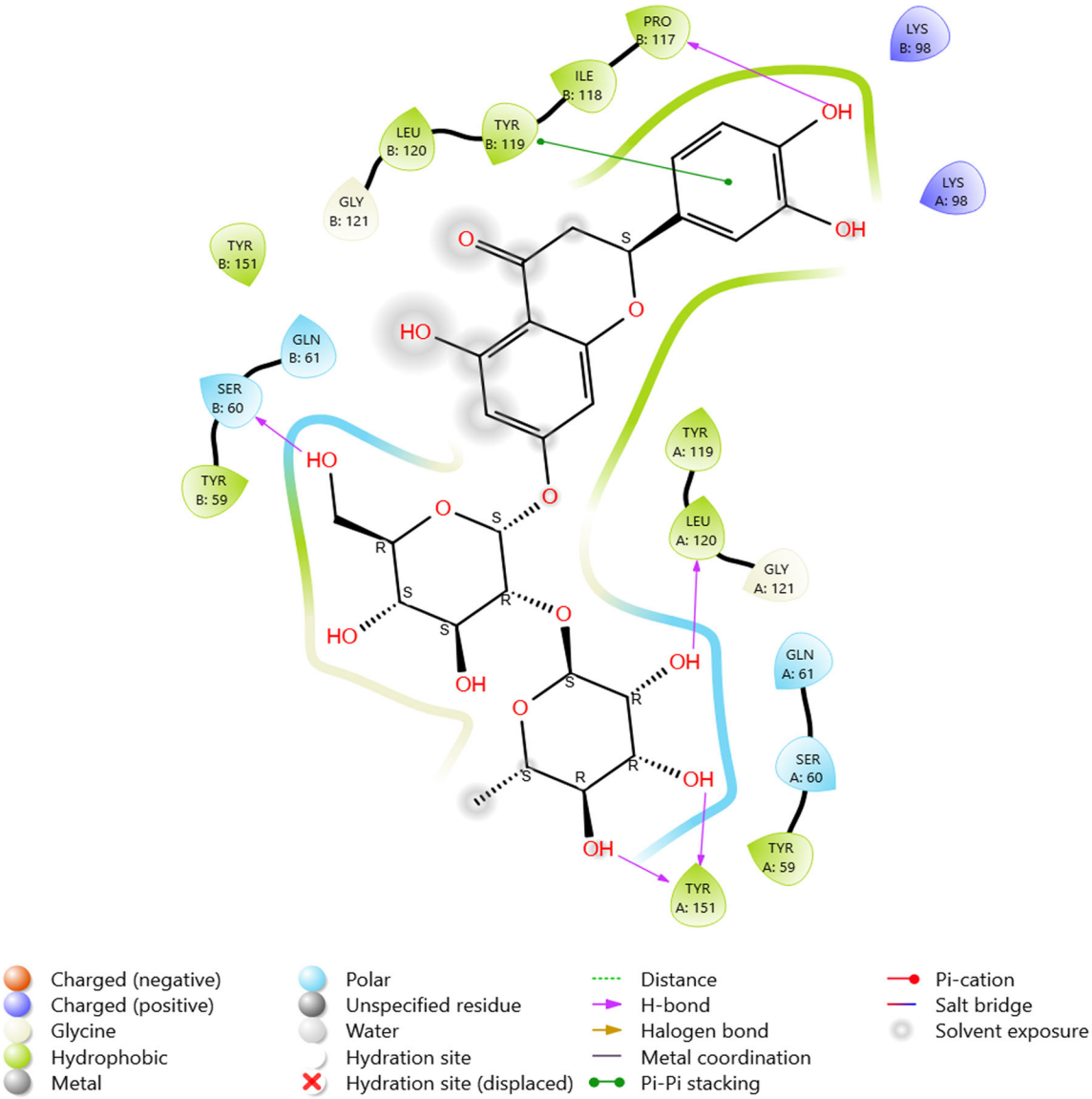

Figure 55: Ligand protein interaction diagram of TNFα V/S Neoeriocitrin.

