

**IMMUNOMODULATORY ACTIVITY AND PHYTOCHEMICAL ANALYSIS OF
SELECTED PLANTS USED IN FOLK MEDICINE OF PALAKKAD FOR POST-
NATAL CARE**

Thesis

Submitted to the University of Calicut for the award of the Degree of

**DOCTOR OF PHILOSOPHY
IN
BOTANY**

BY

JAYALEKSHMI C V

Under the guidance of

Dr. SURESH V



**DEPARTMENT OF BOTANY
GOVERNMENT VICTORIA COLLEGE, PALAKKAD**

JULY 2026



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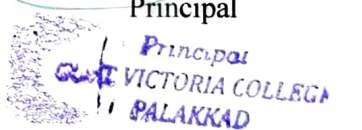
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DECLARATION

I hereby declare that the work presented in the thesis entitled "IMMUNOMODULATORY ACTIVITY AND PHYTOCHEMICAL ANALYSIS OF SELECTED PLANTS USED IN FOLK MEDICINE OF PALAKKAD FOR POST-NATAL CARE" is a genuine record of research work carried out by me at P. G. & Research Department of Botany, Govt. Victoria College, Palakkad under the guidance and supervision of Dr. Suresh V, Professor, P. G. & Research Department of Botany, Govt. Victoria College, Palakkad and has not been included in any other thesis submitted previously for the award of any degree. The contents of the thesis are undergone plagiarism check using iThenticate software at C.H.M.K. Library, University of Calicut, and the similarity index found within the permissible limit. I also declare that the thesis is free from AI generated contents.

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Abbreviations

AFH	- <i>Acalypha fruticosa</i> hexane extract
AFI	- <i>Acalypha fruticosa</i> isopropyl alcohol extract
AFW	- <i>Acalypha fruticosa</i> water extract
ANOVA	- Analysis of variance
ATCC	- American Type Culture Collection
cDNA	- Complementary DNA
CCL-3	- C-C motif chemokine ligand 3
CCL-4	- C-C motif chemokine ligand 4
CCL-5	- C-C motif chemokine ligand 5
CCR-5	- C-C Chemokine receptor type 5
CI	- Consensus Index
Comb	- Combination of plants.
COX-2	- Cyclooxygenase-2
Colo 205	- Colon cancer cells
Ct Value	- Cycle threshold
dH ₂ O	- Distilled water
DMSO	- Dimethyl sulfoxide
dNTP	- Deoxy nucleotide triphosphates
DPPH	- 2,2-Diphenyl-1-picrylhydrazyl
FBS	- Fetal bovine serum
GAPDH	- Glyceraldehyde-3-phosphate dehydrogenase
GVCH	- Government Victoria College Herbarium
HCC-827	- Lung adenocarcinoma cells
HPLC	- High Performance Liquid Chromatography
IC ₅₀	- Inhibitory concentration 50
IFN β	- Interferon β
IL-1 β	- Interleukin-1 β
IL-6	- Interleukin 6
iNOS	- Inducible nitric oxide synthase
JGH	- <i>Justicia gendarussa</i> hexane extract

JGI	- <i>Justicia gendarussa</i> isopropyl alcohol extract
JGW	- <i>Justicia gendarussa</i> water extract
LC ₅₀	- Lethal concentration 50
LPS	- Lipopolysaccharides
MAPK	- Mitogen activated protein kinase
MCP-1	- Monocyte chemoattractant protein 1
MDB	- Membrane Desalting Buffer
mRNA	- Messenger RNA
MTT	-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	- Nuclear factor kappa B
NK cells	- Natural Killer cells
NMR	-Nuclear Magnetic Resonance
NO	- Nitric oxide
OD	- Optical density
OHR LCMS	- Orbitrap High Resolution Liquid Chromatography Mass Spectrometry
PBS	- Phosphate buffered saline
PGE-2	-Prostaglandin E2
PLH	- <i>Piper longum</i> hexane extract
PLI	- <i>Piper longum</i> isopropyl alcohol extract
PLW	- <i>Piper longum</i> water extract
PMA	- Phorbol 12- myristate 13-acetate
PC-3	- Prostate cancer cells
qRTPCR	- Quantitative Real Time Polymerase Chain Reaction
RAW2	- Wash buffer
RA3	- Wash buffer concentrate
RFC	- Relative Frequency of Citation
ROS	- Reactive Oxygen Species
RPMI	- Roswell Park Memorial Institute Medium
RSA%	-Percentage free radical scavenging activity
STAT 1	- Signal transducer and activator of transcription 1
THP1	- Human monocyte leukemia cells

TNF α	- Tumor necrosis factor α
UV	- Use value
VAH	- <i>Vitex altissima</i> hexane extract
VAI	- <i>Vitex altissima</i> isopropyl alcohol extract
VAW	- <i>Vitex altissima</i> water extract
WHO	- World Health Organisation

Glossary

Gotra chikitsa	- Tribal medicine
Kurukkumarunnu	- Medicated porridge used by mothers during post-partum period.
Mangaram	- Mangaram is a fibrous paste like combination of bark of mango tree (<i>Mangifera indica</i>) as a major ingredient and use in the place of soap and scrubber.
Mukkottu	- Combination of coconut oil (<i>Cocos nucifera</i>), gingelly oil (<i>Sesamum indicum</i>) and turmeric powder (<i>Curcuma longa</i>) used for massaging mother and newborn.
Mukkudi	- A special preparation of drug combination used in post-natal care. It is a medicinal drink made up of buttermilk and poly herb combination usually used to cure digestive disorders.
Nalpamara patta	-Bark of four different Ficus plant species. <i>Ficus racemosa</i> , <i>Ficus microcarpa</i> <i>Ficus religiosa</i> and <i>Ficus benghalensis</i>
Nattu chikitsa	- folk medicine
Vayattattie	- Female Folk medicinal practitioner who is specialized in post-natal care.
Vethuvellam	- Medicated water for bathing mother after delivery. The water contains different plant parts like root, stem, leaves, rhizome and petiole of different plant species.
Vevuvellam	- Medicated water for bath in Mahe Union territory.

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Acknowledgements

It is a profound pleasure to convey my deepest gratitude to the individuals and institutions whose unwavering support, wisdom, and encouragement made this dissertation possible. This work represents not just my efforts, but a collective endeavor shaped by the generosity of many. First and foremost, I offer my sincere gratitude and praise to God Almighty for the strength, wisdom, patience, and guidance granted throughout the completion of this doctoral program.

I extend my heartfelt thanks to my dear guide, Dr. Suresh V, whose visionary guidance and intellectual rigor illuminated every step of this research. His ability to challenge my assumptions while nurturing my ideas transformed abstract questions into a coherent thesis. His patience, critical insight, accessibility not only shaped the trajectory of this thesis but also profoundly influenced my development as a researcher. His faith in my abilities, even during the most challenging moments, made this endeavor possible. Without his belief in this project, it would not have reached fruition.

Institutional support played a pivotal role, and I wish to acknowledge the Principal, Government Victoria College Palakkad, for providing all the research infrastructures during the period.

I am equally indebted to Dr. Indulekha C L Pillai, Assistant Professor and Scientist, Stem cell and Regenerative Biology lab, Amrita School of Biotechnology for her expertise in in vitro studies and for providing the critical perspective that refined my analytical framework. Her encouragement during moments of doubt was invaluable, reminding me that perseverance is the cornerstone of discovery. I am also thankful to Dr Bipin Nair, Dean, Amrita School of Biotechnology, for providing all facilities for my cell culture studies.

My sincere thanks to Dr. Dinesh Raj R, Assistant Professor, Department of Botany and Biotechnology, Bishop Moore College Mavelikkara, who first sparked my enduring interest in Botany. I also wish to express my appreciation to all my dear teachers of Bishop Moore College, Mavelikkara and St. Berchman's College Changanassery, whose dedicated teaching and commitment to academic excellence throughout my degree and post-graduation studies provided me with the essential knowledge and analytical tools necessary to undertake this doctoral research.

I would also like to express my heartfelt gratitude to the teachers of Department of Botany, Government Victoria College Palakkad Dr. Rashmi A R, Dr. Sojan Jose, Dr. Sajitha Menon K, Dr. Leeja L, Mr. Alen Alex Philip, Dr. Reedhu Raj for their invaluable support and

contributions throughout my research journey. I also like to express my sincere thanks to Dr. Maya C Nair, Principal, Government Arts and Science College Tholanur for all her support and motivation throughout the research.

This research would not have been possible without the collaborative spirit of my colleagues in the Department of Botany, Government Victoria College, Palakkad. I am thankful to Ambika V, Athira M, Abhilash K S, Gayathri T S, Snehalatha V R, Karthika M, Ashfak Ahmmed O for all the support, encouragement and help during the research. A special thank you to Dr. Maya Ammathil Manoharan, who was always there for me during all the challenges in this period. I also remember the support and suggestions given by Gayathri Vijayakumar, Anisha, Amrutha U, Devika and Durga, Research Scholars, Amrita School of Biotechnology. Their willingness to listen, critique, and share the intensity of the doctoral journey was invaluable to me. My sincere thanks to Ummu Habeeba K, Remya S, Muhsina A A, Athira S, Kanakambika G, Smitha M, Megha B, Navya T, Anila P, Vineesha B, Gopika S Nampoothiri and Drishya D. I am also thankful to Dr. Jisha K former Research Scholar, Government Victoria College Palakkad for all her support and care during the period.

I am indebted to UGC, for providing me with financial assistance as the financial support made this research possible. I extend my deepest gratitude to all the informants took part in my survey and share their knowledge on plants used for post-natal care in folk medicine of Palakkad.

A heartfelt thank you goes to my dear friend, Reshma K Ramesh, a fellow scholar in the department. Beyond the friendship, her insightful discussions, collaborative spirit, and shared understanding of the Ph.D. experience provided both intellectual clarity and continuous emotional reassurance. Our partnership made the isolating moments feel collaborative.

I am greatly indebted to my mother G. Vasanthakumari, my sister Parvathi C V and my grandmother Gourikutty Amma. Your belief in my potential has been the greatest motivator in my life. Thank you for all your sacrifices done for me during the period. I am thankful to my partner Dr. Darshak S for his support, love and patience during the period. A heartfelt thank you to my dear son Ishaan Lekshmi Darshak. This thesis consumed my time, but you filled my spirit. Thank you for your laughter and love. I am thankful to my mother-in-law Deepa S, brother-in-law Deepak S and all my family members for all the support and care throughout this period.

Jayalekshmi C V

PREFACE

This study deals with the immunomodulatory and phytochemical analysis of selected plants used in folk medicine of Palakkad for post-natal care. The study mainly focuses on the plants used for the traditional bath of mother and child after delivery. The water used for this traditional bath is locally called as *vethuvellam*. A survey on plants used for bathing mother and child after delivery was conducted in various parts of Palakkad district and based on the survey four plants were selected for further phytochemical and immunomodulatory studies. This research used available AI tools to improve language and grammar.

The thesis is organised into 8 chapters. The chapter 1 provides an in depth introduction to the research topic. This chapter provides the background of the study, aim and objectives and scope and significance of the study. Chapter 2 provides a review of relevant research literature on the immunomodulation and phytochemical analysis of selected plants used in post-natal care of Palakkad district. It reviews the existing literature on traditional post-natal care practices especially on the plants used for medicated baths of mother and child, pharmacological and phytochemical importance of selected plants used in medicated baths.

The chapter 3 includes various materials and methods used in the research for conducting various experiments. Detailed procedures of survey conducted on plants used for bathing mother and child after delivery, phytochemical analysis of selected plants, bioactivities of selected plants were included in this section. Methodology for preliminary phytochemical analysis, LCMS and TLC of selected plants, *in vitro* macrophage activation studies, antibacterial, antioxidant and antifungal activities of selected plants were included in this section.

In our fourth chapter we provided the results and discussions of all experiments done during the research. The results and discussion on survey of plants used in post-natal care of Palakkad district, preliminary phytochemical analysis and immunostimulation studies of selected plants, immunomodulation studies, antioxidant, antibacterial and antifungal activity of *V. altissima*, LCMS and TLC analysis of *V. altissima* were incorporated in this section.

The chapter 5 deals with the overall discussions of findings of the research. The study concluded that *Vitex altissima* possessed immunomodulatory, antibacterial,

antioxidant and antifungal properties. Chapter 6 summarises the key findings of the present study and its importance. The chapter 7 incorporates the suggestions for future research or practical application based on this study. All references cited in the thesis are listed in Chapter 8

ABSTRACT

The study investigates the immunomodulatory and phytochemical profiling of selected plants used in the folk medicine of Palakkad for postnatal care. The traditional medicinal practices play a vital role in postnatal care of Kerala. Our study mainly focussed on plants used for making *vethuvellam*, a traditional medicated water for bathing mothers after delivery. The study documented the plants used for bathing mothers and infants after delivery. The medicated bath enhances immunity, reduces inflammations and prevents infections. Despite the traditional significance, scientific validation is lacking in this aspect. Our study aimed to document the plants used for traditional bathing of mother and child in Palakkad district, evaluate the bioactivities of selected plants and analyze the phytochemical profile of selected plants.

A survey was conducted on plants used for bathing mother and child in various parts of Palakkad district. 282 informants from the district participated in the survey. 62 plant species used for bathing mother and child after delivery were documented from the survey. The study also reported the various combinations of plants used for making *vethuvellam*. Quantitative ethnobotanical tools like Relative frequency of citation (RFC), Consensus index (CI) and Use value (UV) were used to express the collected data. *Curcuma longa* and *Artocarpus heterophyllus* showed high RFC, CI and UV.

Acalypha fruticosa Forssk., *Justicia gendarussa* Burm. f., *Piper longum* L. and *Vitex altissima* L. f. were selected for further studies based on the survey and prior literature. Leaf extracts of these selected plants in hexane, water and isopropyl alcohol were used in the study. The preliminary phytochemical analysis was done using standard qualitative test. Preliminary phytochemical screening confirmed the presence of alkaloids, flavonoids, tannins, phenolics and saponins across the extracts.

This research investigated the immunomodulation properties of 4 selected plants using *in vitro* macrophage activation studies in THP1 cells. All the four plants modulate the immune system via upregulating or down regulating the expression of IL-1 β , IFN β , TNF α , and CCR-5. *V. altissima* was selected for detailed immunomodulation studies. The hexane (VAH) and isopropyl alcohol (VAI) extract of *V. altissima* upregulated the gene expression of IL-1 β , IFN β , TNF α , and CCR-5 indicating the immunostimulatory property of both extracts.

Our research also studied the antioxidant and antibacterial properties of three different extracts of *V. altissima*. DPPH assay was used to study the antioxidant activity

Our research also studied the antioxidant and antibacterial properties of three different extracts of *V. altissima*. DPPH assay was used to study the antioxidant activity and agar disc diffusion studied against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* was used to explore the antibacterial activity. The *V. altissima* isopropyl alcohol (VAI) extract exhibited superior antioxidant and antibacterial activities.

This study also analysed Orbitrap high resolution liquid chromatography mass spectrometry (OHR-LCMS) for compound identification from *V. altissima* hexane, water (VAW) and isopropyl alcohol extracts. 89 compounds were obtained from VAW, 55 compounds were obtained from VAH and 55 compounds were obtained from VAI. Majority of these compounds possessed many bioactivities.

Our study also highlighted the anti-inflammatory potential of *V. altissima* isopropyl alcohol extract using lipopolysaccharide (LPS) stimulated THP1 derived macrophages. The extract significantly downregulated the expression of IL-1 β , IFN β , TNF α , IL-6 and CCR-5 compared to LPS stimulated control group.

The *V. altissima* isopropyl alcohol extract also exhibited antifungal activity against *Aspergillus niger* studied using food poison technique.

The TLC analysis of VAI extract revealed the presence of seven compounds. The antibacterial activity of TLC fractions was also studied against *B. subtilis*, *E. coli*, *P. aeruginosa* and *S. aureus*. The fractions C5, C6, and C7 showed maximum antibacterial activity against *E. coli* and *B. subtilis*.

This study validates the importance of *vethuvellam* in postnatal care of Palakkad district. *V. altissima* emerges as a leading candidate for drug development due to its immunostimulatory, anti-inflammatory, antibacterial, antioxidant and antifungal activities. The research also supports the folk medicinal use of this plant. The documentation of folk medicinal plants for postnatal care is essential to protect the traditional knowledge.

Keywords: *Vitex altissima*, Antioxidant activity, Inflammation, Post-natal care, Antimicrobial activity.

സംഗ്രഹം

പാലക്കാട് ജില്ലയിൽ പ്രസവരക്ഷയുടെ ഭാഗമായി അമ്മമാർക്കും കുട്ടികൾക്കും കുളിക്കാനായി തയ്യാറാക്കുന്ന 'വേതുവെള്ള'ത്തിൽ ഉപയോഗിക്കുന്ന ഔഷധസസ്യങ്ങളെക്കുറിച്ചും അവയുടെ ശാസ്ത്രീയ ഗുണങ്ങളെക്കുറിച്ചുമുള്ള പഠനമാണിത്. പ്രസവശേഷം അമ്മമാർക്കും കുഞ്ഞുങ്ങൾക്കും രോഗപ്രതിരോധശേഷി നൽകാനും, നീർവീക്കം കുറയ്ക്കാനും, അണുബാധ തടയാനും വേതുവെള്ളം പരമ്പരാഗതമായി ഉപയോഗിച്ചുവരുന്നു. എന്നാൽ ഇതിന് മതിയായ ശാസ്ത്രീയ സാധൂകരണം ലഭിച്ചിട്ടില്ല. ഈ കുറവ് നികത്താനാണ് ഈ പഠനം ലക്ഷ്യമിട്ടത്.

പാലക്കാടിന്റെ വിവിധ ഭാഗങ്ങളിൽ നിന്നായി 282 പേരിൽ നടത്തിയ സർവ്വേയിലൂടെ, പ്രസവരക്ഷയ്ക്കായി ഉപയോഗിക്കുന്ന 62 ഇനം സസ്യങ്ങളെ കണ്ടെത്തി. ഇതിൽ മഞ്ഞളും (*Curcuma longa*), പ്ലാവും (*Artocarpus heterophyllus*) ആണ് ഏറ്റവും കൂടുതലായി ഉപയോഗിക്കുന്നതെന്ന് കണക്കുകൾ വ്യക്തമാക്കുന്നു.

സർവ്വേയുടെ അടിസ്ഥാനത്തിൽ മുഞ്ഞ (*Acalypha fruticosa*), വാതംകൊല്ലി (*Justicia gendarussa*), തിപ്പലി (*Piper longum*), മയിലെള്ള (*Vitex altissima*) എന്നീ നാല് സസ്യങ്ങളെ വിശദമായ പഠനത്തിനായി തിരഞ്ഞെടുത്തു. ഇവയുടെ ഇലകളിൽ ആൽക്കലോയിഡുകൾ, ഫ്ലേവനോയിഡുകൾ തുടങ്ങിയ പ്രധാന രാസഘടകങ്ങൾ അടങ്ങിയിട്ടുണ്ടെന്ന് പരിശോധനയിൽ തെളിഞ്ഞു. ഈ നാല് സസ്യങ്ങളും ശരീരത്തിന്റെ രോഗപ്രതിരോധ സംവിധാനത്തെ (Immune system) ഉത്തേജിപ്പിക്കാൻ സഹായിക്കുന്നവയാണെന്ന് കോശങ്ങളിൽ നടത്തിയ പരീക്ഷണങ്ങളിൽ നിന്ന് വ്യക്തമായി.

ഇതിൽ മയിലെള്ള (*Vitex altissima*) എന്ന സസ്യത്തെക്കുറിച്ച് കൂടുതൽ ആഴത്തിലുള്ള പഠനങ്ങൾ നടത്തി. ഇതിന്റെ സത്തിന് മികച്ച ആന്റിഓക്സിഡന്റ് (Antioxidant) ഗുണങ്ങളും, ബാക്ടീരിയകളെയും ഫംഗസുകളെയും നശിപ്പിക്കാനുള്ള ശേഷിയും (Antibacterial & Antifungal activity) ഉണ്ടെന്ന് തെളിഞ്ഞു. അത്യാധുനിക സാങ്കേതികവിദ്യ (OHR-LCMS) ഉപയോഗിച്ച് നടത്തിയ പരിശോധനയിൽ ഈ സസ്യത്തിൽ ഔഷധഗുണമുള്ള 89-ഓളം രാസസംയുക്തങ്ങൾ ഉണ്ടെന്ന് തിരിച്ചറിഞ്ഞു. കൂടാതെ, ശരീരത്തിലുണ്ടാകുന്ന നീർവീക്കം (Inflammation) കുറയ്ക്കാനും ഇതിന് മികച്ച കഴിവുണ്ട്.

പാലക്കാട്ടെ പരമ്പരാഗത പ്രസവരക്ഷാ രീതികൾക്ക് ശാസ്ത്രീയമായ അടിത്തറയുണ്ടെന്ന് ഈ പഠനം ഉറപ്പിക്കുന്നു. പുതിയ മരുന്നുകൾ വികസിപ്പിക്കാൻ മയിലെള്ള (*Vitex altissima*) എന്ന സസ്യം ഉത്തമമാണെന്നും, ഇത്തരം അമൂല്യമായ നാട്ടുഅറിവുകൾ സംരക്ഷിക്കപ്പെടേണ്ടതാണെന്നും ഈ ഗവേഷണം സമർത്ഥിക്കുന്നു.

സൂചക പദങ്ങൾ: മയിലെള്ള, ആന്റിഓക്സിഡന്റ് ഗുണങ്ങൾ, നീർവീക്കം, പ്രസവരക്ഷ, ആന്റിമൈക്രോബയൽ ഗുണങ്ങൾ.

Chapter 1- Introduction

1.1 Historical and cultural context

Plants have been essential for human well-being for thousands of years, not only providing sustenance but also serving as a rich reservoir of bioactive compounds with medicinal properties. The use of plants as medicine dates back approximately 60,000 years, as evidenced by fossil records (Yuan et al., 2016). Ancient humans developed knowledge about medicinal and edible plants through trial and error, encountering diseases such as diarrhoea, vomiting, constipation, and even death while seeking food and medicine from plants. Over time, they gradually acquired knowledge about edible and medicinal plants.

Globally, traditional medicine remains a cornerstone of healthcare. The World Health Organization (WHO) estimates that 80% of populations in developing countries rely on traditional medicine for primary healthcare, with over 40% of pharmaceutical products derived from plant-based compounds (WHO, 2022). The Sumerian civilization used a wide array of medicinal plants, representing one of the oldest recorded reports on medicinal plant use (Awuchi, 2019). In India, ancient texts like the *Rig Veda* (1500–1000 BC) and *Atharva Veda* (1000–900 BC) document medicinal plant use, emphasizing their role in *Ayurveda* and Folk medicine (Ijnu et al., 2022). Comparatively, ancient Egyptian papyri (e.g., *Ebers Papyrus*, 1550 BC) and Chinese texts (e.g., *Shennong Bencao Jing*, 200–250 AD) reveal parallel developments in plant-based medicine, highlighting a universal reliance on ethnobotanical knowledge (Bou and Richa, 2014; Unschuld, 1986).

In India, traditional systems like *Ayurveda*, *Unani*, *Siddha*, Tribal medicine, *Yoga*, and Naturopathy integrate plants into holistic healthcare. These systems reflect a deep cultural understanding of biodiversity, preserved through oral and written traditions.

1.2 Theoretical framework: ethnobotanical knowledge systems

This study is grounded in ethnobotanical knowledge systems, which emphasize the interplay between human societies, plants, and cultural practices. Ethnobotany examines how communities use plants for medicinal, nutritional, and cultural purposes, often relying on orally transmitted knowledge (Balick and Cox, 2020).

This framework is particularly relevant for studying *vethuvellam*, a practice rooted in Kerala's folk medicine, as it highlights the role of traditional birth attendants (*Vayattaties*) and female informants in preserving plant-based knowledge. By validating these practices scientifically, the study bridges indigenous wisdom with modern pharmacology, contributing to global ethnobotanical research.

1.3 Traditional medicine in Kerala

Kerala, located in South-Western India, is renowned for its biodiversity, driven by a humid tropical climate and its position within the Western Ghats, a UNESCO World Heritage Site and one among the world's biodiversity hotspots (Jaisankar et al., 2018; Kurian et al., 2023). This ecological richness supports a diverse flora, with over 4,500 flowering plant species, many of which are medicinal (Nayar et al., 2014). Kerala's traditional medicine systems, including *Ayurveda*, Folk medicine (*Nattu chikitsa*), and Tribal medicine, capitalize on this biodiversity. Knowledge about these practices is often passed down orally from generation to generation.

Folk medicine, known as *Nattu chikitsa* in Kerala, originated around the 13th century AD (Beegam and Nayar, 2011). It involves the use of plants and other natural products such as milk, ghee, honey, butter, and curd to treat various conditions. Historically, food was considered medicine, with plants integrated into diets for health benefits. Village healers, often women, maintain these practices, particularly in rural areas. Women's role in preserving ethnobotanical knowledge is critical, as they pass down remedies through generations, especially in obstetrics (Mantuan and Sannomiya, 2024).

In ancient periods, folk healers were present in each village to cure various diseases. These folk healers are specialised for treating specific diseases with locally available medicinal plants (Rajasekharan, 2013).

Folk medicine is widely practised in different parts of the world from ancient periods. Primitive humans and animals depend on plants to cure various diseases. Plants have helped humans to support his health from ancient periods. Medicinal plants possessed bioactive compounds with different therapeutic values. Plants possessed anti-inflammatory, analgesic, antimicrobial, anticancer, antioxidant, hepatoprotective, neuroprotective, cardiovascular properties etc. Plant based traditional medicines are cheaper and with no or minimal side effects.

1.4 Obstetrics and post-natal care

Obstetrics is a chief component of folk medicine in Kerala, including pregnancy, childbirth, and postpartum care. The first 6-8 weeks following delivery, known as the post-natal period, are critical for maternal recovery and newborn care. During this time, mothers experience significant physiological, hormonal, and emotional changes (WHO, 2013). Post-natal care is essential for pain management, infection prevention, nutritional support, and restoring physiological functions in mothers. Many herbal preparations are widely used for mothers during this period.

Palakkad is located at the central part of Kerala. It is the second largest district of Kerala and is called the land of Palmyrahs. The unique feature of Palakkad is the Palakkad gap which is the major reason for its rich floristic diversity. In Palakkad, a district renowned for its biodiversity and traditional practices, medicinal plants are widely used for post-natal care. A key practice involves use of *vethuvellam*. It is a medicated water prepared by boiling plant parts, used to bathe mothers after delivery (Rajith et al., 2010). This medicated water is used for newborns also in certain parts of Palakkad district. The plants used in *vethuvellam* vary across Kerala, but the practice remains prevalent in all parts. Mothers are bathed with *vethuvellam* after a massage with *Dhanwantharam kuzhambu* or coconut oil, while virgin coconut oil is applied to the child before bathing. Exposure to volatile compounds and essential oils in these plants is believed to positively affect postpartum health (Anvar and Haneefa, 2015).

In earlier periods women depended on skilled traditional birth attendants for pregnancy, childbirth and postpartum care (Balamurugan et al., 2018). Traditional birth attendants, known as *Vayattaties*, play a crucial role in postpartum care, possessing extensive knowledge of herbal remedies, nutrition and medicated baths (Beegam and Nayar, 2011). They ensure mothers are stress-free by caring for the child, with support from female family members. In some cases, elderly relatives, such as mothers or grandmothers, undertake these responsibilities. These attendants, alongside elderly female family members, possess extensive knowledge of plants used in post-natal care, including medicated baths and dietary preparations that promote maternal recovery, alleviate pain, and enhance lactation. Usually, they stay with mother and child for 28 days after delivery. They had knowledge on plants used for preparing medicated water for baths, plants used to take care of the baby and on the diet of the mother. They prepare special porridges for the mother to improve lactation and also to recover the body of the mother after delivery. These *vayattaties* are still common in rural parts of Kerala.

Currently many ayurveda hospitals provide postpartum care for mothers and children. Even though intake of certain herbal medicines during the postpartum period has declined due to modern medical practices, the tradition of medicated bath or *vethukuli* is still prevalent in many parts of Kerala. The knowledge about the use of plants is limited as the information was passed orally from one generation to the next.

Ethnobotanical research plays a pivotal role in documenting traditional knowledge, which is increasingly at risk due to modernization, urbanization, and cultural shifts. In Kerala, a state renowned for its rich tradition of herbal medicine, such studies are essential for preserving cultural heritage and providing a scientific foundation for validating the therapeutic potential of medicinal plants. This research focuses on a systematic survey conducted in Palakkad district to document plants used in traditional post-natal care practices, specifically for medicated baths. The primary objective of this survey was to identify and catalog the species employed in *vethuvellam* for bathing mothers and infants post-delivery, assess their ethnobotanical significance using quantitative indices and explore their cultural and therapeutic roles in Kerala's folk medicine. This study contributes to the preservation of Kerala's cultural heritage and laying the groundwork for further pharmacological research.

1.5 Immunological changes and immunomodulation during the post-natal period.

Our study aims to document plants used in *vethuvellam* and analyze their immunomodulatory and phytochemical properties. During the postpartum period, significant immunological changes occur in the mother's body. The maternal immune system undergoes complex modulation to tolerate the fetus, which persists into the postpartum period. The maternal immunity undergoes a shift during pregnancy and persists into the postpartum period. Elevated levels of cytokines like IL-6 and TNF α were observed during the postpartum period. Elevated levels of proinflammatory cytokines, such as IL-6 and TNF α , may contribute to postpartum depression, infections, or delayed wound healing (Ross et al., 2022). Increased expression of certain inflammatory markers may lead to the activation of specific inflammatory pathways during the postpartum period (Brann et al., 2019). These inflammatory responses are necessary for recovery but can increase risk of infections, postpartum depressions and delayed tissue healings if not managed. For children, medicated baths aim to enhance immunity against diseases.

Immunomodulation, which alters immune responses, can restore balance, reduce inflammation, and enhance natural defenses during the postpartum period. The traditional treatments like medicated bath and diet during the postpartum period may help mothers to recover faster. Many medicinal plants possess immunomodulatory properties. For instance, the rhizome of *Curcuma longa* and the leaves of *Azadirachta indica* possessed immunostimulatory properties (Parbat et al., 2021). Many bioactive molecules like polysaccharides present in plants produce various immune responses like macrophage stimulation, lymphocyte proliferation and complement activation (Schepetkin and Quinn, 2006).

1.6 Phytochemical studies in plants

Plants play an integral part in the life of humans with an abundant reservoir of bioactive molecules with various medicinal properties. Phytochemicals are produced by plants as a part of their defence mechanisms. They are present in almost all parts. To date more than thousand phytochemicals are reported from different plant species. These phytochemicals defend plants from herbivory, infections and parasites and also provide colour, taste and aroma to plants. Phytochemicals are beneficial to humans as they possess many medicinal properties like antioxidant, antinematicidal, antimicrobial, anti-inflammatory, cardioprotective, neuroprotective, immunostimulants etc (Koche et al., 2016). The bioactive compounds produced by plants include primary metabolites like carbohydrates, proteins, nucleic acid, lipids and secondary metabolites like alkaloids, terpenoids, phenols, saponins, steroids and glycosides (Chinou, 2008).

Many extraction methods are used for the separation of phytochemicals from plants. Maceration, Soxhlet extraction, percolation, Ultrasound Assisted Extraction (UAE), Microwave Assisted Extraction (MAE), Supercritical Fluid Extraction (SFE), Accelerated Solvent Extraction (ASE) are the various types of extraction techniques used for the separation of phytochemicals from plants (De Silva et al., 2017). Different phytochemicals have different affinities for solvents and temperature. Some phytochemicals may degrade in higher temperatures. The selection of right solvents is essential for extraction of phytochemicals. Water, ethanol, methanol, isopropyl alcohol, hexane, acetone, benzene, chloroform, ethyl acetate etc. are the major solvents used for extraction (Lefebvre et al., 2021).

The preliminary phytochemical analysis deals with conventional tests which indicates the presence of various phytochemicals in plants. These preliminary screening tests are cheap, simple and can be done with minimum requirements. These analysis results in creating a chemical profile of the plant extract. Separation techniques like chromatography were employed to analyse the quantification of these phytochemicals.

Chromatography is a method for separation of phytochemicals and the compounds to be separated are placed between a mobile phase and a stationary phase. Mobile phase may be a gas or liquid and the mobile phase passes through the stationary phase. Different types of chromatographic techniques are employed in separation of phytochemicals. TLC (Thin Layer Chromatography) and LCMS (Liquid Chromatography Mass Spectrometry) are widely used chromatographic techniques in separation of phytochemicals. TLC involves the movement of the mobile phase through a stationary phase placed on a strong support. The mobile phase moves through capillary action. The stationary phase may be a thin layer of silica gel or cellulose held on a glass or aluminium or plastic support. The mobile phase may be any organic solvent (Bele and Khale, 2011). In LCMS analysis, the liquid chromatography technique was coupled with mass spectrometry for separation of compounds. LCMS analysis provides a clear idea about the various secondary metabolites present in plant extracts. The analysis of the plant extracts using LCMS provides a chemical basis for various pharmacological effects of plants.

The phytochemicals in plants contribute to the therapeutic efficacy of plants used in traditional medicine, including those employed in Kerala's folk medicine for post-natal care, particularly in the preparation of *vethuvellam*. The systematic analysis of phytochemicals is essential to validate traditional knowledge, standardized herbal formulations, and identify potential drug candidates. This research focussed on the collection, extraction, and phytochemical analysis of four plants *Acalypha fruticosa*, *Justicia gendarussa*, *Piper longum*, and *Vitex altissima*, selected based on their ethnobotanical significance in Palakkad district. The study employs qualitative and quantitative methods, including preliminary phytochemical screening, Thin Layer Chromatography (TLC), and Liquid Chromatography-Mass Spectrometry (LCMS), to characterize the bioactive constituents in selected plants. NMR (Nuclear Magnetic Resonance Spectroscopy) was used to analyse the structure of compounds obtained from TLC. NMR is an effective technique for structure elucidation of unknown compounds. The technique can be used for identification of plant compounds from mixture of different compounds (Mahrous and Farag, 2015).

1.7 Bioactivity studies in plants

Plants are rich in various bioactive molecules which help humans to cure many diseases. These bioactive molecules are used in the production of many drugs. The search for new bioactive molecules from plants is a subject of many global studies.

Bioactivity of plants involves the physiological effects of bioactive compounds present in plants on other living organisms. The bioactive compounds produced by plants exhibit many pharmacological properties like antimicrobial, anti-inflammatory, anticancer, antioxidant, antiviral, antidiabetic etc. The traditional medicinal practices used many plants to treat various diseases. Proper scientific validation of these plants may lead to the production of new drug molecules.

This research also dealt with the bioactivity analysis of selected plants used in the folk medicine of Palakkad district for post-natal care. In our study we analysed the bioactivities like immunomodulation, cytotoxicity, anti-inflammatory, antioxidant and antimicrobial activity analysis of selected plants used for post-natal care. We selected these bioactivities as the traditional bath during postpartum period mainly aimed to reduce inflammations and other diseases and to increase the immunity of mother and child.

Immunomodulation is the modification of the immune response for therapeutic purposes (Gea-Banacloche, 2006). The modification may be initiation, regulation, enhancement or suppression of immune system components. Immunomodulators may be immunosuppressants or immunostimulants. Immunomodulator is a natural or synthetic molecule which can induce, suppress or alter an immune response (Mukherjee et al., 2014). Immunomodulators can alter both innate and adaptive immune response. Many medicinal plants used in traditional medicines possess immunomodulatory properties. Plants exhibited these properties due to the various phytochemicals present in them. These phytochemicals can modulate immune responses by activating T and B cells, macrophages and natural killer cells (Wen et al., 2012). In our study we used macrophages to study the immunomodulation properties of *Acalypha fruticosa*, *Justicia gendarussa*, *Piper longum* and *Vitex altissima*. *In vitro* macrophage activation studies are used to study the immunomodulation properties of selected plants by using THP1 cells.

THP1 cells are monocytes and are produced from children with acute monocytic leukemia and can imitate certain primary responses of macrophages and monocytes (Bosshart and Heinzelmann, 2016; Nascimento et al., 2022). THP1 cells are reliable and practical models for *in vitro* macrophage activation studies due to their ease of use, reproducibility and ethical accessibility. THP1 derived macrophages mimics primary

human macrophages after differentiation using PMA (Phorbol 12-myristate 13-acetate) or other stimuli like LPS (Lipopolysaccharide), IFN γ or IL-4 (Tedesco et al., 2018).

Inflammation is a very essential and complicated reaction of an organism against various stimuli. The stimuli may be physical, chemical or biological. Based on the nature of stimuli the inflammation may be chronic or acute. The acute phase of inflammation showed fever, high levels of acute phase proteins in blood, leukocytosis, enhanced vascular permeability, recruitment of neutrophils and macrophages to the site of infection and the expression of various cytokines like IL-1 β , TNF α and IL-6 (Ahmed, 2011). Cytokines are intracellular or intercellular signaling molecules produced as a result of an infection. They may be proinflammatory or anti-inflammatory. Chronic inflammation leads to tissue damage and fibrosis. Chronic inflammation may lead to diseases like cancer, arthritis, asthma, atherosclerosis, autoimmune diseases etc. (Germolec et al., 2018).

Plants produce many natural products which can be used as medicines to treat a variety of diseases. Flavonoids, terpenoids, tannins, alkaloids, phenolics, essential oils and anthraquinones are used to treat many diseases. Many phytochemicals present in plants possessed anti-inflammatory properties. For example, quercetin and catechins, suppresses the expression of proinflammatory cytokines like IL-1 β and TNF α and stimulates the expression of anti-inflammatory cytokines like IL-10 (Crouvezier et al., 2001; Mucha et al., 2021). The present study aimed to explore the immunostimulatory and anti-inflammatory properties of *V. altissima* (VAI) using THP1 cells.

Antioxidant is a compound that can reduce or prevent the oxidation of a substrate (Gulcin and Alwasel, 2023). These antioxidants help the plant to overcome oxidative stress produced by the environmental factors and metabolic processes (Chaves et al., 2020). These antioxidants are plant derived secondary metabolites or enzymes or vitamins which inhibit the reactive free radicals produced in the body. This property of plants are very useful to humans as it can be used to reduce the oxidative damage related to many chronic diseases. So, the studies on antioxidant potential of plants are essential for the identification of new compounds, medicines or foods. Our study aimed to explore the antioxidant activity analysis of hexane, water and isopropyl alcohol extracts of *V. altissima* using DPPH assay.

Plants have been widely used by humans for centuries in traditional medicine to treat various types of disease conditions. The exploration of novel plant-based antimicrobials is very important as the prevalence of antibiotic resistant microbes are

increasing day by day (Ahmed and Mikail, 2017). The antimicrobial activity of plants are due to the various secondary metabolites produced by plants. These secondary metabolites include alkaloids, flavonoids, tannins, saponins, phenolics and quinones with a wide range of bioactivities making them ideal candidates for the development of new drugs (Abdallah et al., 2023; Jubair et al., 2022). The antibacterial and antifungal activity studies on plants leads to the discovery of new antimicrobial drugs. This study also aimed to explore the antibacterial and antifungal studies on *V. altissima*.

The validation of traditional medicinal practices through scientific evaluation is critical for integrating ethnobotanical knowledge into modern pharmacology. *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima* are used for making *vethuvellam* in various parts of Palakkad district. These plants were valued for their therapeutic properties. These plants were identified through a survey on plants used in post-natal care, are hypothesized to exhibit immunomodulatory, cytotoxicity, antimicrobial, and antioxidant activities, and characterized for phytochemicals aligning with their traditional use in alleviating postpartum ailments, preventing infections, and supporting recovery.

1.8 Rationale for the study

Many traditional post-natal care practices have been followed in various parts of Palakkad district. But there is a lack of documentation of these traditional knowledge and scientific evidence supporting their safety and efficacy. Many plants used in traditional medicinal practices have not been thoroughly investigated for their pharmacological and phytochemical composition. So, there is a need for proper scientific studies to validate the effect of these traditional medicinal plants.

As we had already mentioned, the postpartum period involves several immunological changes in mothers body. So, understanding the pharmacological properties of these plants used in traditional medicine for post-natal care is crucial. These plants may contain compounds that help to alter the immune system and thereby reduce the risk of infections and promote tissue repair.

Phytochemicals are certain bioactive molecules present in plants which possess many medicinal importance. Identifying and characterising these bioactive compounds can provide insights into how these plants exerts their effect on the human body. Flavonoids, alkaloids, phenolics, terpenoids, glycosides, tannins, saponins etc. have its own unique pharmacological properties. Moreover, understanding the phytochemical

composition can help in standardizing herbal formulations and ensuring their quality and safety. Standardization is essential for developing evidence-based herbal medicines and for ensuring consistency in their therapeutic effects.

1.9 Research gap

Despite the prevalence of traditional post-natal care practices in Palakkad, there is a significant lack of documentation and scientific evidence supporting their safety and efficacy. Many plants used in these practices have not been thoroughly investigated for their pharmacological and phytochemical properties. Our study bridges this gap by providing scientific validation for traditional knowledge, contributing to its preservation and application in modern healthcare.

1.10 Aim and objectives of the study

1.10.1 Aim of the study

The present study focuses on the immunomodulatory and phytochemical analysis of selected plants used in folk medicine for post-natal care and infant care in Palakkad district.

1.10.2 Objectives of the study

The main objectives of present study are:

- 1) Survey of Plants used in Folk medicine for post-natal care.
- 2) Collection of selected plants used in folk medicine.
- 3) Quantitative and qualitative phytochemical analysis of selected plants.
- 4) Analysis of immunomodulatory, antimicrobial, antioxidant and cytotoxicity activity of selected plants.

Chapter 2- Review of Literature

2.1 Introduction

This chapter deals with the literature on plant-based folk medicine, with a particular focus on its application in post-natal care in Kerala, India. It explores the historical, cultural, and scientific dimensions of traditional medicinal practices, emphasizing the role of plants in maternal and child health. By integrating global and regional perspectives, this review establishes the theoretical and empirical foundation for the study, which investigates the immunomodulatory and phytochemical properties of selected plants used in Palakkad, Kerala. It also identifies gaps in existing knowledge, such as the need for scientific validation and standardization, which this thesis aims to address.

2.2 Folk medicine

Folk medicine encompasses traditional healing practices that primarily utilize plant-based remedies, often integrated with empirical knowledge and cultural beliefs. These practices, developed through centuries of trial-and-error, remain a cornerstone of primary healthcare in many communities, particularly in developing countries (Balick and Cox, 2020). Globally, plant-based systems such as *Ayurveda* and *Siddha* in India, Traditional Chinese Medicine, and African ethnomedicine rely on ethnobotanical knowledge to address diverse health needs (WHO, 2022; Gurib-Fakim, 2014). Ethnobotanical research has validated numerous folk remedies, leading to the development of modern pharmaceuticals like aspirin (derived from *Salix* and *Spiraea* species) and artemisinin (from *Artemisia annua*) (Vane and Botting, 2003; Tu, 2011). However, challenges such as the erosion of oral traditions and unsustainable harvesting of medicinal plants threaten the preservation of this knowledge (Cunningham, 2014). This study focuses on documenting and scientifically validating plant-based folk medicine in Kerala's post-natal care practices, contributing to the global effort to bridge traditional and modern healthcare.

2.3 Folk medicine of Kerala

Kerala is located in the southern part of India. The state has a geographical area of 38,852 sq km. The state has a forest cover of 21,144.29 sq km based on IRS-

resourcesat-2 LISS III satellite data for December 2017 to March 2018 (India state of forest report, 2019).

Folk medicine is widely practised in certain parts of Kerala and it is one among the plant-based therapies in Kerala. The other two healing practices are *Ayurveda* and tribal medicine. In Kerala, folk medicine is called *Nattu chikitsa* and tribal medicine is called '*Gotra chikitsa*' and *ayurveda* is called *Ayurveda chikitsa* (Beegam and Nayar, 2011).

The folk medicine of Kerala uses local flora to treat various diseases. According to Sasidharan et al., 2024, *Aloe vera*, *Ocimum tenuiflorum* and *Zingiber officinale* were the most commonly used plants in the folk medicinal practices of Palakkad district. 213 plants were documented from the study on folk medicine of Chittur taluk for treating ailments like fever, stomach ache, wounds and skin disease (Jayalekshmi et al., 2023). In both these studies leaves are the most utilised part. The studies on traditional home medicines of Idukki district reveals that 80 local plants were used for treating various diseases (Gaunt, 2015).

All these studies mentioned the importance of documenting traditional knowledge. Socioeconomically, folk medicine remains a cost-effective healthcare option for rural communities with limited access to modern facilities (Poli et al., 2025).

2.4 Use of plants in post-natal care in Kerala

Obstetrics is considered as an important field in folk medicine of Kerala. The *Vayattatties* or the traditional birth attendants took care of mother and child after delivery (Beegam and Nayar, 2011). They had a thorough knowledge on post-natal care, herbal home remedies, food and nutrition of mother, child care and medicated bath.

58 plant species used for prenatal and post-natal care in folk medicine of Kerala was reported by Beegam and Nayar in a study in 2011. Out of 66 plant preparations described in the study, 51 were used for internal administration and 15 preparations were used for external application. From this study *Zingiber officinale*, *Ocimum tenuiflorum* and *Curcuma longa* are the most commonly used plant species for pre and post-natal care.

19 plant species were used for preparing herbal formulations used during post-natal care in Tirunelveli district, Tamil Nadu was reported by Sonitha and Kumar in 2021. These herbal preparations help mothers in their easy recovery during the postpartum period.

Mukkudi, a herbal formulation prepared from a combination of plants, was used in post-natal care in North Kerala (Rose et al., 2021).

52 plant species were documented for their use in post-natal care in rural areas of South Kerala (Rajith et al., 2010). Use of *vethuvellam* and *kurukkumarunnu* (herbal porridge) were reported in this study for postpartum care.

2.5 Medicated bath

The use of medicated water for bathing is still very common in many parts of Kerala. Nowadays the use of herbal formulations for intake for mothers is less as it causes digestion problems in babies. But the use of the medicated bath is very common. The medicated water for bathing is commonly called *vethuvellam* in Kerala. The common name of this medicated bath may vary in different regions. *Vethuvellam* is the primary term used in this thesis for consistency for the medicated bath, while all other names mentioned in the thesis are regional variants. The water is prepared by boiling water with many plant combinations.

Sonitha and Kumar in 2021, reported the use of barks and leaves of *Mangifera indica* and powders of *Curcuma longa* and *Piper nigrum* boiled in water to bath mothers during the postpartum period in Tenkasi district, Tamil Nadu. Before bath, *C. longa* and *P. nigrum* powder and gingelly oil were applied to the stomach. This application strengthens the uterus, restores its natural functions and combats microbes. Using these plants in boiling water is thought to help the mothers to feel refreshed and rejuvenated by removing the wind from her body.

Rajith et al., 2010 reported the use of *vethuvellam* for bathing mothers after delivery in rural areas of South Kerala. The study reported 8 formulations of *vethuvellam* prepared from different combinations of locally available plants. This study also concluded that this medicated bath rejuvenates the body of mothers.

The use of *vevuvellam* was reported by Sivadasan et al., 2014 from Mahe, Union territory of Puducherry. This medicated water is also used to bath mothers after delivery. Several formulations of this medicated water were reported in this study. *Mukkottu* and *Mangaram* were also used before the application of *vevuvellam*.

Anvar and Haneefa in 2015, reported that Zingiberaceae members were predominantly used to prepare medicated baths in various parts of Kozhikode district, Kerala.

The medicated bath during postpartum is also common in certain other parts of the world. A steam bath called *Bakera* prepared by many plants is practised by people of Minahasa (Indonesia) during post-natal care. 60 different plant species are used to prepare *bakera* were documented from the study conducted by Zumsteg and Weckerle, 2007. They reported that this steam bath helps to reduce infections, aids in women's recovery and restores her physical health. The steam bath stimulates blood circulation and removes sweat from the body. It also helps the body to return to pre-pregnancy shape.

Rohmat and Rahayu., 2019 reported the use of 23 plant species for herbal steam bath called *betetimbun* by the Dayak Ngaju tribe in Central Kalimantan.

The herbal bath during the postpartum period is very common among Mien in Northern Thailand. Panyaphu et al., 2011 reported the use of plants like *Acorus*, *Ficus*, *Smilax* & *Schefflera* and *Basella* species in this herbal bath. These herbal baths refresh the body of the mother, relax her muscles and promote the healing of mothers.

The use of herbal baths after delivery are prominent in Malay traditional post-natal care in Malaysia. These baths help to remove body odour, warms the body and also helps in expelling the wind from the body (Jamal et al., 2011).

All these medicated baths help the mothers to recover faster during the postpartum period. The volatile compounds present in these water helps to refresh the body of the mother. These baths also reduce pain, inflammation, bacterial and fungal infections and increase the immunity of mothers.

2.6 Phytochemical analysis of plants used in post-natal care

Plants used in post-natal care are rich in phytochemicals. A systematic literature review by Sibeko et al., 2021 on global plant use by women during postpartum recovery and lactation reported that phytochemicals in plants contribute to postpartum recovery through several ways. The phytochemicals have immunomodulatory, analgesic and anti-inflammatory activity which helps the mother to heal faster after delivery. These phytochemicals help in wound healing and also maintains the uterus to its pre pregnancy shape. The study also reported that the transfer of phytochemicals into breast milk of the mother helps to support the immunity and development of infants (Sibeko et al., 2021; Sibeko and Johns, 2021).

Othman et al., 2017 reported the presence of carotenoids in 20 different plant species used in the traditional Malay post-natal herbal bath. Carotenoids like lutein and

β -carotene possess antioxidant, anti-inflammatory and immunostimulatory effects. These properties help in the recovery and wellbeing of mothers after delivery.

Phytochemical studies on medicinal plants used in herbal baths, used during the postpartum period, in the Mien (Yao) community, revealed the presence of phenolic compounds, flavonoids, triterpenes, glycosides, saponins, tannins and alkaloids in plants used for herbal baths. 10 plants used in herbal baths were selected and studied for its phytochemicals, antioxidant and antimicrobial properties. *Phlogacanthus curviflorus* exhibited high antioxidant activity and *Schefflera bengalensis* and *Plumbago indica* showed maximum antibacterial activity against *Staphylococcus aureus* (Panyaphu et al., 2012).

Jan et al., 2023 reported that secondary metabolites like flavonoids, alkaloids, phytosterols, glycosides, tannins, phenols etc., are present in the medicinal plants used in pregnancy, postpartum care and child birth in Kashmir Himalaya.

Many of the plants used in postpartum care are rich in various types of phytochemicals. For instance, *Curcuma longa*, a widely cited and used plant for postpartum care, is rich in bioactive molecules like phenolic compounds, terpenes, phytosterols and essential oils (Iweala et al., 2023).

2.7 Immunomodulatory activity of plants used in post-natal care

The postpartum period represents a critical window for both maternal and infant health, characterized by significant physiological and immunological adjustments in the mother (Alhazmi et al., 2021). The maternal body undergoes a complex process of restoration to its non-pregnant state, along with her physical recovery and adaptation to the maternal role after delivery (Youn et al., 2021). Traditional postpartum practices, deeply rooted in cultural beliefs and customs, often incorporate the use of plants to promote healing, restore energy, and support lactation (Dennis et al., 2007). These practices highlight the importance of intensive care during this period, not only for physical well-being of the mother but also for her mental and emotional health (Chang, 2018; Seo et al., 2023). The early postpartum days can be particularly stressful for mothers as they have to handle the demands of newborn care alongside their own recovery, which involves considerable physiological and psychological changes (Xiao et al., 2020).

The immunomodulatory potential of plants used in post-natal care is a complex area of research validating comprehensive investigation, as these natural remedies have been employed across diverse cultures for centuries to enhance maternal health and well-being. The utilization of medicinal plants within postpartum care is influenced by different factors including the rich biodiversity of local environments, variations in topography, and deeply rooted cultural traditions (Silalahi, 2020).

The postpartum period of mothers faces many immunological changes. Proinflammatory cytokines and activity of NK and T helper cells increased during the postpartum period (Dye et al., 2022). Studies on women with postpartum depression showed elevated levels of IL-6 and IL-6 receptors in serum (Maes et al., 2000). The proinflammatory proteins like CXCL1, IL-18 and TNF α receptor family are high in the serum of patients with postpartum depression (Brann et al., 2020).

The immune system is affected by plant derived immunomodulators in different ways. They may directly possess antimicrobial or anti-inflammatory properties, indirectly by altering the function of non-immune cells, altering the function of adaptive immunity, influencing cytokine secretion and altering angiogenesis (Lewicka et al., 2019). Plant derived immunomodulators like polyphenols, flavonoids, alkaloids, diterpenes and phytoestrogen act as immunostimulators. They mediate immunomodulation by activating cytokines or influence the immune system through Toll-like receptor (TLR) protein and antigen interactions (Zebeaman et al., 2023).

Many *in vivo* and *in vitro* studies were reported on the immunomodulatory properties of plants. Plants as immunomodulators are more important nowadays due to the growing number of diseases and microbes. Studies on phyto immunomodulators may lead to the production of new drug candidates in future.

2.8 Phytochemical and pharmacological importance of plants selected for the study

We conducted a survey on plants used for bathing mother and child after delivery in various parts of Palakkad district. Based on the survey we selected 4 plants for our study. The 4 plants are *Acalypha fruticosa* Forssk., *Justicia gendarussa* Burm.f., *Piper longum* L. and *Vitex altissima* L.f. Our study focused on the phytochemical profiling, immunostimulatory, anti-inflammatory, antibacterial, antioxidant and antifungal activity of selected plants. So, the reviews of selected plants are written in this context of study.

2.8.1 *Acalypha fruticosa*

A. fruticosa is a shrub belonging to the family Euphorbiaceae. It is commonly called *chinnichedi* or *munja*. The local healers used these plants to treat many diseases in Western districts of Tamil Nadu (Thambiraj et al., 2012). The plant is widely distributed in Southern Western Ghats of India.

The plant had many ethnomedicinal properties. It is widely used in traditional medicine to treat various diseases. Anandakumar et al., 2009 reported the use of this plant to treat cholera, stomach problems, whooping cough, sexually transmitted diseases, fever and cold in various parts of Palakkad district. Thambiraj et al., 2012, reported the use of this plant for treating fever, jaundice and snakebite.

Anandakumar et al., 2009, reported that thin layer chromatographic studies revealed the presence of alkaloids, flavonoids, glycosides and saponins in methanolic and chloroform extracts of leaves of *A. fruticosa*. Flavonoids, carbohydrates, cardiac glycosides, proteins, xanthoproteins, phenols, fatty acids, phlobatannins and emodins were present in the leaves of *A. fruticosa* (Malathi et al., 2018). 2-methyl-5,7-dihydroxychromone 5-O- β -D-glucopyranoside 1, acalypsin 2, apigenin 3 and kaempferol 3-O-rutinoside 4 were reported from the column chromatographic studies of methanolic leaf extract of *A. fruticosa* (Fawzy et al., 2017). Surendhiran et al., 2011 isolated genistein from the plant methanolic extract. Deepaa et al., 2012 reported 40 components by GCMS method from the essential oil isolated from the leaves of the plant. The major component in the oil was caryophellanes.

The plant possessed many pharmacological importance like anti-inflammatory, antimicrobial, antioxidant and cytotoxic properties. *A. fruticosa* methanolic leaf extract showed significant anti-inflammatory action in both chronic model (cotton pellet produced granuloma) and the carrageenan-induced paw edema model. The paw edema model showed 55.2% suppression at 500 mg/kg of plant extract, while the chronic model showed 52.4% less granuloma tissue formation (Gupta et al., 2003). Compounds isolated from *A. fruticosa* showed anti-inflammatory activity by transcriptional activity inhibition of NF- κ B and iNOS (Fawzy et al., 2017).

The antioxidant activity of *A. fruticosa* was reported in many studies. 57 μ g/ml and 10 μ g/ml concentration methanolic extract of *A. fruticosa* exhibited DPPH radical scavenging activity (Mothana et al., 2010). Rajkumar et al., 2010 reported that both aqueous and methanolic extract showed antioxidant activity. Thambiraj et al., 2012

reported that the methanolic leaf extract exhibited antioxidant activity in a dose dependent manner. 92µg/ml, 290µg/ml and 287µg/ml were the IC₅₀ values for DPPH radical scavenging activity, hydroxyl radical scavenging activity and metal chelating activity. Antioxidant activity studies on various fractions of *A. fruticosa* revealed that significant antioxidant activity was observed with ethyl acetate and n-butanol fractions of aerial parts of *A. fruticosa* (Al-Massarani et al., 2019).

Antibacterial activity studies on *A. fruticosa* methanolic extract showed that at 100µl concentration, the plant extract exhibited maximum inhibition against *Klebsiella pneumonia* and *Salmonella typhi* (Surendhiran et al., 2011). Mothana et al., 2010 reported that the antibacterial studies of *A. fruticosa* in different gram positive bacteria and the results showed that the methanolic extract of the plant exhibited maximum zone of inhibition.

A. fruticosa methanolic extract exhibited cytotoxic action against FL cells, with an IC₅₀ value less than 100µg/ml (Mothana et al., 2010). With an IC₅₀ value of 4.81–12.2µg/ml, the *A. fruticosa* chloroform fraction demonstrated cytotoxic activity against all malignant cell lines in an *in vitro* MTT experiment against MCF-7, HCT-116, Hep G-2, and MRC-5 cell lines (Al-Massarani et al., 2019).

2.8.2 *Justicia gendarussa*

J. gendarussa is commonly called *vathamkolli* or water-willow. It is an evergreen plant found distributed to India, China, Sri Lanka and Malaysia. The Indian and Chinese traditional medicine systems use these plants to treat various diseases. The plant belongs to the family Acanthaceae. Habit is a shrub distributed in moist areas.

The plant is commonly used in traditional medicines. The plant is mainly used by traditional healers in treating rheumatism and arthritis. According to Paval et al., 2009, *J. gendarussa* is used to treat a variety of diseases including fever, hemiplegia, rheumatism, arthritis, headache, earache, muscle discomfort, respiratory disorders, and digestive issues. The plant's leaves and branches are diaphoretic. Additionally, the plant is used to cure skin conditions, inflammation, tumors, and liver abnormalities (Jothimanivannan et al., 2010).

Sterols and flavonoids were reported from the ethanolic *J. gendarussa* extract (Paval et al., 2009). Justiprocumins A and B, two anti-HIV compounds, were isolated by Zhang et al., 2017 from the methanolic stem and bark extracts of *J. gendarussa*. Alkaloids, glycosides, triterpenes, flavonoids and phenolic compounds were reported

from the qualitative phytochemical analysis of ethanolic stem and bark extracts of the plant (Jothimanivannan et al., 2010). Uddin et al., 2011, reported that stigmasterol, lupeol and 16- hydroxy lupeol from petroleum ether soluble fractions of methanolic extracts of the aerial part of the plant by column chromatography. Phatangare et al., 2017 reported the presence of β -Sitosterol from the chloroform extract of *J. gendarussa*.

The anti-inflammatory activity studies on the ethanolic aerial parts of plant extract showed that at 500 mg/kg concentration, the extract showed a maximal suppression of 52% in paw edema caused by carrageenan and 45% in dry weight in the formation of cotton pellet granulomas (Jothimanivannan et al., 2010). Phatangare et al., 2017 studied that β -Sitosterol, a molecule that was isolated from *J. gendarussa* chloroform extract, had strong anti-inflammatory properties.

According to Uddin et al., 2011, *J. gendarussa* methanolic whole plant fraction possessed maximum DPPH radical scavenging activity with IC₅₀ value of 18.80 μ g/ml.

Antibacterial activity studies on *J. gendarussa* methanolic whole plant extract fractions against 13 bacterial species showed that the plant extract possessed mild antibacterial activity with 7-10 mm average zone of inhibition (Uddin et al., 2011).

The antifungal activity studies of chloroform, methanol and aqueous extracts of *J. gendarussa* by Sharma et al., 2011, reported the chloroform extract of the plant showed maximum inhibition zone against *Trichophyton mentagrophytes* and *Microsporum fulvum*.

The studies of Uddin et al., 2011, showed that petroleum ether soluble fraction of methanolic extract of *J. gendarussa* exhibited highest toxicity in brine shrimp lethality bioassay with LC₅₀ 1.27 μ g/ml.

The studies of Paval et al., 2009 revealed the anti-arthritic activity of *J. gendarussa* on the Freund's adjuvant induced and collagen induced rat models. The paw edema inhibition rates were 43% and 47% in the FCA induced model and the collagen induced model respectively in extract treated rats.

2.8.3 *Piper longum*

P. longum is commonly called as *thippali* or long pepper. The plant belongs to the family Piperaceae. The plant is a herbaceous vine widely distributed in tropical and subtropical regions. It is mainly found in India, Sri Lanka, Middle East and America. The plant is widely used in many traditional medicinal practices.

The plant is used to cure bronchitis, asthma, cough and cold, an antidote to scorpion and snake bite. The unripe fruits and roots are mainly used to treat these diseases (Kumar et al., 2016). Evans in 2009, reported the use of fruits for respiratory disorders, circulatory stimulants, analgesic and metabolic imbalance problems.

Alkaloids are the most common bioactive compound found in *P. longum*. Piperine is the reason for its hot and spicy flavour. Phytochemical studies on fruits of *P. longum* revealed the presence of 4 alkaloids, 4 coumarins, 6 essential oils, 7 steroids, 13 arbutin, 9 cardiac glycosides and 3 bitter principles (Saraf and Saraf, 2014). Rawat et al., 2022 reported that the GCMS analysis of different extracts of *P. longum* fruits revealed the presence of piperine, caryophyllene, eugenol etc. The study reported 48 compounds from chloroform extract and 26 compounds from hexane extract. The phytochemical analysis of methanolic leaf extracts of *P. longum* revealed the presence of saponins, glycosides, alkaloids and flavonoids (Sultana et al., 2019). GCMS analysis of leaves of *P. longum* revealed the presence of 70 volatile compounds (Bhuiyan et al., 2008).

P. longum possesses many pharmacological properties. Many studies are reported on the pharmacological properties of the plant. Studies on the anti-inflammatory properties of *P. longum*, revealed that supercritical fluid extract (SE) and isolated compounds possessed anti-inflammatory activities. The SE and isolated compounds treated RAW 264.7 cells showed more NO inhibition than indomethacin (Guo et al., 2019). According to Wang et al., 2017, studies on rat models with cerebral ischemia showed that the plant extract treated rats showed a downregulation in expression of IL-1 β , TNF α and IL-6, indicating the anti-inflammatory properties of the plant. Lim et al., 2020 reported that Retrofractamide C, a compound isolated from *P. longum* possessed anti-inflammatory properties. The compound treated LPS induced J774A cells showed a decrease in expression of NO synthase (iNOS) and cyclooxygenase protein.

In vitro studies on *P. longum* showed that the extracts and isolated compounds like piperine possessed antitumor activity against human cancer cells like lung adenocarcinoma (HCC-827), prostate cancer (PC-3) and colon cancer (Colo 205). The plant extract induces cytotoxicity by apoptosis, mitochondrial disruption, inhibiting oxidative stress and influencing cytoskeletal organisation (Biswas et al., 2022; Gaidhani et al., 2013; Lee et al., 2013). *In vivo* studies on *P. longum* also support the antitumor activity of the plant. Many studies reported that the plant extracts or isolated compounds reduces tumor growth and increases the lifespan of affected mice (Subramanian et al., 2010; Sunila and Kuttan, 2004).

Chaudhary et al., 2013 reported the radical scavenging activity of hydro-alcoholic and ethyl acetate fractions of *P. longum* by DPPH assay. In another study, Kumar and Pratyusha, 2019 reported that the methanol extract of leaves possessed antioxidant activity. The extract possessed DPPH, superoxide, hydroxyl lipid peroxide radical scavenging activities. Aung et al., 2020 reported that the ethanol and water extracts of *P. longum* possessed radical scavenging activity.

Studies in methanol, ethanol and acetone extracts of fruits of *P. longum*, showed that 3 extracts inhibit the growth of *Streptococcus mutans* and *S. aureus* (Aneja et al., 2011). Reshmi et al., 2010 reported that methanolic extract of *P. longum* showed antibacterial activity against *Bacillus subtilis*, *Pseudomonas fluorescens*, *Escherichia coli* and *Salmonella typhi*. Agar well diffusion studies on fruit extracts of *P. longum* revealed that the ethyl acetate extract possessed maximum antibacterial activity (Chauhan et al., 2019).

Studies on antifungal activity of *P. longum* fruit extract, hexane fractions and pipernonaline, a compound isolated from the plant against six plant pathogenic fungi, showed that pipernonaline inhibits the growth of *Puccinia recondita* (Lee et al., 2013). Chloroform, ethyl acetate and acetone extracts of fruit *P. longum* showed antifungal activity against *Aspergillus niger* (Khan and Siddiqui, 2007). Methanol, ethanol and acetone fruit extracts of plants showed antifungal activities against *Saccharomyces cerevisiae* and *Candida albicans* (Aneja et al., 2011).

2.8.4 *Vitex altissima*

Vitex altissima is a tree distributed around evergreen and deciduous forests. The plant is commonly called *myeilellu* or peacock chaste tree. The plant belongs to the family Lamiaceae. The plant possessed numerous phytochemicals and ethnomedicinal properties.

V. altissima has many traditional medicinal importance. Vasuki in 2014, documented the use of the plant leaves to treat wounds, ulcer, allergy, inflammation and eczema in Servaragan hill, Salem. Leela et al., 2015, reported the use of plant leaves to heal wounds, allergies, snake bite, scorpion bites and rheumatism. The study also reported the use of stem bark to cure snakebite. According to Bhakshu et al., 2016, for snakebite, bark paste is administered topically and root extract of the plant is taken orally. Augustine et al., 2010 reported the use of the wood and barks of *V. altissima* in boiled water for the medicated baths for mothers after delivery among Mannan tribes of Periyar Tiger

Reserve, Western Ghat. The plant leaves are used by traditional healers to treat inflammation and fungal infections (Padmalatha et al., 2009).

The plant is rich in various phytochemicals. Many studies reported the phytochemical profile of *V. altissima*. Steroids, terpenoids, glycosides, tannins, flavonoids, cardiac glycosides, alkaloids, phenolic compounds and saponins were reported from the qualitative phytochemical analysis of *V. altissima* ethanolic leaf extract (Leela et al., 2015). Phytochemical activity analysis of hydroalcoholic extract of the plant revealed the presence of carbohydrates, terpenoids, phenols, flavonoids, alkaloids, saponins and tannins (Aeman, 2016). Preliminary phytochemical studies in ethanolic stem bark revealed the presence of flavonoids, phenols, alkaloids, tannins and steroids (Ganapathy et al., 2018). Rajesh et al., 2019 reported that the qualitative phytochemical analysis of hexane and chloroform extract of *V. altissima* revealed the presence of phytosterols, saponins, alkaloids and flavonoids. The study also reported that ethyl acetate extract of the plant revealed the presence of phenols, alkaloids and cardiac glycosides, water extract indicated the presence of phenols, saponins, cardiac glycosides, alkaloids and quinones and coumarins, saponins, cardiac glycosides, terpenoids, quinones and alkaloids were present in ethanolic leaf extract of *V. altissima*. The triterpene acids like ursolic acid, corosolic acid, epicorosolic acid, maslinic acid, epimaslinic acid, euscaphic acid, euscaphic acid glucoside ester, 2 α ,3 α ,24-trihydroxyurs-2-en-28-oic acid, and 2 α ,3 α ,24 trihydroxyurs-12,0 (30)-dien-28-oic acid, and flavonoids like vitexin and luteolin-7-O-glucoside were isolated from the leaves of *V. altissima* (Sridhar et al., 2005).

V. altissima possessed many pharmacological properties like antibacterial, antioxidant, anti-inflammatory, antifungal, cytotoxicity etc. Sridhar et al., 2005 reported that the flavonoids from leaves of *V. altissima*, vitexin and luteolin-7-O-glucoside showed mild antioxidant activity in superoxide and in DPPH radical scavenging test. The studies on 3 iridoid glucosides from ethyl acetate leaf extract of the plant showed that these compounds exhibited radical scavenging activity. Compounds 2-4 had corresponding IC₅₀ values of 24.3, 32.0, and 31.9 μ M for the superoxide scavenging assay and 15.2, 10.9, and 11.4 μ M for the DPPH radical scavenging method (Sridhar et al., 2004). Ganapathy et al., 2018 reported that the antioxidant activity studies of ethanolic stem bark of the plant using DPPH assay and Nitric oxide scavenging method. 600 μ g/ml concentration of the extract exhibited maximum radical scavenging activity in both assays. Antioxidant activity studies on nanoparticles isolated from leaf extracts of *V.*

altissima showed that the silver nanoparticles possessed antioxidant activity (Lekkala et al., 2025).

Sridhar et al., 2005 reported that ethyl acetate extract of leaves at 250 mg/kg dose showed anti-inflammatory activity in the carrageenan induced rat paw edema model. In another study, Sridhar et al., 2004 reported that 6^l-O- trans-feruloyl negundoside, isolated from the ethyl acetate leaf extract, at 200 mg/kg dose showed 20% inhibition in rat paw edema model. Ganapathy et al., 2018, reported the anti-inflammatory activity of ethanolic bark extract of the plant by carrageenan induced rat hind paw edema and acetic acid induced vascular permeability in mice. After three hours of carrageenan injection, 74.23% inhibition was achieved at 125 mg/kg dose and 86.35% inhibition at 450 mg/kg dose of extract. Mice's acetic acid-induced vascular permeability was 64.86% reduced by 450 mg/kg of the extract. Lekkala et al., 2025 reported that silver and zinc nanoparticles isolated from leaves of *V. altissima* possessed anti-inflammatory activity. Membrane stabilisation assay and protein denaturation assay was used in this study to explore anti-inflammatory activity. Sundaresan et al., 2020 also reported the anti-inflammatory activity of methanolic leaf extract of the plant using protein denaturation assay.

The antibacterial activity studies of methanolic leaf extract showed that extract significantly inhibits gram positive and gram-negative bacteria. The extract exhibited 14.80 ± 0.793 mm mean zone of inhibition against *Micrococcus luteus* (Kannathasan et al., 2011). Bhavana et al., 2019 reported that nanoparticles (SnO₂NPs) from water extract of the plant exhibited antibacterial activity against *Escherichia coli*, *Pseudomonas desmolyticum* and *Staphylococcus aureus*. Sundaresan et al., 2020, reported that the chloroform and hexane extracts from leaves of *V. altissima* possessed antibacterial activity.

Bhakshu et al., 2016, reported that ethyl acetate and ethanol extract of the plant inhibited the growth of *Candida albicans* with inhibition zone 9mm, 13mm respectively.

Bhavana et al., 2019, reported that nanoparticles (SnO₂NPs) isolated from *V. altissima* water extract showed a concentration dependent cytotoxicity against MCF-7 cell lines.

This review underscores the significance of plant-based folk medicine in Kerala, particularly, for post-natal care. *Vethuvellam* is prepared by utilizing plants like *Curcuma longa*, *Mangifera indica*, *Piper longum* etc. and offers potential immunomodulatory and antimicrobial benefits. These plants are often combined in *vethuvellam*, potentially

enhancing their effects through synergistic interactions. However, clinical studies are needed to confirm efficacy and safety of these preparations.

The selected plants exhibit diverse bioactivities, supporting their traditional use. Despite this, limited scientific validation and standardization challenges persist, which this study addresses through phytochemical and bioactivity analyses. By bridging traditional knowledge with modern pharmacology, this research contributes to preserving Kerala's ethnobotanical heritage and enhancing post-natal care practices.

Chapter 3- Materials and Methods

This chapter includes the various materials and methods used for the study.

3.1 Documentation of folk medicinal knowledge on plants used for post-natal care in Palakkad district

One of the main objectives of our study was the documentation of plants used in folk medicine of Palakkad for post-natal care. Our study mainly focused on plants used for bathing mother and child after delivery. The various materials and methods used for documenting the plants used for bathing mother and child after delivery was given below.

3.1.1 Area of study

The study area selected was Palakkad district of Kerala. The district has no coastal line and Palakkad gap is an important feature of the district. The gap is 32 to 40 km wide and it opens the state to the rest of the country. The unique biogeography and climate of Palakkad is due to the influence of the gap. The district was formed on 1st January 1957 as an administrative unit of Kerala.

It has a total area of 4482 km². The land area is divided into midlands and high lands. Midland occupies valleys and plains and the high land occupies high mountains and dense forests. 1360 km² of the district was covered by dense forest. High mountains and dense forest are located in the eastern part of the district. The forest of the district contains temperate forest, tropical moist deciduous forest and tropical wet evergreen forest (India state of Forest report, 2019).

The district is divided into 7 taluks, 7 municipalities, and 157 villages. The population of the district is 2,809,934. According to the 2011 census, 13,59,478 males and 14,50,456 females were present in the district.

The district is surrounded by other districts, Thrissur in the south, Malappuram in the north and northwest and in the east by Coimbatore of Tamil Nadu. The major rivers of Palakkad district are Bharathapuzha, Gayathripuzha, Thuthapuzha, Chittur puzha, Kannadi puzha, Kalpathy puzha, Bhavani and Shiruvani. The district has a tropical wet dry climate.

Palakkad is one of the important granaries of Kerala. It is called the rice bowl of Kerala. Paddy, tapioca, plantain, arecanut, pepper, ragi, sugarcane and cashew are the

major crops cultivated in the district and the district is also rich in floristic diversity. The district is located between east longitude 76° 28' and 76° 39' and north latitude 10° 46' and 10° 59'. Palakkad, Alathur, Chittur, Mannarkkad, Ottapalam, Shornur and Pattambi are the seven taluks of the district. The map of Palakkad district was given in Plate 3.1.

3.1.2 Data collection

For the documentation of plants used for post-natal care and infant care, frequent field visits were conducted to various parts of Palakkad district. The period of survey was from September 2018 to January 2020. The survey was conducted at 6 taluks of Palakkad. The details on plants used for bathing mother and child during the early stages after delivery was collected from local people and traditional birth attendants of the district. Oral communication with local people was used to document the data. 282 respondents participated in our survey from various parts of Palakkad district. All the 282 informants were females. Two traditional birth attendants also participated in the survey. Informants ranging in age from 25 to 50 took part in the survey, with a majority of them being over the age of 50. Prior to conducting interviews, informed consent was obtained from all participants, ensuring that they understood the study's purpose and their rights. The local name, family, binomial, plant part used and mode of use were documented. The details of informants who took part in the survey were given in the appendix section.

3.1.3 Collection and identification of plants

Plants were collected during field visits with the assistance of informants to ensure accurate identification of species used in traditional practices. Specimens were identified using standard taxonomic literature, including Flora of the Presidency of Madras (Gamble and Fischer, 1936), Flora of Palakkad district including Silent Valley National Park, Kerala (Vajravelu, 1990), Flowering Plants of Kerala (Sasidharan, 2013) and Plants of World Online (POWO, 2024). Voucher specimens of available plants were prepared and deposited at the Government Victoria College Herbarium (GVCH), Palakkad. Photographs of plants were also taken to complement the herbarium records and facilitate visual documentation.

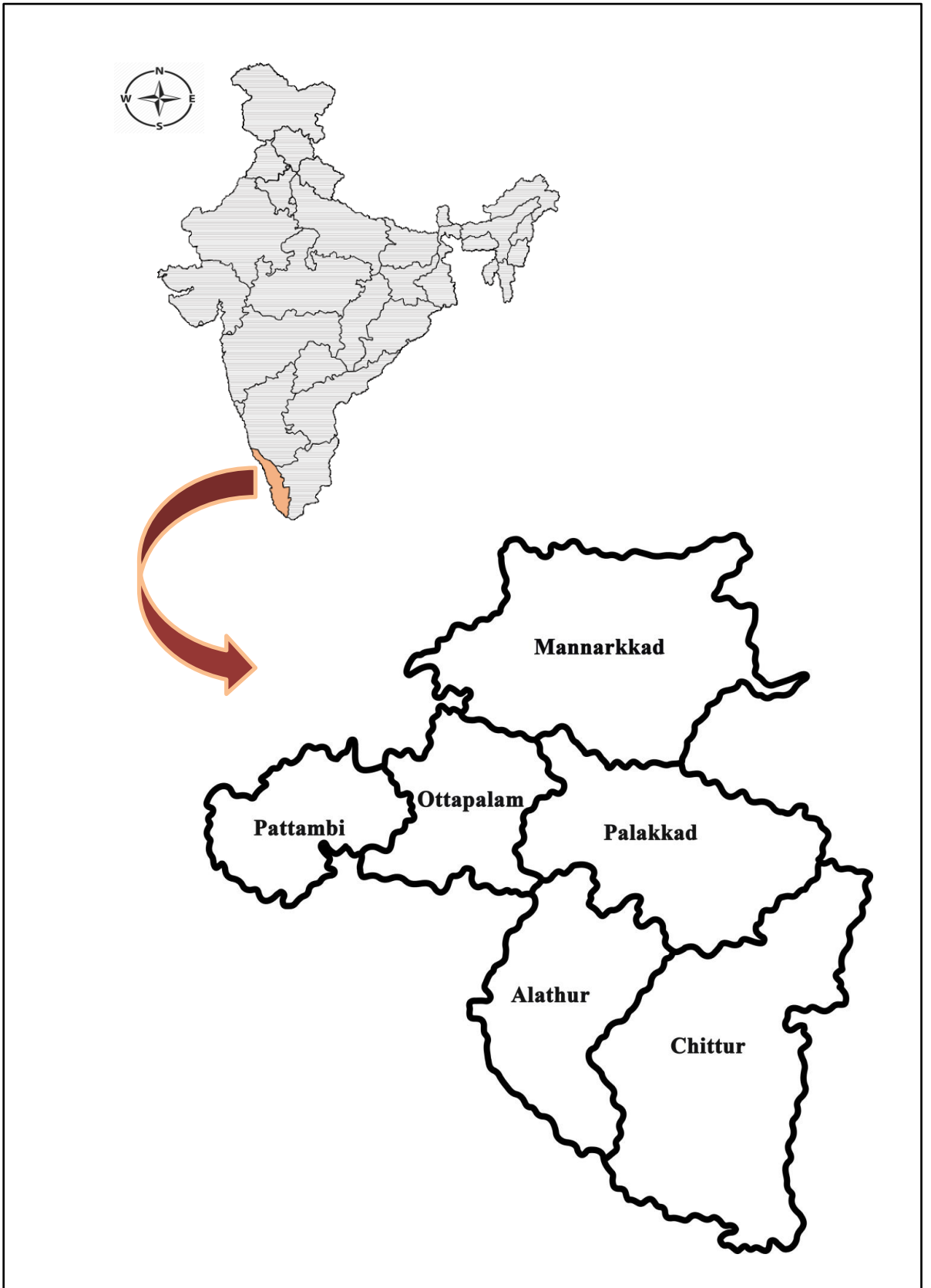


Plate 3.1. Map of study area Palakkad district, Kerala.

3.1.4 Herbarium preparation

Plants were collected from various parts of Palakkad district. Herbs were collected as whole plants. Twigs with flowers, fruits and leaves were collected for shrubs and trees. The plants were collected in polythene bags from the field. The specimens were placed between newspaper sheets and placed in a plant pressor for herbarium preparation. The plant specimens were deposited at GVCH.

3.1.5 Data analysis

Quantitative ethnobotanical tools used in the study

Quantitative ethnobotanical indices were used for the analysis of data obtained from field surveys. The following ethnobotanical indices are used for the study (Vitalini et al., 2013; Kushwaha et al., 2018).

1. RFC (Relative Frequency of Citation):

RFC is used to represent the local importance of each species. RFC is calculated by dividing the frequency of citation, that is the number of people referring to the use of a species, by the total number of informants included in the survey.

$$RFC = FC/N \quad (0 < RFC < 1)$$

where, FC = Frequency of citation

N = Total number of informants

2. Use value (UV)

Use value is the importance of each plant with reference to the number of uses reported.

$$UV = \Sigma U/N$$

where, U = Number of uses reported for a species

N = Total number of informants.

3. Consensus index (CI)

The percentage of informants in an area with knowledge of a specific medicinal use of plants used in folk medicine for treating diseases.

$$CI = (n/N) * 100$$

where n = number of informants citing a medicinal plant

N = Total number of informants.

These indices provide standardized measures of plant importance and informant consensus, enabling comparisons with other ethnobotanical studies and highlighting the most culturally significant species.

3.2 Selection and collection selected of Plants

3.2.1 Selection of plants

Based on the survey conducted for plants used for bathing mother and child after delivery and literature search, four plants were selected for the study. These plants were selected as the immunomodulation and phytochemical studies on these plants were less as compared to frequently cited plant species in the survey. These plants had a strong traditional basis of use but a weak scientific basis for validation. The plants selected were *Acalypha fruticosa*, *Justicia gendarussa*, *Piper longum* and *Vitex altissima*. The plants selected for the study are depicted in Plate 3.2 and Plate 3.3.

1. *Acalypha fruticosa* Forssk.

Acalypha fruticosa belongs to the family Euphorbiaceae. The plant is a shrub distributed in dry deciduous forests and waste lands. It is found in all parts of Kerala. It is commonly called 'birch leaved acalypha'. *Balamunja*, *munja*, *chini*, *kuppameni*, *Sinnimaram* are the commonly used names of *A. fruticosa* in Malayalam. The plant is erect and profusely branched. It is widely used to make fences in various parts of Palakkad district. The leaves are ovate with a truncate or subcordate base, margins of the leaf are crenate or serrate with an acuminate apex and a petiole up to 3 cm long. The leaves are aromatic glandular with hairs present on both sides. Inflorescence spikes which are short and axillary, androgynous. Tepals are tomentose with glands. Numerous stamens with subglobose to oblong anthers. Female flowers are seen below, sessile and with dentate margins. Ovary subglobose to trilobed, styles many. Fruit are loculicidal capsules.

2. *Justicia gendarussa* Burm.f.

Justicia gendarussa belongs to the family Acanthaceae. The plant is distributed in moist deciduous forests and in plains. It is distributed in Tropical Asia and Africa. The plant is commonly called as *Vathamkolli* in Malayalam. It is also called water willow, gandarusa, and daun rusa. Habit shrub, stem dark purple coloured. Leaves are oblong-lanceolate or linear, apex acute or obtuse, base



Plate 3.2. Photographs of plants selected for the study. Figure A, Habit of *Acalypha fruticosa* Forssk. Figure B. Habit of *Justicia gendarussa* Burm.f.



Plate 3.3. Photographs of plants selected for the study. Figure A. Habit of *Piper longum* L. Figure B. Habit of *Vitex altissima* L.f.

acute. Leaves are bluish green in colour with 2-3 mm long petioles. Inflorescence terminal spikes. Flowers are bracteate, petals white with purple streaks.

3. *Piper longum* L.

The plant belongs to the family Piperaceae. The plant is distributed in evergreen, semi-evergreen, moist deciduous forests and waste lands. It is found in the Indo-Malaysian region. It is a climbing or trailing shrub. It is commonly called Indian long pepper and in Malayalam the plant is called *thippali* or *pippali*. Leaves are petiolate with 1-3 cm long petiole, simple, alternate and ovate in shape. The leaf base is cordate and oblique and the leaf apex is acute. Female and male spikes are present. Female spikes are stout and erect with peduncles. Male spikes are erect and slender and up to 7 cm long. Male flowers contain 2 stamens. Fruit is berry with black or deep red colour.

4. *Vitex altissima* L. f.

V. altissima belongs to the family Lamiaceae. It is distributed in moist deciduous and semi-evergreen forest and in sacred groves. It is located in India, Indo-China, Malaysia and Sri Lanka. It is commonly called the ‘peacock chaste tree’. In Malayalam the plant is called *myeilellu*. *V. altissima* is a 30 m tall tree with thick greyish yellow scaly bark. Leaves are compound and opposite with 3-5 leaflets. Leaflets are lanceolate, elliptic lanceolate or oblanceolate with acuminate apex and cuneate or acute base. Young leaflets are winged. Flowers are bisexual in terminal panicles. Petals are white with a slight blue colour. Calyx with 5 lobes and ovate in shape. Corolla bilipped. Stamens 4, didynamous and hairs are present at the base of the filaments. Ovary superior globose with 4 ovules. Filiform style and bifid stigma are present. Fruit is a drupe with blue colour (Gamble and Fischer, 1936; Vajravelu, 1990; Sasidharan, 2013; POWO, 2024).

3.2.2 Collection and authentication of plants

The leaves of *Acalypha fruticosa*, *Justicia gendarussa*, *Piper longum* and *Vitex altissima* were collected from various parts of Palakkad district. The leaves of *A. fruticosa* were collected from Kollengode and chittur taluks, the leaves of *J. gendarussa* and *V. altissima* were collected from Kollengode taluk and the leaves of *P. longum* were collected from Mannarkkad and Palakkad taluks of Palakkad district. The plants were collected from 2019 to 2024. The collected plants were identified and authenticated using

floras and taxonomic literature. Voucher specimens of plants were deposited at Government Victoria College Herbarium (GVCH).

3.2.3 Cleaning, drying and powdering of plants

The collected plants were washed with running tap water to remove dust and other substances. The leaves were peeled from the stem and the leaves were kept at room temperature for drying. Drying was done by placing the plants in shade or by keeping them in a hot air oven at around 37°C. The dried leaves were ground to powder using electric grinders. The powders were sieved through filters and the fine powders were stored at air tight containers for further analysis.

3.3 Preparation of plant extracts

The plants were extracted in isopropyl alcohol, distilled water and hexane by Soxhlet extraction method. 25 gm of each plant powder was extracted in Soxhlet apparatus for 48 hours using isopropyl alcohol, hexane and distilled water. The crude extract was then evaporated to remove the solvents. The solvent free extracts were weighed and stored in sterilised airtight glass tubes at 4°C. These extracts were used for further analysis.

3.4 Preliminary phytochemical analysis of *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima*

Qualitative phytochemical analysis of leaves of *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima* were done by methods described below. 1 g leaf powder was placed in 10 ml water, hexane and isopropyl alcohol for 24 hours and the extracts were filtered. The filtrate was used for further phytochemical analysis. Results were recorded as present (+) or absent (-) (Doss, 2009; Auwal et al., 2014; Balamurugan et al., 2019).

The analysis for the following phytochemicals were done, viz Alkaloids, Phenols, Flavonoids, Saponins, Coumarins, Glycosides, Tannins, and Steroids.

1 Test for alkaloids

3 ml of plant extract was treated with 1 ml Hydrochloric acid (HCl) and placed in a water bath for 20 minutes. The mixture was filtered and to the filtrate Hager's reagent or Wagner's reagent was added.

a. Hager's test

A few drops of Hager's reagent were added to the filtrate. Hager's reagent is an alkaline picric acid solution. Hager's reagent is prepared by dissolving 1 g picric acid in 100 ml distilled water. Bright yellow colour indicated the presence of alkaloids.

b. Wagner's test

Wagner's reagent is prepared by dissolving 2g Iodine and 6g Potassium iodide in distilled water. A few drops of Wagner's reagent were added to the filtrate. Brown reddish ppt indicated the presence of alkaloids.

2. Test for phenols

1 ml of plant extract was treated with 4 drops of alcoholic Ferric chloride (FeCl_3) solution. Formation of bluish black colour indicated the presence of phenols.

3. Test for flavonoids

Alkaline reagent test was used for the detection of flavonoids. 1 ml extract was treated with 10% Sodium hydroxide (NaOH) solution. Initially a bright yellow colour was formed but it disappeared on adding a few drops of HCl indicating the presence of flavonoids.

4. Test for saponins

5 ml of extract was treated with 20 ml of distilled water and mixed well. Persistent foam indicated the presence of saponins.

5. Test for coumarins

2 ml of plant extract was treated with 10% Sodium hydroxide (NaOH) solution. Formation of yellow colour indicated the presence of coumarins.

6. Test for glycosides

1 ml plant extract was treated with 2 ml glacial acetic acid and a few drops of ferric chloride (FeCl_3) solution. The presence of a brown colour ring indicated the presence of glycosides.

7. Test for tannins

4 ml plant extract was treated with 4 ml of Ferric chloride (FeCl_3). Green colour indicated the presence of tannins.

8. Test for steroids (Salkowski's test)

1 ml of plant extract was treated with 10 ml Chloroform and 10 ml concentrated Sulphuric acid (conc. H_2SO_4). Formation of 2 layers, an upper red layer and a lower layer with yellow green colour indicated the presence of steroids.

3.5 Immunomodulation properties of selected plants

After preliminary phytochemical analysis, we studied the immunomodulation properties of hexane, water and isopropyl alcohol extracts of *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima*.

3.6 Plant extract preparation for immunomodulation studies of selected plants

The dried extracts were dissolved in Dimethyl sulphoxide (DMSO) or Phosphate buffered saline (PBS) for *in vitro* macrophage activation studies. The hexane and isopropyl alcohol extracts of plants were dissolved in DMSO and the water extracts of all plants were dissolved in PBS. The dissolved extracts were filtered using a 0.22 μm syringe filter. The filtered extracts were labelled and stored in centrifuges tubes at -20°C .

3.7 Cell culture

Human monocytes THP1 cell line was used for *in vitro* macrophage activation studies. The cells were obtained from ATCC, Rockville, MD USA. The ATCC number for THP1 cells is TIB 202. THP1 cells are widely used as an alternative to primary human macrophages due to its consistent, controllable and ethically accessible properties. THP1 cells can be differentiated to macrophages by reagents like PMA, mimicking primary human macrophages. Primary macrophages induce experimental variability as it is collected from different donors while THP1 cells provide consistency and reproducibility with stable genetic and phenotypic traits. Primary human macrophages are isolated from blood which is ethically regulated. THP1 cells are easily available and can be cultured indefinitely (Chanput et al., 2014; Tedesco et al., 2018).

The immunomodulation, cytotoxicity and anti-inflammatory studies were done at Stem cell and Regenerative Biology Lab, School of Biotechnology, Amrita Vishwa Vidyapeetham, Vallikavu, Kollam, Kerala. RPMI-1640 medium with 10% fetal bovine

serum (FBS) was used to culture THP1 cells. The cells were kept in T75 flasks with 10 to 15 ml media and maintained at 37°C, 5% CO₂. The THP1 cells are free floating cells. These cells are converted to attached macrophages by treating with 10 ng phorbol 12-myristate 13-acetate (PMA).

3.8 *In vitro* macrophage activation studies to explore the immunomodulation properties of selected plants

The THP1 cells were first converted to macrophages and used for the study. 10 ng of PMA was treated with RPMI medium and was used for cell culture. The cells were cultured in 60mm dishes for *in vitro* macrophage activation studies. The cells were seeded at a seeding density of 3x10⁶ cells per plate. 3ml RPMI media treated with PMA was added to each plate. The cells were incubated for 24 hours. The media was replaced with fresh 3 ml RPMI and incubated for 48 hours at 37°C, 5% CO₂. After 48 hours the cells were treated with different concentrations of plant extracts with fresh RPMI media and incubated at 37°C, 5% CO₂ for 24 hours. *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima* 10 µg/ml hexane extracts and 25 µg/ml water and isopropyl alcohol extracts were used for the preliminary screening. DMSO and PBS were added to control groups. The dishes were incubated 37°C, 5% CO₂ for 24 hours. The cells were lysed and RNA was isolated for cDNA synthesis. The expression of IL-1β, TNF-α, CCR-5 and IFN-β were analysed with internal control GAPDH using qRT-PCR (Quantitative Real Time Polymerase Chain Reaction). The method is a very powerful tool for gene expression studies. The various steps in the procedure for finding gene expression were mentioned below.

i. RNA isolation

The extract treated cells were lysed and lysate from the cells was collected for RNA isolation. Nucleospin Lysis buffer was used to lyse cells. The plates were washed with 1 ml PBS and a 350µl lysis buffer was added to each plate. The lysate was collected and stored in sterile eppendorf tubes at -80°C for RNA isolation. The isolation of RNA was done using Macherry-Nagel Nucleospin RNA isolation kit as per manufacturer's instructions. The purity and concentration of the isolated RNA was quantified using the Nanodrop spectrophotometer. The isolated RNA was stored at -80°C.

The steps involved in RNA isolation procedure by Macherry-Nagel Nucleospin RNA isolation kit are given below.