4.4.2 Discussion

Inflammation is a critical immune system response; while it was previously believed that inflammation was linked to immune system diseases, it is now recognised that inflammation is indicative of several kinds of diseases (Ahmed, 2011; Schmid-Schönbein, 2006). The role of oxidative stress and inflammation is impeccable in the onset of these diseases. Oxidative stress, the imbalance between the generation of reactive molecules and its scavenging, plays significant roles in non-communicable diseases. Together with this, numerous studies have established that inflammation has a role in the progression of different diseases (Sidhic *et al.*, 2023). The link between inflammatory response and the onset of different cancers such as ovarian cancer and pancreatic cancer has been studied well. According to researches, persistent, unresolved inflammation is associated with a variety of health complications and may even contribute to the development of cancer (Allavena *et al.*, 2008). Inflammation is key symptom of many diseases, greatly affecting overall health and quality of life (Gregory *et al.*, 2013). Chronic pain and inflammation can lead to emotional distress, fatigue, and severe health complications, including organ damage, heart disease, and cancer, ultimately affecting overall well-being and longevity (Chrousos, 2000; Jarosz *et al.*, 2020; Manabe, 2011). Treatment processes often relies on analgesics and anti-inflammatory drugs, but their use is linked to various health concerns. As a result, there is a growing shift toward herbal remedies as safer alternatives (Botz *et al.*, 2017; Singh *et al.*, 2008).

The present study evaluated the anti-inflammatory potential of *Humboldtia sanjappae* extract (HBA) in both acute and chronic inflammation models, comparing its effects to the standard anti-inflammatory drug, Indomethacin. The results indicate that HBA, particularly at a higher dosage (200 mg/kg), exhibits a significant anti-inflammatory response, comparable to Indomethacin.

The histopathological analysis of paw tissues in both acute and chronic inflammation models revealed distinct patterns of inflammation and recovery. Table 25 summarizes these observations. The differences in histopathological observations between acute and chronic inflammation might be primarily due to the duration of the immune reaction associated with each type of inflammation. Inflammation is the body's

protective response to harmful stimuli, involving increased blood flow, vascular permeability, and immune cell migration, leading to pain, swelling, redness, heat, and functional impairment of the affected tissue (Chen *et al.*, 2017; Harvanová & Duranková; Ji *et al.*, 2023). These signs were persistent in the case of chronic inflammation. Which was indicated by elevated hyperplasia, wide spread inflammatory cell infiltration more disrupted ductal architecture. But all these symptoms were less severe in the case of acute model.

COX-2 is an essential enzyme that is linked to tumorigenesis and inflammation. It regulates cell proliferation, differentiation, and apoptosis (Tsyndrenko *et al.*, 2023). Proinflammatory cytokines and COX-2 can be upregulated by the inflammatory microenvironment, which can contribute to malignancy (Wang *et al.*, 2004). Previous report suggest that the COX-2 expression has role in the severity of the hyperplasic condition (Tsyndrenko *et al.*, 2023). Our study supports this observation, as the elevated COX-2 expression in the acute inflammation model contributed to hyperplasia in the inflamed paw tissues. This effect intensified in the chronic disease model, likely due to prolonged immune activation. Additionally, cytokine levels remained significantly elevated, intensified the inflammatory response.

Table 25: Histopathology observation comparison of two different inflammation models

Feature	**Acute Model**	**Chronic Model**
Epidermal hyperplasia	Moderate to severe	Severe and persistent
Dermal hyperplasia	Mild to moderate	More pronounced and sustained.
Inflammatory cell infiltration	High initially, reducing over time	Persistent and widespread
Vascular changes	Minimal or mild congestion	Increased size of blood vessels with haemorrhage.
Ductal architecture	Slightly altered	More disrupted, depending on severity.
Response to treatment	Faster resolution with anti-inflammatory drugs	Slow improvement, requiring long-term treatment.

In acute inflammation, treatment helps lower gene expression and reduce inflammation effectively. However, in chronic inflammation, inflammatory genes stay elevated despite treatment, showing ongoing immune activation. HBA (200 mg/kg) and Indomethacin reduce inflammation in both models, but they work better in acute cases. The inflammatory process begins with an external stimulus, such as injury, infection, or toxins, which triggers immune cell activation (macrophages, neutrophils, and mast cells) (Harvanová & Duranková). These activated immune cells then release pro-inflammatory cytokines, including IL-6, TNF-α, and IL-1β**,** which further amplify inflammation (Mukai *et al.*, 2018; Zaporowska-Stachowiak *et al.*, 2024).

COX-2 and TNF-α remain high in chronic inflammation, suggesting the need for longer treatment to control it fully. The link between COX-2 and TNF-α is evident from the study. They act in a positive feedback loop that amplifies inflammation (Mark *et al.*, 2001; Medeiros *et al.*, 2010)**.** IL-6 and IL-1β has role in promoting the inflammation. The persistent activation of these cytokines in chronic inflammation has been linked to cancer development and progression**.** Additionally, COX-2 overexpression and sustained TNF-α signalling create a pro-tumorigenic environment (Cruceriu *et al.*, 2020; Hugo *et al.*, 2015).