1. Added the cell lysate to the Nucleospin violet filter with a 2 ml collection tube with column capacity 700µl. Centrifuged at 11000g for one minute.
2. The violet filter was discarded and 350µl 70% ethanol was added to the lysate and mixed well by pipetting up and down 5 times.
3. The lysate was then loaded to Nucleospin light blue column with column capacity 750µl. The column was centrifuged at 11000g for 30 seconds. The collection tube was discarded and the column was placed in a new collection tube.
4. 350µl MDB (Membrane Desalting Buffer) was added to the column and the column was centrifuged at 11000g for 1 minute. Place the tube back after discarding the flowthrough.
5. DNase reaction mixture was prepared by adding 10µl reconstituted rDNase to 90µl Reaction Buffer for rDNase in a sterile 1.5ml tube. 95µl rDNase reaction mixture was added to the column and incubated for 15 minutes at room temperature.
6. 200µl buffer RAW2 was added to the column and centrifuged for 30 seconds at 11000g. Place the column in a new collection tube.
7. 600µl buffer RAW3 was added to the column and centrifuged for 30 seconds at 11000g. The flowthrough was discarded and the tube was placed back.
8. 250µl RAW3 was added to the column and centrifuged for 2 minutes at 11000g. Placed the column in a 1.5 ml vial.
9. 40µl RNase free water was added to the column for elution and centrifuged at 11000g for 1 minute.
10. The RNA isolated was quantified using a Nanodrop Spectrophotometer and stored at -80°C.

ii. cDNA synthesis

Takara PrimeScript 1st strand cDNA synthesis kit was utilised for cDNA synthesis. The kit consists of all reagents for the synthesis of cDNA. PrimeScript RTase was derived from M-MLV (Moloney Lkemia Virus) reverse transcriptase. The RTase is able to synthesise up to 12 kb cDNA.

Reagents used: 5X PrimerScript Buffer, dNTPs, Random 6 mers, PrimeScript RTase, RNase inhibitor, RNase free water.

Protocol:

1. Prepared the following reagent mixture in a PCR tube.

Random 6 mers (50 μ M)	- 1 μ l
RNA	- 1000 ng
RNase free dH ₂ O	- make up to 14 μ l
Total	- 14 μ l

2. The reaction mixture was incubated at 65°C for 5 minutes. The mixture was cooled immediately by placing it in ice.

3. Added the following reagents to the reaction mixture.

5X PrimeScript Buffer	- 4 μ l
dNTPs	- 1 μ l
RTase	- 0.5 μ l
RNase inhibitor	- 0.5 μ l

4. The reaction mixture was placed in a PCR machine for cDNA synthesis. The cDNA was diluted with 20 μ l RNase free water and kept at -20°C for storage.

iii Genes selected for the study:

IL-1 β , TNF α , CCR-5 and IFN β were the genes selected for the study. The genes selected for the study are involved in innate immunity response and immune cell trafficking. These genes are involved in physiological changes and complications like inflammatory responses, infection, mood changes, depressions associated with postpartum period. So, selection of these genes validates the immunomodulation properties of selected plants.

IL-1 β is a potent pro-inflammatory cytokine, a key initiator of the inflammatory cascade. It is involved in fever induction, activation of lymphocytes, and recruitment of other immune cells to the site of infection or injury. Its production is tightly regulated, and overproduction is linked to chronic inflammatory diseases (Wijdan et al., 2025).

TNF α is another major pro-inflammatory cytokine with pleiotropic effects (Tan et al., 2014; Gough and Myles, 2020). It plays a critical role in systemic inflammation, apoptosis, and the acute phase response (Gough and Myles, 2020). The gene is essential for defense, but its sustained overproduction contributes to tissue damage and chronic inflammatory conditions (Silva et al., 2019). Elevated TNF α is also noted during the

postpartum period and can be linked to complications like postpartum depression (Ross et al., 2022).

IFN β is a Type I interferon, classically known for its potent antiviral activity (Abdolvahab et al., 2016). It also has complex immunomodulatory roles, including the activation of macrophages and NK cells, and can influence both pro and anti-inflammatory pathways (McNab et al., 2015; Bolivar et al., 2018). It has roles in bacterial infections and general inflammation (Kovarik et al., 2016; Kopitar-Jerala, 2017).

CCR-5 is a chemokine receptor expressed on various immune cells, including T-cells, macrophages, and dendritic cells. It plays a crucial role in the recruitment of these cells to sites of inflammation by binding to chemokines like CCL3, CCL4, and CCL5 (Viola et al., 2008; Shi and Pamer, 2011; Namas et al., 2015). Overexpression of CCR-5 promotes macrophage accumulation, IL-1 β generation and induction of inflammation (Chen et al., 2021; Zhang et al., 2020). The primers used for genes are given in Table 3.1.

Table 3.1. Primers of IL-1 β , TNF α , CCR-5 and IFN β used in immunomodulation studies.

Sl. No.	Genes		Sequence 5' to 3'
1	IL- 1 β	Forward primer	CCACAGACCTTCCAGGAGAATG
		Reverse primer	GTGCAGTTCAGTGATCGTACAGG
2	TNF- α	Forward primer	GCCAGAGGGCTGATTAGAGA
		Reverse primer	GCCAGAGGGCTGATTAGAGA
3	IFN- β	Forward primer	AAACTCATGAGCAGTCTGCA
		Reverse primer	AAACTCATGAGCAGTCTGCA
4	CCR-5	Forward primer	TCTCTTCTGGGCTCCCTACAAC
		Reverse primer	CCAAGAGTCTCTGTACACCTGCA

iv Gene expression

The gene expression analysis of IL-1 β , TNF α , CCR-5 and IFN β were analysed using qRT-PCR. The instrument used for the analysis was QuantStudio 5 (Applied Biosystems). The reaction mixture of RT-qPCR contains the following reagents.

SYBR GREEN- 10 μ l

Forward primer- 1 μ l

Reverse primer- 1 μ l

ROX dye - 0.4 μ l

Molecular grade dH₂O- 5.6 μ l

cDNA - 2µl

20µl was the total volume of reaction mixture. The samples were run in triplicates. GAPDH gene was used as the internal control. The fold gene expression values of TNFα, IFNβ, IL-1β and CCR-5 were calculated using the $\Delta\Delta C_t$ method. The method analyses qPCR data to calculate the gene expression of target genes. The expression of target gene was expressed as relative fold change of target gene from the experimental group compared to control group. $\Delta\Delta C_t$ is calculated from C_t value (cycle Threshold) obtained from qPCR data. The target gene C_t values are normalised against a reference gene (internal control). This value is called ΔC_t and these values were then normalised against the control sample ($\Delta\Delta C_t$). The fold change of target gene was calculated using 2 raises to minus power of delta delta C_t ($2^{-\Delta\Delta C_t}$) (Livak and Schmittgen, 2001).

3.9 Viability of cells treated with *V. altissima*

The viability of cells treated with plant extract was studied using the Origin MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay kit. The THP1 cells were first converted to macrophages and used for the study. 10 ng of PMA was treated with RPMI medium and was used for cell culture. For MTT assay 96 well plates were used. The cells were seeded at a seeding density of 1×10^4 cells per well. 100µl RPMI media treated with PMA was added to each well. The cells were incubated at 37°C, 5% CO₂ for 24 hours. The media was replaced with fresh RPMI and incubated for 48 hours at 37°C, 5% CO₂. The cells were then treated with different concentrations of hexane, water and isopropyl alcohol extracts of *V. altissima* and incubated at 37°C, 5% CO₂ for 24 hours. The plant extracts from 15µg/ml to 100µg/ml were used for the study. DMSO and PBS were added to the control group. 90µl RPMI and 10µl MTT reagent was added to each well after 24 hrs of extract treatment and incubated for 4 hours at 37°C, 5% CO₂. MTT assay is a colorimetric assay which measures the formazan crystals formed by the cleavage of the tetrazolium ring of MTT by mitochondrial dehydrogenase enzyme. Only live cells can convert MTT to formazan crystals. 100µl solubilisation solution was added to each well after 4 hours and the plates were kept in a shaker for 30-45 minutes to dissolve the formazan crystals. The absorbance was measured at 490 nm in a microplate reader. The following equation was used to calculate the viability percentage (Chan et al., 2015; Sharif et al., 2017).

$$\text{Cell Viability \%} = \left(\frac{A_s}{A_c} \right) * 100$$

where, A_c = Absorbance of control cells

A_s = Absorbance of extract treated cells

3.10 *In vitro* macrophage activation on *V. altissima* treated THP1 cells to study the immunostimulatory property.

The THP1 cells were first converted to macrophages and used for the study. 10 ng of PMA was treated with RPMI medium and was used for cell culture. The cells were cultured in 60mm dishes for *in vitro* macrophage activation studies. The cells were seeded at a seeding density of 3×10^6 cells per plate. 3ml RPMI media treated with PMA was added to each plate. The cells were incubated at 37°C, 5% CO₂ for 24 hours. The media was replaced with fresh 3 ml RPMI and incubated for 48 hours at 37°C, 5% CO₂. After 48 hours the cells were treated with different concentrations of plant extracts with fresh RPMI media and incubated at 37°C, 5% CO₂ for 24 hours. Hexane, water and isopropyl alcohol extracts *V. altissima* at concentrations 10 µg/ml, 15 µg/ml, and 25 µg/ml were used for the study. DMSO and PBS were added to control plates. The dishes were incubated at 37°C, 5% CO₂ for 24 hours. The expression of IL-1β, TNFα, CCR-5 and IFNβ was analysed with internal control GAPDH. Hexane, water and isopropyl alcohol extracts *V. altissima* are denoted as VAH, VAW and VAI respectively. The procedure of RNA isolation, cDNA synthesis and gene expression studies were already mentioned in section 3.8.

3.11 Antioxidant activity of *V. altissima*:

The antioxidant activity of hexane, isopropyl alcohol and water extracts of *V. altissima* was studied using DPPH assay. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method was a colorimetric method for determining the antioxidant activity of plant extracts. The method is easy, affordable and quick. For the antioxidant activity analysis, 0.1mM DPPH solution was prepared. 100% ethanol was used to prepare the DPPH solution. 0.25 mg/ml, 0.5mg/ml, 0.75mg/ml and 1 mg/ml extracts of *V. altissima* were used for the study. 5, 10, 15, 20 µl plant extracts were used for the study. The plant extracts were made up to 40µl with DMSO. 2.96 ml DPPH solution was added to the plant extracts. 3ml DPPH solution was taken as the control. The mixture was incubated for 20 minutes and the absorbance was measured at 517 nm using a UV Visible Spectrophotometer. 0.025

mg/ml, 0.05mg/ml, 0.075mg/ml and 0.1mg/ml Ascorbic acid was used as the standard. The RSA% (Percentage Radical Scavenging Activity) was calculated using the formula given below (Gulcin and Alwasel, 2023; Mensor et al., 2001).

$$RSA\% = \left(\frac{A_{control} - A_{sample}}{A_{control}} \right) * 100$$

where, A control= Absorbance of the control

A sample = Absorbance of the sample

3.12 Antibacterial activity of *V. altissima* against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Antibacterial activity of hexane, water and isopropyl alcohol extracts of *V. altissima* were studied using disc diffusion method. The microorganisms were procured from Agharkar Research Institute, Pune and from School of Biosciences, Mahatma Gandhi University, Kottayam. The bacteria were cultured in nutrient agar slants as mother culture and were stored at 4° C for further studies. The different bacterial strains used for the study were given in Table 3.2.

Table 3.2. Bacterial test organisms used in the study with their accession numbers.

Sl. No.	Bacteria species	Gram stain	Type
1	<i>Bacillus subtilis</i>	Gram positive	MCMB 310
2	<i>Escherichia coli</i>	Gram negative	ATCC 35218
3	<i>Pseudomonas aeruginosa</i>	Gram negative	MCMB 407
4	<i>Staphylococcus aureus</i>	Gram positive	ATCC 25923

3.12.1 Preparation of stock solution

100mg/ml hexane, water and isopropyl alcohol extract of *V. altissima* dissolved in DMSO were used as the stock for preliminary antibacterial assay. 10, 20, 30 and 40 µl extract was added to each disc. The antibacterial assay was repeated with 200mg/ml stock concentration of isopropyl alcohol extract of *V. altissima*. For this assay 20, 30, 40 and 50 µl extract was used. The extracts were dissolved in DMSO and used for the study.

3.12.2 Preparation of bacterial inoculum from mother culture

Nutrient agar around 20-25 ml was poured into a culture tube under aseptic conditions. A loopful of test organisms was added to the culture tube and the tubes were labelled and incubated for 24 hours.

3.12.3 Preparation of Nutrient agar medium for plates

28g of nutrient agar and 12g agar were dissolved in 1000 ml distilled water and 15 ml of the medium were poured into each sterile petri dish. The plates were incubated around 15-20 minutes at room temperature.

3.12.4 Inoculation of Plates

Sterile Cotton swabs were used to inoculate the plates. Cotton swab was dipped in the bacterial inoculum and the swab was streaked through the nutrient agar medium in the petri dishes. Separate cotton swabs were used for each test organism.

3.12.5 Agar disc diffusion method

Disc diffusion is a conventional, standard and cost-effective method for determining the antibacterial activity of plants (Valgas et al., 2007). It is also called the Kirby-Bauer test. The technique measures the zone of inhibition around the disc with antimicrobial compounds.

Sterile disc from Himedia was used for the antibacterial activity analysis. Streptomycin 10 mcg susceptibility disc from Himedia was used as the positive control. DMSO treated disc was set as negative control. The plant extracts at different concentrations were added to the sterile disc and the discs were allowed to evaporate. The evaporated disc with different concentration of extracts was added to the inoculated petri plates with test organisms. The positive and negative control discs are also added to the petri plates. The plates were labelled and placed in an incubator at 37°C for 24 hours. After 24 hours the plates were measured for the diameter of the zone of inhibition of bacterial growth. The zone of inhibition was measured in mm. All experiments were done in triplicates (Hemeg et al., 2020; Sbhatu and Abraha, 2020; Subramaniam et al., 2020).

3.13 Liquid Chromatography Mass Spectrometry (LCMS)

We had selected *V. altissima* for detailed phytochemical analysis based on our immunostimulatory studies on all the four selected plants. The phytochemical profile of crude extracts of *V. altissima* was analysed using Orbitrap high resolution liquid

chromatography mass spectrometry (OHR-LCMS). The analytical instrument is Q Exactive plus Biopharma, Thermo Scientific Xcalibur version 4.2.28.14. Two outer electrodes and one central electrode act as the analyzer and the detector. For the direct infusion of ions both negative and positive ionisation modes with 50 to 8000 amu mass ranges were used. Electrodynamics squeezing is the method used for capturing the ions entering into the orbitrap. These ions oscillate around the core and outer electrodes. The separation is done as different ions oscillate at various frequencies. Image current detection was used to obtain the mass spectra of ions by calculating the oscillation frequencies induced by the ions on the peripheral electrodes. The LCMS technique merges HPLC (High Performance Liquid Chromatography) with mass spectroscopy.

0.1% formic acid in Milli-Q water (solvent A), methanol (solvent B) and acetonitrile (solvent D) were the mobile phase compounds. The run was set for 35 minutes. Thermo compound Discoverer 3.2 and BioPharma Finder software was used for compound identification. The further analysis and processing of data was done with Compound Discoverer 3.2. The LCMS analysis was done at Sophisticated Analytical Instrument Facility (SAIF), Indian Institute of Technology (IIT) Mumbai. Search engines like Pub-Chem, Chem-spider and Google Scholar were used to identify the biological properties of the identified chemical compounds in hexane, water and isopropyl alcohol extracts of *V. altissima*.

3.14 *In vitro* macrophage activation of *V. altissima* isopropyl alcohol extract (VAI) in LPS treated THP1 cells

The THP1 cells were treated with RPMI supplemented with 10% FBS and 10 ng PMA. 4×10^6 cells were cultured in a 60mm dish and incubated at 37°C, 5% CO₂ for 24 hours. After 24 hours the medium was replaced by fresh RPMI and incubated for 48 hours. The media was replaced by RPMI media mixed with 10 ng LPS.

LPS is lipopolysaccharides present in the outer membrane of gram-negative bacteria. LPS binds to cell surface receptors like Toll-like receptors and activates signaling cascades like NF- κ B which leads to the production of pro-inflammatory cytokines like TNF α , IL-1 β , IL-6 etc (Honda and Inagawa, 2023). When LPS is added to cells like macrophages they mediate the expression of pro-inflammatory cytokines.

The macrophages were incubated for 24 hours at 37°C, 5% CO₂. After 24 hours 10 μ g/ml, 15 μ g/ml and 25 μ g/ml VAI in RPMI medium was added to the dishes and the dishes were incubated for 24 hours at 37°C, 5% CO₂. Macrophages without any

treatments, LPS treated macrophages and DMSO treated macrophages were also maintained. After 24 hours the cells were lysed for RNA isolation. The expression levels of IL-1 β , TNF α , IL-6, IFN β and CCR-5 was analysed with internal control GAPDH in treated and untreated plates. The expression of IL-6 was also analysed in LPS treated cells.

IL-6 is a pro-inflammatory cytokine. It is a key mediator of the acute phase response and is involved in B-cell differentiation and T-cell activation (Tanaka et al, 2014). Elevated IL-6 is commonly observed in inflammation and infection, during the postpartum period, contributing to postpartum depression (Liu et al., 2016). The primer of IL-6 was given in Table 3.3.

Table 3.3. Primer of IL-6 gene studied in anti-inflammatory studies.

Sl. No.	Genes		Sequence 5' to 3'
1	IL-6	Forward primer	AGTCCTGATCCAGTTCCTGC
		Reverse primer	CTGGCATTGTGGTTGGGTC

The procedure RNA isolation, cDNA synthesis and gene expression studies were already mentioned in section 3.8.

3.15 Antifungal activity of Isopropyl alcohol extract of *V. altissima* against *Candida albicans* and *Aspergillus niger*

Antifungal activity of *V. altissima* was studied using agar well diffusion method and by food poisoning method. Potato Dextrose Agar (PDA) medium was used to culture fungal species. *V. altissima* isopropyl alcohol extract was used to study the antifungal activity. The test organism used for the study was given in Table 3.4. Itraconazole was used as the positive control and isopropyl alcohol was used as the negative control.

Table 3.4. Fungal species used for the study with their accession numbers.

Sl. No.	Fungal species	Type
1	<i>Aspergillus niger</i>	MTCC 1344
2	<i>Candida albicans</i>	NFCC 15123

3.15.1 Agar well diffusion method

Agar well diffusion technique was used to study the antifungal activity of *V. altissima* isopropyl alcohol extract against *C. albicans*. 1 ml fungal suspension of *C. albicans* was mixed with PDA media and poured into sterile petri dishes under aseptic conditions. 4mm diameter wells were made on the solidified media with a cork borer. Plant extract at 20, 40 and 60µl concentrations was added to each well. DMSO was added to the negative control plate and Itraconazole was added to the positive control plate. The plates were incubated at 28°C for 48-72 hours. The inhibition zone was measured after 48-72 hours and compared with that of the control plates. All the experiments were done in triplicates and the mean values were taken for antifungal activity analysis (Gizaw et al., 2022; Raj et al., 2021; Sales et al., 2016).

3.15.2 Food poison method

Food poisoning technique was used to study the antifungal activity of plant extract against *A. niger*. The technique prevents the volatilisation of compounds present in the plant extract. *V. altissima* isopropyl alcohol extract at concentration 500 mg/ml was used for the study. 1ml plant extract was dissolved in 9 ml PDA media and was poured into a 90mm petri dish under aseptic conditions. The media was spread over the entire plate. The mycelium of *A. niger* was cut into 4 mm size agar blocks from 7 day old mother culture and inoculated in the petri dish with plant extract and PDA medium. The plates were incubated at 27°C for 7 days (Erhonyota et al., 2023). The hyphal growth of fungi was measured after 2 days. The plate with PDA media and fungal mycelium served as negative control. Itraconazole treated plates served as positive control. The experiments were done in triplicates and the mean values were compared with control plates. The inhibition percentage of the fungus was calculated using the formula given below (Anil Kumar and Raj Kumar, 2015).

$$L = [(C - T)/C] * 100$$

where, L= Inhibition Percentage,

C = Radius of colony in control plate

T = Radial growth of fungi in plant extract treated plates

3.16 Thin layer Chromatography (TLC)

We had selected *V. altissima* isopropyl alcohol (VAI) extract based on our immunostimulatory and anti-inflammatory studies for TLC analysis. Silicagel coated

Aluminium plates were used for the study. The plates were cut into 7 x 2 cm small pieces and used for the study. Isopropyl alcohol extract of *V. altissima* was used to perform TLC. A capillary tube was used as the spotter. The spotter was placed in plant extracts. The extract filled spotter was placed about 1 cm above from the base of the plate and allowed the solvent to evaporate.

Butanol, acetic acid and water in the ratio 7:2:1 was taken in a beaker and mixed thoroughly. This was the solvent system used for the TLC. The prepared solvents were covered with aluminium foil to prevent the evaporation of solvents.

TLC was done in a closed beaker. The solvent system was added to the beaker and the spotted plate was slowly placed in the beaker with its base dipped in the solvent system. The solvent passed through the plate by capillary action and the sample was carried towards the upper end of the plate. The plate was removed when the solvent reached about 1 cm from the upper end of the plate and marked the solvent front position. The plates were placed in a UV transilluminator after the solvents evaporated.

3.17 Preparative thin layer chromatography for purification and isolation of compounds

Preparative TLC was done for the purification and isolation of compounds separated by TLC. The various steps involved in this method were,

1. Preparation of plates

25 g silica gel G for thin layer chromatography was mixed with 75 ml distilled water with a glass rod. The solution was thoroughly mixed and the slurry was poured on to a 20 x 20 cm pre cleaned glass plate at 2 mm thickness. The plate is slightly tilted for the evenly spreading of the slurry over the whole plate. The plates were placed at around 25°C for 2 hours. The plates were then placed in a shelf to dry by overnight incubation. The plates were dried slowly by evaporation. After overnight incubation the plates were placed at 130°C for 2 hours for activation. The plates were then cooled slowly at around 25°C.

2. Sample loading

Capillary tubes are used to apply plant extract to the TLC plates. The extracts were added above 1 cm from the base of the plate.

3. Solvent system

The solvent system was prepared by mixing Butanol, acetic acid and water in the ratio 7:2:1. The prepared solvents were covered with aluminium foil to prevent the evaporation of solvents.

4. Plate development

The solvent system was added to the beaker and the spotted plate was slowly placed in the beaker with its base dipped in the solvent system. The solvent passed through the plate by capillary action and the sample was carried towards the upper end of the plate. The plate was removed when the solvent reached about 1 cm from the upper end of the plate and marked the solvent front position. The nature of each compound in TLC was determined by its Retention factor (R_f) and is calculated by the formula given below (Bele and Khale, 2011).

$$R_f = \frac{\text{Distance travelled by the compound from the spotting point}}{\text{Distance travelled by the solvent}}$$

5. Compound detection and isolation

The separated compounds were detected by placing the plates in a UV chamber with visible, short and long UV range. The located bands were scrapped using a clean spatula. The scrapped compounds were dissolved in solvents. The adsorbent was removed from the compounds by filtration and centrifugation.

3.18 Antibacterial activity of fragments separated by TLC of isopropyl alcohol extract of *V. altissima* (VAI)

TLC analysis of VAI revealed the presence of seven compounds. The compounds were denoted as C1- C7. The compounds were isolated and dissolved in ethyl acetate. Agar disc diffusion method was used to test the antibacterial activity of isolated compounds. The antibacterial activity of compounds was analysed against *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis*. Amoxicillin 15 mcg was used as the positive control and ethyl acetate was used as the negative control. 20µl of each compound was added to the disc. The evaporated disc was added to the inoculated petri plates with test organisms. The positive and negative control discs are also added to the petri plates. The plates were labelled and placed in an incubator at 37°C for 24 hours. After 24 hours the plates were measured for the diameter of the zone of inhibition of bacterial growth. The zone of inhibition was measured in mm.

3.19 NMR analysis of TLC isolated compounds

NMR analysis exploits the magnetic properties of atom nuclei to elucidate the structure of compounds. The technique analyses the physical and chemical characteristics of molecules. The NMR analysis was done at CLIF (Central Laboratory for Instrumentation and Facilitation), University of Kerala, Thiruvananthapuram. Bruker Avance 400MHz FT-NMR spectrometer was used for the analysis. The compounds obtained from preparative TLC was dissolved in deuterated chloroform (CDCl_3) and used for the analysis. The compounds C7 and C5 were analysed using NMR spectrometer.

3.20 Statistical Analysis

All experiments were performed in triplicate, and data are expressed as mean $\pm$ standard deviation (SD). Statistical significance was determined using one-way ANOVA with a p-value threshold of <0.05 followed by Turkey's HSD post hoc analysis using Python 3.10 and GraphPad Prism 10.4.1 software.

Chapter 4 - Results and Discussion

This chapter includes the results and discussions of all studies conducted during the research.

4.1 Folk medicinal survey on plants used for post-natal care in Palakkad district

The survey on plants used for bathing mother and child after delivery was conducted across six taluks of Palakkad district. 282 informants from the district participated in the study. The study resulted in the documentation of 62 plant species belonging to 28 plant families. Moraceae, Zingiberaceae, Euphorbiaceae and Meliaceae were the commonly cited plant families. Plants cited in the study are used either as single preparations or compound preparations for the medicated bath. Table 4.1 depicts the plants cited in the study with its family, binomial, mode of use and common name. Plate 4.1 and 4.2 depicts the photographs of selected plants cited in the study.

4.2 Medicated bath after delivery for mothers and infants

The medicated bath after delivery is considered as very important in various parts of Kerala. Plants are boiled with water to prepare *vethuvellam* and are used for bathing mothers after delivery. The *vayattaties* are highly specialised in taking care of mother and child for 28 days after delivery. The water used for the medicated bath is rich in various phytochemicals. This water is prepared a day before the medicated bath. The plants were boiled in water and kept for some hours. The water is boiled again to a temperature that the body can bear on the day of bathing. Various plant parts like leaves, stem and roots were used for this medicated bath. In some places the lukewarm medicated water is used to bathe babies also (Jayalekshmi et al., 2022).

This medicated bath is considered as important for postpartum recovery in various parts of the world. *Batak* people of North Sumatra, Indonesia use 59 plant species for steam bathing for 7-14 days after childbirth (Silalahi and Nisyawati, 2019). A study on medicinal plants of Mien for postpartum care reported the use of plants for herbal baths for mothers. 5 to 9 species of plants were used in these herbal formulations. This herbal bath promotes blood circulation and regains the health of the mother (Panyaphu et al., 2011). 23 plant species were used for a steam bath called *betetimbun* by the *Dayak Ngaju* tribe of Kalimantan. This bath is very helpful for the recovery of the mother after delivery (Rohmat and Rahayu, 2019). *Oukup* is a steam bath used by the *Karo* community for the

recovery of postpartum mothers (Nasution, 2009). *Bakera* is another steam bath practised by people of Minahasa, Indonesia for post-natal care. The study documented the use of 60 plant species for *bakera* (Zumstug and Weckerle, 2007). Neem leaves (*Azadirachta indica*) boiled in water were used for bathing mothers after delivery in Maharashtra (Mangulkar et al., 2012).

The plants used for bathing mother and child were also reported from different parts of Kerala. 8 formulations of vethuvellam were used in South Kerala for bathing mothers after delivery (Rajith et al., 2010). Beegam and Nayar, 2009 also reported the use of plants for the medicated bath. Zingiberaceae members are commonly used for the preparation of the medicated water in various parts of Kozhikode district (Anvar and Haneef, 2015). *Vevuvellam* is another type of medicated bath practised in Mahe for post-natal care (Sivadasan et al., 2014). Some of the plants reported in our study for making the medicated water were similar to these reported studies.

Coconut (*Cocos nucifera*) oil, gingelly (*Sesamum indicum*) oil or *Dhanwantharam thailam* was applied to the body of the mother before this medicated bath. Virgin coconut oil was preferred for babies. Rhizome paste of *Curcuma longa* was also applied to the body of mother and child in some places. This paste was not recommended for male babies as it prevents body hair growth. Green gram (*Vigna radiata*) seed powder was used instead of soap for bathing. Leaves and flowers of *Hibiscus rosa-sinensis* were used to wash the hair of mothers. The period of this bath may be 7,9 or 28 days for the mother. This bath helps in skin brightening, reduces body pain and brings back the body to pre-pregnancy condition.



Plate 4.1. Plants cited in study for bathing mother and child after delivery. Figure A. *Justicia gendarussa* Burm.f. Figure B. *Vitex altissima* L.f. Figure C. *Ocimum gratissimum* L. Figure D. *Clerodendrum infortunatum* L. Figure E. *Justicia adhatoda* L. Figure F. *Pongamia pinnata* (L.) Pierre. Figure G. *Calotropis gigantea* (L.) W.T.Aiton. Figure H. *Ficus religiosa* L. Figure I. *Moringa oleifera* Lam. Figure J. *Sida acuta* Burm.f. Figure K. *Piper longum* L. Figure L. *Acalypha fruticosa* Forssk.



Plate 4.2. Plants cited in the study for bathing mother and child after delivery. Figure A. *Azadirachta indica* A. Juss. Figure B. *Hibiscus x rosa-sinensis* L. Figure C. *Mangifera indica* L. Figure D. *Coffea arabica* L. Figure E. *Artocarpus heterophyllus* Lam. Figure F. *Ficus benghalensis* L. Figure G. *Ficus microcarpa* L.f. Figure H. *Psidium guajava* L. Figure I. *Glycosmis pentaphylla* (Retz.) DC. Figure J. *Ficus racemosa* L. Figure K. *Ocimum tenuiflorum* L. Figure L. *Leucas aspera* (Willd.) Link.

Table 4.1. List of plants cited in the study for bathing mother and child after delivery with its binomial, common name, family, mode of use and part used.

SL. No.	Plants cited in the study	Common name	Family	Part Used	Mode of use	Used to Care
1	<i>Acalypha fruticosa</i> Forssk.	Munja	Euphorbiaceae	Leaf	Leaf paste applied to the body	Mother and Child
2	<i>Achyranthes aspera</i> L.	Kadaladi	Amaranthaceae	Leaf	Used in boiling water	Mother
3	<i>Acorus calamus</i> L.	Vyambu	Acoraceae	Leaf	Paste applied to body	Child
4	<i>Anacardium occidentale</i> L.	Kasumavu	Anacardiaceae	Leaf	Used in boiling water	Mother
5	<i>Anethum graveolens</i> L.	Sathakuppa	Apiaceae	Leaf	Used in boiling water	Mother
6	<i>Artocarpus heterophyllus</i> Lam.	Plavu	Moraceae	Yellow Leaf	Used in boiling water	Mother
7	<i>Azadirachta indica</i> A. Juss.	Aryaveppu	Meliaceae	Leaf and bark	Used in boiling water	Mother
8	<i>Borassus flabellifer</i> L.	Karimbana	Arecaceae	Root	Used in boiling water	Mother
9	<i>Calotropis gigantea</i> (L.) W.T.Aiton	Eriku	Apocynaceae	Leaf	Used in boiling water	Mother
10	<i>Causonis trifolia</i> (L.) Mabb. & J.Wen	Vathakkodi	Vitaceae	Leaf and stem	Used in boiling water	Mother
11	<i>Centella asiatica</i> (L.) Urb.	Kudakan	Apiaceae	Leaf	Used in boiling water	Mother

12	<i>Chrysopogon zizanioides</i> (L.) Roberty	Ramacham	Poaceae	Root	Used in boiling water	Mother
13	<i>Clerodendrum infortunatum</i> L.	Peruku	Lamiaceae	Leaf	Used in boiling water	Mother
14	<i>Clitoria ternatea</i> L.	Sankupushpam	Fabaceae	Root	Used in boiling water	Mother
15	<i>Cocos nucifera</i> L.	Thengu	Arecaceae	Rachis	Used in boiling water	Mother
16	<i>Coffea arabica</i> L.	Kappi	Rubiaceae	Leaf	Used in boiling water	Mother
17	<i>Coleus amboinicus</i> Lour.	Panikurka	Lamiaceae	Leaf	Used in boiling water	Mother
18	<i>Croton persimilis</i> Mull. Arg.	Thorayam	Euphorbiaceae	Leaf	Used in boiling water	Mother
19	<i>Curcuma longa</i> L.	Manjal	Zingiberaceae	Rhizome	Paste applied to the body	Mother and child
20	<i>Cymbopogon flexuosus</i> (Nees ex Steud.) Will. Watson	Theruvappullu	Poaceae	Leaf	Used in boiling water	Mother
21	<i>Eucalyptus globulus</i> Labill.	Yucali	Myrtaceae	Leaf	Used in boiling water	Mother
22	<i>Ficus benghalensis</i> L.	Peraal	Moraceae	Bark	Used in boiling water	Mother
23	<i>Ficus microcarpa</i> L.f.	Ethi	Moraceae	Bark	Used in boiling water	Mother
24	<i>Ficus racemosa</i> L.	Athi	Moraceae	Bark	Used in boiling water	Mother

25	<i>Ficus religiosa</i> L.	Arayal	Moraceae	Bark	Used in boiling water	Mother
26	<i>Getonia floribunda</i> Roxb.	Pullanji	Combretaceae	Leaf	Used in boiling water	Mother
27	<i>Glycosmis pentaphylla</i> (Retz.) DC.	Panal	Rutaceae	Leaf	Used in boiling water	Mother
28	<i>Hamelia patens</i> Jacq.	Thechipoo	Rubiaceae	Leaf, Stem Flower	Used in boiling water	Mother
29	<i>Hibiscus</i> × <i>rosa-sinensis</i> L.	Chembarathi	Malvaceae	Leaf	Used to prepare shampoo	Mother
30	<i>Ixora coccinea</i> L.	Thechipoo	Rubiaceae	Leaf	Used in boiling water	Mother
31	<i>Jatropha hernandiifolia</i> Vent.	Kottathari	Euphorbiaceae	Bark	Used in boiling water	Mother
32	<i>Justicia adhatoda</i> L.	Adalodakam	Acanthaceae	Leaf	Used in boiling water	Mother
33	<i>Justicia gendarussa</i> Burm.f.	Vathamkolli	Acanthaceae	Leaf	Used in boiling water	Mother
34	<i>Leucas aspera</i> (Willd.) Link.	Thumba	Lamiaceae	Whole plant	Used in boiling water	Mother
35	<i>Mangifera indica</i> L.	Mavu	Anacardiaceae	Leaf and Bark	Used in boiling water	Mother
36	<i>Moringa oleifera</i> Lam.	Muringa	Moringaceae	Bark	Used in boiling water	Mother
37	<i>Musa</i> × <i>paradisiaca</i> L.	Vazha	Musaceae	Leaf	Used in boiling water	Mother

38	<i>Mussaenda frondosa</i> L.	Vellila	Rubiaceae	Leaf	Used in boiling water	Mother
39	<i>Ocimum gratissimum</i> L.	Kattutulsi	Lamiaceae	Leaf	Leaf extract mixed with water	Child
40	<i>Ocimum tenuiflorum</i> L.	Tulsi	Lamiaceae	Leaf	Leaf extract mixed with water	Child
41	<i>Piper longum</i> L.	Thipali	Piperaceae	Leaf and stem	Used in boiling water	Mother
42	<i>Piper nigrum</i> L.	Kurumulaku	Piperaceae	Leaf and stem	Used in boiling water	Mother
43	<i>Plumbago zeylanica</i> L.	Koduveli	Plumbaginaceae	Root	Used in boiling water	Mother
44	<i>Pongamia pinnata</i> (L.) Pierre	Ungu	Fabaceae	Leaf	Used in boiling water	Mother
45	<i>Premna serratifolia</i> L.	Munja	Lamiaceae	Leaf	Used in boiling water	Mother
46	<i>Pseudarthria viscida</i> (L.) Wight & Arn.	Moovila	Fabaceae	Leaf	Used in boiling water	Mother
47	<i>Psidium guajava</i> L.	Pera	Myrtaceae	Leaf	Used in boiling water	Mother
48	<i>Pterocarpus marsupium</i> Roxb.	Venga	Fabaceae	Bark	Used in boiling water	Mother
49	<i>Ricinus communis</i> L.	Avanaku	Euphorbiaceae	Leaf and Bark	Used in boiling water	Mother
50	<i>Samadera indica</i> Gaertn.	Karigotta	Simaroubaceae	Leaf	Used in boiling water	Mother

51	<i>Schleichera oleosa</i> (Lour.) Oken	Poovam	Sapindaceae	Leaf	Used in boiling water	Mother
52	<i>Senegalia caesia</i> (L.) Maslin, Seigler & Ebinger	Incha	Fabaceae	Bark	Used as scrubber for bathing	Mother
53	<i>Sida acuta</i> Burm.f.	Kurumthotti	Malvaceae	Root	Used in boiling water	Mother
54	<i>Stereospermum colais</i> (Buch.-Ham. ex Dillwyn) Mabb.	Karingazha	Bignoniaceae	Leaf	Used in boiling water	Mother
55	<i>Strychnos nux-vomica</i> L.	Kanjiram	Loganiaceae	Leaf	Used in boiling water	Mother
56	<i>Tamarindus indica</i> L.	Puli	Fabaceae	Leaf	Used in boiling water	Mother
57	<i>Terminalia paniculata</i> B.Heyne ex Roth	Maruthu	Combretaceae	Leaf	Used in boiling water	Mother
58	<i>Thespesia populnea</i> (L.) Sol. ex Correa	Poovarasu	Malvaceae	Bark	Used in boiling water	Mother
59	<i>Vigna radiata</i> (L.) R. Willczek	Cherupayar	Poaceae	Seed	Paste applied to the body	Mother
60	<i>Vitex altissima</i> L.f.	Mayilellu	Lamiaceae	Leaf	Used in boiling water	Mother
61	<i>Vitex negundo</i> L.	Karinochi	Lamiaceae	Leaf	Used in boiling water	Mother
62	<i>Xylia xylocarpa</i> (Roxb.) W.Theob.	Iruvullu	Fabaceae	Leaf	Used in boiling water	Mother

4.2.1 Single preparations and compound preparations.

i Single preparation.

This type of preparation involves use of one plant species for bathing mother and child after delivery. *Coleus amboinicus*, *Ocimum tenuiflorum*, *Acalypha fruticosa* and *Vigna radiata* were used as single preparations. *C. amboinicus*, and *O. tenuiflorum* were used to bathe babies. *A. fruticosa* was used for both mothers and babies. *V. radiata* was used for mothers. The plants used as single preparations were given in Table 4.2.

Table 4.2. Plants used as single preparations for bathing mother and child after delivery.

SL. No.	Scientific Name	Common name	Family	Part used	Mode of use
1	<i>Coleus amboinicus</i> Lour.	Panikurka	Lamiaceae	Leaf	Leaf extract mixed with warm water for bathing
2	<i>Ocimum tenuiflorum</i> L.	Tulsi	Lamiaceae	Leaf	Leaf extract mixed with warm water for bathing
3	<i>Acalypha fruticosa</i> Forssk.	Munja	Euphorbiaceae	Leaf	Leaf paste applied to the body
4	<i>Vigna radiata</i> (L.) R. Wilczek	Cherupayar	Fabaceae	Seed	Seed paste applied to body

ii Compound Preparations:

This type of preparation involves use of two or more plants for the preparation of medicated water. 16 combinations of different plants were used for the preparation of medicated baths. Comb 15 was used for bathing babies and all other combinations were used to bathe mothers. The combinations varied across taluks, indicating regional diversity in plant selection and preparation methods. The different combination of plants used for the preparation of medicated water for bath was given in Table 4.3.

Table 4.3. Plants used in different formulations for bathing mother and child after delivery.

Combination	Scientific name	Common name	Family	Plant part used	Mode of use
Comb 1	<i>Ficus racemosa</i> L.	Athi	Moraceae	Bark	Used in boiling water
	<i>Ficus microcarpa</i> L.f.	Ethi			
	<i>Ficus religiosa</i> L.	Arayal			
	<i>Ficus benghalensis</i> L.	Peral			
Comb 2	<i>Azadirachta indica</i> A. Juss.	Aryaveppu	Meliaceae	Leaf and Bark	Used in boiling water
	<i>Jatropha hernandiifolia</i> Vent.	Kottathari	Euphorbiaceae	Bark	
	<i>Pterocarpus marsupium</i> Roxb.	Venga	Fabaceae	Bark	
Comb 3	<i>Vitex altissima</i> L.f.	Mayilellu	Verbenaceae	Leaf	Used in boiling water
	<i>Causonis trifolia</i> (L.) Mabb. & J.Wen	Vathakkodi	Vitaceae	Leaf and Stem	
	<i>Getonia floribunda</i> Roxb.	Pullanni	Combretaceae	Leaf	
	<i>Artocarpus heterophyllus</i> Lam.	Plavu	Moraceae	Leaf	
Comb 4	<i>Ficus benghalensis</i> L.	Peral	Moraceae	Bark	Used in boiling water
	<i>Pterocarpus marsupium</i> Roxb.	Venga	Fabaceae	Bark	
	<i>Azadirachta indica</i> A. Juss.	Aryaveppu	Meliaceae	Bark	
	<i>Jatropha hernandiifolia</i> Vent.	Kottathari	Euphorbiaceae	Bark	
	<i>Moringa oleifera</i> Lam.	Muringa	Moringaceae	Bark	
Comb 5	<i>Artocarpus heterophyllus</i> Lam.	Plavu	Moraceae	Leaf	Used in boiling water
	<i>Azadirachta indica</i> A. Juss.	Aryaveppu	Meliaceae	Leaf	
Comb 6	<i>Azadirachta indica</i> A. Juss.	Aryaveppu	Meliaceae	Leaf	Used in boiling water
	<i>Ixora coccinea</i> L.	Chethi	Rubiaceae	Leaf and Flower	
	<i>Mangifera indica</i> L.	Maavu	Anacardiaceae	Bark	
	<i>Azadirachta indica</i> A. Juss.	Aryaveppu	Meliaceae	Bark and Leaf	Used in boiling water
	<i>Ixora coccinea</i> L.	Chethi	Rubiaceae	Leaf and Flower	
	<i>Mangifera indica</i> L.	Maavu	Anacardiaceae	Bark	

Comb 7	<i>Ficus racemosa</i> L.	Aathi	Moraceae	Bark	
	<i>Ficus microcarpa</i> L.f.	Ethi	Moraceae	Bark	
	<i>Ficus religiosa</i> L.	Arayal	Moraceae	Bark	
	<i>Ficus benghalensis</i> L.	Peraal	Moraceae	Bark	
	<i>Eucalyptus globulus</i> Labill.	Eucalyptus	Myrtaceae	Leaf	
Comb 8	<i>Samadera indica</i> Gaertn.	Karigotta	Simaroubaceae	Leaf	Used in boiling water
	<i>Mangifera indica</i> L.	Mavu	Anacardiaceae	Leaf	
	<i>Thespesia populnea</i> (L.) Sol. ex Correa.	Poovarasu	Malvaceae	Leaf	
	<i>Artocarpus heterophyllus</i> Lam.	Plavu	Moraceae	Leaf	
Comb 9	<i>Artocarpus heterophyllus</i> Lam.	Plavu	Moraceae	Yellow leaf	Used in boiling water
	<i>Justicia adhatoda</i> L.	Adalodakam	Acanthaceae	Leaf	
	<i>Azadirachta indica</i> A. Juss.	Aryaveppu	Meliaceae	Leaf	
	<i>Moringa oleifera</i> Lam.	Muringa	Moringaceae	Bark	
	<i>Jatropha hernandiifolia</i> Vent.	Kottathari	Euphorbiaceae	Bark	
	<i>Thespesia populnea</i> (L.) Sol. ex Correa.	Poovarasu	Malvaceae	Bark	
Comb 10	<i>Azadirachta indica</i> A. Juss.	Aryaveppu	Meliaceae	Leaf and Bark	Used in boiling water
	<i>Artocarpus heterophyllus</i> Lam.	Plavu	Moraceae	Leaf	
	<i>Moringa oleifera</i> Lam.	Muringa	Moringaceae	Bark	
Comb 11	<i>Justicia adhatoda</i> L.	Adalodakam	Acanthaceae	Leaf	Used in boiling water
	<i>Ixora coccinea</i> L.	Thechi	Rubiaceae	Leaf	
	<i>Jatropha hernandiifolia</i> Vent.	Kottathari	Euphorbiaceae	Bark	
	<i>Justicia gendarussa</i> Burm.f.	Vathamkolli	Acanthaceae	Leaf	
	<i>Artocarpus heterophyllus</i> Lam.	Plavu	Moraceae	Leaf	
	<i>Vitex negundo</i> L.	Karinochi	Lamiaceae	Leaf	
	<i>Moringa oleifera</i> Lam.	Muringa	Moringaceae	Bark	Used in boiling water
	<i>Mangifera indica</i> L.	Mavu	Anacardiaceae	Leaf	
	<i>Justicia gendarussa</i> Burm.f.	Vathamkolli	Acanthaceae	Leaf	

Comb 12	<i>Jatropha hernandiifolia</i> Vent.	Kottathari	Euphorbiaceae	Bark	
	<i>Azadirachta indica</i> A. Juss.	Aryaveppu	Meliaceae	Leaf	
Comb 13	<i>Ficus benghalensis</i> L.	Peraal	Moraceae	Bark	Used in boiling water
	<i>Borassus flabellifer</i> L.	Pana	Arecaceae	Bark	
	<i>Vitex negundo</i> L.	Karinochi	Lamiaceae	Leaf	
	<i>Achyranthes aspera</i> L.	Kadaladi	Amaranthaceae	Leaf	
Comb 14	<i>Artocarpus heterophyllus</i> Lam.	Plavu	Moraceae	Leaf	Used in boiling water
	<i>Azadirachta indica</i> A. Juss.	Aryaveppu	Meliaceae	Bark	
	<i>Mangifera indica</i> L.	Mavu	Anacardiaceae	Leaf	
	<i>Tamarindus indica</i> L.	Puliyila	Fabaceae	Leaf	
Comb 15	<i>Acalypha fruticosa</i> Forssk.	Chinnayila (munja)	Euphorbiaceae	Leaf	Paste applied to the body
	<i>Acorus calamus</i> L.	Vayambu	Araceae	Leaf	
	<i>Curcuma longa</i> L.	Manjal	Zingiberaceae	Rhizome	
Comb 16	<i>Psidium guajava</i> L.	Pera	Myrtaceae	Leaf	Used in boiling water
	<i>Coffea arabica</i> L.	Kappi	Rubiaceae	Leaf	
	<i>Mangifera indica</i> L.	Mavu	Anacardiaceae	Leaf	
	<i>Clerodendrum infortunatum</i> L.	Peruku	Lamiaceae	Leaf	
	<i>Artocarpus heterophyllus</i> Lam.	Plavu	Moraceae	Yellow leaf	
	<i>Getonia floribunda</i> Roxb.	Pullanji	Combretaceae	Leaf	
	<i>Mussaenda frondosa</i> L.	Vellila	Rubiaceae	Leaf	
	<i>Piper nigrum</i> L.	Kurumulaku	Piperaceae	Leaf and stem	
	<i>Piper longum</i> L.	Thippali	Piperaceae	Leaf and stem	
	<i>Vitex altissima</i> L.f.	Mayilellu	Lamiaceae	Leaf	
	<i>Schleichera oleosa</i> (Lour.) Merr.	Poovam	Sapindaceae	Leaf	

*Comb: Combination of plants

37 plant species used for making *vethuvellam* were first reported from our study. 25 plant species reported in our study were previously documented in other studies for the preparation of medicated water. *Xylia xylocarpa*, *Vitex altissima*, *Thespesia populnea*, *Terminalia paniculata*, *Schleichera oleosa*, *Sida acuta*, *Stereospermum colais*, *Strychnos nux-vomica*, *Ricinus communis*, *Pseudarthria viscida*, *Plumbago zeylanica*, *Pongamia pinnata*, *Premna serratifolia*, *Pterocarpus marsupium*, *Coleus amboinicus*, *Piper longum*, *Ocimum gratissimum*, *Moringa oleifera*, *Mussaenda frondosa*, *Leucas aspera*, *Jatropha hernandifolia*, *Justicia gendarussa*, *Hamelia patens*, *Eucalyptus globulus*, *Chrysopogon zizanioides*, *Cymbopogon flexuosus*, *Croton persimilis*, *Coffea arabica*, *Clitoria ternatea*, *Centella asiatica*, *Causonis trifolia*, *Borassus flabellifer*, *Anethum graveolens*, *Acorus calamus*, *Achyranthes aspera*, *Acalypha fruticosa*, *Senegalia caesia* were the newly reported plant species for preparing medicated bath mother and child after delivery.