The gene expression analysis revealed a substantial downregulation of inflammatory markers**,** particularly COX-2 and pro-inflammatory cytokines, aligning with the molecular docking studies**.** The strong binding energies and docking scores indicate that the phytochemicals present in HBA may effectively inhibit inflammatory enzymes and cytokines, contributing to their potential anti-inflammatory effects. Neoeriocitrin displayed the strongest interactions with TNF-α, suggesting their possible role in inflammation modulation. The docking results further support the use of these compounds in targeting key pathways of inflammation. In our study, COX-1 important for normal body functions was inhibited, but no negative effects were seen. This might be because the treatment acted like a non-selective COX inhibitor, similar to Indomethacin. Most anti-inflammatory drugs work by blocking both COX-1 and COX-2 rather than just COX-2 (Lucas, 2016). Thus, the findings of our study align with the mechanistic pathway represented in the Figure 56, further validating the anti-

inflammatory potential of compounds from Ethanol extract of *H. sanjappae* bark in regulating inflammation through cytokine suppression and COX inhibition.

4.4.3. Conclusion

HBA exhibits significant anti-inflammatory properties in both acute and chronic inflammation models. The dose-dependent reduction in paw thickness, histopathological improvements, and gene expression modulation confirm its therapeutic efficacy. Molecular docking studies further support the potential of HBA-derived bioactives in targeting key inflammatory mediators, demonstrating strong ligand-protein interactions. These findings suggest that HBA could serve as a promising natural anti-inflammatory agent, warranting further investigation in clinical settings.

The experimental and computational findings suggest that HBA exhibits strong anti-inflammatory activity, likely through COX inhibition and cytokine modulation. Molecular docking results further support its ability to target key inflammatory pathways, making it a promising natural anti-inflammatory agent. Further Bioassay guided fractionation and purification can yield important pharmacologically active anti-inflammatory agents.