The plants used for the medicated bath were rich in various phytochemicals. These phytochemicals have various pharmacological properties. These phytochemicals provide a good odour to this water. Inhaling of these vapours are also considered as essential for the smooth recovery of mother after delivery (Panyaphu et al., 2011).

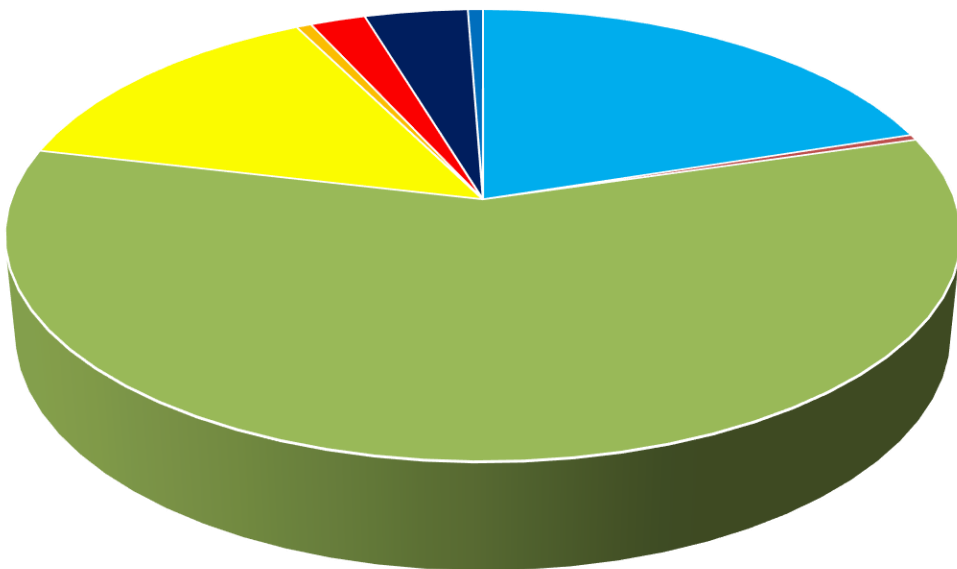
4.2.2 Plant Parts Used

The different plant parts used for the preparation of *vethuvellam* were roots, leaf, fruits, flowers and bark. The leaves were the most commonly used plant parts followed by barks. This distribution likely reflects the accessibility and abundance of leaves, as well as their high content of bioactive compounds. The leaves of *Artocarpus heterophyllus* were the most frequently cited plant part. Yellow leaves of *A. heterophyllus* were used with boiling water to bathe mothers. Nalpamara patta was another most cited plant. Nalpamara patta includes the bark of four different plants. *Ficus racemosa*, *F. microcarpa*, *F. religiosa* and *F. benghalensis* were the four different plants. The rhizome of *Curcuma longa* was another commonly cited plant. The pie-chart representing different plant parts used were given in Plate 4.3.

4.3 Use value, Consensus Index and Relative Frequency of Citation of plants cited in the survey.

The use value, consensus index and Relative frequency of citation was high for *Curcuma longa* followed by *Artocarpus heterophyllus*. The RFC for *C. longa* was 0.4964 and for *A. heterophyllus* was 0.4539. The CI for *C. longa* was 49.64% and for *A. heterophyllus* was

PLANT PART USED



- Bark
- Flower
- Leaf
- Rhizome
- Root
- Seed
- Stem
- Whole plant

Plate 4.3. Pie diagram showing percentage of plant parts used reported from the survey on plants used for bathing mother and child after delivery.

45.39%. The use value for *C. longa* was 0.482 and for *A. heterophyllus* was 0.447. The use value, CI and RFC were given in Table 4.4.

High RFC, CI and UV was for *Curcuma longa* followed by *Artocarpus heterophyllus*. *C. longa* is rich in various phytochemicals. Curcuminoids, sesquiterpenes and essential oils were the major class of compounds present in *C. longa* (Sambhav et al., 2014; Omosa et al., 2017). This plant possessed anticancer, antidiabetic, anti-inflammatory, antimicrobial, cardiovascular and neuroprotective activities (Omosa et al., 2017; Verma et al., 2018; Chanda et al., 2019; Jyotirmayee et al., 2022). *A. heterophyllus* possessed antibacterial, antioxidant, antidiabetic (Indrianingsih, 2024), analgesic (Praveen et al., 2016) and wound healing (Periyanayagam & Karthikeyan, 2013) properties. Many studies reported on the use of these plants in post-natal care. The use of *A. heterophyllus* and *C. longa* for making *vethuvellam* in South Kerala was stated by Rajith et al., 2010. The study documented 8 formulations which include different plants for making *vethuvellam*. Sonitha and Kumar, 2021 also described the use of *C. longa* for medicated bath in Triunelveli district, Tamil Nadu. External application of *C. longa* helps to regain the shape of the uterus to the normal and the antimicrobial properties of the plant helps to prevent further infections. *C. long* and *A. heterophyllus* were used for medicated bath in Mahae, Puducherry Union territory (Sivadasan et al, 2014). All these studies mentioned the importance of these two plants in post-natal care. In our study also, *C. longa* and *A. heterophyllus* were reported to be the most frequently used plants for the medicated bath.

Nalpamara patta, the bark of 4 *Ficus* species, was another commonly cited plant for bathing mother and child after delivery. The medicated water prepared from the barks of these plants prevents inflammation during pregnancy (Sreelakshmi & Muthusamy, 2021). Nalpamara was used to cure vaginal diseases, nervous disorders, skin diseases and diabetes. It is also used for cleaning vagina (Babu et al., 2010). All the plant species reported in the study possessed different pharmacological properties. So, proper documentation and studies on these plants are essential to protect this traditional knowledge.

Table 4.4. Consensus Index (CI), Relative Frequency of Citation (RFC) and Use Value (UV) of plants cited in the study for bathing mother and child after delivery.

SL. No.	Plants used for the study	Consensus Index	Relative Frequency of Citation	Use Value
1	<i>Acalypha fruticosa</i> Forssk.	23	0.23	0.227
2	<i>Achyranthes aspera</i> L.	0.35	0.0035	0.0035
3	<i>Acorus calamus</i> L.	1.77	0.0177	0.0177
4	<i>Anacardium occidentale</i> L.	0.35	0.0035	0.0035
5	<i>Anethum graveolens</i> L.	0.35	0.0035	0.0035
6	<i>Artocarpus heterophyllus</i> Lam.	45.39	0.4539	0.447
7	<i>Azadirachta indica</i> A. Juss.	32.27	0.3227	0.3227
8	<i>Borassus flabellifer</i> L.	2.83	0.0283	0.0283
9	<i>Calotropis gigantea</i> (L.) W.T.Aiton	0.35	0.0035	0.0035
10	<i>Causonis trifolia</i> (L.) Mabb. & J.Wen	6.737	0.06737	0.0638
11	<i>Centella asiatica</i> (L.) Urb.	1.418	0.01418	0.01418
12	<i>Chrysopogon zizanioides</i> (L.) Roberty	0.35	0.0035	0.0035
13	<i>Clerodendrum infortunatum</i> L.	4.6	0.046	0.0425
4	<i>Clitoria ternatea</i> L.	0.35	0.0035	0.0035
15	<i>Cocos nucifera</i> L.	6.03	0.0603	0.0567
16	<i>Coffea arabica</i> L.	1.77	0.0177	0.0177
17	<i>Coleus amboinicus</i> Lour.	0.35	0.0035	0.0035
18	<i>Croton persimilis</i> Mull. Arg.	1.06	0.0106	0.0106
19	<i>Curcuma longa</i> L.	49.64	0.4964	0.482
20	<i>Cymbopogon flexuosus</i> (Nees ex Steud.) Will. Watson	0.35	0.0035	0.0035
21	<i>Eucalyptus globulus</i> Labill.	0.7	0.007	0.007
22	<i>Ficus benghalensis</i> L.	29.1	0.291	0.2837
23	<i>Ficus microcarpa</i> L.f.	28.36	0.2837	0.2765
24	<i>Ficus racemosa</i> L.	28.36	0.2837	0.2765

25	<i>Ficus religiosa</i> L.	28.36	0.2837	0.2765
26	<i>Getonia floribunda</i> Roxb.	3.9	0.039	0.039
27	<i>Glycosmis pentaphylla</i> (Retz.) DC.	0.35	0.0035	0.0035
28	<i>Hamelia patens</i> Jacq.	0.7	0.007	0.007
29	<i>Hibiscus</i> × <i>rosa-sinensis</i> L.	3.19	0.0319	0.0319
30	<i>Ixora coccinea</i> L.	7.09	0.0709	0.0709
31	<i>Jatropha hernandiifolia</i> Vent.	2.84	0.0284	0.0284
32	<i>Justicia adhatoda</i> L.	17.7	0.177	0.174
33	<i>Justicia gendarussa</i> Burm.f.	2.12	0.021	0.021
34	<i>Leucas aspera</i> (Willd.) Link.	6.7	0.067	0.067
35	<i>Mangifera indica</i> L.	5.67	0.0567	0.0567
36	<i>Moringa oleifera</i> Lam.	1.418	0.01418	0.01418
37	<i>Musa</i> × <i>paradisiaca</i> L.	0.35	0.0035	0.0035
38	<i>Mussaenda frondosa</i> L.	0.7	0.007	0.007
39	<i>Ocimum gratissimum</i> L.	5.3	0.053	0.053
40	<i>Ocimum tenuiflorum</i> L.	6.02	0.0602	0.0567
41	<i>Piper longum</i> L.	7	0.07	0.0674
42	<i>Piper nigrum</i> L.	4.6	0.046	0.046
43	<i>Plumbago zeylanica</i> L.	0.7	0.007	0.007
44	<i>Pongamia pinnata</i> (L.) Pierre	0.35	0.0035	0.0035
45	<i>Premna serratifolia</i> L.	0.35	0.0035	0.0035
46	<i>Pseudarthria viscida</i> (L.) Wight & Arn.	3.19	0.0319	0.0319
47	<i>Psidium guajava</i> L.	1.4	0.014	0.014
48	<i>Pterocarpus marsupium</i> Roxb.	1.4	0.014	0.014
49	<i>Ricinus communis</i> L.	0.35	0.0035	0.0035
50	<i>Samadera indica</i> Gaertn.	2.83	0.028	0.028
51	<i>Schleichera oleosa</i> (Lour.) Oken	1.06	0.0106	0.0106
52	<i>Senegalia caesia</i> (L.) Maslin, Seigler & Ebinger	2.83	0.0283	0.0283

53	<i>Sida acuta</i> Burm.f.	0.7	0.007	0.007
54	<i>Stereospermum colais</i> (Buch.-Ham. ex Dillwyn) Mabb.	0.35	0.0035	0.0035
55	<i>Strychnos nux-vomica</i> L.	7.8	0.078	0.078
56	<i>Tamarindus indica</i> L.	0.35	0.0035	0.0035
57	<i>Terminalia paniculata</i> B.Heyne ex Roth	3.19	0.0319	0.0319
58	<i>Thespesia populnea</i> (L.) Sol. ex Correa	0.35	0.0035	0.0035
59	<i>Vigna radiata</i> (L.) R. Willczek	7.8	0.078	0.078
60	<i>Vitex altissima</i> L.f.	9.2	0.092	0.092
61	<i>Vitex negundo</i> L.	2.13	0.0213	0.0213
62	<i>Xylia xylocarpa</i> (Roxb.) W.Theob.	0.35	0.0035	0.0035

Plants and plant-based treatments are frequent in various parts of the world. Use of folk medicine for post-natal care is widely practised in different parts of the world. Pregnancy and delivery are considered as important milestones in the lifespan of a woman. Pre and post-natal care is essential for the smooth recovery of the mother. Medicinal plants used during post-natal care possessed many pharmacological properties (Jain et al., 2011). The present study dealt with the use of plants for bathing mother and child after delivery. The study documented 62 plant species used for the medicated bath of mother and child. The medicated water called *vethuvellam* restores the body of the mother. Bathing with these water increases disease resistance in mother and child. This bath improves blood circulation, reduces inflammation, helps the body to regain the shape, helps in skin brightening and reduces body pain in mothers. The medicated bath for babies increases the immunity and also helps in drying of umbilical cord. Oil massaging helps in the restoration of abdominal muscles of the mother and in shaping the body of the infant. This traditional knowledge about medicated baths was passed from generation to generation as a mouthpiece. The plants used for this medicated bath may vary in different parts. All the plants used for this medicated bath were locally available.

4.4 Preliminary Phytochemical analysis of hexane, water and isopropyl alcohol extracts of *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima*

The preliminary phytochemical analysis of hexane, water and isopropyl alcohol extracts of *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima* were done using various qualitative tests mentioned in chapter 3. The hexane extract of *A. fruticosa* showed the presence of alkaloids, flavonoids and steroids. The water and isopropyl alcohol extract of *A. fruticosa* revealed the presence of alkaloids, flavonoids, phenols, glycosides, tannins, coumarins, saponins and steroids. The hexane extract of *J. gendarussa* disclosed the presence of alkaloids, saponins and steroids. Alkaloids, flavonoids, phenols, glycosides, tannins, coumarins, saponins and steroids were present in the water extract of *J. gendarussa*. The isopropyl alcohol extract of *J. gendarussa* showed the presence of alkaloids, flavonoids, phenols, glycosides, saponins and steroids. The hexane extract of *P. longum* revealed the presence of alkaloids, flavonoids, saponins and steroids. The water and isopropyl alcohol extract of *P. longum* revealed the presence of alkaloids, flavonoids, phenols, glycosides, tannins, coumarins, saponins and steroids. The hexane extract of *V. altissima* disclosed the presence of alkaloids, flavonoids, saponins and steroids. Alkaloids, flavonoids, phenols, glycosides, tannins, coumarins, saponins and steroids were present in water and isopropyl alcohol extract of *V. altissima*. The tables and

photographs of preliminary phytochemical analysis were given in Table 4.5, 4.6, 4.7 and 4.8 and Plate 4.4, 4.5 and 4.6 respectively.

Table 4.5. Preliminary phytochemical analysis of hexane, water and isopropyl alcohol extracts of *Acalypha fruticosa*.

SL. No.	Phytochemicals	Test	Hexane	Water	Isopropyl alcohol
1	Alkaloids	Wagner's test	+	+	+
		Hager's test	+	+	+
2	Flavonoids	Alkaline reagent test	+	+	+
3	Phenols	Ferric chloride test	-	+	+
4	Saponins	Froth Test	-	+	+
5	Coumarins	Sodium hydroxide test	-	+	+
6	Glycosides	Ferric chloride test	-	+	+
7	Tannins	Ferric chloride test	-	+	+
8	Steroids	Salkowski's test	+	+	+

Table 4.6. Preliminary phytochemical analysis of hexane, water and isopropyl alcohol extracts of *Justicia gendarussa*.

SL. No.	Phytochemicals	Test	Hexane	Water	Isopropyl alcohol
1	Alkaloids	Wagner's test	+	-	+
		Hager's test	+	+	+
2	Flavonoids	Alkaline reagent test	-	+	+
3	Phenols	Ferric chloride test	-	+	+
4	Saponins	Froth Test	+	+	+
5	Coumarins	Sodium hydroxide test	-	+	-

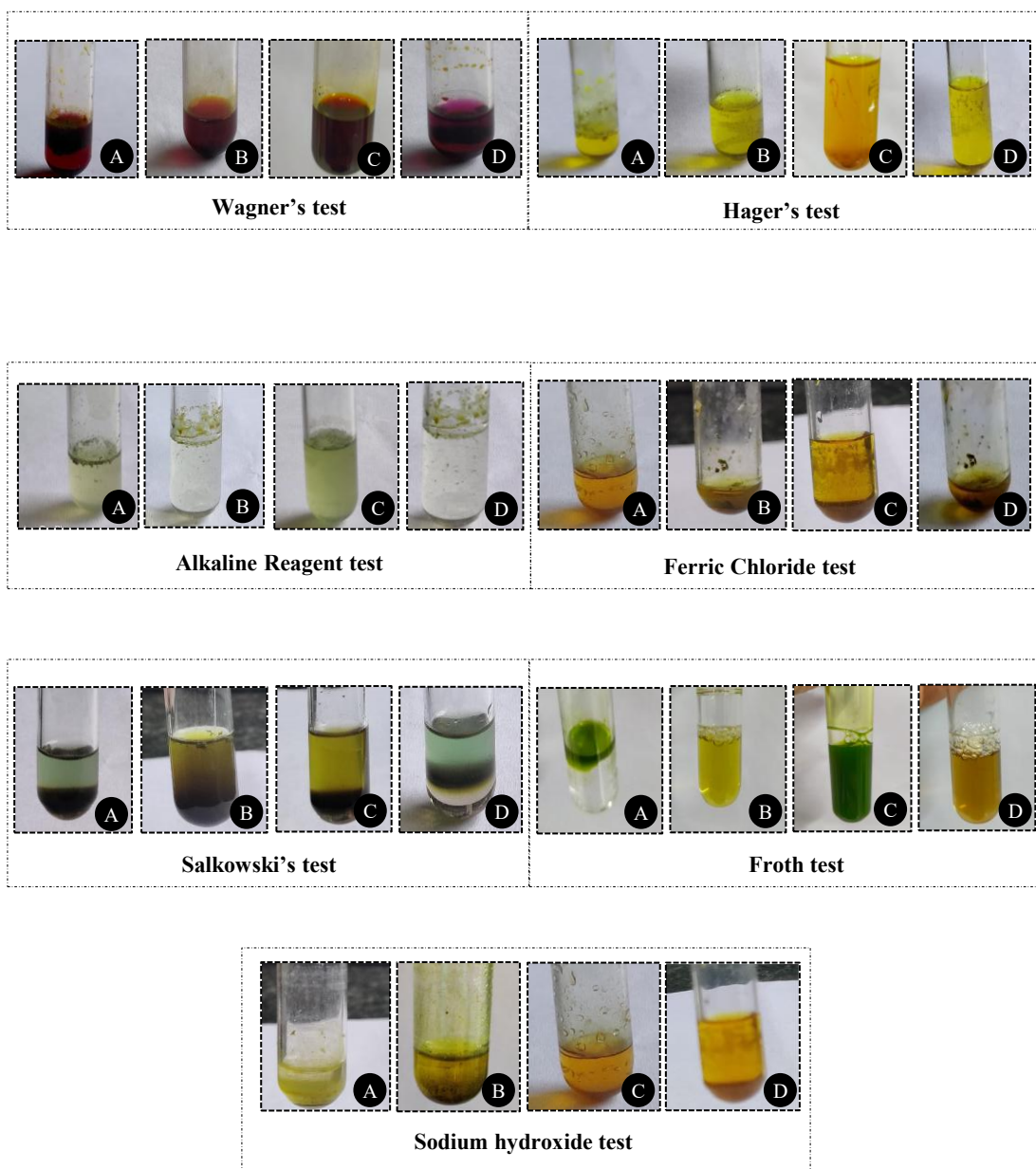


Plate 4.4. Preliminary Phytochemical analysis of hexane extracts of A. *Acalypha fruticosa*, B. *Justicia gendarussa*, C. *Piper longum*, D. *Vitex altissima*.



Plate 4.5. Preliminary Phytochemical analysis of water extracts of A. *Acalypha fruticosa*, B. *Justicia gendarussa*, C. *Piper longum*, D. *Vitex altissima*.

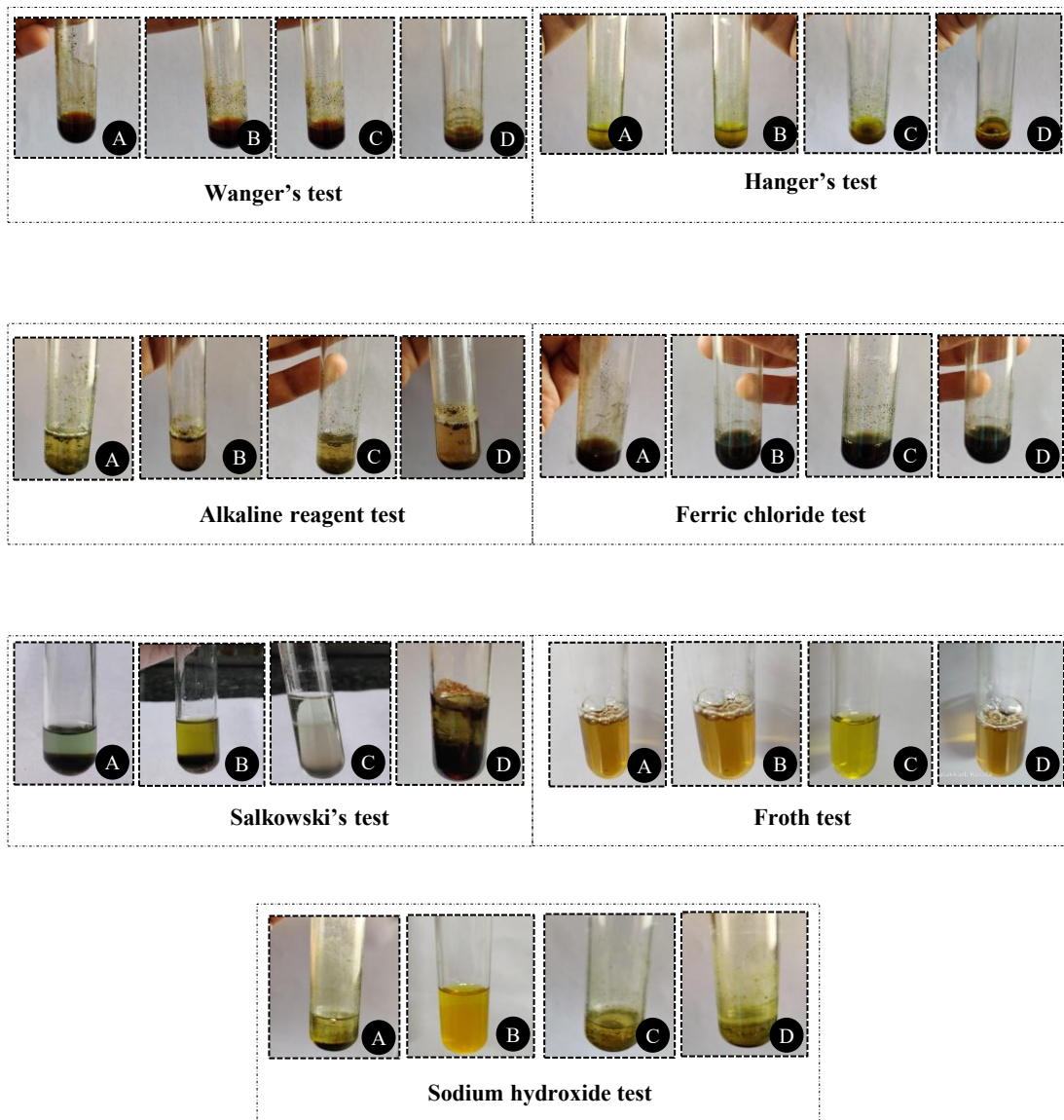


Plate 4.6. Preliminary Phytochemical analysis of isopropyl alcohol extracts of A. *Acalypha fruticosa*, B. *Justicia gendarussa*, C. *Piper longum*, D. *Vitex altissima*.

6	Glycosides	Ferric chloride test	-	+	+
7	Tannins	Ferric chloride test	-	+	-
8	Steroids	Salkowski's test	+	+	+

Table 4.7. Preliminary phytochemical analysis of hexane, water and isopropyl alcohol extracts of *Piper longum*.

SL. No.	Phytochemicals	Test	Hexane	Water	Isopropyl alcohol
1	Alkaloids	Wagner's test	+	+	+
		Hager's test	+	+	+
2	Flavonoids	Alkaline reagent test	+	+	+
3	Phenols	Ferric chloride test	-	+	+
4	Saponins	Froth Test	+	+	+
5	Coumarins	Sodium hydroxide test	-	+	+
6	Glycosides	Ferric chloride test	-	+	+
7	Tannins	Ferric chloride test	-	+	+
8	Steroids	Salkowski's test	+	+	+

Table 4.8. Preliminary phytochemical analysis of hexane, water and isopropyl alcohol extracts of *Vitex altissima*.

SL. No.	Phytochemicals	Test	Hexane	Water	Isopropyl alcohol
1	Alkaloids	Wagner's test	+	+	+
		Hager's test	+	+	+
2	Flavonoids	Alkaline reagent test	+	+	+
3	Phenols	Ferric chloride test	-	+	+
4	Saponins	Froth Test	+	+	+

5	Coumarins	Sodium hydroxide test	-	+	+
6	Glycosides	Ferric chloride test	-	+	+
7	Tannins	Ferric chloride test	-	+	+
8	Steroids	Salkowski's test	+	+	+

The preliminary phytochemical analysis of hexane, water and isopropyl alcohol extracts of *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima* showed the presence of many bioactive secondary metabolites like flavonoids, phenols, saponins, tannins, alkaloids etc. Flavonoids, cardiac glycosides, phenols, fatty acids and proteins were reported from the qualitative phytochemical analysis of various extracts of *A. fruticosa* (Malathi et al., 2018). Our study also showed the presence of flavonoids, phenols, glycosides, saponins and steroids from the hexane, water and isopropyl alcohol extract of *A. fruticosa*. The preliminary phytochemical analysis of aqueous, methanol and ethanol extract of *J. gendarussa* disclosed the presence of alkaloids, glycosides, saponins, terpenoids, flavonoids and steroids (Ali et al., 2023). In another study on preliminary phytochemical analysis of ethanolic and aqueous extract of leaves and stem of *J. gendarussa* revealed the presence of tannins, saponins, terpenoids, glycosides, steroids, phenolics, flavonoids and starch (Subramanian et al., 2012). Our preliminary phytochemical analysis results of hexane, water and isopropyl alcohol extracts supported these findings. Tannins, alkaloids, phenols, flavonoids and steroids were reported from ethyl alcohol extract of *P. longum* (Suman et al., 2021). The results from our qualitative phytochemical analysis of hexane, water and isopropyl alcohol extract confirmed these findings. Saponins, flavonoids, alkaloids, phenols and phytosteroids were reported from various extracts of *V. altissima* (Rajesh et al., 2019). Carbohydrates, starch, reducing sugar, proteins, amino acids, hexose sugar, tyrosine and steroids were present in aqueous ethanolic extract of *V. altissima* (Leela et al., 2015). The phytochemical analysis of hexane, chloroform and methanol extracts of *V. altissima* disclosed the presence of carbohydrates, flavonoids, saponins, phenolic acids and terpenoids (Sundaresan et al., 2020). The preliminary phytochemical analysis data obtained from hexane, water and isopropyl alcohol extracts of *V. altissima* align with these conclusions. The secondary metabolites produced by plants possess diverse medicinal properties like anticancer, antimicrobial, neuroprotective, anti-inflammatory, immunomodulation, and cardioprotective properties (Elshafie et al., 2023).

4.5 Immunomodulatory activity of Selected Plants

The immunomodulatory activity of selected plants used for post-natal care in folk medicine of Palakkad was studied using *in vitro* macrophage activation. THP1 cells were converted to macrophages and used for the study. The plants selected for the study were *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima*. Hexane, water and isopropyl alcohol leaf extracts of these plants were used for the study.

4.5.1 *In vitro* macrophage activation studies on THP1 cells treated with 10µg/ml hexane extracts of *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima*

The THP1 cells were converted to macrophages by treating with PMA reagent. The macrophages were treated with 10µg/ml of hexane extracts of *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima* and incubated for 24 hours. The RNA was isolated from these cells to prepare cDNA and the relative fold gene expression level of IL-1β, IFNβ, TNFα and CCR-5 were analysed with internal control GAPDH using qRT-PCR. $\Delta\Delta C_t$ method was used for result analysis. The fold change of genes in extract treated group was calculated relative to the control group with normalisation by the internal control gene. The hexane extract of *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima* were denoted as AFH, JGH, PLH, and VAH respectively. DMSO treated cells were used as the control.

The one-way ANOVA results authenticated that significant differences in the expression of IL-1β, IFNβ, TNFα and CCR-5 across the tested groups with $p < 0.001$. The results suggested that the 10µg/ml of hexane extracts modulate the expression of these genes compared to control.

The results of post hoc analysis revealed that treatment of PLH and VAH significantly upregulated the gene expression of IL-1β and TNFα ($p < 0.001$). AFH and JGH treated groups showed a non-significant fold change of IL-1β and TNFα compared to the control. AFH exhibited a mild upregulation of IL-1β (fold gene expression- 1.09 ± 0.039) and JGH downregulated the expression of IL-1β (fold gene expression- 0.25 ± 0.127).

The VAH extract showed significant upregulation in the expression of IFNβ and CCR-5 with $p < 0.001$. PLH treated cells exhibited a mild induction in expression of IFNβ whereas the JGH and AFH treated cells showed decrease in fold expression of IFNβ. The post hoc analysis indicated that the JGH, AFH and PIH induced expression of IFNβ was not significant compared to control. The gene expression of CCR-5 was downregulated in AFH, JGH and

PLH treated cells. The relative fold gene expression of IL-1 β , IFN β , TNF α and CCR-5 are summarized in Table 4.9 and Plate 4.7.

Table 4.9. Relative fold gene expression of IL-1 β , IFN β , TNF α and CCR-5 in AFH, JGH, PLH, and VAH treated macrophages.

Sl. No.	Treatment	IL-1 β Mean fold expression $\pm$ SD	IFN β Mean fold expression $\pm$ SD	TNF α Mean fold expression $\pm$ SD	CCR-5 Mean fold expression $\pm$ SD
1	Control	1	1	1	1
2	AFH	1.09 $\pm$ 0.039 ^{ns}	0.535 $\pm$ 0.08 ^{ns}	1.56 $\pm$ 0.06 ^{ns}	0.53 $\pm$ 0.29 ^{ns}
3	JGH	0.25 $\pm$ 0.127 ^{ns}	0.44 $\pm$ 0.167 ^{ns}	0.37 $\pm$ 0.19 ^{ns}	0.612 $\pm$ 0.10 ^{ns}
4	PLH	5.62 $\pm$ 0.287 ^{**}	1.33 $\pm$ 0.128 ^{ns}	14.2 $\pm$ 0.4 ^{**}	0.327 $\pm$ 0.21 [*]
5	VAH	3.13 $\pm$ 0.568 ^{**}	3.42 $\pm$ 0.415 ^{**}	4.13 $\pm$ 0.75 ^{**}	2.7 $\pm$ 0.08 ^{**}

The data are expressed as mean $\pm$ SD with n=3. Values bearing superscript** in rows (comparison with Control group) differ significantly with p<0.001 and with superscript* differ significantly with p<0.05. Values bearing superscript ns in rows were non-significant.

In summary, VAH has the ability to upregulate IFN β , which is a component of antimicrobial immunity. It also upregulates pro-inflammatory cytokines IL-1 β and TNF α , which helps in immune activation. Detailed studies are needed to fully understand the VAH's immunomodulatory effects.

4.5.2 *In vitro* macrophage activation studies on THP1 cells treated with 25 μ g/ml water extracts of *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima*

PMA reagent was used to convert THP1 cells to macrophages. The macrophages were then treated with 25 μ g/ml of water extracts of *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima* and incubated for 24 hours. DMSO treated cells were used as the control. cDNA was prepared from the isolated RNA and the fold expression level of IL-1 β , IFN β , TNF α and CCR-5 were analysed with internal control GAPDH using qRTPCR. $\Delta\Delta$ Ct method was used for result analysis. The water extract of *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima* were denoted as AFW, JGW, PLW and VAW respectively.

The one-way ANOVA confirmed significant differences in the expression of IL-1 β , IFN β , TNF α and CCR-5 across the tested groups with p<0.001. The results suggested that the 25 μ g/ml of water extracts modulate the expression of these genes compared to control.

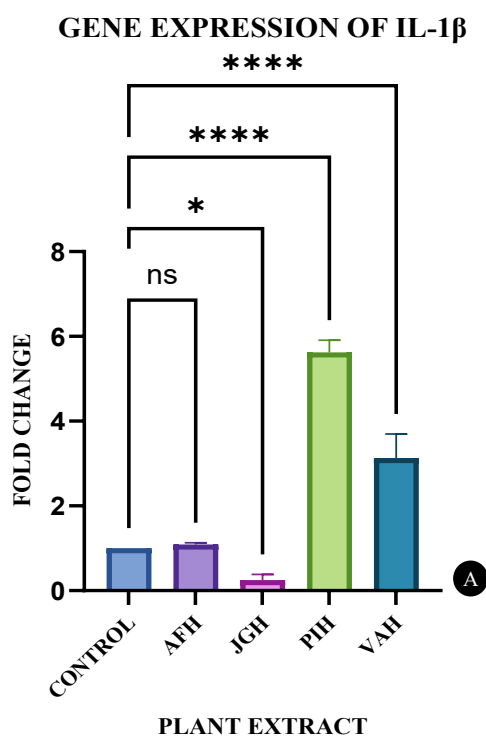
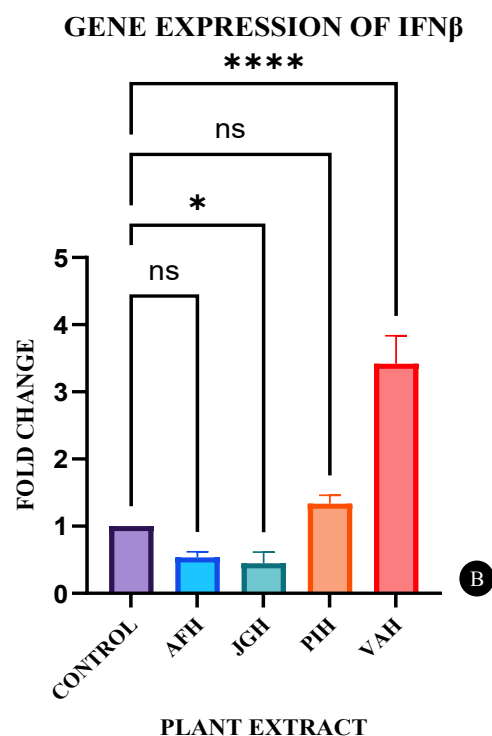
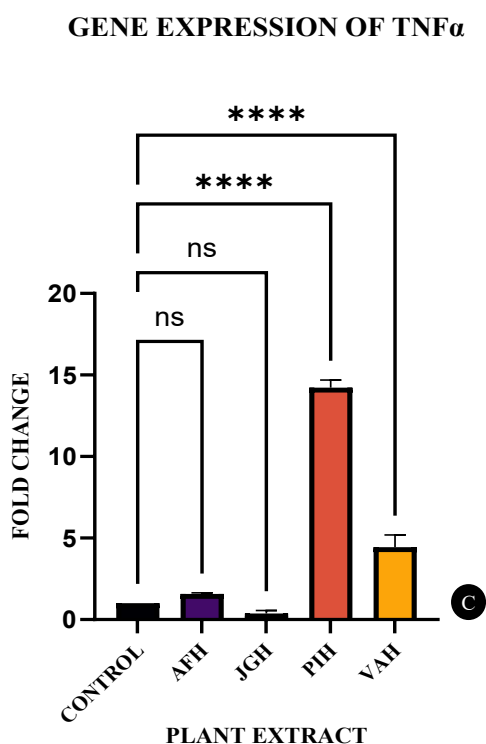
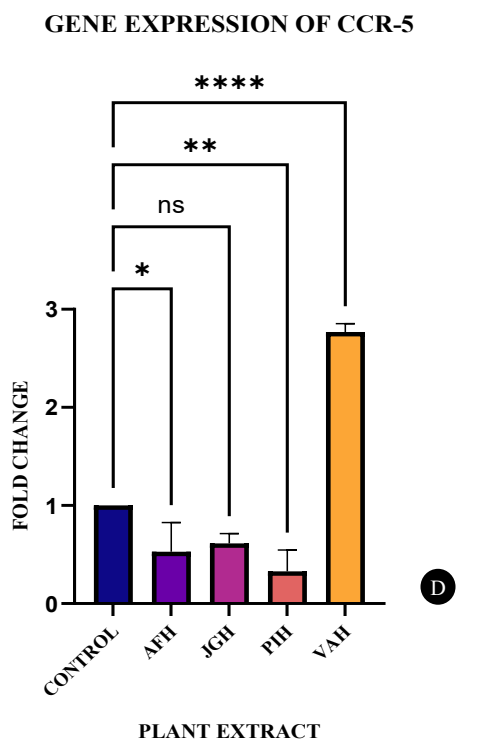


Plate 4.7. Figure A-D. Relative gene expression of IL-1 β , IFN β , TNF α and CCR-5 in 10 μ g/ml hexane extracts of *A. fruticosa* (AFH), *J. gendarussa* (JGH), *P. longum* (PLH) and *V. altissima* (VAH) treated macrophages. The data are expressed as mean $\pm$ SD with n=3. **** indicates p<0.0001, *** p<0.001, ** p<0.01, *p<0.05. ns indicates p>0.05. * indicates significant difference from control group.

The treatment of AFW significantly upregulated the gene expression of IL-1 β and TNF α with $p < 0.001$. The fold change for TNF α in AFW treated cells was 126.02 ± 35.48 . The PLW treated cells showed a slight upregulation of IL-1 β and TNF α . The JGW and VAW treated cells downregulates the gene expression of IL-1 β and showed a slight upregulation in the expression of TNF α .

PLW and AFW treated cells showed a slight increase in fold expression of IFN β whereas the JGW and VAW treated cells showed decrease in fold expression of IFN β . The AFW treated group exhibited significant upregulation of IFN β when compared with control.

The relative fold expression of CCR-5 was downregulated in AFW, JGW, PLW and VAW treated cells. AFW, JGW and VAW showed significant downregulation in expression of CCR-5 with $p < 0.05$. The mean fold change of IL-1 β , IFN β , TNF α and CCR-5 are summarised in Table 4.10 and Plate 4.8.

Table 4.10. Relative fold gene expression of IL-1 β , IFN β , TNF α and CCR-5 in AFW, JGW, PLW, and VAW treated macrophages.

Sl. No.	Treatment	IL-1 β Mean fold expression $\pm$ SD	IFN β Mean fold expression $\pm$ SD	TNF α Mean fold expression $\pm$ SD	CCR-5 Mean fold expression $\pm$ SD
1	Control	1	1	1	1
2	AFW	3.454 ± 0.598 **	1.37 ± 0.14 2*	126.02 ± 35.48 **	0.174 ± 0.17 *
3	JGW	0.344 ± 0.205 ns	0.948 ± 0.0 3 ^{ns}	1.424 ± 0.178 ^{ns}	0.518 ± 0.225 *
4	PLW	1.44 ± 0.889 ⁿ s	1.15 ± 0.08 2 ^{ns}	1.44 ± 0.205 ^{ns}	0.582 ± 0.203 ^{ns}
5	VAW	0.105 ± 0.08 ⁿ s	0.92 ± 0.03 8 ^{ns}	1.38 ± 0.136 ^{ns}	0.447 ± 0.192 *

The data are expressed as mean $\pm$ SD with $n=3$. Values bearing superscript** in rows (comparison with Control group) differ significantly with $p < 0.001$ and with superscript* differ significantly with $p < 0.05$. Values bearing superscript ns in rows were non-significant.

We selected VAW for further studies due to its ability to downregulate the pro-inflammatory cytokine IL-1 β . VAW also downregulates the expression of CCR-5 indicating potential for modulating immune cell migration, which is relevant in various diseases. Also, VAW slightly upregulates TNF α , but it is less potent than other extracts, offering a favourable balance.

4.5.3 *In vitro* macrophage activation studies on THP1 cells treated with 25µg/ml isopropyl alcohol extracts of *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima*

PMA reagent was used to convert THP1 cells to macrophages. The macrophages were then treated with 25µg/ml of isopropyl alcohol extracts of *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima* and incubated for 24 hours. DMSO treated cells were used as the control. cDNA was prepared from the isolated RNA and the fold expression level of IL-1 β , IFN β , TNF α and CCR-5 were analysed with internal control GAPDH using qRTPCR. $\Delta\Delta C_t$ method was used for result analysis. The isopropyl alcohol extract of *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima* were denoted as AFI, JGI, PLI and VAI respectively.

The one-way ANOVA results confirmed that significant differences exist among the different treated groups. The treatment of AFI, JGI and VAI upregulates the gene expression of IL-1 β . The post hoc analysis indicated that JGI significantly upregulated the expression of IL-1 β ($p < 0.001$) compared to control.

All the four plant extracts upregulated the expression of TNF α . JGI and PLI significantly upregulated the expression of TNF α ($p < 0.05$) compared to control.

JGI and VAI upregulated the expression of IFN β significantly with $p < 0.001$ compared to the control group. The PLI treated cells downregulates the gene expression of IL-1 β and PLI and AFI treated cells downregulates the fold expression of IFN β .

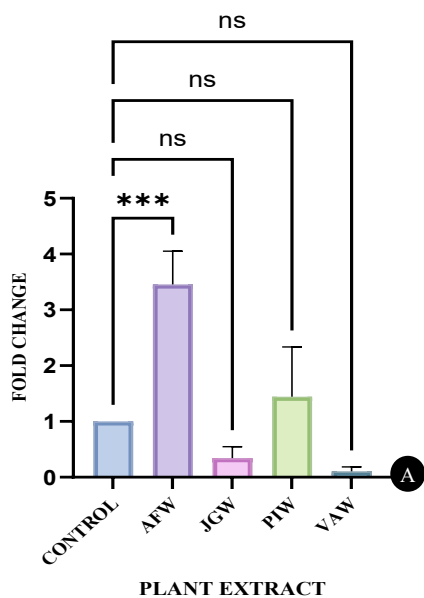
The relative fold expression of CCR-5 was downregulated in JGI and VAI treated cells significantly with control with $p < 0.05$. The relative fold expression of IL-1 β , IFN β , TNF α and CCR-5 are summarised in Table 4.11 and Plate 4.9.

Table 4.11. Relative fold gene expression of IL-1 β , IFN β , TNF α and CCR-5 in AFI, JGI, PLI, and VAI treated macrophages.

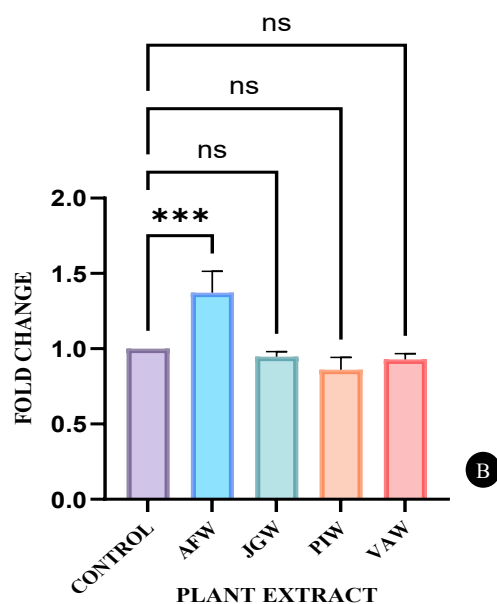
Sl. No.	Treatment	IL-1 β Mean fold expression $\pm$ SD	IFN β Mean fold expression $\pm$ SD	TNF α Mean fold expression $\pm$ SD	CCR-5 Mean fold expression $\pm$ SD
1	Control	1	1	1	1
2	AFI	3.18 $\pm$ 0.10 ^{ns}	0.39 $\pm$ 0.108 ^{ns}	1.89 $\pm$ 0.4 ^{ns}	1.23 $\pm$ 0.12 ^{ns}
3	JGI	30.85 $\pm$ 5.7 ^{**}	2.57 $\pm$ 0.432 ^{**}	7.48 $\pm$ 0.88 [*]	0.24 $\pm$ 0.06 [*]
4	PLI	0.93 $\pm$ 0.03 ^{ns}	0.84 $\pm$ 0.032 ^{ns}	2.44 $\pm$ 0.67 [*]	1.27 $\pm$ 0.139 ^{ns}
5	VAI	2.51 $\pm$ 0.21 ^{ns}	2.32 $\pm$ 0.31 ^{**}	1.86 $\pm$ 0.23 ^{ns}	0.27 $\pm$ 0.152 [*]

The data are expressed as mean $\pm$ SD with n=3. Values bearing superscript^{**} in rows (comparison with Control group) differ significantly with $p < 0.001$ and with superscript^{*} differ significantly with $p < 0.05$. Values bearing superscript ns in rows were non-significant.