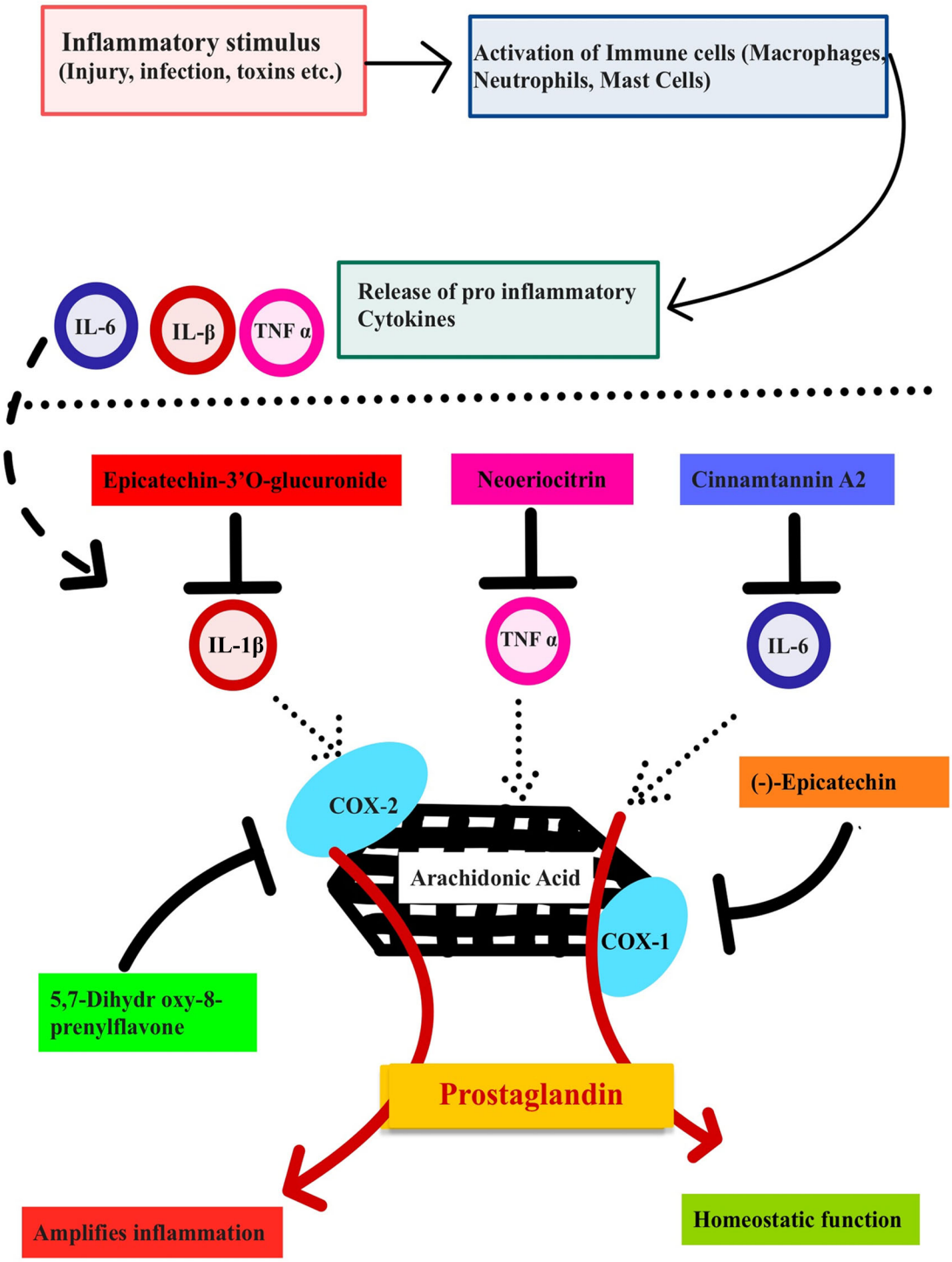

Figure 56: The probable mechanism of inhibition and reduction in inflammation.

Chapter 5

SUMMARY AND CONCLUSIONS

The increasing interest in medicinal plants has led to extensive research on their phytochemical composition and pharmacological properties. *Humboldtia sanjappae*, a lesser-known species, belongs to the family Leguminosae and is endemic to Western ghats. Despite its traditional uses, scientific validation of its therapeutic potential remains largely unexplored. Phytochemical studies on this genus are scarce, making it vital to investigate its bioactive compounds and their pharmacological relevance.

Natural products derived from plants have been widely recognized for their anti-inflammatory, antioxidant, and pharmacokinetic benefits. *H. sanjappae* is believed to contain diverse bioactive compounds that may contribute to its medicinal value. Given the increasing burden of inflammation-related diseases, exploring natural anti-inflammatory agents has become a priority in drug discovery.

The objectives of the study include,

1. Phytochemical evaluation of *H. sanjappae* using various chromatographic and spectroscopic techniques
2. Pharmacological evaluation of *H. sanjappae* using *in silico, in vitro and in vivo* models.

The study is structured into four major results.

I. Effect of extraction solvents and plant part on phytochemical composition and antioxidant potential.
II. Comprehensive Analysis of Bioactive Compounds from ethanol extract of *H. sanjappae* bark Using LC-MS/MS Analysis, ADMET Based Pharmacokinetics Prediction and *in vivo* Acute Oral Toxicity Assessment.
III. Network pharmacology and *In vitro* analysis of ethanol extract of *H. sanjappae* bark in treating inflammation.
IV. *In vivo* evaluation of anti-inflammatory potential and molecular docking analysis of key phytochemicals.

By integrating these findings, this study provides a comprehensive understanding of *H. sanjappae*'s pharmacological potential and its possible role in managing inflammation.

I. Effect of extraction solvents and plant part on phytochemical composition and antioxidant potential

The study aimed to determine the optimal concentration and combination of extraction solvent and plant part (leaf or bark) of *H. sanjappae* through preliminary qualitative analysis, High Performance Thin Layer Chromatography (HPTLC) fingerprinting, Total Phenolic Content (TPC), Total Flavonoid Content (TFC), antioxidant potential (DPPH and FRAP), and extract yield. Ethanol was identified as the most effective extraction solvent, and the bark exhibited superior results compared to the leaves. The positive correlation between TPC, TFC, and antioxidant activity, along with the unique, rich HPTLC bands in the bark extract, justified selecting ethanol extract of *H. sanjappae* bark for further studies.

II. Comprehensive Analysis of Bioactive Compounds from ethanol extract of *H. sanjappae* bark Using LC-MS/MS Analysis, ADMET Based Pharmacokinetics Prediction and *In vivo* Acute Oral Toxicity Assessment.

The bark of this plant has abundant bioactive chemicals, promoting it as a prospective target for pharmaceutical research. This study utilises LC-MS/MS analysis to characterise the phytochemicals, subsequently employing ADMET-based pharmacokinetics prediction to evaluate their drug-likeness. An *in vivo* acute oral toxicity study was conducted to assess its safety for potential therapeutic applications.

a) LC-MS/MS Metabolite Identification

HBA underwent comprehensive metabolite identification via LC-MS/MS. This is the first report of a detailed metabolite analysis in this genus. A total of 57 phytochemicals were identified, categorized into 14 groups based on chemical nature. Flavones represented the major class (21.05%), followed by phenols and catechins (14.035% each). Minor categories included betalains, pyridine derivatives, benzopyrans, and steroid lactones, consistent with previous findings in the family.

b) Drug-Likeness and Pharmacokinetics

Drug-likeness analysis using Lipinski's rule revealed that 38 out of 57 compounds exhibited drug-like properties. The crude extract's multiple bioactive compounds

likely act synergistically, enhancing therapeutic potential while minimizing toxicity. The following parameters were studied for understanding the compound's pharmacokinetic properties.

- Absorption: Synergistic action of compounds improved bioavailability and reduced the accumulation.
- Distribution: Optimal plasma protein binding and controlled tissue accumulation was observed.
- Metabolism: Predominantly metabolized via Cytochrome P450 enzymes, promoting detoxification.
- Excretion: Balanced renal and biliary elimination ensured proper clearance.
- Toxicity: *In silico* toxicity assessment highlighted the need for *in vivo* screening.

c) *In vivo* Toxicity Study

The animal experimental procedures were carried out with the approval of IAEC, JSS College of Pharmacy, Ooty, India (IAEC (118/PO/ReBiRc/S/1999/CPCSEA)) under permission number JSSCP/OT/IAEC/24/2024-2025.

Acute oral toxicity in Swiss albino mice (OECD 423) classified HBA as GHS category 4 (LD50 >300-2000 mg/kg).

III. Network pharmacology and *In vitro* analysis of ethanol extract of *H. sanjappae* bark in treating inflammation.

Network pharmacology is a new field that studies how drugs interact with multiple targets (usually protein) in the body. Network pharmacology use computational methods to investigate the characteristics of pharmaceuticals.

a) Network Pharmacology

Using Swiss Target Prediction, STITCH, OMIM, and Gene Cards, 117 common targets were identified between *H. sanjappae* bark extract's phytochemicals and inflammation-related genes. The compound-target network comprised 145 nodes and

226 edges, with luteolin and CYP19A1 as the nodes with maximum interactions. CYP19A1 was linked to tumor inflammation, obesity-linked inflammation, and neuroinflammation.

Protein-protein interaction (PPI) analysis using STRING data base revealed 117 nodes, 1230 edges, and a mean node degree of 21, with significant clustering (0.657) and p-value < 1.0e-16.

The results indicate that the phytochemicals of *H. sanjappae* interact with various inflammation-associated genes, endorsing a multi-target strategy for the modulation of inflammatory pathways. The pivotal function of key targets in chronic inflammatory diseases underscores their potential as treatment possibilities. The remarkable connections within the PPI network further demonstrates the functional relevance of these targets in inflammation-related pathways. The higher clustering coefficient and statistical significance suggest that the targets function in a coordinated manner, hence augmenting the therapeutic potential of *H. sanjappae* phytochemicals in managing inflammation.

b) Gene ontology and Pathway enrichment analysis

Gene Ontology (GO) is a structured framework that categorizes genes based on their biological roles, molecular functions, and cellular localization. It helps in understanding gene-pathway interactions by classifying genes into three main domains, such as biological processes, cellular components and molecular functions (Thomas, 2017).

The results suggest that, 117 targets are involved in, 395 biological processes (e.g., cellular response to hypoxia, reactive oxygen species, and nitric oxide biosynthesis), 148 molecular functions (e.g., enzyme binding and zinc ion binding) and located in 64 cellular components (e.g., cytosol, nucleus, and plasma membrane).

Pathway enrichment analysis (DAVID, ShinyGO) linked 149 biological pathways to the targets, including pathways in cancer, AGE-RAGE signaling, steroid biosynthesis, and PI3K-Akt signalling; all associated with inflammation.

c) *In vitro* Anti-Inflammatory Activity

Ethanol extract of *H. sanjappae* bark exhibited dose-dependent anti-inflammatory activity in RAW 264.7 macrophages by reducing IL-1β, IL-6, TNF-α, and nitric oxide levels. The highest inhibition was observed at 20 µg/mL, approaching the standard aspirin response.