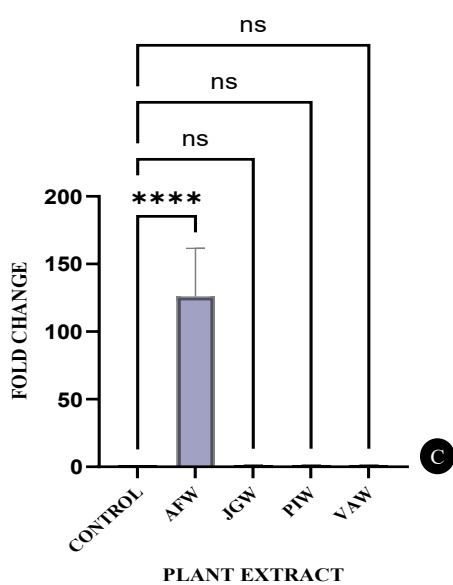
GENE EXPRESSION OF IL-1 β



GENE EXPRESSION OF IFN β



GENE EXPRESSION OF TNF α



GENE EXPRESSION OF CCR-5

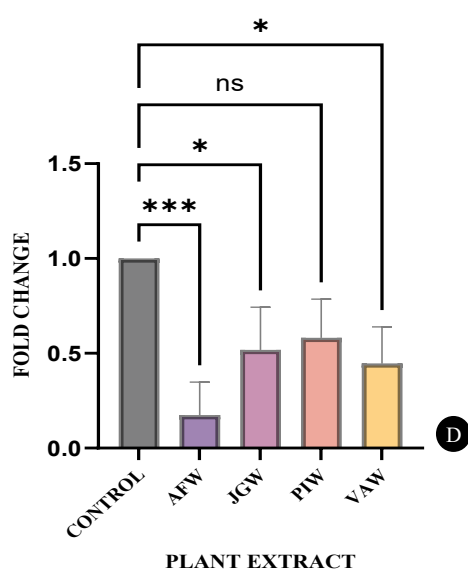


Plate 4.8. Figure A-D. Relative gene expression of IL-1 β , IFN β , TNF α and CCR-5 in 25 μ g/ml water extracts of *A. fruticosa* (AFW), *J. gendarussa* (JGW), *P. longum* (PLW) and *V. altissima* (VAW) treated macrophages. The data are expressed as mean $\pm$ SD with n=3. **** indicates p<0.0001, *** p<0.001, ** p<0.01, *p<0.05. ns indicates p>0.05. * indicates significant difference from control group.

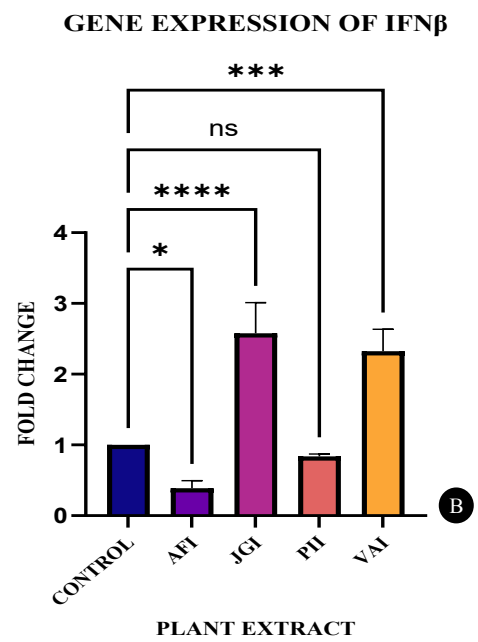
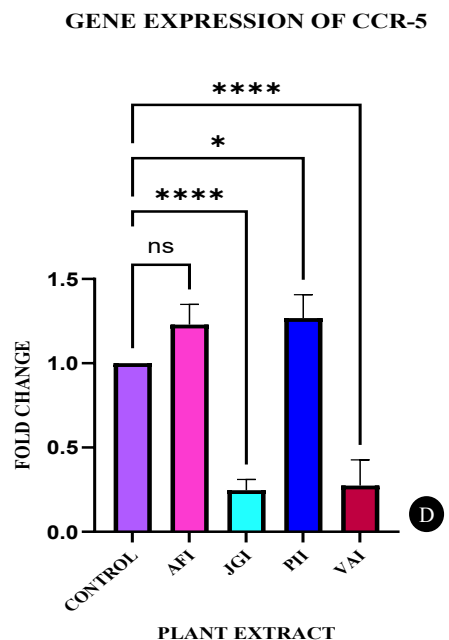


Plate 4.9. Figure A-D. Relative fold gene expression of IL-1 β , IFN β , TNF α and CCR-5 in 25 μ g/ml isopropyl alcohol extracts of *A. fruticosa* (AFI), *J. gendarussa* (JGI), *P. longum* (PLI) and *V. altissima* (VAI) treated macrophages. The data are expressed as mean $\pm$ SD with n=3. **** indicates p<0.0001, *** p<0.001, ** p<0.01, *p<0.05. ns indicates p>0.05. * indicates significant difference from control group.

VAI is a strong candidate for further studies due to its dual action of upregulating IFN β , IL-1 β and TNF α and downregulating CCR-5. This result indicates a balanced immune activation rather than purely harmful inflammation. Its unique profile suggests potential for specific immunomodulatory applications.

Based on these results and literature support, *V. altissima* was selected for further analysis due to its consistent immunomodulatory effects across extracts. THP1 cells were used to study the immunomodulatory properties of these plants. The THP1 cells are commonly using monocytic cell lines for *in vitro* experiments. THP1 cell line was an appropriate *in vitro* model to study the responses of monocytes and macrophages (Chanput et al., 2014).

4.6 Viability assay to choose the concentration of *V. altissima* extract with more than 80% cell viability

Next, we need to study the immunomodulation properties of hexane, water and isopropyl alcohol extracts of *V. altissima* at different concentrations. To study the viability of macrophages treated with hexane, water and isopropyl alcohol extracts of *V. altissima* (VAH, VAW and VAI), MTT assay was used. 15, 25, 50, 100 μ g/ml hexane, water and isopropyl alcohol extract was used for viability assay. The cytotoxicity of VAH, VAW and VAI was analysed with THP1 derived macrophages.

From our study we found that VAH treated macrophages exhibit viability below 70% at 50 μ g/ml and 100 μ g/ml extract concentration. VAI treated macrophages exhibit viability below 70% at 100 μ g/ml extract concentration. VAW treated cells exhibit viability above 70% in all treated concentrations. All the extracts showed a decrease in viability percentage with increase in concentration of extract. The least cytotoxic effect was exhibited by VAW. The LC₅₀ value was also calculated for VAH, VAW and VAI. The LC₅₀ value of VAH was 100.67 μ g/ml, VAW was 281.51 μ g/ml and for VAI was 130.11 μ g/ml.

The one-way ANOVA results indicated that VAH, VAW and VAI exhibited dose dependent cytotoxicity exists between extracts. Turkey's HSD post hoc analysis revealed that percentage viability of VAH, VAW and VAI showed significant reduction at 15 μ g/ml, 25 μ g/ml, 50 μ g/ml and 100 μ g/ml concentration with $p < 0.001$. Significant differences existed between 25 μ g/ml-50 μ g/ml ($p < 0.001$), 50 μ g/-100 μ g/ml ($p < 0.001$) concentration treated groups of VAI. The viability percentage of VAH, VAW and VAI was given in Table 4.12 and photographs and graphs of MTT assay were given in Plate 4.10-4.11.

Table 4.12. Percentage viability of macrophages treated with 15 to 100µg/ml concentration of VAH, VAW AND VAI.

SL. No.	Sample Concentration	% Viability VAH	% Viability VAW	% Viability VAI
1	CONTROL	100	100	100
2	15µg/ml	91.70±0.66*	95.96±1.39*	91.25±0.12**+
3	25µg/ml	88.81±3.22**	91.96±2.5*	88.71±1.78**
4	50µg/ml	67.64±4.88**+	86.43±1.89**+	74.68±3.45**++
5	100µg/ml	53.21±2.02**++	82.19±2.14**	62.97±0.84**++

The data are expressed as mean ± SD with n=3. Values bearing superscript** in rows (comparison with Control group) differ significantly with p<0.001 and with superscript* differ significantly with p<0.05. Values bearing superscript ++ in rows (comparison with treated group) differ significantly with p<0.001 and with superscript + differ significantly with p<0.05.

Our results showed that the viability percentage decreases with increase in concentration of extract. Viability below 70% can be considered as cytotoxic (Standard, 2009). We selected concentrations showing above 80% viability of cells for our further studies. VAH and VAI extract at higher concentrations was more cytotoxic than VAW. We selected 10, 15 and 25µg/ml extract concentrations for our further studies. All concentrations up to 25µg/ml showed above 80% viability.

The cytotoxic properties of VAH, VAW and VAI can be used to study further anticancer properties of *V. altissima*. However, our assumption requires further verification with *in vitro* and *in vivo* cancer models.

4.7 Immunomodulatory activity of *V. altissima*

4.7.1 *In vitro* macrophage activation studies on THP1 cells treated with 10, 15 and 25µg/ml *V. altissima* hexane extract (VAH)

To study the immunomodulatory property of VAH, the THP-1-derived macrophages were treated with 10, 15 and 25µg/ml extracts. The macrophages treated with DMSO served as the control. RNA was isolated from the treated macrophages and cDNA was prepared from the isolated RNA. The fold expression level of IL-1β, IFNβ, TNFα and CCR-5 were analysed with housekeeping GAPDH using qRT-PCR. ΔΔCt method was used for result analysis.

We found a concentration dependent increase in expression of IL-1β, IFNβ, TNFα and CCR-5. The fold change of IL-1β increases from 1.622±0.004 at 10µg/ml (p<0.05) to

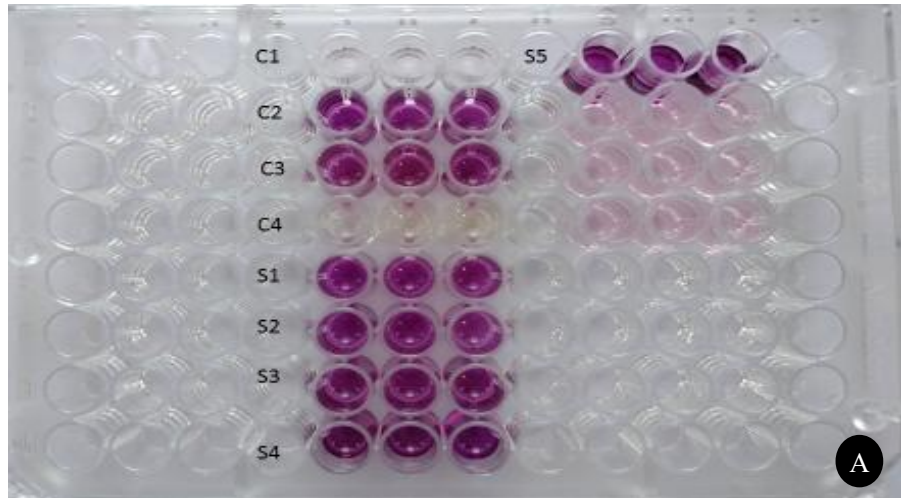
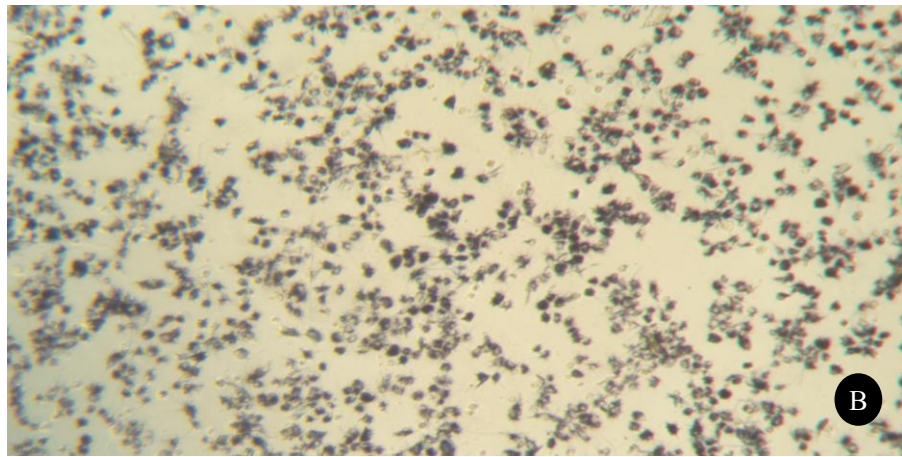
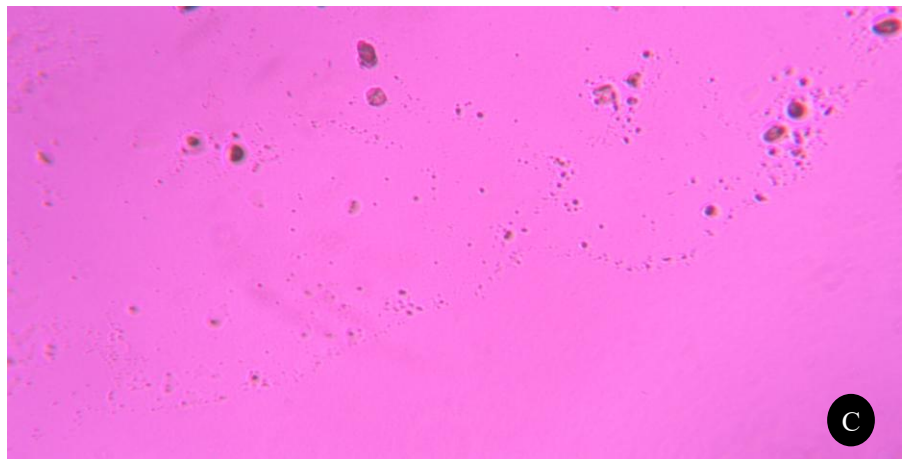


Plate 4.10. MTT assay of *V. altissima* treated macrophages. Figure A. *V. altissima* extract at different concentrations treated cells treated with MTT reagent in 96 well plates. S1, S2, S3 and S4 represents sample concentrations 15, 25, 50 and 100 μ g/ml. C1, C2, C3, C4 are control plates. C1 – only cell culture media, C2- cells and cell culture media, C3- cells with cell culture media with DMSO (negative control), C4- cells treated with Triton X 100 (positive control). Figure B. Microscopic view of formation of formazan crystals in MTT treated cells. Figure C. Microscopic view of dissolution of formazan crystals by solubilisation reagent.

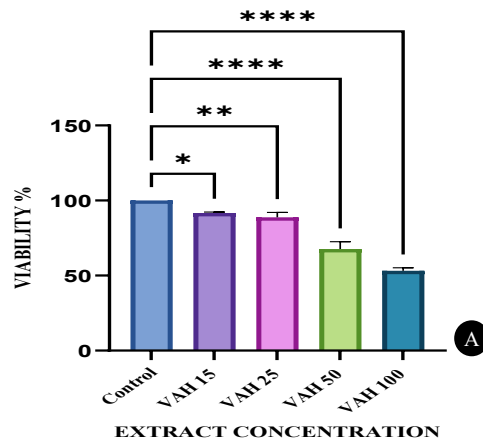
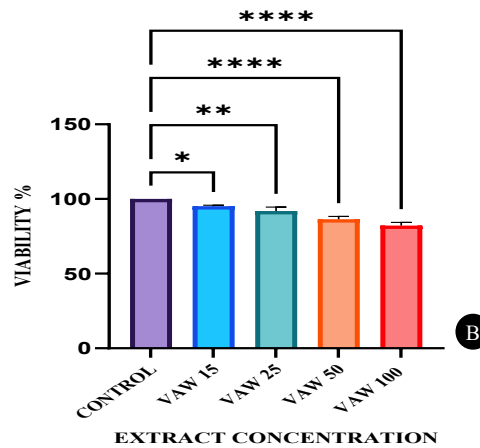
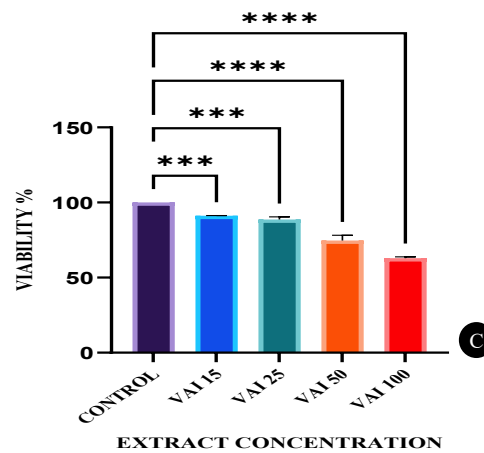


Plate 4.11. MTT assay of *V. altissima* treated macrophages. Figure A. Viability percentage of macrophages treated with 15, 25, 50 and 100µg/ml concentration of *V. altissima* hexane extract. Figure B. Viability percentage of macrophages treated with 15 to 100µg/ml concentration of *V. altissima* water extracts. Figure C. Viability percentage of macrophages treated with 15 to 100µg/ml concentration of *V. altissima* isopropyl alcohol extract. The data are expressed as mean $\pm$ SD with n=3. **** indicates $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$. ns indicates $p>0.05$. * indicates significant difference from control group.

4.35±0.173 at 25µg/ml (p<0.001 compared to control) concentration of VAH. For IFNβ, fold change increases from 2.22±0.189 at 10µg/ml to 4.65±0.33 at 25µg/ml concentration of VAH with p<0.001. At 10µg/ml concentration of VAH, the fold change was 1.67±0.12 (p<0.05) and at 25µg/ml the fold change was 3.57±0.168 (p<0.001) for TNFα. For CCR-5, the fold change was 2.31±0.058 at 10µg/ml concentration and 3.212±0.18 at 25µg/ml with p<0.001. Significant differences were observed between the treated groups with p<0.001 for the expression of IL-1β, TNFα and CCR-5 and with p<0.05 for IFNβ. The relative fold gene expression of IL-1β, IFNβ, TNFα and CCR-5 are summarised in Table 4.13 and Plate 4.12.

Table 4.13. Relative fold gene expression of IL-1β, IFNβ, TNFα and CCR-5 in VAH treated macrophages.

Sl. No.	Treatment	IL-1β Mean fold expression ± SD	IFNβ Mean fold expression ± SD	TNFα Mean fold expression ± SD	CCR-5 Mean fold expression ± SD
1	Control	1	1	1	1
2	10µg/ml	1.622±0.04*	2.22±0.189* *	1.67±0.12*	2.31±0.058**
3	15µg/ml	3.41±0.29*** ++	3.4±0.297** +	3.29±0.28** ++	3.01±0.132*** +
4	25µg/ml	4.35±0.173*** +	4.65±0.33** +	3.57±0.168*** +	3.212±0.18*** +

The data are expressed as mean ± SD with n=3. Values bearing superscript** in rows (comparison with Control group) differ significantly with p<0.001 and with superscript* differ significantly with p<0.05. Values bearing superscript ++ in rows (comparison with treated group) differ significantly with p<0.001 and with superscript + differ significantly with p<0.05. Values bearing superscript ns are non-significant.

4.7.2 *In vitro* macrophage activation studies on THP1 cells treated with 10, 15 and 25µg/ml *V. altissima* water extract (VAW).

The THP-1-derived macrophages were treated with 10, 15 and 25µg/ml extract concentration to study the immunomodulatory property of VAW. The DMSO treated macrophages were taken as the control. cDNA was prepared from the isolated RNA from treated macrophages. qRT-PCR was used to analyse the expression of IL-1β, IFNβ, TNFα and CCR-5.

The VAW treated cells showed a decrease in expression of IL-1 β with increase in concentration of extract. The fold change of IL-1 β was 2.84 $\pm$ 0.002 at 10 μ g/ml concentration and reduced to 1.33 $\pm$ 0.05 at 25 μ g/ml concentration of VAW with $p < 0.001$ compared to control. The VAW significantly downregulates the expression of IFN β at all concentrations with $p < 0.001$. There is significant downregulation in the expression of TNF α at 10 μ g/ml compared to control in VAW treated cells. For CCR-5, the fold change was 2.33 $\pm$ 0.15 at 25 μ g/ml concentration. 15 μ g/ml and 25 μ g/ml concentration of VAW upregulates the expression of CCR-5 with $p < 0.001$. Significant differences were observed between the treated groups in the expression of IL-1 β , IFN β , TNF α and CCR-5 with $p < 0.001$. The relative fold change of IL-1 β , IFN β , TNF α and CCR-5 are presented in Table 4.14 and Plate 4.13.

Table 4.14. Relative fold gene expression of IL-1 β , IFN β , TNF α and CCR-5 in VAW treated macrophages.

Sl. No.	Treatment	IL-1 β Mean fold expression $\pm$ SD	IFN β Mean fold expression $\pm$ SD	TNF α Mean fold expression $\pm$ SD	CCR-5 Mean fold expression $\pm$ SD
1	Control	1	1	1	1
2	10 μ g/ml	2.84 $\pm$ 0.002 **	0.66 $\pm$ 0.002**	0.915 $\pm$ 0.007*	0.75 $\pm$ 0.006*
3	15 μ g/ml	1.69 $\pm$ 0.046 ***+	0.949 $\pm$ 0.008* *++	1.06 $\pm$ 0.013** ++	1.85 $\pm$ 0.027***++
4	25 μ g/ml	1.33 $\pm$ 0.05** ++	1.209 $\pm$ 0.134* *++	1.21 $\pm$ 0.062** ++	2.33 $\pm$ 0.15***++

The data are expressed as mean $\pm$ SD with n=3. Values bearing superscript** in rows (comparison with Control group) differ significantly with $p < 0.001$ and with superscript* differ significantly with $p < 0.05$. Values bearing superscript ++ in rows (comparison with treated group) differ significantly with $p < 0.001$ and with superscript + differ significantly with $p < 0.05$. Values bearing superscript ns are non-significant.

4.7.3 *In vitro* macrophage activation studies on THP1 cells treated with 10, 15 and 25 μ g/ml *V. altissima* isopropyl alcohol extract (VAI).

For studying the immunomodulation property of VAI, 10, 15 and 25 μ g/ml extract concentration was treated with THP1 derived macrophages. The macrophages treated with DMSO were used as the control. The RNA was isolated from treated macrophages and cDNA

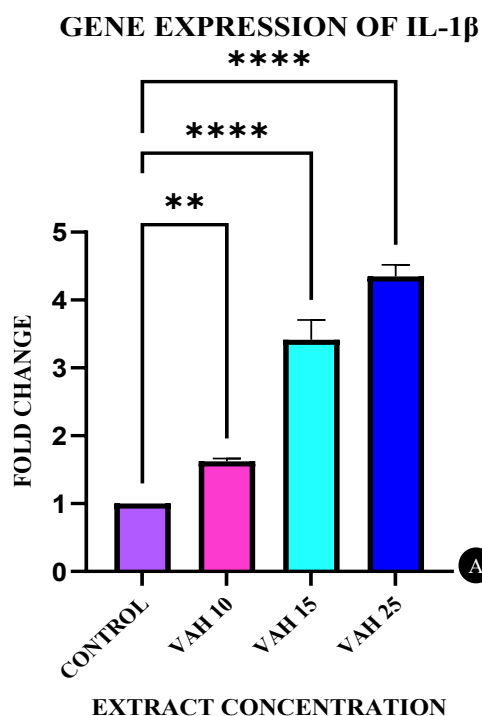
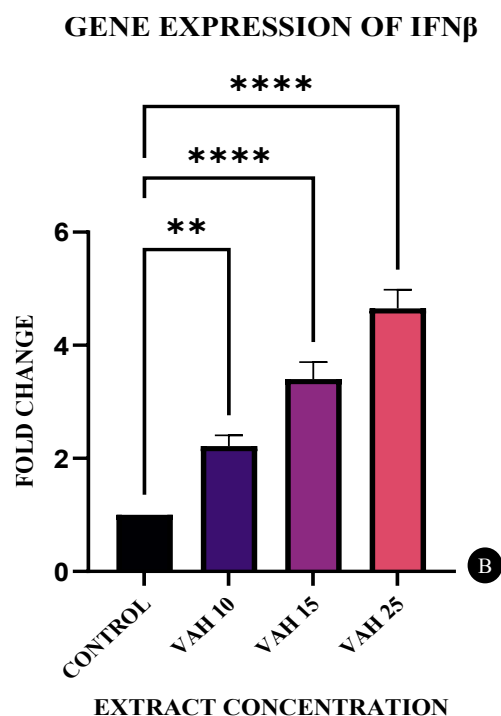
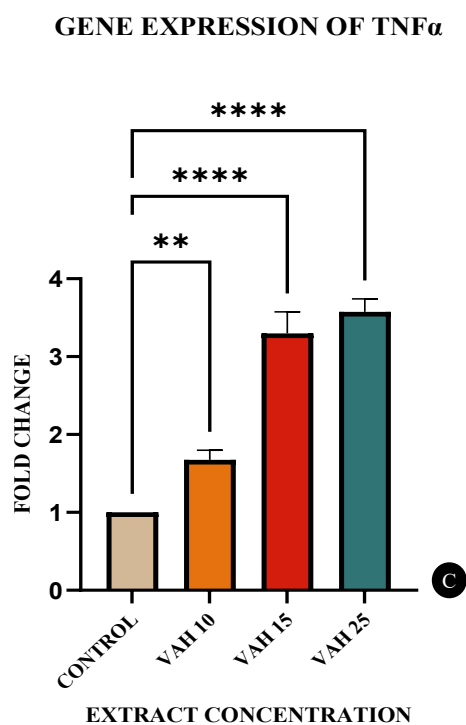
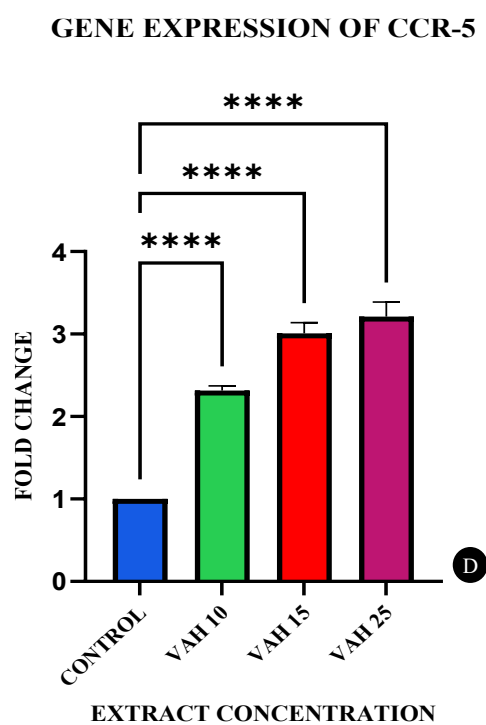


Plate 4.12. *In vitro* macrophage activation studies in *V. altissima* hexane extract (VAH) treated cells. Figure A-D. Relative gene expression of IL-1 β , IFN β , TNF α and CCR-5 in 10, 15 and 25 μ g/ml *V. altissima* hexane extract treated macrophages. The data are expressed as mean $\pm$ SD with n=3. **** indicates p<0.0001, *** p<0.001, ** p<0.01, *p<0.05. ns indicates p>0.05. * indicates significant difference from control group.

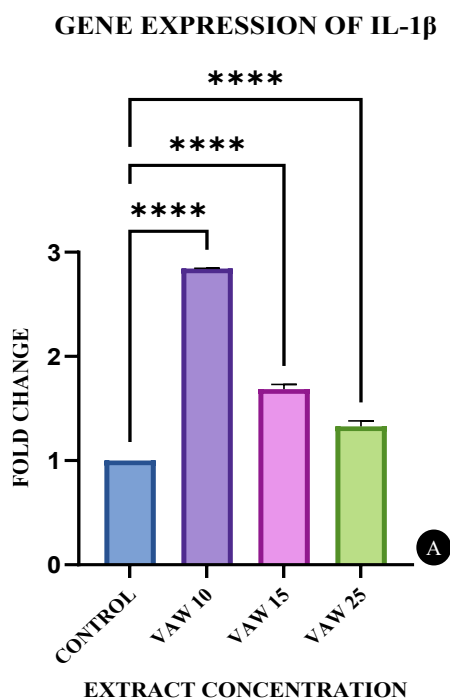
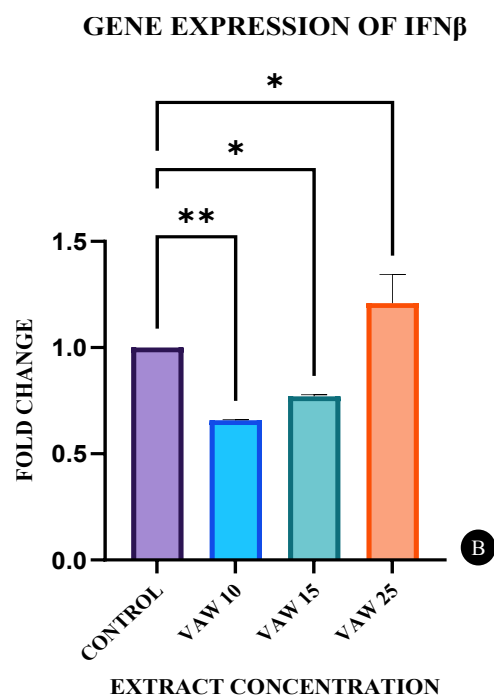
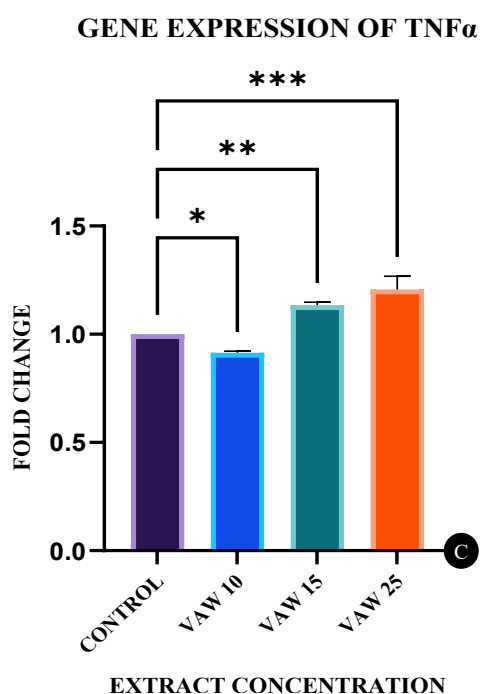
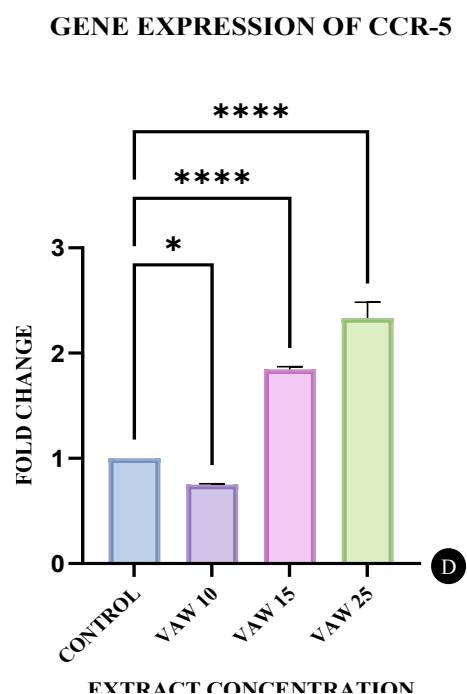


Plate 4.13. *In vitro* macrophage activation studies in *V. altissima* water extract (VAW) treated cells. Figure A-D. Relative gene expression of IL-1 β , IFN β , TNF α and CCR-5 in 10, 15 and 25 μ g/ml *V. altissima* water extract treated macrophages. The data are expressed as mean $\pm$ SD with n=3. **** indicates p<0.0001, *** p<0.001, ** p<0.01, *p<0.05. ns indicates p>0.05. * indicates significant difference from control group.

was prepared for qRT-PCR. The gene expression of IL-1 β , IFN β , TNF α and CCR-5 were analysed and compared with GAPDH.

The VAI treated cells showed a decrease in expression of all genes with increase in concentration of extract. The fold change of IL-1 β significantly reduced from 3.13 $\pm$ 0.189 at 10 μ g/ml concentration to 2.10 $\pm$ 0.06 at 25 μ g/ml concentration of VAI with $p < 0.001$ when compared with control. The fold change of IFN β for VAI treated macrophages reduced from 3.4 $\pm$ 0.15 ($p < 0.001$), at 10 μ g/ml concentration to 1.8 $\pm$ 0.06 at 25 μ g/ml. For TNF α the mRNA expression was 2.74 $\pm$ 0.43 ($p < 0.001$) at 10 μ g/ml and 0.342 $\pm$ 0.018 fold at 25 μ g/ml concentration. The fold gene expression of CCR-5 was 2.19 $\pm$ 0.012 at 10 μ g/ml and 0.53 $\pm$ 0.07 at 25 μ g/ml concentration of VAI. The mean fold change of IL-1 β , IFN β , TNF α and CCR-5 are presented in Table 4.15 and Plate 4.14.

Table 4.15. Relative fold gene expression of IL-1 β , IFN β , TNF α and CCR-5 in VAI treated macrophages

Sl. No.	Treatment	IL-1 β Mean fold expression $\pm$ SD	IFN β Mean fold expression $\pm$ SD	TNF α Mean fold expression $\pm$ SD	CCR-5 Mean fold expression $\pm$ SD
1	Control	1	1	1	1
2	10 μ g/ml	3.13 $\pm$ 0.189 ^{**+}	3.4 $\pm$ 0.15 ^{**}	2.74 $\pm$ 0.43 ^{**}	2.19 $\pm$ 0.012 ^{**}
3	15 μ g/ml	2.57 $\pm$ 0.15 [*] ₊	2.9 $\pm$ 0.305 ^{**+}	2.02 $\pm$ 0.24 ⁺	2.059 $\pm$ 0.29 ^{**+}
4	25 μ g/ml	2.10 $\pm$ 0.06 [*] ₊	1.8 $\pm$ 0.06 ⁺	0.342 $\pm$ 0.18 ⁺	0.53 $\pm$ 0.07 ⁺⁺

The data are expressed as mean $\pm$ SD with n=3. Values bearing superscript^{**} in rows (comparison with Control group) differ significantly with $p < 0.001$ and with superscript^{*} differ significantly with $p < 0.05$. Values bearing superscript ++ in rows (comparison with treated group) differ significantly with $p < 0.001$ and with superscript + differ significantly with $p < 0.05$. Values bearing superscript ns are non-significant.

From the results we found that VAI showed a concentration-dependent upregulation of all tested genes IL-1 β , IFN β , TNF α , and CCR-5. This indicates VAI is a potent immunostimulatory agent, capable of inducing key pro-inflammatory cytokines (IL-1 β , TNF α) and IFN β . Furthermore, the dose-dependent increase in CCR-5 expression implies VAI may also enhance macrophage recruitment or modulate their migratory behavior. Overall, VAI exhibits an immunomodulatory profile.

VAW was an interesting candidate for modulating IL-1 β -driven inflammation without strongly activating TNF α pathways, and suppressing IFN β and enhancing CCR-5. This extract possessed anti-inflammatory effects particularly targeting IL-1 β .

VAI immunomodulatory properties are concentration-dependent, with a trend towards suppressing or reducing the expression of both pro-inflammatory (IL-1 β , TNF α), IFN β and CCR-5 at higher doses. These results suggested VAI could be a promising candidate for anti-inflammatory therapies. At its lowest tested concentration (10 μ g/ml), VAI acts as an immunostimulant, upregulating IL-1 β , IFN β , TNF α , and CCR-5. However, as the concentration increases to 15 and 25 μ g/ml, VAI's effect shifts towards suppressing the expression of these genes, with TNF α and CCR-5 expression being significantly downregulated below control at the highest dose. This dose-dependent decrease in expression, particularly for pro-inflammatory markers, suggests that VAI might have a dual role, with lower concentrations stimulating and higher concentrations potentially having a moderating or anti-inflammatory effect.

4.8 LCMS analysis of *V. altissima*

OHR-LCMS analysis of hexane, water and isopropyl alcohol extracts were done at SAIF, IIT Bombay. The list of compounds with its bioactivity obtained from LCMS analysis of *V. altissima* hexane (VAH), water (VAW) and isopropyl alcohol (VAI) extract was given in Table 5, 6 and 7 respectively. The isopropyl alcohol extract of *V. altissima* LCMS analysis revealed the presence of 55 compounds, hexane extract revealed the presence of 55 compounds and water extract revealed the presence of 89 compounds.

33 phenolics, 13 flavonoids, 8 alkaloids, 2 anthocyanins, 1 glycoside, 4 terpenoids, 3 coumarins, 1 glycoside, 3 tannin and 1 lignan were present in water extract of *V. altissima*. Phenolics were the dominating group of secondary metabolites in water extracts. 5 phenols, 5 terpenoids, 19 fatty acids, 5 alkaloids, 1 lignan, 1 flavonoid and 1 coumarin were present in the hexane extract of *V. altissima*. 5 phenols, 16 flavonoids, 2 anthraquinones, 4 terpenoids, 13 fatty acids and 3 coumarins were obtained from the LCMS analysis of isopropyl alcohol extract of *V. altissima*. The chromatogram of LCMS analysis and radial tree diagram representing compounds with bioactivity obtained from OHR-LCMS analysis of hexane, water and isopropyl alcohol extract of *V. altissima* are given in Plate 4.15-4.23.

LCMS analysis of plant extracts provides a more comprehensive characterisation of phytochemicals present in the plants. The LCMS analysis of plant extracts led to the identification of various secondary metabolites like phenols, flavonoids, alkaloids, tannins etc.

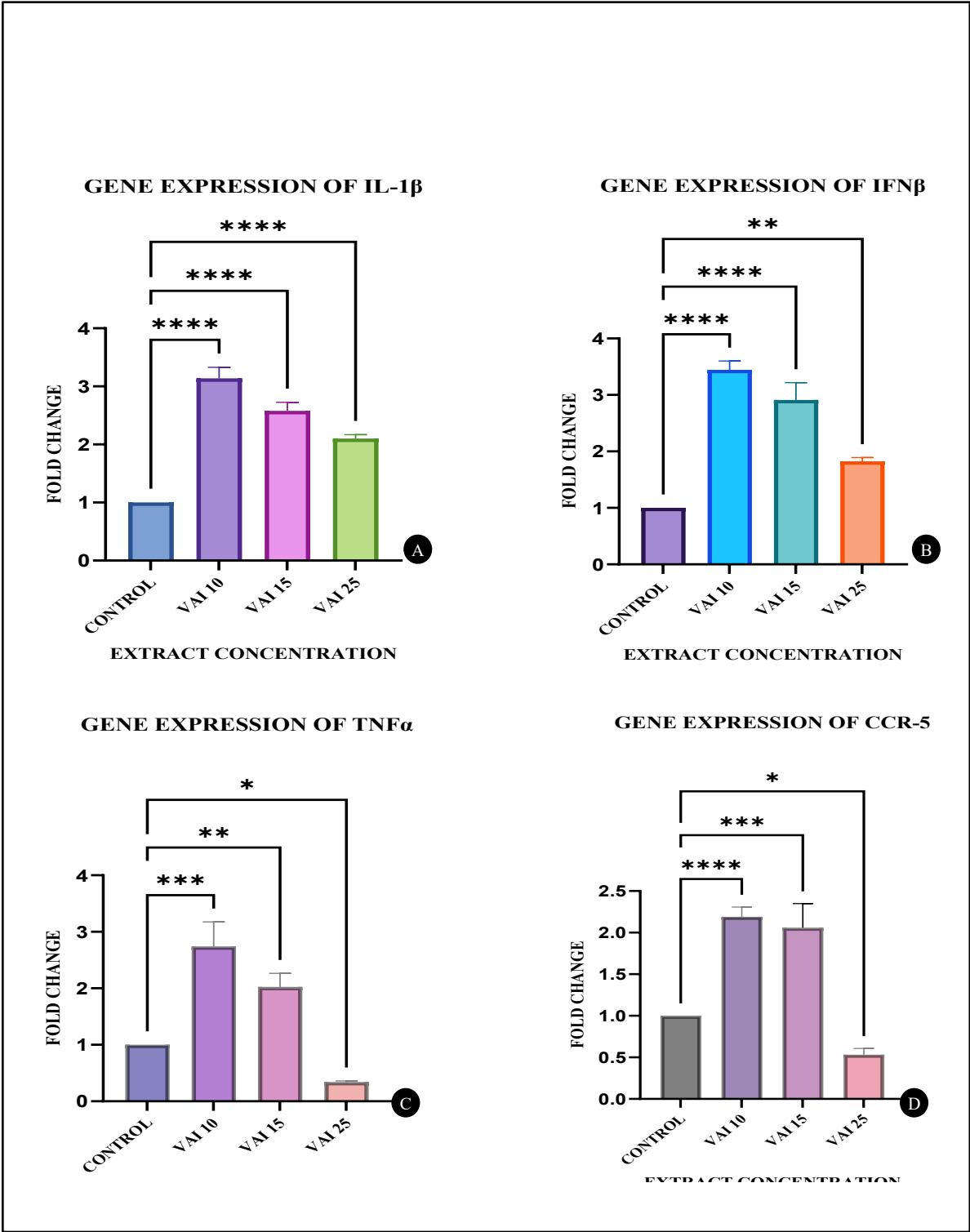


Plate 4.14. *In vitro* macrophage activation studies in *V. altissima* isopropyl alcohol extract (VAI) treated cells. Figure A-D. Relative gene expression of IL-1 β , IFN β , TNF α and CCR-5 in 10, 15 and 25 μ g/ml *V. altissima* isopropyl alcohol extract treated macrophages. The data are expressed as mean $\pm$ SD with n=3. **** indicates p<0.0001, *** p<0.001, ** p<0.01, *p<0.05. ns indicates p>0.05. * indicates significant difference from control group.

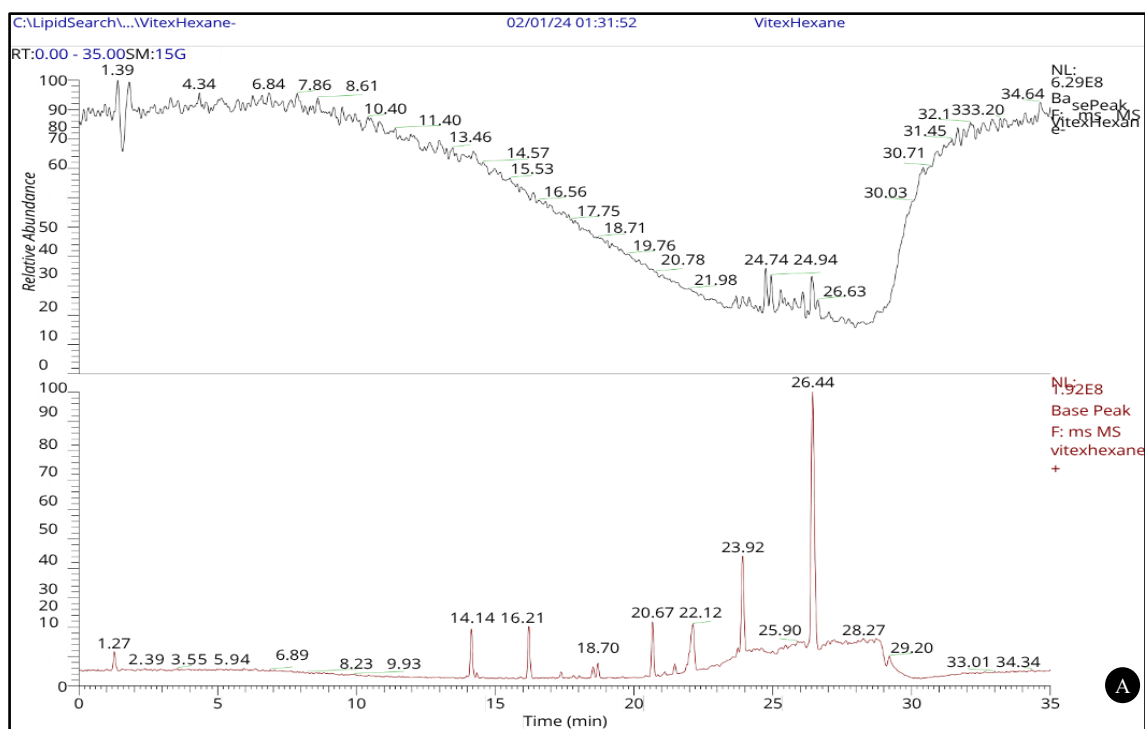
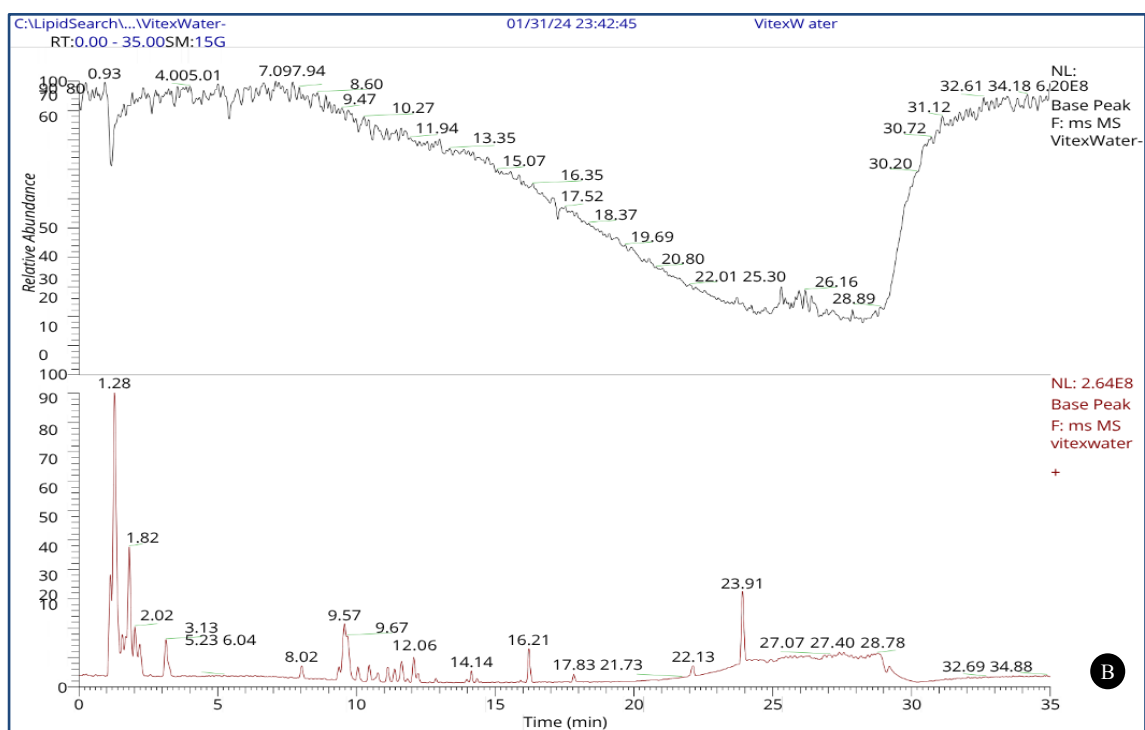


Plate 4.15. Chromatogram of *Vitex altissima*. Figure A. Chromatogram of *V. altissima* hexane extract (VAH) obtained from OHR LCMS analysis. Figure B. Chromatogram of *V. altissima* water extract (VAW) obtained from OHR LCMS analysis.