NF-κB and COX-2 expression analysis confirmed significant downregulation in the treatment groups.

IV. *In vivo* evaluation of anti-inflammatory potential and molecular docking analysis of key phytochemicals.

The experiment assesses the *in vivo* anti-inflammatory activity of ethanol extract of *H. sanjappae* bark through established swiss albino mice to determine its effectiveness in mitigating inflammation. Two different studies (acute and chronic inflammation) were performed to understand the effectiveness of the extract against inflammation. Acute inflammation is an immediate immunological reaction that occurs rapidly following injury or infection, marked by irritation, oedema, and discomfort, usually subsiding within hours or days (Bello *et al.*, 2020; Boddawar *et al.*, 2016). Conversely, chronic inflammation is an extended response that endures for weeks or months, frequently resulting in tissue damage and exacerbating problems such as arthritis or autoimmune disorders.

In the acute inflammation study, carrageenan was used to induce inflammation, and the experiment lasted for six hours, beginning with the pre-treatment of mice using the ethanol extract of *H. sanjappae* bark. The chronic inflammation study involved inducing inflammation with formalin solution, followed by a post-treatment regimen with the extract, and was carried out over five days. After both studies, inflamed tissues were collected for gene expression analysis of key inflammatory markers, including COX-1, COX-2, IL-6, IL-1β, and TNF-α**,** along with histopathological examinations to assess tissue-level changes.

In addition, molecular docking studies were conducted to assess the influence of the phytochemicals in the expression of the key inflammatory markers COX-1, COX-2, IL-6, IL-1β, and TNF-α**.**

a) Acute and Chronic Inflammation Models

Carrageenan-induced paw edema and formalin-induced inflammation models in mice demonstrated significant inflammation reduction with HBA (200 mg/kg), comparable to indomethacin. The lower dose (100 mg/kg) also showed notable anti-inflammatory effects.

b) Gene Expression Analysis

The treatment with ethanol extract of *H. sanjappae* bark significantly reduced the expression of pro-inflammatory genes COX-1, COX-2, IL-1β, IL-6, and TNF-α in both acute and chronic inflammation models. In the carrageenan-induced acute inflammation, pre-treatment with the extract diminished the initial inflammatory response, restricting cytokine expression and avoiding excessive tissue damage. In the chronic inflammation, where formalin has been used to create chronic inflammation, post-treatment with the extract markedly inhibited inflammatory gene expression, suggesting its capacity to modulate long-term inflammatory mechanisms.

c) Histopathology

The treated groups demonstrated decreased epidermal hyperplasia, inflammatory cell infiltration, and angiogenesis, with in the high-dose group exhibiting nearly normal tissue architecture. In the acute inflammation induced mices, treatment with ethanol extract of *H. sanjappae* bark significantly reduced inflammation, leading to tissue anatomy which was identical to that of the normal control group. In the chronic inflammation model, inflammation decreased in a dose-dependent manner; yet, full restoration of normal tissue architecture was not attained within the five-day treatment duration. The findings indicate that extended treatment may be essential for the full resolution of chronic inflammation, underscoring the potential of ethanol extract of *H. sanjappae* bark as a long-term therapeutic option.

Molecular docking analyses demonstrated significant binding affinities of phytochemicals from ethanol extract of *H. sanjappae* bark to critical inflammatory targets, indicating their potential effective inhibitors. Of these, (-)-Epicatechin demonstrated the greatest binding affinity with COX-1 (docking score: -11.9751,

MMGBSA dG Bind: -49.13), signifying its robust interaction in regulating the biosynthesis of prostaglandins. 5,7-Dihydroxy-8-prenylflavone exhibited a notable binding affinity for COX-2 (docking score: -10.0586, MMGBSA dG Bind: -46.30), indicating its potential in mitigating inflammation-induced discomfort and oedema. Cinnamtannin A2 interacted with IL-6 (docking score: -7.30883, MMGBSA dG Bind: -30.41), a pivotal cytokine implicated in chronic inflammation and immunological responses. (-)-Epicatechin-3'-O-glucuronide exhibited a significant affinity for IL-1β (docking score: -7.9686, MMGBSA dG Bind: -44.87), underscoring its potential to modulate inflammatory pathways. Furthermore, Neoeriocitrin demonstrated significant binding affinity with TNF-α (docking score: -11.0504, MMGBSA dG Bind: -50.33), indicating its potential in obstructing a principal inflammatory mediator.

Hydrogen bonding, hydrophobic interactions, π- cation interactions and π-π stacking played key roles in the strong binding of the ligand. The ligand fit well within the binding site without steric clashes, ensuring stable interaction. Notably, the absence of salt bridges and metal coordination suggested that non-electrostatic forces primarily governed the binding.

These findings highlight the potential of phytochemicals present in ethanol extract of *H. sanjappae* bark extract in addressing numerous inflammatory pathways, hence enhancing their therapeutic significance in inflammation treatment.

Conclusion

The present study provides insights into the phytochemical composition and pharmacological potential of *H. sanjappae*. Preliminary phytochemical analysis identified a variety of bioactive chemicals, such as phenols, flavonoids, saponins, triterpenoids, and other secondary metabolites. Among the solvents tested, ethanol was identified as the most effective extraction solvent. Additionally, the bark extract exhibited superior antioxidant activity and phytochemical contents compared to the leaf extract. Plant-derived antioxidants, particularly polyphenolic compounds, have gained significant attention for their potential health benefits, and the strong correlation among Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and

antioxidant activity, along with the presence of distinctive HPTLC bands in the ethanol extract of *H. sanjappae* bark, substantiated its selection for further investigation. The LC-MS/MS analysis of the ethanol extract revealed 57 phytochemicals, classified into 14 categories, with flavones being the most prevalent class (21.05%), succeeded by phenols and catechins (14.035% each). ADMET-based drug-likeness assessments and *in vivo* acute oral toxicity evaluations revealed advantageous pharmacokinetic characteristics and a safe toxicity profile, with 38 of 57 compounds conforming to Lipinski's rule of five. Network pharmacology and *in vitro* cell line investigations validated the extract's multi-target anti-inflammatory efficacy, demonstrating robust interactions with critical inflammatory pathways and substantial decreases in IL-1β, IL-6, TNF-α, and nitric oxide levels in RAW 264.7 macrophages. The study of NF-κB and COX-2 expression corroborated a notable downregulation in the treated groups. *In vivo* studies such as acute and chronic inflammation on swiss albino mice confirmed its anti-inflammatory efficacy, demonstrating significant decreases in paw oedema and inflammation comparable to diseased groups. Histopathological examination revealed less tissue damage and inflammatory cell infiltration, and gene expression as well as molecular docking investigations validated the extract's capacity to downregulate critical inflammatory indicators (COX-1, COX-2, IL-6, IL-1β, and TNF-α) via efficient ligand-target interactions. These results not only validate the traditional claims of *H. sanjappae* but also open doors for the translation of these findings into drug discovery and nutraceutical development, highlighting its potential as a natural anti-inflammatory therapeutic.

Chapter 6

RECOMMENDATIONS

The research carried out on *Humboldtia sanjappae*, an endemic and underexplored leguminous plant, has yielded insights into its phytochemical wealth and therapeutic potential. In view of the findings, several future directions are proposed that could contribute to both scientific advancement and sustainable resource management.

- Phytopharmaceutical development.
- Clinical translation of the current study.
- Gut microbiome interaction studies.
- Resource base enhancement through tissue culture.
- Enhanced production of secondary metabolites through biotechnological approaches.

Chapter 7

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ANNEXURE

PUBLICATIONS

Research Papers (Journal)