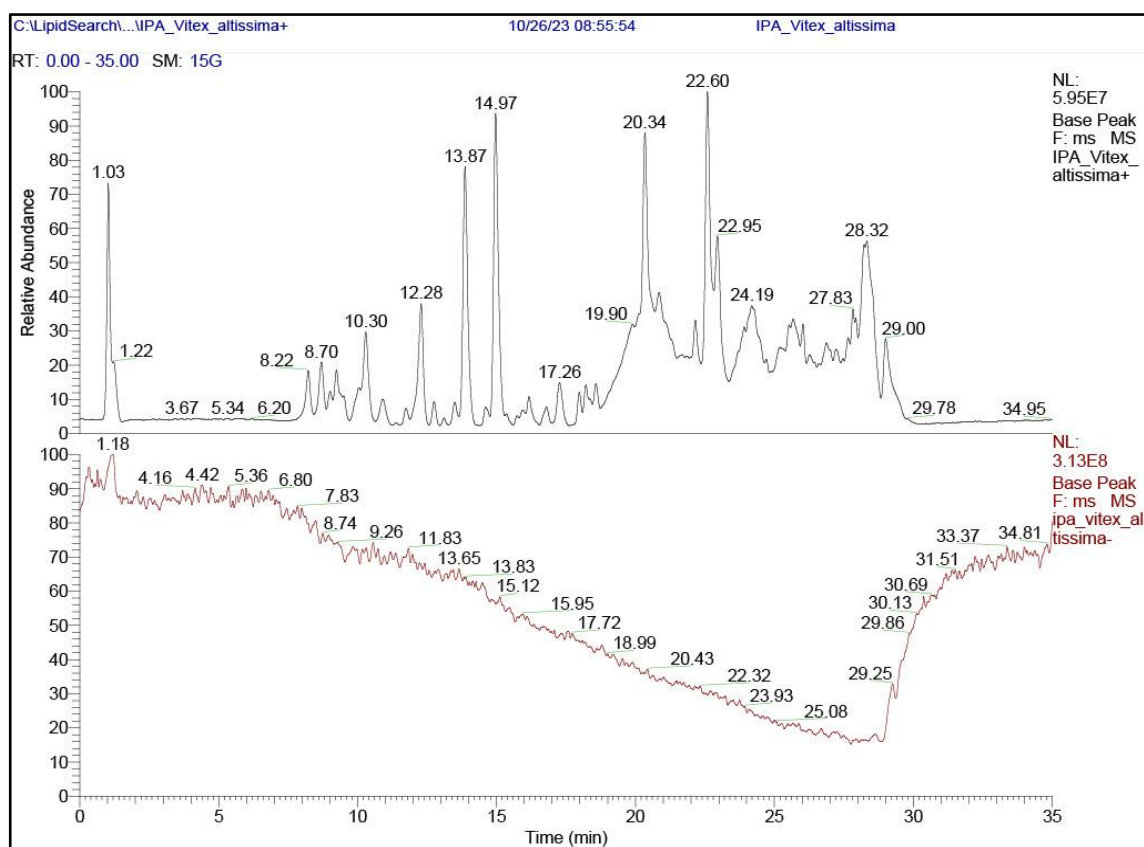


Plate 4.16. Chromatogram of *V. altissima* isopropyl alcohol extract (VAI) obtained from OHR LCMS analysis.

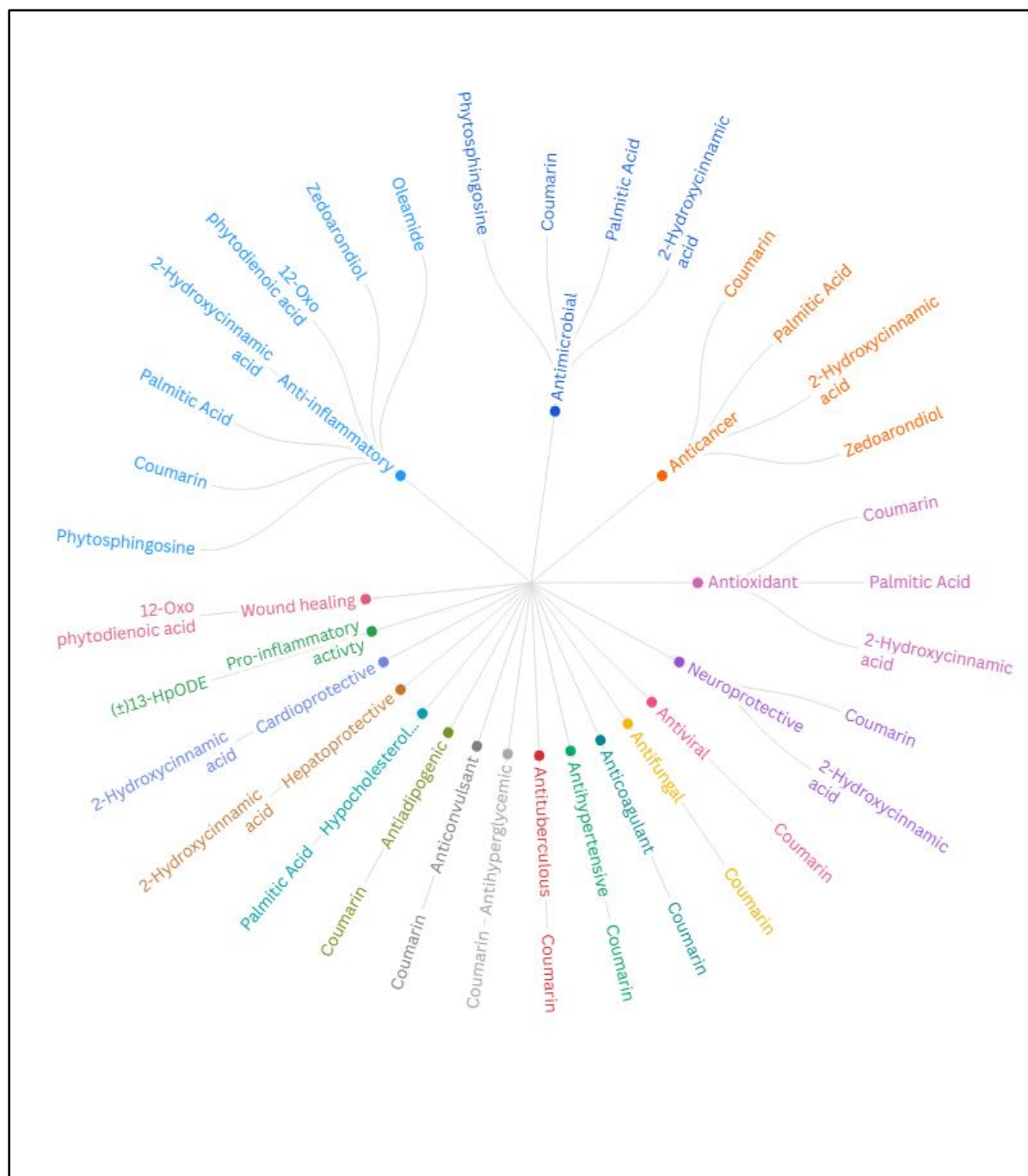


Plate 4.17. Compounds with bioactivity obtained from OHR LCMS analysis of hexane extract of *V. altissima*.

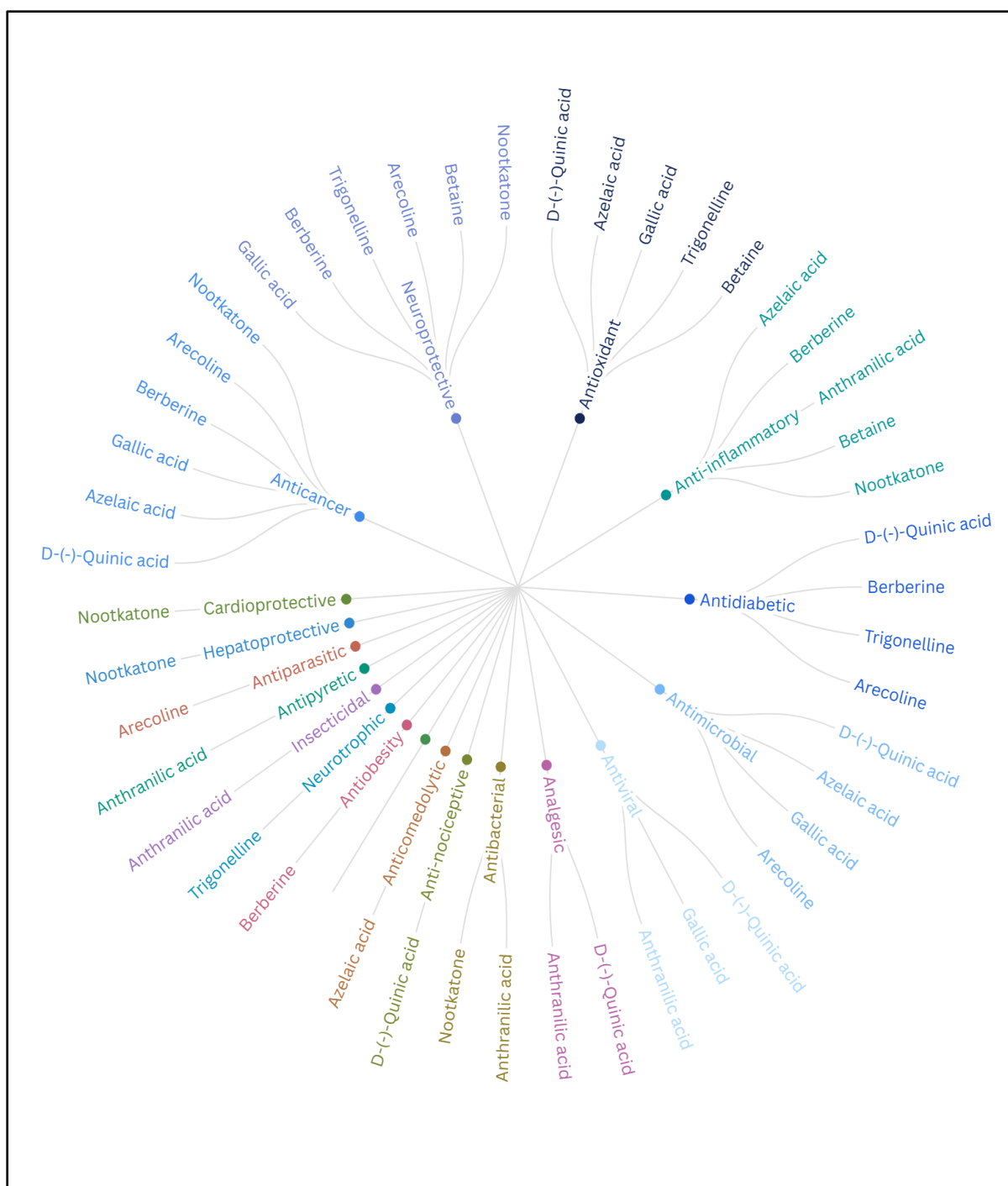


Plate 4.18. Compounds with bioactivity obtained from OHR LCMS analysis of hexane extract of *V. altissima*.

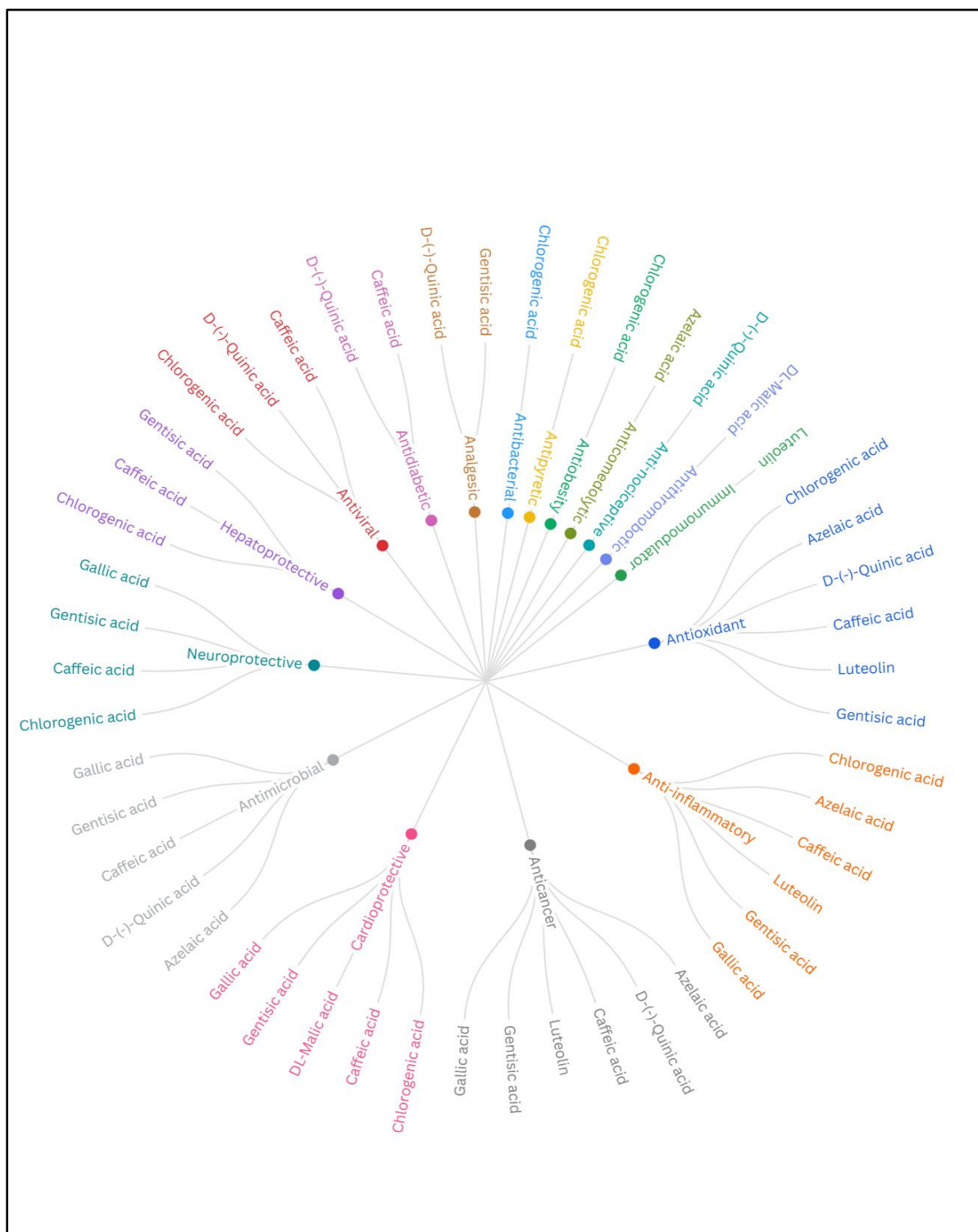


Plate 4.20. Compounds with bioactivity obtained from OHR LCMS analysis of water extract of *V. altissima*.

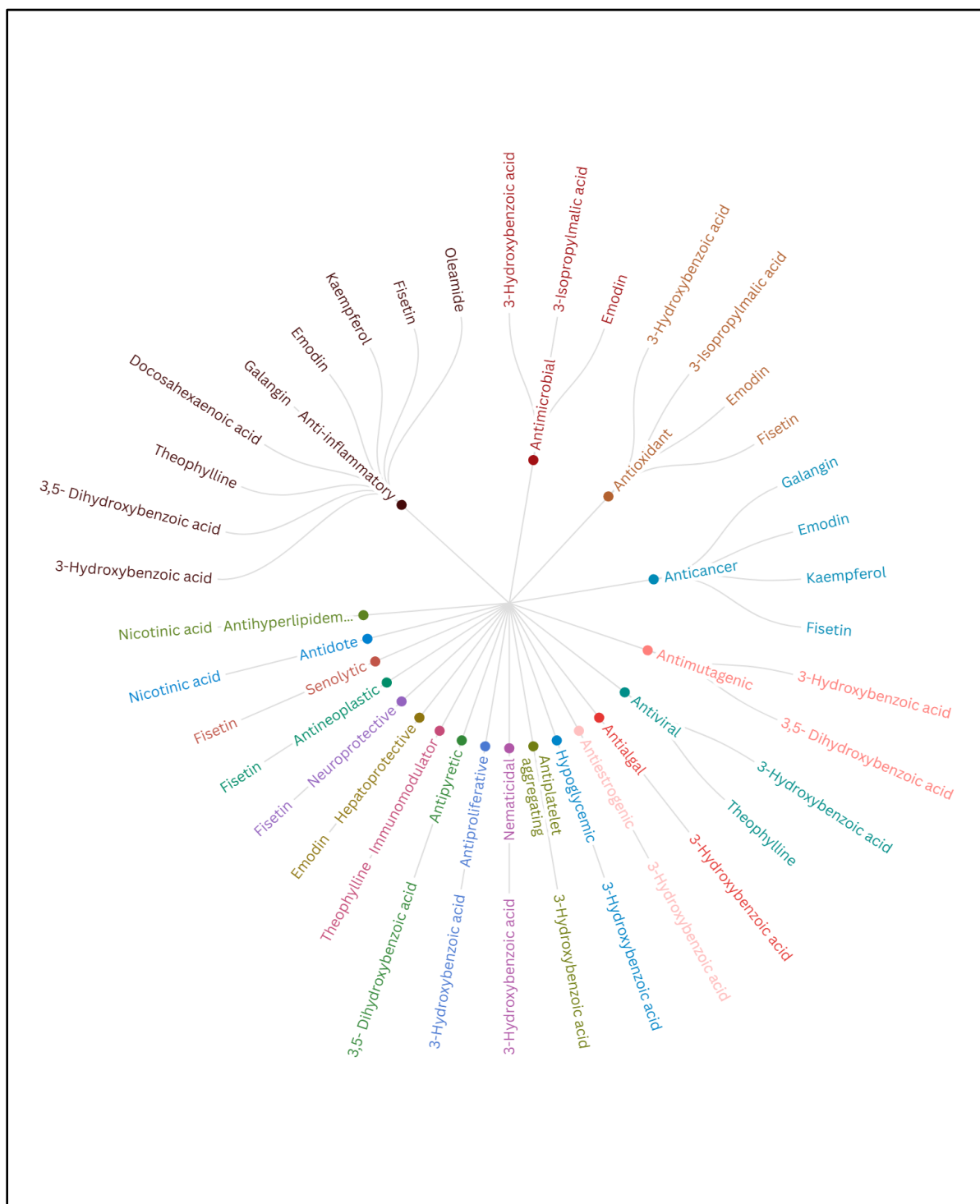


Plate 4.22. Compounds with bioactivity obtained from OHR LCMS analysis of water extract of *V. altissima*.

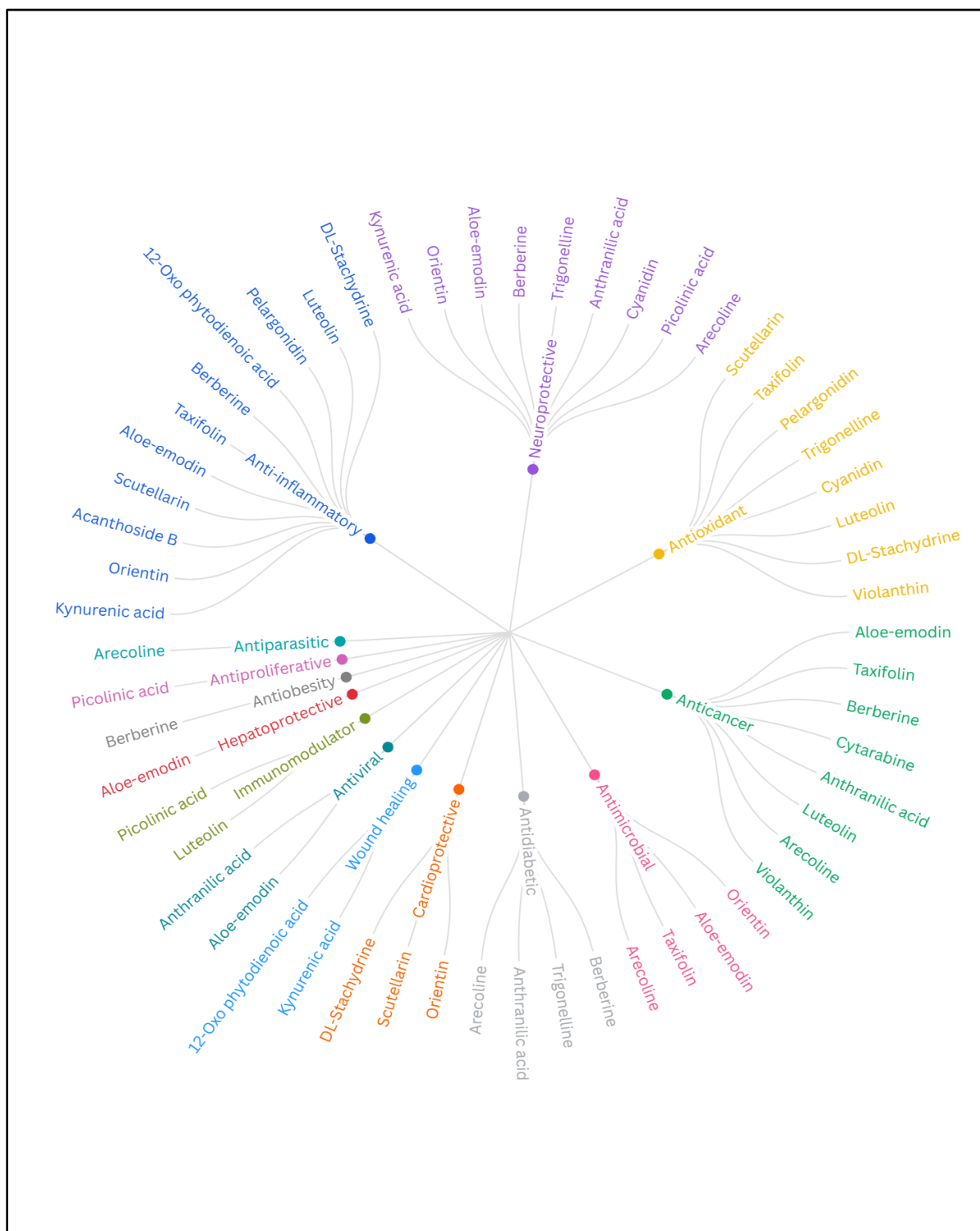


Plate 4.23. Compounds with bioactivity obtained from LCMS analysis of water extract of *V. altissima*.

Our study revealed the presence of 89, 55 and 55 compounds from water, hexane and isopropyl alcohol extracts of *V. altissima*. Phenolics were more prevalent in VAW, VAH exhibited a higher content of fatty acids and flavonoids were the dominating group in VAI. All these compounds possessed various bioactivities like antioxidant, antitumor, anti-inflammatory, neuroprotective, antimicrobial etc. as summarised in Table 4.16-4.18.

Table 4.16. List of compounds from OHR LCMS analysis of *V. altissima* hexane extract with reported bioactivity.

Sl. No.	Name Of Compounds	Formula	Class Of Compounds	Bioactivity	References
1	D- (-) - Quinic acid	C ₇ H ₁₂ O ₆	Phenols	Antioxidant, antidiabetic, anticancer, antimicrobial, antiviral, analgesic, anti-nociceptive and activity	(Benali et al., 2022)
2	NP-014839 [8-(4-Hydroxyphenyl)-3- methoxy-6,7-dimethyl-5,6,7,8-tetrahydro naphthalen-2-ol]	C ₁₉ H ₂₂ O ₃	Unknown	Unknown	-
3	NP-021701 [8,9-Dihydroxy-2,10,10-trimethyl tricyclo[6.3.0.01,5] undec-6-ene-6-carboxylic acid]	C ₁₅ H ₂₂ O ₄	Phenolic compound	Unknown	-
4	NP-012534 ([5-Hydroxy-3-(hydroxymethyl)-2-oxo-6-propan-2-ylcyclohex-3-en-1-yl] 3-methylbutanoate)	C ₁₅ H ₂₄ O ₅	Monoterpenoid	Unknown	-
5	NP-006255(2-(6-Hydroxy-1-methyl-2-methylidene-9-propan-2-yl-5-oxatricyclo [5.4.0.03,8] undecan-4-yl) acetic acid)	C ₁₇ H ₂₆ O ₄	Unknown	Unknown	-
6	NP-021050 ((1S,2R,4aS,6aS,6bR,10S,11R,12aR,14bS)-10,11-dihydroxy-1,2,6a,6b,9,9,12a-heptamethyl-2,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydro-1H-picene-4a-carboxylic acid)	C ₃₀ H ₄₈ O ₄	Phenolic compound	Unknown	-
7	NP-021023 [(E)-6-(1,3-dihydroxy-4,4,8,10,14-pentamethyl-2,3,5,6,7,9,11,15,16,17-decahydro-1H-cyclopenta[a] phenanthren-17-yl)-2-methylhept-2-enoic acid]	C ₃₀ H ₄₈ O ₄	Unknown	Unknown	-

8	Azelaic acid	C ₉ H ₁₆ O ₄	Phenols	Anti-inflammatory, antimicrobial, antioxidant, anticomedolytic and anticancer activities	(Kumar et al., 2020)
10	cis, cis-Muconic acid	C ₆ H ₆ O ₄	Dicarboxylic acid	Unknown	-
11	NP-018716 (2-Hexylpentanedioic acid)	C ₁₁ H ₂₀ O ₄	Unknown	Unknown	-
12	Gallic acid	C ₇ H ₆ O ₅	Phenolics	Antimicrobial, antioxidant, anticancer, anti-inflammatory and antiviral activities	(Hadidi et al., 2024)
13	NP-003294	C ₁₈ H ₁₆ O ₇	Unknown	Unknown	-
14	Stearoyl ethanol amide	C ₂₀ H ₄₁ NO ₂	Fatty acid amide	Unknown	-
15	NP-001798	C ₁₆ H ₁₇ NO ₂	Unknown	Unknown	-
16	Cannabicitran	C ₂₁ H ₃₀ O ₂	Terpenoids	Unknown	-
17	Hexadecanamide	C ₁₆ H ₃₃ NO	Fatty acid amide	Unknown	-
18	NP-016582((2E,4E,10Z)-NSS-(2-methylpropyl) hexadeca-2,4,10-trienamide)	C ₂₀ H ₃₅ NO	Fatty acid	Unknown	-
19	9-Oxo-10(E),12(E)- octadecadienoic acid	C ₁₈ H ₃₀ O ₃	Fatty acid	Anticancer, lipid metabolism	(Kim et al., 2011)
20	Berberine	C ₂₀ H ₁₇ NO ₄	Alkaloid	Anticancer, antidiabetic, anti-obesity, anti-inflammatory, neuroprotective properties	(Och et al., 2020)

21	Trigonelline	C ₇ H ₇ NO ₂	Alkaloid	Antioxidant, Antidiabetic, Neuroprotection and Neurotrophic	(Liang et al., 2023; Zia et al., 2023)
22	4-Aminobenzoic acid	C ₇ H ₇ NO ₂	Folate	Neurotransmitter modulator, anti-inflammatory, Antioxidant	(Chen et al., 2025)
23	Anthranilic acid	C ₇ H ₇ NO ₂	Alkaloid	Antibacterial, antiviral, insecticidal, anti-inflammatory, analgesic and antipyretic	(Milusheva et al., 2023)
24	Arecoline	C ₈ H ₁₃ N O ₂	Alkaloid	Anticancer, antimicrobial, neuroprotective, antiparasitic and antidiabetic	(Badawy et al., 2023)
25	N, N Dimethyl sphingosine	C ₂₀ H ₄₁ N O ₂	Lipid	Anticancer	(Kim et al., 2009)
26	NP-004713 ((4S,4AR)-4-(Hydroxymethyl)-3,4A,8,8-tetramethyl-5,6,7,8A-tetrahydro-4H-naphthalen-1-one)	C ₁₅ H ₂₄ O ₂	Cyclohexenone	Unknown	-
27	9-Oxo-ODE	C ₁₈ H ₃₀ O ₃	Fatty acid	Unknown	-
28	Betaine	C ₅ H ₁₁ NO ₂	Amino acid	Antioxidant, neuroprotective, anti-inflammatory,	(Kaur et al., 2019)
29	NP-020241	C ₁₈ H ₁₉ N O ₄	-	-	-

30	Nootkatone	C ₁₅ H ₂₂ O	Sesquiterpenoid	Anti-inflammatory, anticancer, antibacterial, hepatoprotective, neuroprotective and cardioprotective	(Jha et al., 2022)
31	Octadec-9-ynoic acid (Stearolic acid)	C ₁₈ H ₃₂ O ₂	Fatty acid	Unknown	-
32	NP-008095(6-Hydroxy-4-(hydroxymethyl)-3,4A,8,8-tetramethyl-5,6,7,8A-tetrahydro-4H-naphthalen-1-one)	C ₁₅ H ₂₄ O ₃	-	Unknown	-
33	2-Amino-1,3,4-octadecanetriol (Phytosphingosine)	C ₁₈ H ₃₉ N O ₃	Fatty acid	Antimicrobial and anti-inflammatory	(Pavicic et al., 2007)
34	2-(2,6-dimethoxyphenyl)-5,6-dimethoxy-4 H chromen-4-one	C ₁₉ H ₁₈ O ₆	Flavonoid	Unknown	-
35	DL-Stachydrine	C ₇ H ₁₃ N O ₂	Alkaloid	Cardioprotective, neuroprotective, anti-inflammatory, anticancer and antiobesity	(He et al., 2024)
36	7-(acetyloxy)-6-hydroxy-2-methyl 10-oxo-3,6,7,8,9,10-hexahydro-2H oxecin-3-yl (2E)- but-2-enoate	C ₁₆ H ₂₂ O ₇	Lipid	Unknown	-
37	NP-012616	C ₁₅ H ₂₄ O ₂	-	Unknown	-
38	Coumarin	C ₉ H ₆ O ₂	Coumarin	Anti-inflammatory, anticoagulant, antibacterial, antifungal, antiviral, anticancer, antihypertensive, antituberculous, anticonvulsant, antiadipogenic, antihyperglycemic,	(Sharifi-Rad et al., 2021)

				antioxidant and neuroprotective	
39	Palmitic Acid	$C_{16} H_{32} O_2$	Fatty acid	Anti-inflammatory, antioxidant, hypocholesterolemic, antiandrogenic, hemolytic, antipsychotic, antibacterial, antifungal and cytotoxic activities. 5-Alpha reductase inhibitor	(Yff et al., 2002)
40	2-Hydroxycinnamic acid	$C_9 H_8 O_3$	Phenolic compound	Antioxidant, antimicrobial, anticancer, anti-inflammatory, hepatoprotective, cardioprotective and neuroprotective	(Sova and Saso, 2020)
41	(±)13-HpODE	$C_{18} H_{32} O_4$	Fatty acid	Pro-inflammatory activity	(Rohr et al., 2020)
42	Tetranor-12(S)- HETE	$C_{16} H_{26} O_3$	Fatty acid	Unknown	-
43	(2E,4E)-N-(2-methylpropyl) deca-2,4-dienamide (Pellitorine)	$C_{14} H_{25} N O$	Fatty amide	Insecticidal property	(Eddy-Doh et al., 2020)
44	13(S)-HOTrE	$C_{18} H_{30} O_3$	Fatty acid	Anti-inflammatory	(Kumar et al., 2016)
45	NP-008993 (8-Hydroxy-9,10-epoxystearic acid)	$C_{18} H_{34} O_4$	Fatty acid	Unknown	-
46	Tetrahydromagnolol	$C_{18} H_{22} O_2$	Lignan	Anti-inflammatory, antitumor, antioxidant, hypoglycemic, cardiovascular protection,	(Lin et al., 2021)

				antiangiogenesis, and antibacterial	
47	12-oxo Phytodienoic Acid	C ₁₈ H ₂₈ O ₃	Fatty acid (Precursor of Jasmonic acid pathway)	Wound healing activity, Anti-inflammatory activity, defence responses	(Taki et al., 2005)
48	2-[(2R,4aR,8R,8aR)-8-hydroxy-4a,8-dimethyldecahydronaphthalen-2-yl] prop-2-enoic acid	C ₁₅ H ₂₄ O ₃	Terpenoids	Unknown	-
49	1,4-dihydroxy-1,4- dimethyl-7-(propan2-ylidene)-decahydroazulen-6- one (Zedoarondiol)	C ₁₅ H ₂₄ O ₃	Sesquiterpenoid	Anti-inflammatory, anticancer	(Cho et al., 2009; Ganur et al., 2021)
50	THC (Tetrahydrocannabinol, Dronabinol)	C ₂₁ H ₃₀ O ₂	Terpenophenolic compound	Antipsychotic, antibiotic activity,	(Flemming et al., 2007)
51	Urocanic acid	C ₆ H ₆ N ₂ O ₂	Carbohydrate	application in sun protection and skin disease therapy, antioxidant, immunomodulation	(Musa et al., 2025)
52	Oleamide	C ₁₈ H ₃₅ N O	fatty acid amide	Anti-inflammatory activity	(Ameamsri et al., 2021)
53	NP-014287 ((9E,11Z)-8-hydroxyoctadeca-9,11-dienoic acid)	C ₁₈ H ₃₂ O ₃	Fatty acid	Unknown	-
54	NP-020521 (Beta-dimorphecolic acid)	C ₁₈ H ₃₂ O ₃	Fatty acid	Unknown	-
55	KKK (6-Amino-2-[[6-amino-2-(2,6-diamino hexanoyl amino) hexanoyl] amino] hexanoic acid)	C ₁₈ H ₃₈ N ₆ O ₄	-	Unknown	-

Table 4.17. List of compounds from OHR LCMS analysis of *V. altissima* water extract with its reported bioactivity.

Sl. No.	Name Of Compounds	Formula	Class of Compounds	Bioactivity	References
1	Citric acid	C ₆ H ₈ O ₇	Phenolic compound	Antimicrobial and antioxidant activity	(Sharma et al., 2018)
2	Chlorogenic acid	C ₁₆ H ₁₈ O ₉	Tannin	Antioxidant, antibacterial, hepatoprotective, cardioprotective, anti-inflammatory, anti-pyretic, neuroprotective, antiobesity and antiviral	(Naveed et al., 2018)
3	Azelaic acid	C ₉ H ₁₆ O ₄	Phenols	Anti-inflammatory, antimicrobial, antioxidant, anticomedolytic and anticancer activities	(Kumar et al., 2020)
4	D-(-)-Quinic acid	C ₇ H ₁₂ O ₆	Phenols	Antioxidant, antidiabetic, anticancer activity, antimicrobial, antiviral, aging, protective, anti-nociceptive and analgesic activity	(Benali et al., 2022)
5	NP-014839 (8-(4-Hydroxyphenyl)-3-methoxy-6,7-dimethyl-5,6,7,8-tetrahydronaphthalen-2-ol)	C ₁₉ H ₂₂ O ₃	-	Unknown	-

6	Catechol	$C_6 H_6 O_2$	Phenolic compound	Antimicrobial, antioxidant, antitumor, antiviral, antineoplastic	(Surana et al., 2023)
7	NP-021701 (8,9-Dihydroxy-2,10,10-trimethyltricyclo [6.3.0.01,5] undec-6-ene-6-carboxylic acid)	$C_{15} H_{22} O_4$	Phenolic compound	Unknown	-
8	Caffeic acid	$C_9 H_8 O_4$	Phenolic compound	Antioxidant, anti-inflammatory, anti-cancer, cardioprotective, antidiabetic, hepatoprotective, antimicrobial, antiviral and Neuroprotective.	(Aijaz et al., 2022)
9	DL-Malic acid	$C_4 H_6 O_5$	Phenolic compound	Cardioprotective and antithrombotic	(Tang et al., 2013; Zhang et al., 2014)
10	NP-006255 (2-(6-Hydroxy-1-methyl-2-methylidene-9-propan-2-yl-5-oxatricyclo[5.4.0.03,8] undecan-4-yl) acetic acid)	$C_{17} H_{26} O_4$	-	Unknown	-
11	Luteolin	$C_{15} H_{10} O_6$	Flavonoids	Antioxidant, anti-inflammatory, immune system modulator and anticancer agent	(Imran et al., 2019a; López-Lázaro, 2009)
12	Gentisic acid	$C_7 H_6 O_4$	Phenolic compound	Antioxidant, Anticancer, Hepatoprotective, Analgesic, anti-inflammatory, antimicrobial, neuroprotective, cardioprotective	(Abedi et al., 2020)

13	Gallic acid	C ₇ H ₆ O ₅	Phenolic compound	Anti-inflammatory, antimicrobial, anticancer, cardioprotective, neuroprotective	(Kahkeshani et al., 2019)
14	4- Hydroxyphenyl acetic acid	C ₈ H ₈ O ₃	Phenolic compound	Antioxidant	(Zhao et al., 2018)
15	Genistein	C ₁₅ H ₁₀ O ₅	Flavonoids	Anti-oxidant, cardioprotective, anti-cancer, hepatoprotective and antimicrobial	(Islam et al., 2020)
16	Apigenin	C ₁₅ H ₁₀ O ₅	Flavonoids	Anti-inflammatory, Antiproliferative, Anti-cancer (Skin cancer)	(Salehi et al., 2019; Zhou et al., 2017)
17	1,3,5-trihydroxy-4- {[(2E)-3-(3-hydroxy4-methoxyphenyl) prop-2- enoyl]oxy} cyclohexane-1- carboxylic acid	C ₁₇ H ₂₀ O ₉	Phenolic compound	Unknown	-
18	L-(+)-Tartaric acid	C ₄ H ₆ O ₆	Phenolic compound	Antihypertensive and vasorelaxant	(Kousar et al., 2022)
19	3,3-Dimethylglutaric acid	C ₇ H ₁₂ O ₄	Phenolic compound	Unknown	-
20	3-Anisic acid	C ₈ H ₈ O ₃	Phenolic compound	Antifungal activity	(Bala and Paul, 2012)
21	NP-018716 (2-Hexylpentanedioic acid)	C ₁₁ H ₂₀ O ₄	Fatty acid	Unknown	-
22	Resorcinol	C ₆ H ₆ O ₂	Phenolic compound	Anti-inflammatory, antidiabetic, antimelanogenic and cytotoxicity	(Beaumet et al., 2024; Shahinozzaman et al., 2019; Topal, 2018)
23	3- Hydroxyphenyl acetic acid	C ₈ H ₈ O ₃	Phenolic compound	Blood pressure reducing agent	(Dias et al., 2022)

24	Sorbic acid	$C_6H_8O_2$	Fatty acid	Antimicrobial activity	(Wei et al., 2018)
25	Salicylic acid	$C_7H_6O_3$	Phenolic compound	Anti-pyretic, anti-inflammatory, antimicrobial and analgesic properties	(Sykes et al., 2024)
26	4-Hydroxybenzoic acid	$C_7H_6O_3$	Phenolic compound	Antimicrobial, antialgal, antimutagenic, antiestrogenic, hypoglycemic, anti-inflammatory, anti-platelet aggregating, nematocidal, antiviral, antioxidant	(Manuja et al., 2013)
27	2-Isopropylmalic acid	$C_7H_{12}O_5$	Phenolic compound	Antibacterial	(Ricciutelli et al., 2020)
28	2,5- Dihydroxy benzaldehyde	$C_7H_6O_3$	Phenolic compound	Anti-inflammatory, anti-nociceptive and anti-angiogenic activity	(Jung et al., 2009)
29	3-Hydroxybenzoic acid	$C_7H_6O_3$	Phenolic compound	Antimicrobial, antialgal, antimutagenic, antiestrogenic, hypoglycemic, anti-inflammatory, anti-platelet aggregating, nematocidal, antiviral, antioxidant, antiproliferative	(Mahdi et al., 2013; Satpute et al., 2018)

30	3,5- Dihydroxybenzoic acid	$C_7 H_6 O_4$	Phenolic compound	Gastrointestinal hemorrhage, Antimutagenic, Necrosis, Kidney function stimulant, Anti-inflammatory, Antipyretic, Glucose oxidase inhibitor,	(Annapoorani and Manimegalai, 2013)
31	(2R)-2,3- Dihydroxy propanoic acid (Glyceric acid)	$C_3 H_6 O_4$	Phenolic compound	Unknown	-
32	3-Isopropylmalic acid	$C_7 H_{12} O_5$	Fatty acid	Anti-oxidant and anti-microbial activities	(Ricciutelli et al., 2020)
33	Galangin	$C_{15} H_{10} O_5$	Flavonoids	Anticancer agent, anti-inflammatory activity	(Rampogu et al., 2021)
34	Emodin	$C_{15} H_{10} O_5$	Anthraquinones	Anti-inflammatory, hepatoprotective, anticancer, antioxidant and antimicrobial activity	(Dong et al., 2016; Hsu and Chung, 2012)
35	Kaempferol	$C_{15} H_{10} O_6$	Flavonoids	Anticancer and anti-inflammatory activity	(Devi et al., 2015; Imran et al., 2019b)
36	Fisetin	$C_{15} H_{10} O_6$	Flavonoids	Antioxidant, neuroprotective, anti-inflammatory, antineoplastic, anticancer, senolytic, and longevity promoting activities	(Kim et al., 2015; Pal et al., 2016)
37	Theophylline	$C_7 H_8 N_4 O_2$	Alkaloids	Immunomodulatory, antiviral and anti-inflammatory	(Cvietusa et al., 1996; Montaña et al., 2022)

38	Docosaheptaenoic acid	C ₂₂ H ₃₂ O ₂	Fatty acid	Anti-inflammatory activity	(Li et al., 2021)
39	(2E)-3- [(1R,4S,7R,7aR)-1- hydroxy-3,7-dimethyl2,4,5,6,7,7ahexahydro-1Hinden-4-yl]-2-methylprop-2-enoic acid	C ₁₅ H ₂₂ O ₃	-	Unknown	-
40	(1r,3R,4s,5S)-4- {[(2E)-3-(3,4-dihydroxyphenyl) prop-2-enoyl] oxy}-1,3,5-trihydroxycyclohexane-1-carboxylic acid	C ₁₆ H ₁₈ O ₉	Phenolic compound	Unknown	-
41	Trihydroxycyclohexane-1-carboxylic acid (2S,3S,4S,5R,6S)- 6- {[5,7-dihydroxy-2- (4-hydroxyphenyl)- 4-oxo-4H-chromen3-yl]oxy}-3,4,5- trihydroxyoxane-2- carboxylic acid	C ₂₁ H ₁₈ O ₁₂	Phenolic compound	Unknown	-
42	6-hydroxy-4a- (hydroxymethyl)-5- methyl-3- (prop-1- en-2-yl)- 2,3,4,4a,5,6,7,8-octahydronaphthalen-2-one	C ₁₅ H ₂₂ O ₃	-	Unknown	-
43	2- {[7-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-3 (hydroxymethyl)-6,8-dimethoxy1,2,3,4-tetrahydronaphthalen-2-yl]methoxy}-6- (hydroxymethyl)oxane-3,4,5-triol	C ₂₈ H ₃₈ O ₁₃	-	Unknown	-
44	(3S,4aR,5R,6R)-3, 6-dihydroxy-4a,5-dimethyl-3- (prop-1-en-2-yl)-2,3,4,4a,5,6,7,8-octahydro naphthalen-2-one	C ₁₅ H ₂₂ O ₃	-	Unknown	-
45	Nicotinic acid	C ₆ H ₅ N O ₂	Alkaloids	Antidote and antilipemic drug. A vasodilator agent, nicotinamidase inhibitor and antihyperlipidemic activity	(Kim et al., 2023)

46	NP-005128 ((2R,3R,4S,5S,6R)-2-[[6-hydroxy-4-(4-hydroxy-3,5-dimethoxyphenyl)-3-(hydroxymethyl)-5,7-dimethoxy-1,2,3,4-tetrahydronaphthalen-2-yl] methoxy]-6-(hydroxymethyl) oxane-3,4,5-triol	C ₂₈ H ₃₈ O ₁₃	Unknown	-	-
47	Oleamide	C ₁₈ H ₃₅ N O	fatty acid amide	Anti-inflammatory activity	(Ameamsri et al., 2021)
48	Pelargonidin	C ₁₅ H ₁₀ O ₅	Anthocyanin	Antioxidant and anti-inflammatory activity	(Duarte et al., 2018; Noda et al., 2002)
49	Trigonelline	C ₇ H ₇ NO ₂	Alkaloid	Antioxidant, Antidiabetic, Neuroprotection and Neurotrophic	(Liang et al., 2023; Zia et al., 2023)
50	Anthranilic acid	C ₇ H ₇ NO ₂	Alkaloid	Antibacterial, antiviral, insecticidal, anti-inflammatory, analgesic and antipyretic	(Milusheva et al., 2023)
51	Luteolin	C ₁₅ H ₁₀ O ₆	Flavonoids	Antioxidant, anti-inflammatory, immune system modulator and anticancer agent	(Imran et al., 2019a)
52	Cyanidin	C ₁₅ H ₁₀ O ₆	Anthocyanin	Antioxidant and neuroprotective agent	(Stintzing et al., 2002)
53	Picolinic acid	C ₆ H ₅ NO ₂	Phenolic compound	Neuroprotective, immunomodulator and antiproliferative activities	(Grant et al., 2009)
54	(11E,15Z)-9,10,13- trihydroxyoctadeca11,15-dienoic acid	C ₁₈ H ₃₂ O ₅	Fatty acid	Unknown	-

55	Hexadecanamide	C ₁₆ H ₃₃ N O	Fatty acid amide	Unknown	-
56	Arecoline	C ₈ H ₁₃ N O ₂	Alkaloid	Anticancer, antimicrobial, neuroprotective, antiparasitic and antidiabetic	(Badawy et al., 2023)
57	Xanthurenic acid	C ₁₀ H ₇ N O ₄	Phenolic compound	Neuroprotective	(Forino et al., 2019)
58	2-[(1S,2S,4aR, 8aS)-1-hydroxy-4amethyl-8-methylidenedecahydronaphthal en-2-yl]prop-2-enoic acid	C ₁₅ H ₂₂ O ₃	Sesquiterpenoid	Unknown	-
59	NP-014517 (3-Methoxy-4-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxybenzoic acid)	C ₁₄ H ₁₈ O ₉	Tannin	Unknown	-
60	NP-014517 (3-Methoxy-4-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxybenzoic acid)	C ₁₄ H ₁₈ O ₉	Tannin	Unknown	-
61	3-Pyridylacetic acid	C ₇ H ₇ NO ₂	Phenolic compound	Unknown	-
62	4-(4-hydroxy-2,6,6- trimethyl-3- {[(2R,3R,4S,5S, 6R)-3,4,5- trihydroxy-6-(hydroxymethyl) oxan-2- yl]oxy} cyclohex-1- en-1-yl)butan-2-one	C ₁₉ H ₃₂ O ₈	Terpenoid	Unknown	-
63	(1R,2R,6R,9R)- 2,11,11-trimethyl-3- oxotricyclo [4.3.2.0Âa, âµ]undecane-9- carboxylic acid	C ₁₅ H ₂₂ O ₃	Phenolic compound	Unknown	-
64	(2E,4E)-N-(1,3- dihydroxy-3- methylpentan-2-yl)- 2-methylhexa-2,4- dienamide	C ₁₃ H ₂₃ N O ₃	Fatty acid	Unknown	-
65	DL-Stachydrine	C ₇ H ₁₃ N O ₂	Alkaloid	Cardioprotective, anti-inflammatory and antioxidant	(He et al., 2024)