1. **Jameema Sidhic,** M. K. Aswathi, Aparna Prasad, Alby Tom, Pooja Mohan, Paromita Sarbadhikary, Arunaksharan Narayanankutty, Satheesh George, Heidi Abrahamse, and Blassan P. George. "Advancements in Metal and Metal Oxide Nanoparticles for Targeted Cancer Therapy and Imaging: Mechanisms, Applications, and Safety Concerns." Journal of Drug Delivery Science and Technology (2025): 106622. **(IF- 4.9)**
2. **Jameema Sidhic** †, Renny, A†., Alby Tom, Aswathi Moothakoottil Kuttithodi, Joice Tom Job, Rajakrishnan Rajagopal, Ahmed Alfarhan, and Arunaksharan Narayanankutty. "Methanol Extract of Thottea siliquosa (Lam.) Ding Hou Leaves Inhibits Carrageenan-and Formalin-Induced Paw Edema in Mice." Molecules 29, no. 20 (2024): 4800. **(IF 4.6)**
3. **Jameema Sidhic**., George, S., Alfarhan, A., Rajagopal, R., Olatunji, O. J., & Narayanankutty, A. (2023). Phytochemical Composition and Antioxidant and Anti-Inflammatory Activities of Humboldtia sanjappae Sasidh. & Sujanapal, an Endemic Medicinal Plant to the Western Ghats. *Molecules*, *28*(19), 6875. **(IF 4.6)**
4. **Jameema Sidhic.,** George, S., & Narayanankutty, A. (2024). Traditional Medicinal Plants in Cancer Therapy and Chemoprevention: A Review of Preclinical and Clinical Studies. *Current Nutrition & Food Science*, *20*(6), 703-715. **(IF 1.1)**
5. Prasad, A., **Jameema Sidhic.,** Sarbadhikary, P. *et al.* Role of metal nanoparticles in organogenesis, secondary metabolite production and genetic transformation of plants under *in vitro* condition: a comprehensive review. *Plant Cell Tiss Organ Cult* **158**, 33 (2024). **(IF 2.4)**
6. Prasad, A., **Jameema Sidhic**., Mohan, C. K., Sarbadhikary, P., George, S., Narayanankutty, A., & Abrahamse, H. (2025). Biogenic synthesis of metal oxide nanoparticles for the elicitation of secondary metabolites in the *in vitro* propagated plantlets of Rubia cordifolia L. Plant Cell, Tissue and Organ Culture (PCTOC), 160(3), 1-17. **(IF 2.4)**

Chapter in Book

1. **Jameema Sidhic,** Athira, C. K., Narayanankutty, A., & George, S. (2024). Ayurveda-Inspired Reverse Pharmacology in Drug Discovery. In *Drugs from Nature: Targets, Assay Systems and Leads* (pp. 121-134). Singapore: Springer Nature Singapore.
2. **Jameema Sidhic,** Akshaya Prakash, C., Sarath, N. G., Narayanankutty, A., George, S., & Sebastian, D. P. (2024). CRISPR-Based Plant Improvements for Boosting the Natural Products. In *Biosynthesis of Natural Products in Plants: Bioengineering in Post-genomics Era* (pp. 125-139). Singapore: Springer Nature Singapore.
3. **Jameema Sidhic,** Aparna Prasad, Arunaksharan Narayanankutty, Satheesh George, and Blassan George. "Antioxidant-Oxidative Stress Balance in Chemoprevention of Cancer." In *Bioactive Compounds from Medicinal Plants for Cancer Therapy and Chemoprevention*, pp. 76-102. Bentham Science Publishers, 2024.
4. Prakash, C. A., **Jameema Sidhic,** Sarath, N. G., Sebastian, D. P., & George, S. (2023). RNA Interference: Novel Technique for Enhancing Secondary Metabolite Production in Medicinal Plants. In *Genetic Manipulation of Secondary Metabolites in Medicinal Plant* (pp. 25-44). Singapore: Springer Nature Singapore.

Research Papers (Seminar/Poster)

1. **Jameema Sidhic,** Satheesh George and Arunaksharan Narayanakutty. 2023**,** Identification of the best extraction solvent of an unexplored medicinal plant *Humboldtia sanjappae* Sasidh.& Sujanapal in terms of phytochemical components and pharmacological properties. National conference on Phyto medicine Current Challenges and future Perspectives. From 5 th- 6 th January 2023. Organized by Department of Botany, Bharathiar University, Coimbatore, Tamilnadu. In collaboration with National Medicinal Plants Board, Ministry of Ayush, Govt. Of India New Delhi.
2. **Jameema Sidhic,** Arunaksharan Narayanakutty and Satheesh George. 2023 Identification of the best extraction solvent for *Humboldtia sanjappae*, Elucidation of its anti-inflammatory potential, and LC-MS screening of major phytocompounds. National conference on Interdisciplinary Academic Translational Research and Innovation. 7 th – 8th February 2023 MES ASMABI college, Kodungallur, Thrissur.
3. **Jameema Sidhic**., & Satheesh George (2025). Computational insights into the antidiabetic potential of compounds from *Humboldtia sanjappae*. 37th Kerala Science congress, Kerala Agricultural University, Thrissur, Kerala.
4. **Jameema Sidhic**., Arunaksharan N., & Satheesh George (2024). Bioassay Guided fractionation and pharmacological evaluation of *Humboldtia sanjappae.* National Conference on Omics in redefining Healthcare-ORAH 2024, Jubilee centre for medical Research, Thrissur, Kerala.