66	(2S,3R,4S,5S,6R)-2- {4- [(1S,3aR,4S,6aR)- 4-(4-hydroxy-3,5- dimethoxyphenyl)-hexahydrofuro [3,4- c] furan-1-yl]-2,6- dimethoxyphenoxy}-6- (hydroxymethyl) oxane-3,4,5-triol (Acanthoside B)	C ₂₈ H ₃₆ O ₁₃	Glycosides	Anti-cholinergic, anti-neuroinflammatory and anti-amnesic effects	(Karthivashan et al., 2019)
67	4-methoxy-7- ({[(2R,3R,4S,5S, 6R)-3,4,5-trihydroxy-6- (hydroxymethyl) oxan-2-yl]oxy } methyl)-5Hfuro[3,2-g]chromen5-one	C ₁₉ H ₂₀ O ₁₀	-	Unknown	-
68	NP-020902 (2-hydroxy-3-methoxy-4-(1-methoxypropan-2-yl)-2H-furan-5-one)	C ₉ H ₁₄ O ₅	-	Unknown	-
69	Scutellarin	C ₂₁ H ₁₈ O ₁₂	Flavonoid	Anti-inflammatory, anti-oxidant, vascular relaxation and myocardial protection	(Wang and Ma, 2018)
70	Berberine	C ₂₀ H ₁₇ N O ₄	Alkaloid	Anticancer, antidiabetic, anti-obesity, anti-inflammatory, neuroprotective properties	(Och et al., 2020)
71	Aloe-emodin	C ₁₅ H ₁₀ O ₅	Anthraquinones	Anti-inflammatory, antiviral, anticancer, antibacterial, neuroprotective and hepatoprotective activity	(Dong et al., 2020)
72	(1S,4aS,6S,7R, 7aS)-6-hydroxy-7- methyl-1- {[(2S,3R,4S,5S, 6R)-3,4,5- trihydroxy-6- (hydroxymethyl) oxan-2-yl] oxy }-1H,4aH,5H,6H,7H, 7aH cyclopenta[c]pyran4-carboxylic acid	C ₁₆ H ₂₄ O ₁₀	Terpenoid	Unknown	-

73	NP-020232 (3-(2,4-Dihydroxyphenyl)-5-hydroxy-6-(2-hydroxy-3-methylbut-3-enyl)-8,8-dimethyl-4a,5,6,6a,10a,10b-hexahydropyrano[2,3-h]chromen-4-one	C ₂₅ H ₂₄ O ₇	Flavonoids	Unknown	-
74	Orientin	C ₂₁ H ₂₀ O ₁₁	Flavonoids	Antioxidant, antiaging, antiviral, antibacterial, anti-inflammation, vasodilatation, cardioprotective, radiation protective, neuroprotective, antidepressant, antiadipogenesis, and antinociceptive activity	(Lam et al., 2016)
75	Kynurenic acid	C ₁₀ H ₇ N O ₃	Phenolic compound	Antioxidant, anti-inflammatory, wound healing and neuroprotective properties	(Wróbel-Kwiatkowska et al., 2024)
76	Taxifolin	C ₁₅ H ₁₂ O ₇	Flavonoids	Antioxidant, anti-inflammatory, antimicrobial, anticancer activity	(Das et al., 2021)
77	5,7-dihydroxy-2-(4-hydroxyphenyl)-6-[(2S,3R,4R,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]-8-[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]-4H-chromen-4-one (Violanthin)	C ₂₇ H ₃₀ O ₁₄	Flavonoids	Antioxidant, antisorbitol dehydrogenase, anti-alpha amylase, anticancer activity	(Zhong et al., 2023)
78	4-Guanidinobutyric acid	C ₅ H ₁₁ N ₃ O ₂	Alkaloid	Gastric lesions inhibitor	(Hwang and Jeong, 2012)

79	4,8-dihydroxy-6,6, 8-trimethyl1H,3H,4H,4aH,5H, 6H,7H,7aH,8H,9H azuleno[5,6- c] furan-1-one	C ₁₅ H ₂₂ O ₄	Terpenoid	Unknown	-
80	4-Hydroxybenzoic acid	C ₇ H ₆ O ₃	Phenolic compound	Antimicrobial, anti-inflammatory, nematocidal, antiviral, antiatherogenic	(Manuja et al., 2013)
81	12-Oxo phytodienoic acid	C ₁₈ H ₂₈ O ₃	Fatty acid (Precursor of Jasmonic acid pathway)	Wound healing activity, Anti-inflammatory activity, defense responses	(Taki et al., 2005)
82	Vitexin	C ₂₁ H ₂₀ O ₁₀	Flavonoids	Antioxidant, anti-inflammatory, antihyperalgesic, anti-cancer and neuroprotective activity	(He et al., 2016)
83	4-Methoxycinnamic acid	C ₁₀ H ₁₀ O ₃	Phenols	Antifungal, anti-inflammatory, antibacterial, anti-cancer and neuroprotective activity	(Wang et al., 2023)
84	Palmitic Acid	C ₁₆ H ₃₂ O ₂	Fatty acid	Antibacterial activity	(Yff et al., 2002)
85	7- Hydroxycoumarine (Umbelliferone)	C ₉ H ₆ O ₃	Coumarin	Anti-inflammatory, Antidiarrheal, antibacterial, antiulcerogenic effects and antioxidant activity	(Cruz et al., 2020; Mazimba, 2017)
86	4-Hydroxycoumarin	C ₉ H ₆ O ₃	Coumarin	Anti-coagulant and antimicrobial activity	(Završnik et al., 2008)

87	Coumarin	$C_9 H_6 O_2$	Coumarin	Anti-inflammatory, anticoagulant, antibacterial, antifungal, antiviral, anticancer, antihypertensive, antituberculosis, anticonvulsant, antiadipogenic, antihyperglycemic, antioxidant and neuroprotective	(Sharifi-Rad et al., 2021)
88	Tetrahydromagnolol	$C_{18} H_{22} O_2$	Lignan	Anti-inflammatory, antitumor, antioxidant, hypoglycemic, cardiovascular protection, antiangiogenesis, and antibacterial	(Lin et al., 2021)
89	Apocynin	$C_9 H_{10} O_3$	Phenolic compound	Antioxidant, anti-inflammatory, cardioprotective, neuroprotective, Anticancer	(Boshtam et al., 2021; Savla et al., 2021)

Table 4.18. List of compounds from OHR LCMS analysis of *V. altissima* isopropyl alcohol extract with its reported bioactivity

SL. No.	Compound	Formula	Class of compound	Bioactivity	Reference
1	D - (-)- Quinic acid	C ₇ H ₁₂ O ₆	Phenolic compound	Antioxidant, antidiabetic, anticancer, antimicrobial, antiviral, anti-nociceptive and analgesic activity	(Benali et al., 2022)
2	Luteolin	C ₁₅ H ₁₀ O ₆	Flavonoids	Antioxidant, anti-inflammatory, immune system modulator and anticancer agent	(Imran et al., 2019a)
3	Apigenin	C ₁₅ H ₁₀ O ₅	Flavonoids	Anti-inflammatory, antiproliferative and anticancer activity	(Salehi et al., 2019)
4	Genistein	C ₁₅ H ₁₀ O ₅	Flavonoids	Antioxidant, anti-helminthic, antineoplastic, anticancer, antiangiogenic and immunosuppressive activities. Phytoestrogen	(Polkowski and Mazurek, 2000)
5	4-(3,4-dihydroxyphenyl)-7-hydroxy-5- {[(2S, 3R, 4S, 5S, 6R)-3, 4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl] oxy}- 2 Hchromen - 2-one	C ₂₁ H ₂₀ O ₁₁	Flavonoids	Unknown	-
6	α, α-Trehalose	C ₁₂ H ₂₂ O ₁₁	Disaccharide	Plant- microbe interaction, Stress tolerance	(Almeida et al., 2007; El-Bashiti et al., 2005)

7	Cynaroside	C ₂₁ H ₂₀ O ₁₁	Flavonoids	Antioxidant, antimicrobial, antileishmanial, hepatoprotective, antidiabetic, anti-inflammatory and anticancer activity	(Bouyahya et al., 2023)
8	NP-005397(12-Hydroxy-13-(hydroxymethyl)-3,5,7-trimethyl-2,4-tetradecadienedioic acid)	C ₁₈ H ₃₀ O ₆	Polyketide	Unknown	-
9	Apigetrin	C ₂₁ H ₂₀ O ₁₀	Flavonoids (Flavone)	Anti-inflammatory, anticancer and antioxidant activity	(Patel and Patel, 2021)
10	NP-004626	C ₂₆ H ₄₀ O ₁₂	-	Unknown	-
11	(±)12(13)- Di HOME	C ₁₈ H ₃₄ O ₄	Lipid	Unknown	-
12	9,10-dihydroxy12Z-octadecenoic acid	C ₁₈ H ₃₄ O ₄	Fatty acid	Unknown	-
13	(±)13-HpODE	C ₁₈ H ₃₂ O ₄	Fatty acid	Pro-inflammatory activity	(Rohr et al., 2020)
14	(±)9-HpODE	C ₁₈ H ₃₂ O ₄	Fatty acid	Unknown	-
15	Trifolin	C ₂₁ H ₂₀ O ₁₁	Flavonoids	Antifungal and anticancer activity	(Kim et al., 2016)
16	Pelargonidin	C ₁₅ H ₁₀ O ₅	Anthocyanin	Antioxidant and anti-inflammatory activity	(Duarte et al., 2018; Noda et al., 2002)
17	Galangin	C ₁₅ H ₁₀ O ₅	Flavonoids	Anticancer agent, anti-inflammatory activity	(Rampogu et al., 2021)

18	Emodin	C ₁₅ H ₁₀ O ₅	Anthraquinones	Anti-inflammatory, hepatoprotective, anticancer, antioxidant and antimicrobial activity	(Dong et al., 2016; Hsu and Chung, 2012)
19	Kaempferol	C ₁₅ H ₁₀ O ₆	Flavonoids	Anticancer and anti-inflammatory activity	(Devi et al., 2015; Imran et al., 2019b)
20	Fisetin	C ₁₅ H ₁₀ O ₆	Flavonoids	Antioxidant, neuroprotective, anti-inflammatory, antineoplastic, anticancer, senolytic, and longevity promoting activities	(Kim et al., 2015; Pal et al., 2016)
21	NP-020752	C ₁₅ H ₁₀ O ₅	-	Unknown	-
22	5,7-dihydroxy-2-(3-hydroxy-4- {[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl] oxy} phenyl)-4Hchromen-4-one	C ₂₁ H ₂₀ O ₁₁	Flavonoid	Unknown	-
23	Kuromanin	C ₂₁ H ₂₀ O ₁₁	Anthocyanin	Antioxidant and neuroprotective activity	(Winter et al., 2017)
24	Genistin	C ₂₁ H ₂₀ O ₁₀	Flavonoid	Anti-oxidant, cardioprotective, anti-cancer, hepatoprotective and antimicrobial activity	(Islam et al., 2020)
25	5-hydroxy-3-(4-hydroxyphenyl)-7- {[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}-4Hchromen-4-one	C ₂₁ H ₂₀ O ₁₀	Flavonoid	Unknown	-
26	Cyanidin	C ₁₅ H ₁₀ O ₆	Anthocyanin	Antioxidant and neuroprotective agent	(Stintzing et al., 2002)

27	Scutellarin	C ₂₁ H ₁₈ O ₁₂	Flavonoid	Anti-inflammatory, anti-oxidant, vascular relaxation and myocardial protection	(Wang and Ma, 2018)
28	2-hydroxy-4-methoxy-3-(3- methylbut-2-en-1-yl)-6-(2- phenylethyl) benzoic acid (Amorfrutin A)	C ₂₁ H ₂₄ O ₄	Terpenoid	Antimicrobial, anti-HCV (hepatitis C virus), NF-κB inhibitory activity, PPARγ (peroxisome proliferator-activated receptor gamma) modulating activity	(Fujita et al., 2020)
29	(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl(4aS,6aS,6bR,9R,10R,11R,12aR)-10,11-dihydroxy-9-(hydroxymethyl)-2,2,6a,6b,9,12a hexamethyl-2,3,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,13,14 bicosahdropicene-4a-carboxylate	C ₃₆ H ₅₈ O ₁₀	Terpenoid	Unknown	-
30	NP-014517 (3-Methoxy-4-[3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl] oxybenzoic acid)	C ₁₄ H ₁₈ O ₉	Tannin	Unknown	-
31	Aloe-emodin	C ₁₅ H ₁₀ O ₅	Anthraquinones	Anti-inflammatory, antiviral, anticancer, antibacterial, neuroprotective and hepatoprotective activity	(Dong et al., 2020)
32	NP-021050 (1S,2R,4aS,6aS,6bR,10S,11R,12aR,14bS)-10,11-dihydroxy-1,2,6a,6b,9,9,12a-heptamethyl-2,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydro-1H-picene-4a-carboxylic acid)	C ₃₀ H ₄₈ O ₄	Phenolic compound	Unknown	-

33	NP-010026	C ₁₃ H ₁₂ O ₆	-	Unknown	-
34	Taxifolin	C ₁₅ H ₁₂ O ₇	Flavonoids	Antioxidant, anti-inflammatory, antimicrobial, anticancer activity	(Das et al., 2021)
35	NP-001928	C ₁₉ H ₁₈ O ₈	-	Unknown	-
36	9-Oxo-10(E),12(E)- octadecadienoic acid	C ₁₈ H ₃₀ O ₃	Fatty acid	Anticancer, lipid metabolism	(Kim et al., 2011)
37	NP-019748	C ₃₀ H ₄₈ O ₅	-	Unknown	-
38	Vitexin	C ₂₁ H ₂₀ O ₁₀	Flavonoids	Antioxidant, anti-inflammatory, antihyperalgesic, anti-cancer and neuroprotective activity	(He et al., 2016)
39	9-Oxo-ODE	C ₁₈ H ₃₀ O ₃	Fatty acid	Unknown	-
40	4-Methoxycinnamic acid	C ₁₀ H ₁₀ O ₃	Phenols	Antifungal, anti-inflammatory, antibacterial, anti-cancer and neuroprotective activity	(Wang et al., 2023)
41	3,4,5-trihydroxy-6- (hydroxymethyl) oxan-2-yl2-(prop-1-en-2-yl)-2,3-dihydro-1- benzofuran-5- carboxylate	C ₁₈ H ₂₂ O ₈	Phenolic compound	Unknown	-
42	Estriol	C ₁₈ H ₂₄ O ₃	Oestrogen	Phytoestrogen	(Bradbury and White, 1954)

43	NP-012042(6-[(E)-3,4-dihydroxypent-1-enyl]pyran-2-one)	C ₁₀ H ₁₂ O ₄	Polyketides	Unknown	-
44	Tetranor-12(S)- HETE	C ₁₆ H ₂₆ O ₃	Lipid	Unknown	-
45	Palmitic Acid	C ₁₆ H ₃₂ O ₂	Fatty acid	Antibacterial activity	(Yff et al., 2002)
46	NP-008993(8-Hydroxy-9,10-epoxystearic acid)	C ₁₈ H ₃₄ O ₄	Fatty acid	Unknown	-
47	7-Hydroxycoumarine (Umbelliferone)	C ₉ H ₆ O ₃	Coumarin	Anti-inflammatory, Antidiarrheal, anti-bacterial, antiulcerogenic effects and antioxidant activity	(Cruz et al., 2020; Mazimba, 2017)
48	4-Hydroxycoumarin	C ₉ H ₆ O ₃	Coumarin	Anti-coagulant and antimicrobial activity	(Završnik et al., 2008)
49	NP-014287 ((9E,11Z)-8-hydroxyoctadeca-9,11-dienoic acid)	C ₁₈ H ₃₂ O ₃	Fatty acid	Unknown	-
50	3-Hydroxy-2-methyl-4-[(7-oxo7H-furo[3,2-g]chromen-4-yl)oxy]-2-butanyletadglucopyranoside	C ₂₂ H ₂₆ O ₁₁	Coumarin	Unknown	-
51	NP-020521(9-Hydroxy-10,12-octadecadienoic acid)	C ₁₈ H ₃₂ O ₃	Fatty acid	Increasing platelet adenylate cyclase activity	(Henry et al., 1987)
52	12-Oxo phytodienoic acid	C ₁₈ H ₂₈ O ₃	Fatty acid (Precursor of Jasmonic acid pathway)	Wound healing activity, Anti-inflammatory activity, defense responses	(Taki et al., 2005)

53	18-β-Glycyrrhetic acid	C ₃₀ H ₄₆ O ₄	Triterpenoid	Antiasthmatic, hepatoprotective, anticancer, nephroprotective, antidiabetic, antileishmanial, antiviral, antibacterial, antipsoriasis, antiosteoporosis, antiepileptic, antiarrhythmic, and anti-inflammatory	(Shinu et al., 2023)
54	2-Amino-1,3,4-octadecanetriol	C ₁₈ H ₃₉ N O ₃	Phyto- sphingosine	Unknown	-
55	3-(4-hydroxy-3-methoxyphenyl) propanoic acid	C ₁₀ H ₁₂ O ₄	Phenolic compound	Antidiabetic, Antioxidant, antiobesity and anticancer	(Ohue-Kitano et al., 2023)

The LCMS analysis of the *V. altissima* extracts led to the identification of several interesting compounds, including various flavonoids, terpenoids, and phenolic acids. These secondary metabolites possessed many potential therapeutic applications. For instance, the presence of flavonoids, such as apigenin and luteolin derivatives, in the extracts was significant, as these compounds have been associated with antioxidant, anti-inflammatory, and anticancer activities (Salehi et al., 2019; Imran et al., 2019a). Compounds like D-(-)- quinic acid, genistein, apigetrin, pelargonidin, cynaroside, emodin, fisetin, kuromanin, genistin, taxifolin, cyanidin, scutellarin, vitexin etc possessed antioxidant activities (Benali et al., 2022; Polkowski and Mazurek, 2000; Bouyahya et al., 2023; Patel and Patel, 2021; Duarte et al., 2018; Noda et al., 2002; Dong et al., 2016; Kim et al., 2015; Winter et al., 2017; Stintzing et al., 2002; Islam et al., 2020; Wang and Ma, 2018; He et al., 2016). Compounds like D-(-)- quinic acid, apigetrin, pelargonidin, cynaroside, emodin, fisetin, aloe-emodin, taxifolin, scutellarin, vitexin, kaempferol etc possessed anti-inflammatory activities (Benali et al., 2022; Bouyahya et al., 2023; Patel and Patel, 2021; Duarte et al., 2018; Noda et al., 2002; Dong et al., 2016; Kim et al., 2015; Wang and Ma, 2018; He et al., 2016; Das et al., 2021; Dong et al., 2020; Devi et al., 2015; Rampogu et al., 2021). Compounds like D-(-)- quinic acid, pelargonidin, cynaroside, emodin, aloe-emodin, taxifolin, genistin etc possessed anti-microbial activities (Benali et al., 2022; Bouyahya et al., 2023; Patel and Patel, 2021; Dong et al., 2016; Das et al., 2021; Dong et al., 2020; Islam et al., 2020).

The identification of potent anti-inflammatory, antioxidant and antimicrobial bioactive molecules like luteolin, apigenin, aloe-emodin etc in the plant extracts provides a direct chemical explanation for the anti-inflammatory, antioxidant and antimicrobial effects observed in our bioassays.

Our findings are consistent with the other reported LCMS studies on *Vitex* species. 73 compounds were reported from the LCMS analysis of leaves of *V. negundo* (Dwivedi et al., 2021). The UPLC- ESI- QTOF-MS/MS analysis of bark and leaves of *V. pubescens* identified the presence of 71, 43 and 51 compounds from methanolic extract, ethyl acetate and n-butanol fractions (Abdelbaset et al., 2024). The GCMS analysis studies on methanolic leaf extract of *V. negundo* revealed the presence of 32 bioactive phytochemicals (Alans et al., 2024).

4.9 Antioxidant activity of *V. altissima*

The DPPH radical scavenging activity was used to study the antioxidant potential of *V. altissima* hexane (VAH), water (VAW) and isopropyl alcohol (VAI) extracts. Ascorbic acid was used as the standard for DPPH assay. The percentage of radical scavenging activity of

ascorbic acid is presented in Table 9 and figure 9. The maximum DPPH radical scavenging activity was exhibited by VAI. The percentage radical scavenging activity for VAI at 1mg/ml was 47.25 ± 0.84 . VAH extract showed least antioxidant activity compared to VAI and VAW.

VAH, VAW and VAI at all the tested concentrations showed significant radical scavenging activity compared to Ascorbic acid ($p < 0.001$).

The IC_{50} values for VAH, VAW and VAI was 7.15mg/ml, 2.34mg/ml and 1.23mg/ml respectively. The IC_{50} value for ascorbic acid was 0.397 mg/ml. IC_{50} value is the half maximal inhibitory concentration and lower IC_{50} value indicates higher antioxidant potential. Our results concluded that VAI had higher antioxidant activity.

The percentage radical scavenging activity of ascorbic acid, VAH, VAW and VAI are given in Table 4.19 and 4.20 and graphs are given in Plate 4.24.

Table 4.19. Percentage Radical Scavenging Activity of Ascorbic acid.

SL. No.	Concentration of Ascorbic acid	Percentage Radical Scavenging Activity
1	0.025mg/ml	47.68 ± 0.469
2	0.05mg/ml	47.79 ± 0.51
3	0.075mg/ml	47.9 ± 0.517
4	0.1mg/ml	48.17 ± 0.528

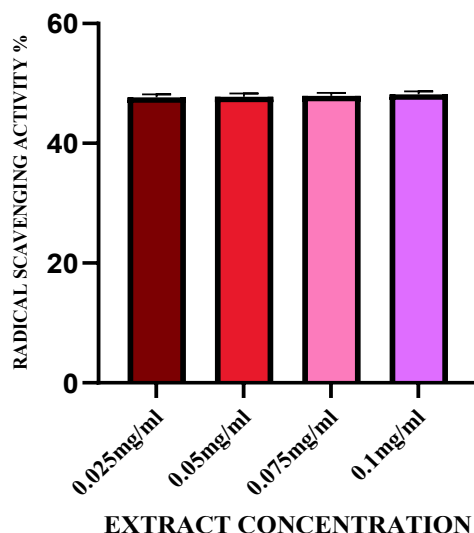
The data are expressed as mean $\pm$ SD.

Table 4.20. Percentage Radical Scavenging Activity of *V. altissima* hexane (VAH), water (VAW) and isopropyl alcohol (VAI) extracts.

SL. No.	Concentration of Plant Extract	Percentage Radical Scavenging Activity of VAH	Percentage Radical Scavenging Activity of VAW	Percentage Radical Scavenging Activity of VAI
1	0.25mg/ml	$21.81 \pm 0.27^{**}$	$41.58 \pm 0.81^{**}$	$39.19 \pm 0.615^{**}$
2	0.5mg/ml	$22.68 \pm 0.31^{**}$	$42.54 \pm 0.609^{**}$	$43.31 \pm 0.42^{**}$
3	0.75mg/ml	$23.67 \pm 0.45^{**}$	$43.65 \pm 0.598^{**}$	$45.05 \pm 0.721^{**}$
4	1mg/ml	$24.89 \pm 0.33^{**}$	$44.56 \pm 0.55^{**}$	$47.25 \pm 0.84^{**}$

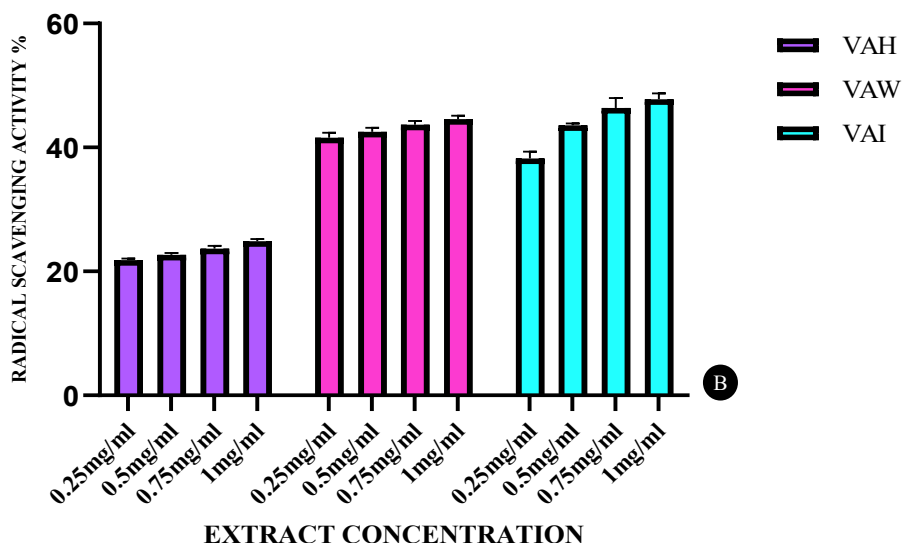
The data are expressed as mean $\pm$ SD with $n=3$. Values bearing superscript** in rows (comparison with ascorbic acid) differ significantly with $p < 0.001$ and with superscript* differ significantly with $p < 0.05$. Values bearing superscript ns in rows were non-significant.

DPPH RADICAL SCAVENGING ACTIVITY OF ASCORBIC ACID



A

DPPH RADICAL SCAVENGING ACTIVITY OF *V. altissima*



B

Plate 4.24. DPPH free radical scavenging activity of Ascorbic acid and *V. altissima*. Figure A. Percentage free Radical Scavenging Activity of Ascorbic acid at 0.025, 0.05, 0.075 and 0.1 mg/ml concentration. Figure B. Percentage free Radical Scavenging Activity of 0.25, 0.5, 0.75 and 1 mg/ml concentration of *V. altissima* hexane extract (VAH), *V. altissima* water extract (VAW) and *V. altissima* isopropyl alcohol extract (VAI).

Our studies on the antioxidant properties of *V. altissima* revealed that isopropyl alcohol extract (VAI) possessed more DPPH radical scavenging activity than water (VAW) and hexane (VAH) extracts. The order of antioxidant activity of three extracts was VAI > VAW > VAH. The least antioxidant activity was observed with hexane extracts. The percentage radical scavenging activity of 1 mg/ml VAI was equivalent to 0.025 mg/ml of ascorbic acid. The bioactive molecules present in plants exhibit these antioxidant properties. Antioxidants inhibit reactive free radicals. They either donate electrons or scavenge free radicals or quench singlet oxygen (Sirivibulkovit et al., 2018). DPPH assay was a widely used assay technique to study the antioxidant properties of plant extracts (Munteanu and Apetrei, 2021). It is a colorimetric assay in which the violet colour of DPPH is reduced to a pale yellow colour. The DPPH assay with methanolic extracts of leaves of *V. altissima* exhibited antioxidant activities (Sundaresan et al., 2020). These studies support our findings on the antioxidant properties of *V. altissima*.

4.10 Antibacterial activity of *V. altissima* against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus subtilis*.

The antibacterial activity of *V. altissima* hexane (VAH), water (VAW) and isopropyl alcohol (VAI) extracts was studied using agar disc diffusion method against *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis*. 100mg/ml extract was used as the stock. 10µl, 20µl, 30µl and 40µl VAH, VAW and VAI extracts were used to study the antibacterial activity. The extracts significantly showed antibacterial activity against *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis* with $p < 0.001$ compared to positive control streptomycin. The VAH extract showed maximum antibacterial activity at 40µl concentration against *B. subtilis* with zone of inhibition 9.6 ± 0.577 mm with $p < 0.001$. The maximum zone of inhibition for VAW was 10.83 ± 0.289 mm ($p < 0.001$) against *E. coli* at 40µl concentration. The maximum zone of inhibition for VAI was 14.167 ± 0.289 mm ($p < 0.001$) at 40µl concentration against *E. coli*. The VAI extract showed maximum antibacterial activity against all the four tested bacteria. The results of antibacterial activity of VAH, VAW and VAI were summarised in Table 4.21, 4.22 and 4.23 respectively. The photographs are given in Plate 4.25-4.27.

Table 4.21. Zone of inhibition in mm against *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis* for *V. altissima* hexane extract.

SAMPLE	ZONE OF INHIBITION DIAMETER (mm)			
	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>
CONTROL (DMSO)	0	0	0	0
STREPTOMYCIN	29.83±0.289	34.3±0.57	24.3±0.57	27.3±0.57
VAH 10µl	0 ^{**}	0 ^{**}	0 ^{**}	0 ^{**}
VAH 20µl	0 ^{**}	0 ^{**}	7.16±0.289 ^{**}	0 ^{**}
VAH 30µl	7.16±0.289 ^{**}	7.33±0.289 ^{**}	7.83±0.289 ^{**}	7.1±0.173 ^{**}
VAH 40µl	9.6±0.577 ^{**}	8.5±0.5 ^{**}	8.83±0.289 ^{**}	8.16±0.287 ^{**}

The data are expressed as mean ± SD with n=3. Values bearing superscript^{**} in rows (comparison with streptomycin) differ significantly with p<0.001 and with superscript^{*} differ significantly with p<0.05. Values bearing superscript ns in rows were non-significant.

Table 4.22. Zone of inhibition in mm against *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis* for water extract of *V. altissima*.

SAMPLE	ZONE OF INHIBITION DIAMETER (mm)			
	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>
CONTROL (DMSO)	0	0	0	0
STREPTOMYCIN	29.83±0.289	34.3±0.57	24.3±0.57	27.3±0.57
VAW 10µl	0 ^{**}	0 ^{**}	0 ^{**}	7±0.5 ^{**}
VAW 20µl	7.16±0.289 ^{**}	7.33±0.289 ^{**}	6.67±0.288 ^{**}	8.167±0.289 ^{**}
VAW 30µl	8.16±0.289 ^{**}	9.33±0.289 ^{**}	8.167±0.289 ^{**}	9.167±0.288 ^{**}
VAW 40µl	9.167±0.289 ^{**}	10.83±0.289 ^{**}	9.33±0.289 ^{**}	10.167±0.289 ^{**}

The data are expressed as mean ± SD with n=3. Values bearing superscript^{**} in rows (comparison with streptomycin) differ significantly with p<0.001 and with superscript^{*} differ significantly with p<0.05. Values bearing superscript ns in rows were non-significant.

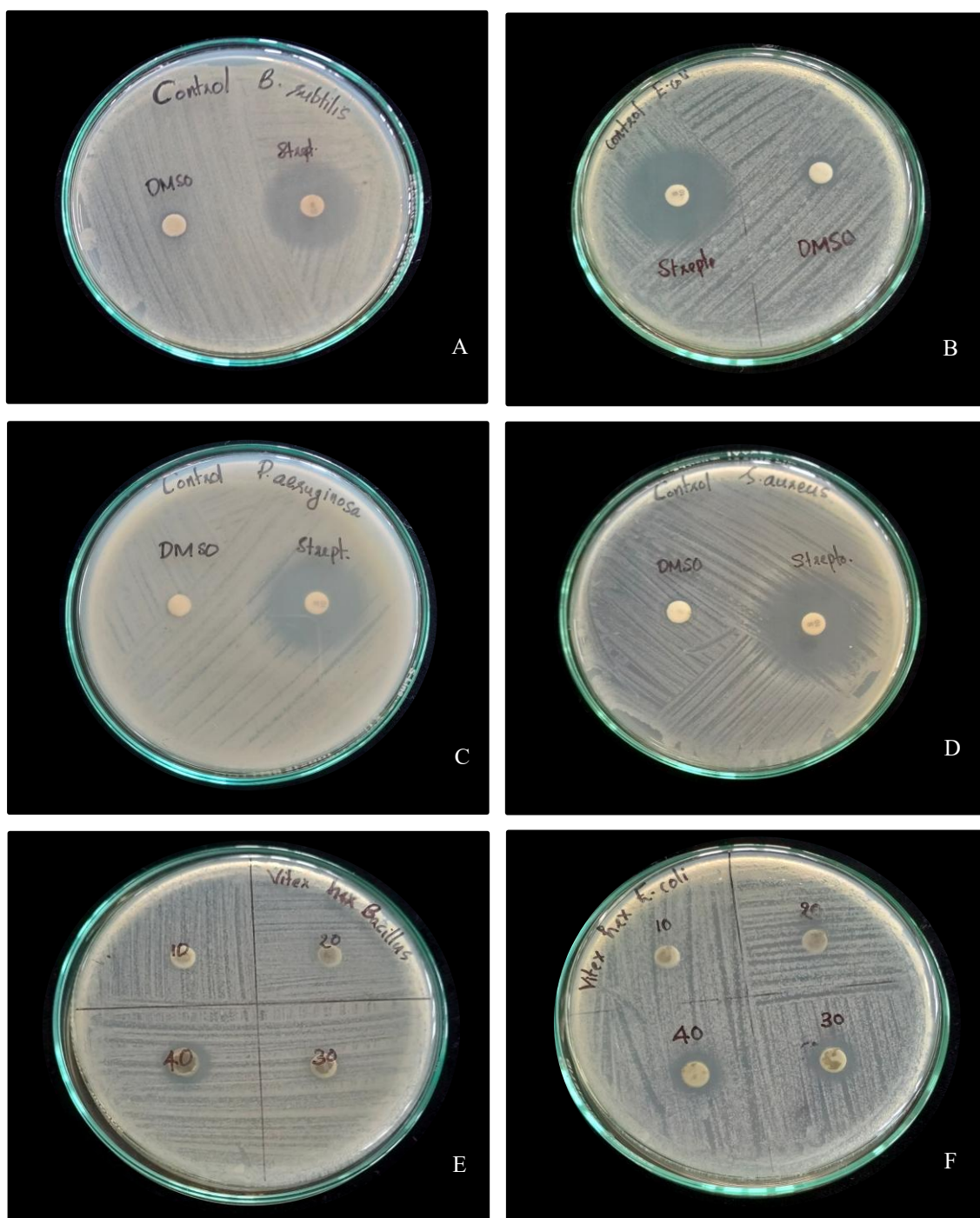


Plate 4.25. Antibacterial activity of *Vitex altissima*. Figure A. Zone of inhibition produced by DMSO and Streptomycin against *B. subtilis*. Figure B. Zone of inhibition produced by DMSO and Streptomycin against *E. coli*. Figure C. Zone of inhibition produced by DMSO and Streptomycin against *P. aeruginosa*. Figure D. Zone of inhibition produced by DMSO and Streptomycin against *S. aureus*. Figure E. Zone of inhibition produced by 10, 20, 30, 40µl *V. altissima* hexane extracts (VAH) concentrations against *B. subtilis*. Figure F. Zone of inhibition produced by 10, 20, 30, 40µl *V. altissima* hexane extracts (VAH) concentrations against *E. coli*.

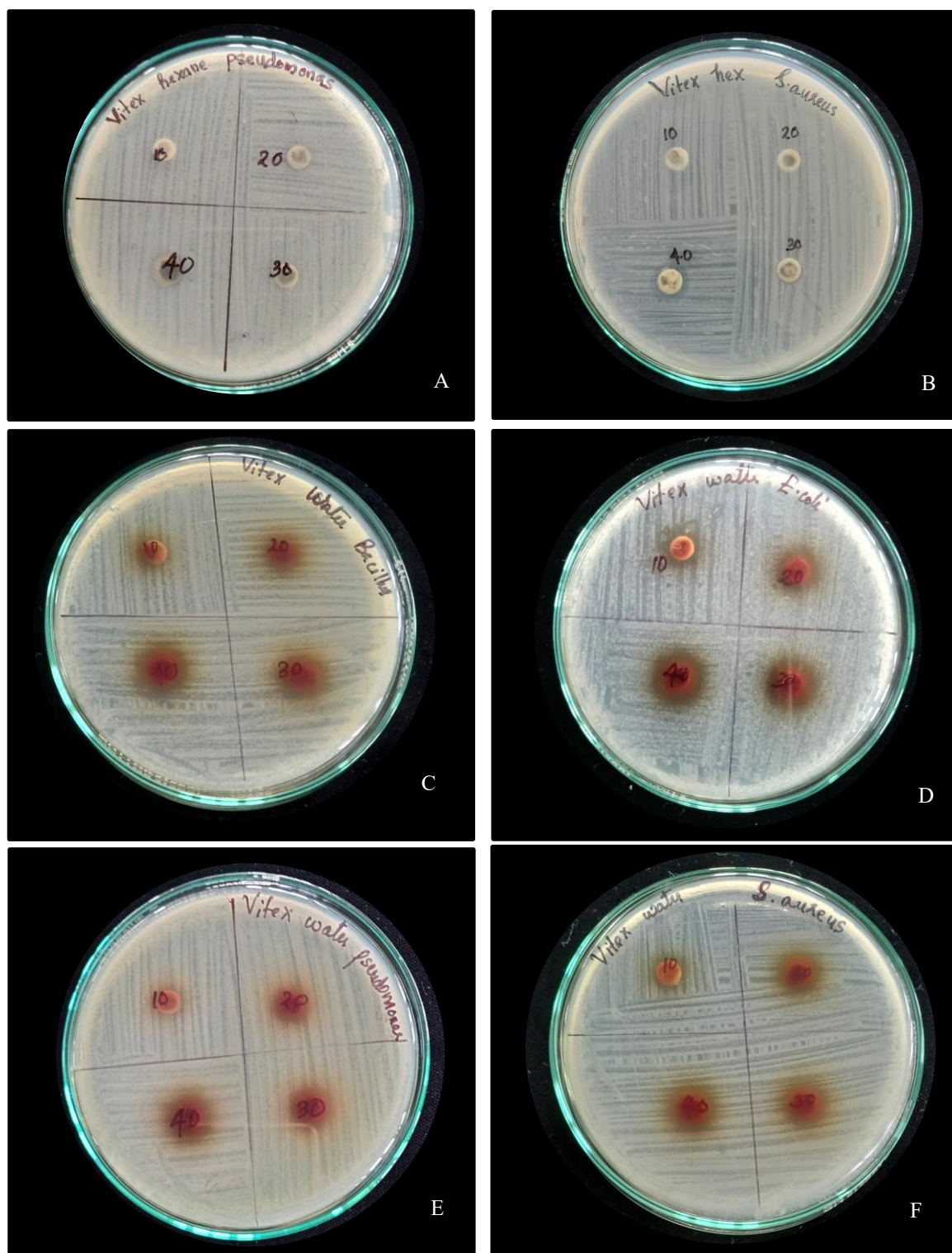


Plate 4.26. Antibacterial activity of *Vitex altissima*. Figure A. Zone of inhibition produced by 10, 20, 30, 40µl *V. altissima* hexane extracts (VAH) concentrations against *P. aeruginosa*. Figure B. Zone of inhibition produced by 10, 20, 30, 40µl *V. altissima* hexane extracts (VAH) concentrations against *S. aureus*. Figure C. Zone of inhibition produced by 10, 20, 30, 40µl *V. altissima* water extracts (VAW) concentrations against *B. subtilis*. Figure D. Zone of inhibition produced by 10, 20, 30, 40µl *V. altissima* water extracts (VAW) concentrations against *E. coli*. Figure E. Zone of inhibition produced by 10, 20, 30, 40µl *V. altissima* water extracts (VAW) concentrations against *P. aeruginosa*. Figure F. Zone of inhibition produced by 10, 20, 30, 40µl *V. altissima* water extracts (VAW) concentrations against *S. aureus*.

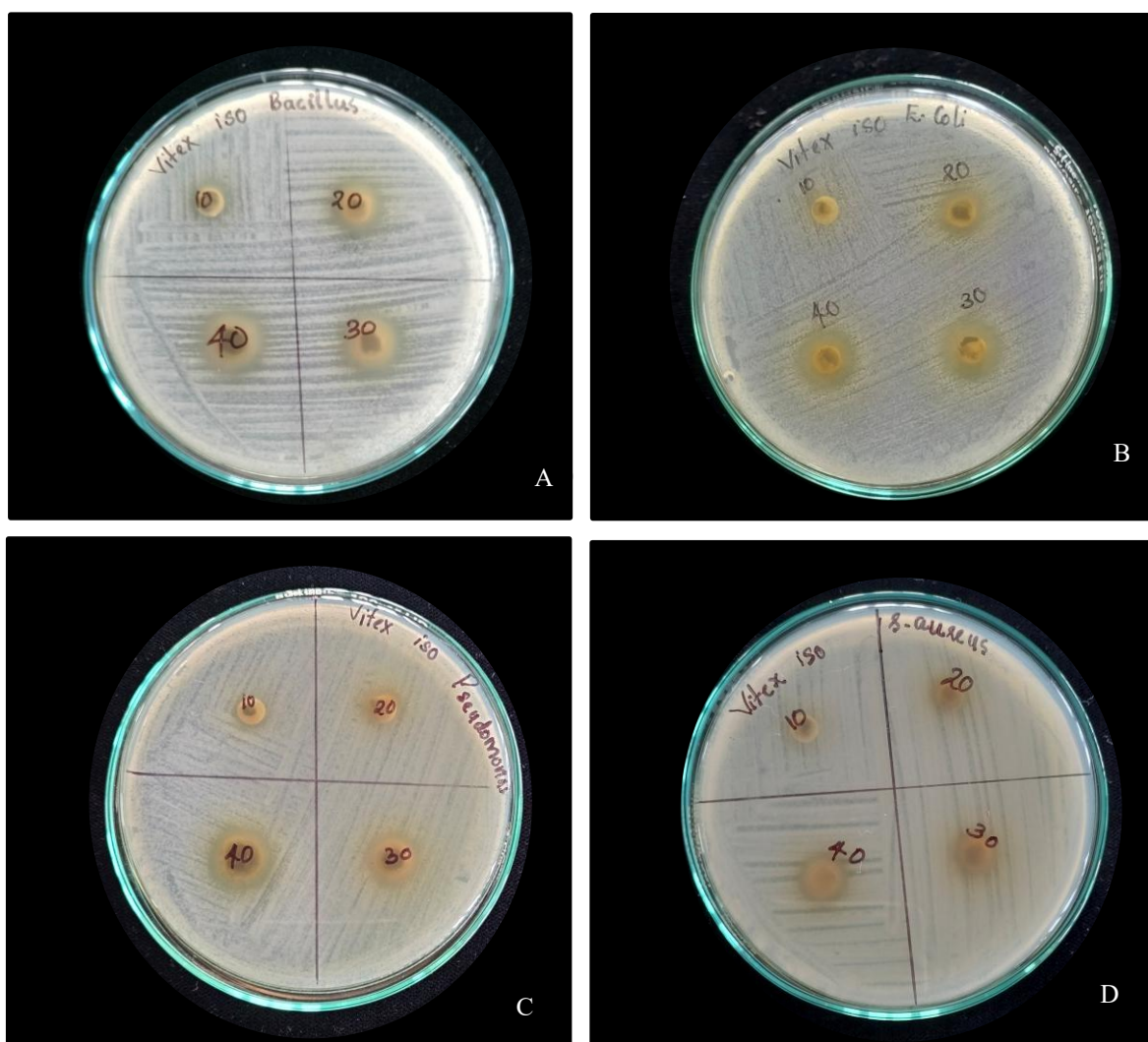


Plate 4.27. Antibacterial activity of *V. altissima* isopropyl alcohol extract. Figure A. Zone of inhibition produced by 10, 20, 30, 40 μ l *V. altissima* isopropyl alcohol extracts (VAI) concentrations against *B. subtilis*. Figure B. Zone of inhibition produced by 10, 20, 30, 40 μ l *V. altissima* isopropyl alcohol extracts (VAI) concentrations against *E. coli*. Figure C. Zone of inhibition produced by 10, 20, 30, 40 μ l *V. altissima* isopropyl alcohol extracts (VAI) concentrations against *P. aeruginosa*. Figure D. Zone of inhibition produced by 10, 20, 30, 40 μ l *V. altissima* isopropyl extracts (VAI) concentrations against *S. aureus*.

Table 4.23. Zone of inhibition in mm against *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis* for isopropyl alcohol extract of *V. altissima*.

SAMPLE	ZONE OF INHIBITION DIAMETER (mm)			
	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>
CONTROL (DMSO)	0	0	0	0
STREPTOMYCIN	29.83±0.289	34.3±0.57	24.3±0.57	27.3±0.57
VAI 10µl	0**	0**	0**	0**
VAI 20µl	9.167±0.28**	10.166±0.76**	9.167±0.76**	0**
VAI 30µl	10.67±0.57**	12.33±0.577**	10.83±0.76**	9.167±0.288**
VAI 40µl	12.33±0.58**	14.167±0.28**	12.33±0.57**	11.16±0.287**

The data are expressed as mean $\pm$ SD with n=3. Values bearing superscript** in rows (comparison with streptomycin) differ significantly with $p<0.001$ and with superscript* differ significantly with $p<0.05$. Values bearing superscript ns in rows were non-significant.

4.11 Antibacterial activity of *V. altissima* isopropyl alcohol extract (VAI)

From the preliminary screening for antibacterial activity, the maximum activity was observed with VAI extracts. So, we repeated the antibacterial activity studies of VAI with 200mg/ml stock. 20µl, 30µl, 40µl and 50µl extract concentrations were taken for the study. We observed an increase in antibacterial activity of VAI against the tested microorganisms. The maximum zone of inhibition for VAI was 15.233±0.4 ($p<0.001$) and 15.2±0.35 ($p<0.001$) against *B. subtilis* and *P. aeruginosa* respectively. The results of antibacterial activity of VAI were summarised in Table 4.24. Photographs of antibacterial activity of VAH, VAI and VAW are given in Plate 4.28.

Our antibacterial studies showed that the VAI extract showed maximum antibacterial activity against the tested organisms. We used disc diffusion assay to test the antibacterial activity of extracts. The maximum antibacterial activity of VAI was against *B. subtilis*. VAH and VAW showed moderate antibacterial activity compared to VAI. Agar disc diffusion is a widely used technique for antibacterial activity since the method is simple and cost effective (Tan and Lim, 2015). The studies on antibacterial activity of *V. altissima* aqueous ethanolic bark extract showed that the extract possessed maximum antibacterial activity against *Streptococcus pneumoniae* and *Streptococcus pyogenes* at 250mg/ml (Thakur et al., 2025). Studies on ethyl acetate extract of leaves of *V. altissima* showed antibacterial activity against

B. subtilis with zone of inhibition 19.67 ± 1.53 mm at 4 mg/ml extract concentration (Irulandi et al., 2017). The antibacterial activity studies on hydroethanolic extract of *V. altissima* revealed that the extract possessed antibacterial activity against *Klebsiella pneumonia* with zone of inhibition 1.25cm (Leela et al., 2015). All these studies support our studies on antibacterial activity of *V. altissima*.

Table 4.24. Zone of inhibition in mm against *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis* for *V. altissima* isopropyl alcohol extract.

SAMPLE	ZONE OF INHIBITION DIAMETER (mm)			
	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>
CONTROL (DMSO)	0	0	0	0
STREPTOMYCIN	29.75 ± 0.35	34.3 ± 0.57	24.3 ± 0.57	27.3 ± 0.57
VAI 20 μ l	$11.06 \pm 0.115^{**}$	$9.83 \pm 0.289^{**}$	$8.167 \pm 0.28^{**}$	$10.167 \pm 0.289^{**}$
VAI 30 μ l	$13.33 \pm 0.289^{**}$	$12.33 \pm 0.577^{**}$	$11.33 \pm 0.28^{**}$	$11.167 \pm 0.289^{**}$
VAI 40 μ l	$14.4 \pm 0.36^{**}$	$13.67 \pm 0.289^{**}$	$13.167 \pm 0.3^{**}$	$13.33 \pm 0.288^{**}$
VAI 50 μ l	$15.233 \pm 0.4^{**}$	$14.83 \pm 0.28^{**}$	$15.2 \pm 0.35^{**}$	$14.83 \pm 0.764^{**}$

The data are expressed as mean $\pm$ SD with n=3. Values bearing superscript** in rows (comparison with streptomycin) differ significantly with $p < 0.001$ and with superscript* differ significantly with $p < 0.05$. Values bearing superscript ns in rows were non-significant.

4.12 VAI treatment with LPS-treated macrophages downregulates the expression of IL-1 β , IFN β , TNF α , IL-6 and CCR-5: Evidence for anti-inflammatory activity of VAI

In order to investigate the effect of VAI in macrophage activation, we treated THP1 derived human macrophages with 10 ng LPS and 10, 15 and 25 μ g/ml concentration of extracts for 24 hours. Cells treated with DMSO were taken as the control.

The ANOVA results showed a highly significant p value ($p < 0.05$) for the selected genes. We observed 6.52 ± 0.14 fold increase in expression of IL-1 β , 3.11 ± 0.063 fold increase for IFN β , 2.33 ± 0.26 fold increase for TNF α , 5.76 ± 0.162 fold increase for CCR-5 and 3.26 ± 0.189 fold increase for IL-6 in LPS treated macrophages without plant extract. All these fold expressions were significant with $p < 0.001$ compared to the control group. In LPS treated macrophages with VAI at 10, 15 and 25 μ g/ml concentration, we observed the fold change of TNF α was decreased from 1.15 ± 0.05 to 0.513 ± 0.002 , IL-1 β was decreased from 1.12 ± 0.063 to 0.357 ± 0.006 , IL-6 was decreased from 0.55 ± 0.01 to 0.389 ± 0.002 , IFN β was decreased from

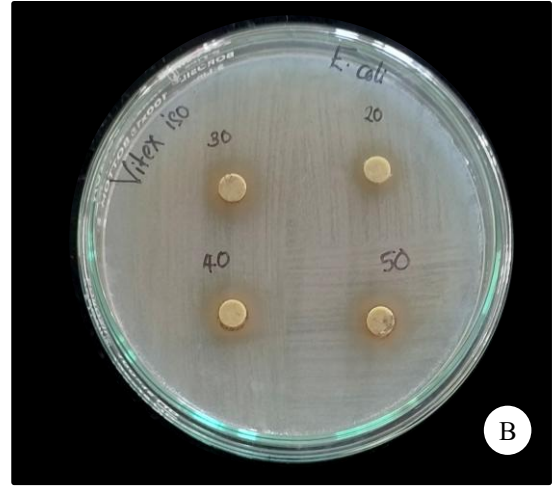
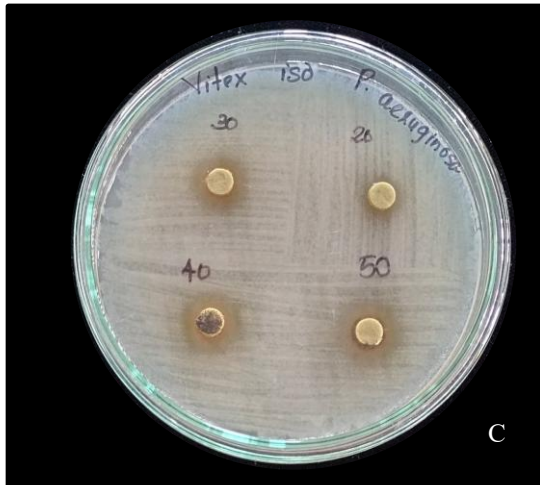
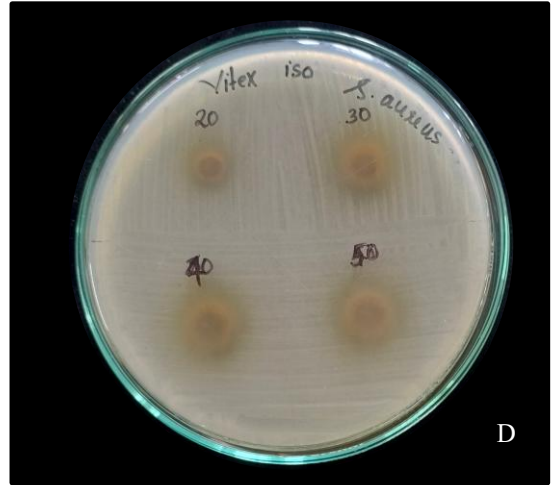


Plate 4.28. Antibacterial activity of *V. altissima* isopropyl alcohol extract. Figure A. Zone of inhibition produced by 20, 30, 40 and 50 μ l *V. altissima* isopropyl alcohol extracts (VAI) concentrations against *B. subtilis*. Figure B. Zone of inhibition produced by 20, 30, 40, 50 μ l *V. altissima* isopropyl alcohol extracts (VAI) concentrations against *E. coli*. Figure C. Zone of inhibition produced by 20, 30, 40, 50 μ l *V. altissima* isopropyl alcohol extracts (VAI) concentrations against *P. aeruginosa*. Figure D. Zone of inhibition produced by 20, 30, 40, 50 μ l *V. altissima* isopropyl extracts (VAI) concentrations against *S. aureus*.

0.66±0.008 to 0.43±0.017 and CCR-5 was decreased from 0.88±0.046 to 0.34±0.002 with p<0.001.

The LPS and VAI treated cells showed a prominent decrease in IL-1 β , TNF α , IL-6, IFN β and CCR-5 gene expression. The LPS alone treated macrophages showed an upregulation in gene expression for all the genes. Gene expression levels were reduced concentration dependently in cells treated with LPS and VAI. The relative fold gene expression of IL-1 β , IFN- β , TNF- α , CCR-5 and IL-6 are summarized in Table 4.25 and Plate 4.29.

Table 4.25. Relative fold gene expression of IL-1 β , IFN β , TNF α and CCR-5 in VAI and LPS treated macrophages.

Sl . No.	Treatme nt	IL-1 β Mean fold expressi on $\pm$ SD	IFN β Mean fold expression $\pm$ SD	TNF α Mean fold expression $\pm$ SD	CCR-5 Mean fold expression $\pm$ SD	IL-6 Mean fold expression $\pm$ SD
1	Control	1	1	1	1	1
2	Control with LPS	6.52 $\pm$ 0. 14	3.11 $\pm$ 0.06 3	2.33 $\pm$ 0.26	5.76 $\pm$ 0.162	3.26 $\pm$ 0.18 9
3	10 μ g/ml	1.12 $\pm$ 0. 063**	0.66 $\pm$ 0.00 8**	1.15 $\pm$ 0.05 1**	0.88 $\pm$ 0.046**	0.55 $\pm$ 0.01 **
4	15 μ g/ml	0.746 $\pm$ 0 .011***++	0.57 $\pm$ 0.01 1***++	0.85 $\pm$ 0.01 75**	0.869 $\pm$ 0.0 1**	0.46 $\pm$ 0.03 5**
5	25 μ g/ml	0.357 $\pm$ 0 .006***++	0.43 $\pm$ 0.01 7***++	0.513 $\pm$ 0.0 018***+	0.34 $\pm$ 0.002***+	0.388 $\pm$ 0.0 02**

The data are expressed as mean $\pm$ SD with n=3. Values bearing superscript** in rows (comparison with Control with LPS group) differ significantly with p<0.001 and with superscript* differ significantly with p<0.05. Values bearing superscript ++ in rows (comparison with treated group) differ significantly with p<0.001 and with superscript + differ significantly with p<0.05. Values bearing superscript ns are non-significant.

Our studies confirmed that the *V. altissima* possesses immunomodulatory activity. In unstimulated cells the plant extracts (VAH and VAI) showed immunostimulatory and in LPS stimulated cells the plant extract (VAI) possessed anti-inflammatory activity by upregulation or down regulation of cytokines. Cytokines are soluble molecules released in response to an infection. Macrophages release cytokines in response to an infection to activate other cells. Production of cytokines in large amounts at the site of infection may lead to cancer.

The inflammatory cytokines activate other cytokines, macrophages and other genes in case of acute and chronic inflammation to eliminate the pathogen from the body.

The upregulation of TNF α and IL-1 β has been documented in many studies on inflammatory processes (Gayathri et al., 2007). Overproduction of TNF α in response to an infection result in the production of IL-6, COX-2, PGE-2, NO, iNOS and ROS which activates inflammation (Gayathri et al., 2007; Medeiros et al., 2010). COX-2 expression was also induced by IL-1 β (Huang et al., 2000). IL-6 was produced in response to an infection by either immune or non-immune cells and it modulates various immunological and acute phase responses, chronic inflammations and cytokine storms (Hirano, 2021).

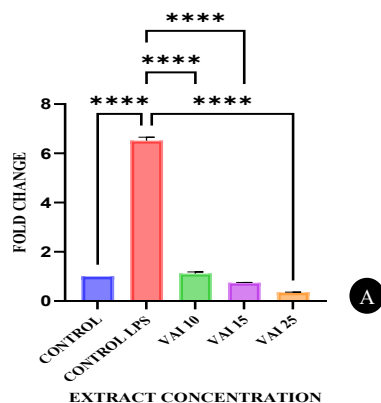
TNF α , IL-6 and IL-1 β activates NF- κ B, MAPK and JAK/STAT signalling pathways which results in apoptosis and chronic inflammation (Kim et al., 2010; Maity et al., 2012; Oluwamodupe et al., 2024).

IFN β is produced in response to pathogens like bacteria, virus, fungi, parasites etc. IFN β possesses both pro and anti-inflammatory activity. IFN β expression leads to the phosphorylation of STAT 1. In LPS treated RAW 264.7, STAT 1 functions as a transcriptional factor for iNOS (Toshchakov et al., 2002). In cardiac fibroblast cells, IP-10 and MCP-1 were produced by the activation of STAT 1 which in turn increases neutrophil migration (Bolívar et al., 2018). So, these studies concluded that IFN β activates STAT 1 which finally leads to chronic inflammation.

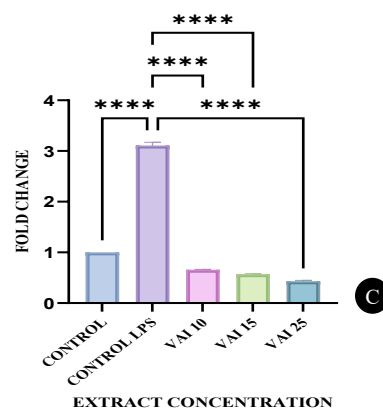
CCR- 5 is a G-protein coupled receptor and regulates immune response by CCL3, CCL4 and CCL5 (C-C chemokine ligands) (Samson et al., 1996). It is expressed in monocytes and macrophages. The over-expression of CCR-5 promotes the macrophage accumulation and generation of IL-1 β . The down regulation of CCR-5 leads to reduced inflammation (Chen et al., 2021). The overexpression of IL-1 β , IFN β , TNF α , IL-6 and CCR-5 leads to chronic inflammations.

Our studies are in agreement with the latest anti-inflammatory activity studies on *V. altissima*. The anti-inflammatory activity of methanolic extract of leaves of *V. altissima* was reported by Sundaresan et al., 2020. Protein denaturation assay was used to study the anti-inflammatory properties. Studies on anti-inflammatory properties of methanolic extract of *V. altissima* revealed that extract significantly reduce the activity of inflammatory genes COX (Cyclooxygenase), 5-LOX (Lipoxygenase-5) and MPO (myeloperoxidase) in LPS treated RAW 264.7 cells (Azeeza et al, 2023).

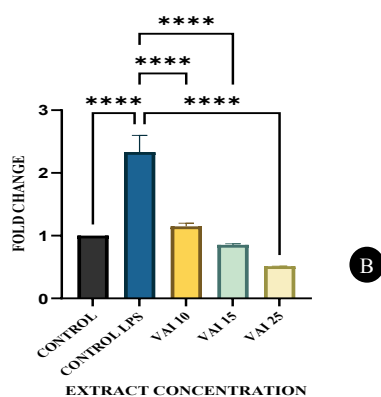
GENE EXPRESSION OF IL-1 β



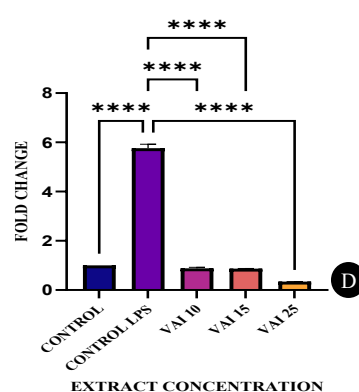
GENE EXPRESSION OF IFN β



GENE EXPRESSION OF TNF α



GENE EXPRESSION OF CCR-5



GENE EXPRESSION OF IL-6

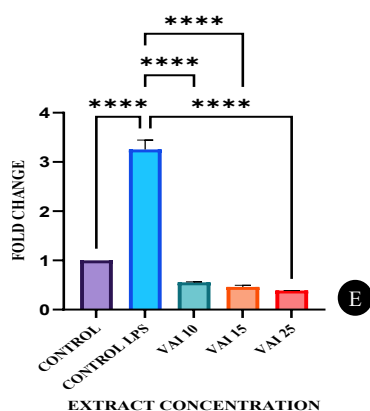


Plate 4.29. *In vitro* macrophage activation studies in LPS and *V. altissima* treated macrophages. Figure A-E. Relative fold gene expression of IL-1 β , IFN β , TNF α , CCR-5 and IL-6 in LPS and 10, 15, 25 μ g/ml *V. altissima* isopropyl alcohol extract treated macrophages. The data are expressed as mean $\pm$ SD with n=3. **** indicates $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. ns indicates $p > 0.05$. * indicates significant difference from control group.

4.13 Antifungal activity of *V. altissima* Isopropyl alcohol extract (VAI)

Antifungal activity of VAI was studied using disc diffusion method for *C. albicans* and food poisoning method for *A. niger*. Itraconazole was used as the positive control and isopropyl alcohol was used as the negative control. The diameter of the zone of inhibition was measured for antifungal activity against *C. albicans*. 20, 40 and 60µl VAI extract was used to test the antifungal activity against *C. albicans*. Inhibition percentage was used to calculate the antifungal activity of VAI against *A. niger*. From the results, we observed that VAI extract had no effect against *C. albicans*. The antifungal activity of *C. albicans* against Itraconazole was 24.5 ± 2.8 .

The VAI extract at 500mg/ml concentration showed significant antifungal activity against *A. niger* with an inhibition percentage of 41.79 ± 0.05 % ($p < 0.001$). Itraconazole showed 93.47 ± 0.89 % inhibition against *A. niger*. Photographs of antifungal activity are given in Plate 4.30.

Our studies on antifungal activity on *V. altissima* isopropyl alcohol extract revealed that VAI possessed antifungal activity against *A. niger*. VAI had no antifungal activity against *C. albicans*. Only a few studies are reported on antifungal activity of *V. altissima*. The antifungal activity of *V. altissima* ethyl acetate and ethanol extracts was reported with 9mm and 13mm zone of inhibition respectively against *Candida albicans* (Bhakshu et al., 2016). Further studies on the antifungal activity of VAI may result in the production of new drugs with antifungal activities.

4.14 TLC and preparatory TLC of isopropyl alcohol extract of *V. altissima*

The TLC analysis of *V. altissima* isopropyl alcohol extract revealed the presence of 7 bands. The separated bands were detected using UV chambers with visible, short and long UV range. Preparative TLC was done to isolate the separated bands. The bands were scratched from the plate, dissolved in ethyl acetate and centrifuged to remove silica particles. The retention values of each band were given in Table 4.26. The dissolved compounds at 10mg/ ml concentration were further tested for its antibacterial properties. The photographs of TLC fractions are given in Plates 4.31- 4.32.

Table 4.26 Retention factor values from preparatory TLC analysis of *V. altissima* isopropyl alcohol extract

SL. No.	TLC Band Number	Retention Factor (Rf) value
1	1	0.52
2	2	0.47
3	3	0.4
4	4	0.38
5	5	0.33
6	6	0.27
7	7	0.05

The TLC studies on plant extracts helped us to get an overview of various phytochemicals present in plants. Seven spots were obtained from the TLC studies on isopropyl alcohol extracts of *V. altissima*. The TLC of ethanolic extract of *V. altissima* showed 2 spots at 254 nm and 4 spots at 366 nm UV short wavelength at 2µl concentration and 4 spots and 5 spots at 254 nm and 366 nm respectively at 6µl concentration (Rajesh et al., 2019).

4.15 Antibacterial activity of compounds isolated from TLC

The antibacterial activity of compounds separated by TLC from *V. altissima* isopropyl alcohol (VAI) extract was studied using agar disc diffusion method against *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis*. We found that C5, C6 and C7 showed antibacterial activity against the test microorganisms. C7 exhibited a maximum zone of inhibition against *B. subtilis*, with 41mm. C5 and C6 also showed antibacterial activity against *E. coli* with zone of inhibition 35mm and 33mm. There is no antibacterial activity observed against *P. aeruginosa* and *S. aureus*. The results of antibacterial activity of compounds separated from TLC are given in Table 4.27 and photographs are given in Plate 4.33.

The antibacterial activity of TLC fractions showed that fraction C7 exhibited maximum antibacterial activity against *B. subtilis*. Fraction C5 and C6 also showed antibacterial activity against *E. coli*. The diameter of the zone of inhibition for C7, C5 and C6 at 20µl concentration was higher than the positive control Amoxicillin (15 mcg).

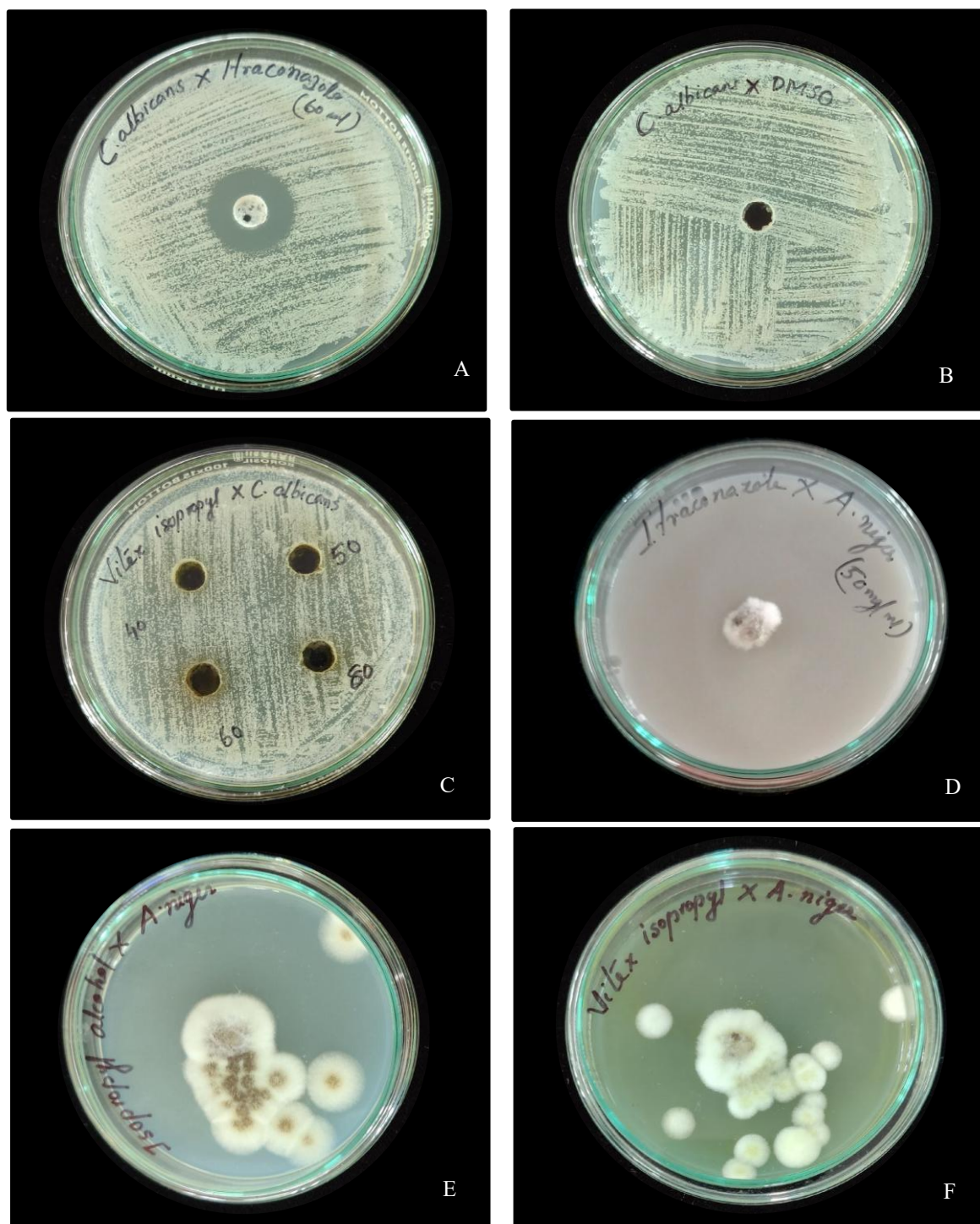


Plate 4.30. Antifungal activity of *V. altissima*. Figure A. Inhibitory effects of Itraconazole against *C. albicans*. Figure B. Inhibitory effects of DMSO against *C. albicans*. Figure C. Inhibitory effects of *V. altissima* isopropyl alcohol (VAI) extract against *C. albicans*. Figure D. Inhibitory effects of Itraconazole against *A. niger*. Figure E. Inhibitory effects of isopropyl alcohol against *A. niger*. Figure F. Inhibitory of *V. altissima* isopropyl alcohol (VAI) extract against *A. niger*.

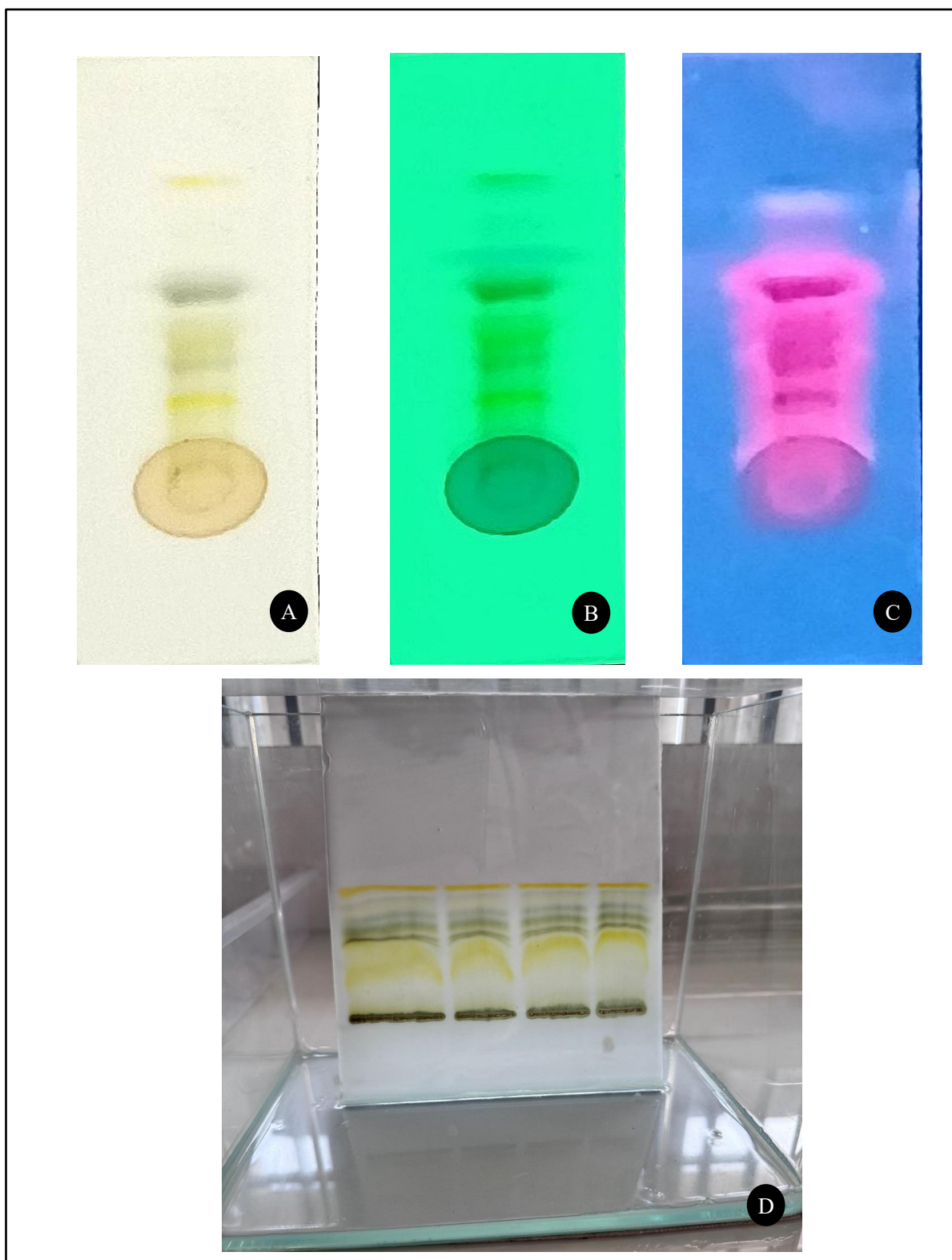


Plate 4.31. TLC images of *V. altissima*. Figure A. TLC image of *V. altissima* isopropyl alcohol extract under visible light. Figure B. TLC image of *V. altissima* isopropyl alcohol extract under UV 365nm. Figure C. TLC image of *V. altissima* isopropyl alcohol extract under UV 254nm. Figure D. Preparatory TLC of *V. altissima* isopropyl alcohol extract.

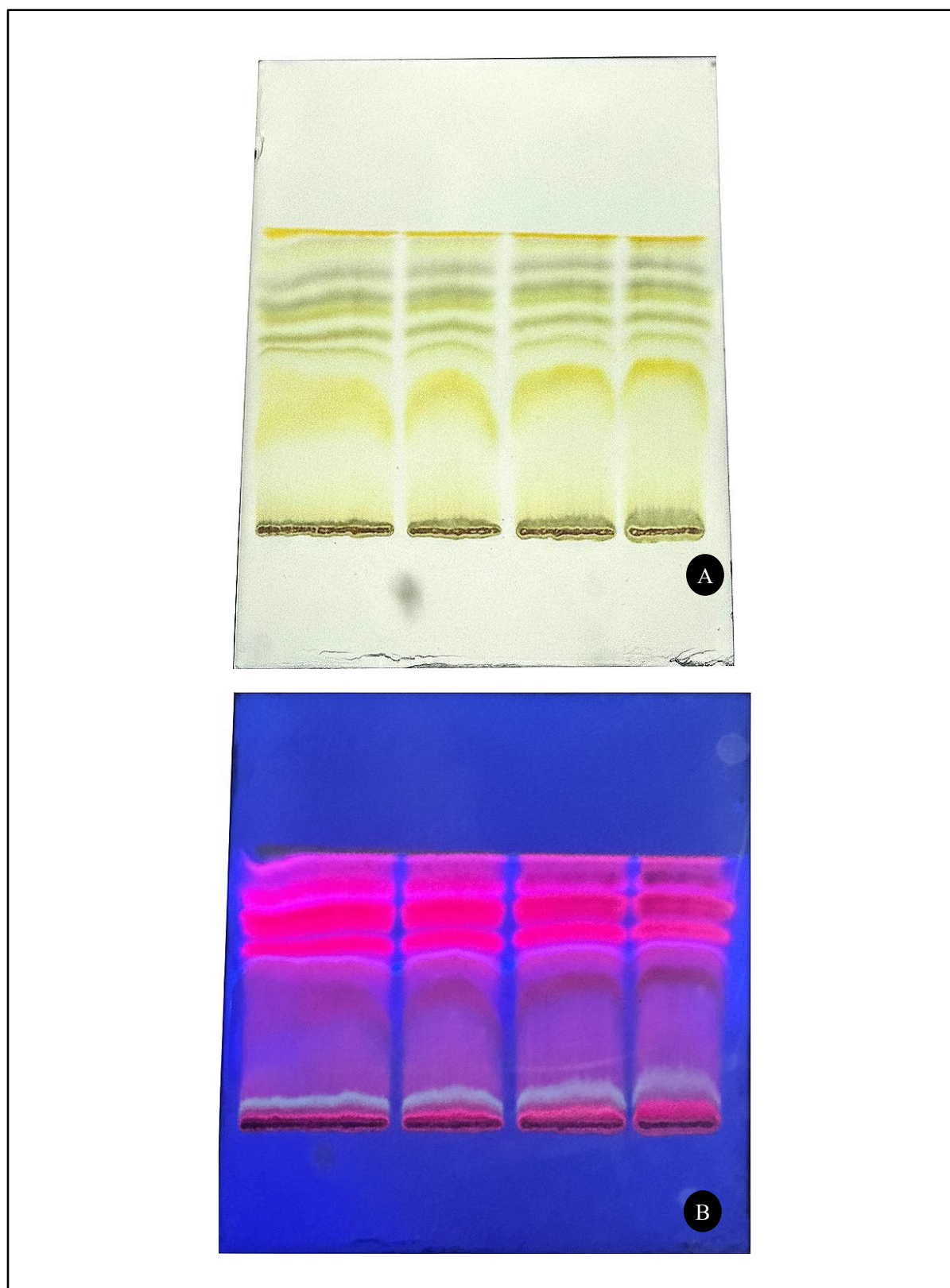


Plate 4.32. Preparatory TLC (pTLC) images of *V. altissima*. Figure A. pTLC image of *V. altissima* isopropyl alcohol extract under visible light. Figure B. pTLC image of *V. altissima* isopropyl alcohol extract under UV 254nm.

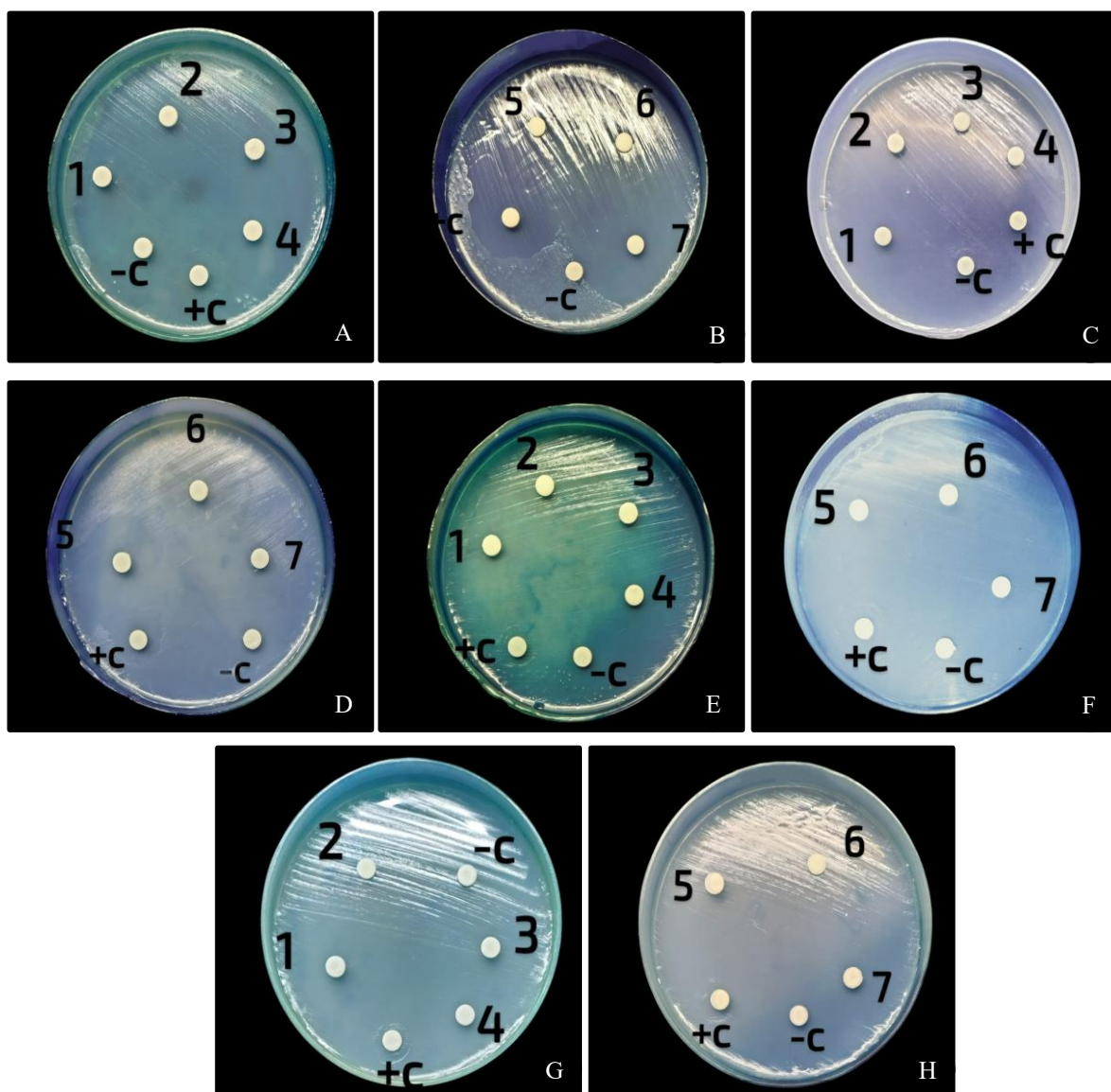


Plate 4.33. Antibacterial activity of TLC isolated compounds of *V. altissima* isopropyl alcohol extract (VAI). Figure A. Zone of inhibition of compounds 1-4 and control against *B. subtilis*. Figure B. Zone of inhibition of compounds 5-7 and control against *B. subtilis*. Figure C. Zone of inhibition of compounds 1-4 and control against *E. coli*. Figure D. Zone of inhibition of compounds 5-7 and control against *E. coli*. Figure E. Zone of inhibition of compounds 1-4 and control against *P. aeruginosa*. Figure F. Zone of inhibition of compounds 5-7 and control against *P. aeruginosa*. Figure G. Zone of inhibition of compounds 1-4 and control against *S. aureus*. Figure H. Zone of inhibition of compounds 5-7 and control against *S. aureus*.

Table 4.27. Zone of inhibition in mm against *E. coli* and *B. subtilis* for compounds separated from TLC of VAI.

Sample	Zone of Inhibition Diameter (Mm)	
	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>
CONTROL (Ethyl acetate)	0	0
Amoxicillin	39	22
C5 20µl	0	35
C6 20µl	0	33
C7 20µl	41	10

4.16 NMR analysis of TLC isolated compounds.

The antibacterial results suggest that compound Compound 5 (C5) and compound 7 (C7) possessed potent antibacterial activity. So, we done the NMR analysis of the sample. The ¹H NMR spectrum of compound 5 displayed peak for monosubstituted benzene ring and aliphatic protons. Minor peaks were also observed which may indicate impurities. We could not able to identify the compound from the peak. The ¹H NMR spectrum of compound 5 (C5) was given in Plate 4.34. The NMR spectrum of compound C7 also exhibited the same results indicating an issue with sample preparation and concentration.

Plants and plant-based treatments are frequent in various parts of the world. Use of folk medicine for post-natal care is widely practised in different parts of the world. Pregnancy and delivery are considered as important milestones in the lifespan of a woman. Pre and post-natal care is essential for the smooth recovery of the mother. Medicinal plants used during post-natal care possessed many pharmacological properties (Jain et al., 2011). The present study dealt with the use of plants for bathing mother and child after delivery. The study documented 62 plant species used for the medicated bath of mother and child. The medicated water called *vethuvellam* restores the body of the mother. Bathing with these water increases disease resistance in mother and child. This bath improves blood circulation, reduces inflammation, helps the body to regain the shape, helps in skin brightening and reduces body pain in mothers. The medicated bath for babies increases the immunity and also helps in drying of umbilical cord. Oil massaging helps in the restoration of abdominal muscles of the mother and in shaping the body of the infant. This traditional knowledge about medicated baths was passed from generation to generation as a mouthpiece. The plants used for this medicated bath may vary in different parts and all the plants used for this medicated bath were locally available.

The preliminary phytochemical analysis confirms that *A. fruticosa*, *J. gendarussa*, *P. longum*, and *V. altissima* are rich in bioactive compounds, supporting their use in *Vethuvellam* for post-natal care. The preliminary immunomodulatory studies in *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima* hexane, water and isopropyl alcohol extracts revealed that all the extracts had upregulated or downregulated the mRNA expression of IL-1 β , IFN β , TNF α and CCR-5. We had selected *V. altissima* for our further studies. We found that the treatment with VAH and VAI in THP1 derived macrophages upregulates the expression of IL-1 β , IFN β , TNF α and CCR-5 in unstimulated cells. These results suggest an inherent immunostimulatory activity of *V. altissima*. Our antioxidant and antibacterial studies confirmed that VAI exhibited maximum antioxidant and antibacterial activity.

The LCMS profiling of *V. altissima* revealed the presence of 55 compounds from VAH, 89 compounds from VAH and 55 compounds from VAI. The presence of various bioactive compounds are the key determinants of pharmacological activity of plants. For example, the presence of flavonoids, such as apigenin and luteolin, in the plants have been associated with antioxidant, anti-inflammatory, and anticancer activities (Imran et al., 2019b; Salehi et al., 2019). Similarly, the presence of phenolics like gentisic acid and caffeic acid in plants account for its antimicrobial, anti-inflammatory, antioxidant, cardioprotective and hepatoprotective properties (Abedi et al., 2020; Aijaz et al., 2022). The detailed phytochemical analysis of the *Vitex altissima* extracts provides a foundation for further investigation of its biological activities and potential applications of this plant species.

VAI treated cells downregulates the expression of IL-1 β , IFN β , TNF α , IL-6 and CCR-5 in LPS stimulated macrophages at different concentrations. IL-1 β , IFN β , TNF α , IL-6 and CCR-5 were involved in inflammatory responses. The downregulation of these genes in LPS stimulated cells strongly indicates the anti-inflammatory potential of *V. altissima*. Our antifungal activity studies showed that VAI significantly inhibited the growth of *A. niger*. The TLC isolated compounds C5 and C7 also exhibit potent antibacterial activities. So, our studies concluded that *V. altissima* possessed immunomodulatory, antioxidant, antibacterial and antifungal properties supporting the use of the plant in folk medicine of Palakkad for postnatal care.

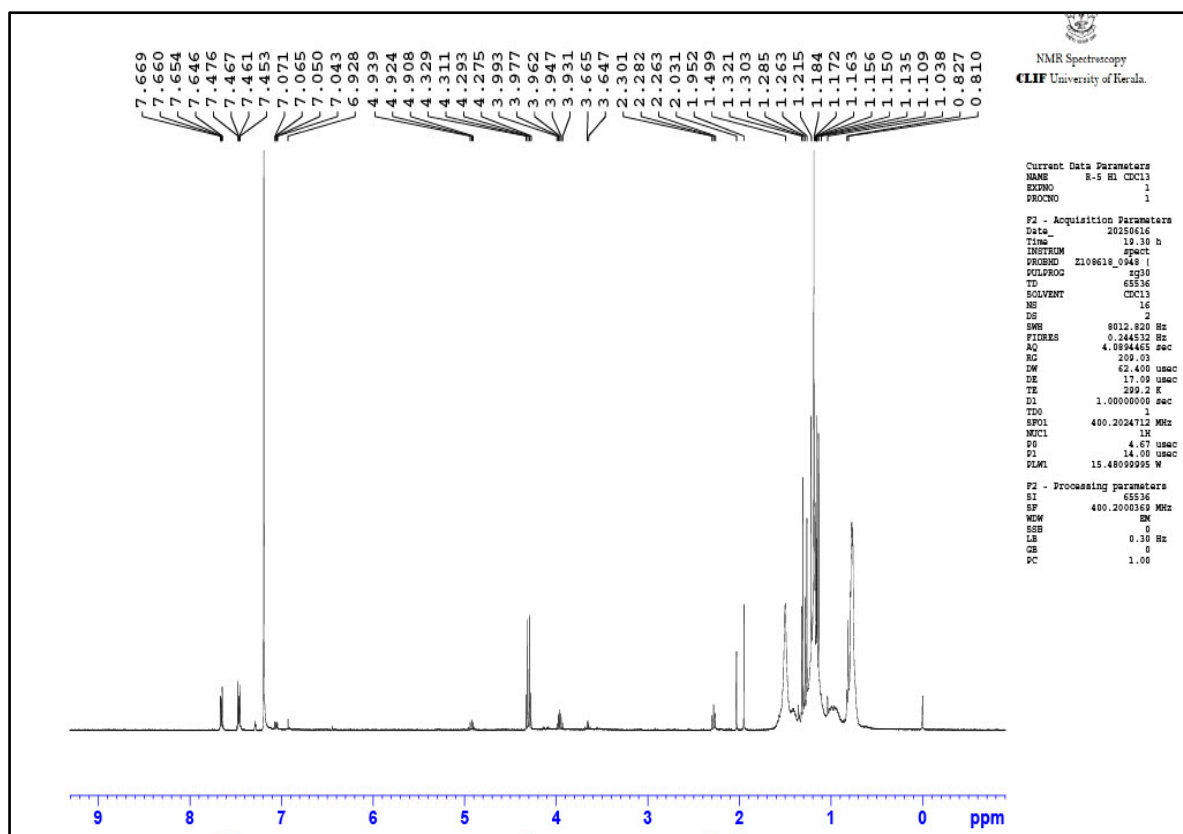


Plate 4.34. ^1H NMR spectrum of compound C5 isolated from TLC.

Chapter 5- General Discussions

The main aim of this study was to conduct a systematic and precise investigation into the traditional post-natal care practices involving *vethuvellam* within the Palakkad district of Kerala. This work aimed to bridge the gap between folk medicinal practices and contemporary scientific validation, thereby offering a comprehensive understanding of these folk medicinal remedies. By progressing from the ethnobotanical documentation to detailed phytochemical characterization and pharmacological assessment, the study has yielded significant insights into the therapeutic rationale behind these traditional practices, contributing to growing evidence that supports the integration of traditional medicine into modern healthcare practices.

The ethnobotanical survey is the basis of this research. The documentation of 62 plant species used in *vethuvellam* preparations is a proof of the rich biodiversity exploited by local communities and emphasises the importance of preserving such knowledge, especially in an era of rapid environmental and cultural change. The identification of 37 species not previously recorded for this specific postpartum application represents a substantial contribution to the regional and global ethnopharmacological study. The high Relative Frequency of Citation (RFC) and Use Value (UV) was observed for plants such as *C. longa* and *A. heterophyllus*. These findings indicate their local importance and their well-established roles in broader traditional medicine systems. *C. longa*, with its principal curcuminoids, is extensively recognized for its potent anti-inflammatory, antioxidant, and wound-healing properties, mechanisms crucial for postpartum recovery (Iweala et al., 2023; Verma et al., 2018; Chanda et al., 2019). Similarly, *A. heterophyllus* has documented antibacterial, antioxidant, and wound healing activities, further supporting its traditional use (Indrianingsih, 2024; Periyamayagam & Karthikeyan, 2013). The practice of using specific plant combinations, as documented, also hints at a traditional understanding of potential synergistic effects, a concept gaining increasing attention in modern pharmacology (Gilbert & Alves, 2003; Parasuraman et al., 2014).

The preliminary phytochemical analysis of the four selected plants, *Acalypha fruticosa*, *Justicia gendarussa*, *Piper longum*, and *Vitex altissima* confirmed that these plants are rich reservoirs of different secondary metabolites.

The most striking and significant finding was its dual immunomodulatory capacity. In the unstimulated THP-1 macrophages, both the hexane (VAH) and isopropyl alcohol (VAI) extracts showed immunostimulatory effects, by the upregulation of proinflammatory cytokines such as IL-1 β and TNF- α . The results suggested that *V. altissima* enhances the maternal basal

immune system, enhancing its readiness to combat opportunistic infections, which represent a significant concern during the physiologically demanding and often immunologically dynamic postpartum period (Alhazmi et al., 2021). Such an immune-priming effect, potentially mediated by pattern recognition receptor activation by plant-derived compounds, would be invaluable for maintaining maternal health and preventing puerperal sepsis, a major cause of maternal mortality worldwide (Say et al., 2014).

The LCMS analysis of *V. altissima* revealed several bioactive compounds. The identification of 89 distinct compounds in the water extract (VAW), 55 in the hexane extract (VAH), and 55 in the isopropyl alcohol extract (VAI) provided a detailed outlook on its chemical profile. The prevalence of phenolics (e.g., D-(-)-Quinic acid, Gallic acid, Caffeic acid) in the water extract and a rich diversity of flavonoids (e.g., Luteolin, Apigenin, Vitexin, Scutellarin) in the isopropyl alcohol and water extracts is of paramount pharmacological relevance. For instance, the presence of Luteolin and Apigenin is highly significant, as these flavonoids are extensively documented in scientific literature for their broad-spectrum biological activities, including potent anti-inflammatory (acting via NF- κ B and MAPK pathways), antioxidant, neuroprotective, and anticancer effects (Imran et al., 2019a; López-Lázaro, 2009; Salehi et al., 2019). Caffeic acid, another prominent phenolic identified, is recognized for its antioxidant, anti-inflammatory, and antimicrobial activities, often attributed to its ability to modulate cellular signaling pathways and enzyme activities (Aijaz et al., 2022; Espíndola et al., 2019). This detailed chemical fingerprinting provides a clear molecular explanation for the diverse biological activities observed in the subsequent bioassays, highlighting the plant's potential for multiple therapeutic actions.

The significant antioxidant activity demonstrated by the VAI extract, which was comparable to that of the standard, ascorbic acid, at certain concentrations, represents another important dimension of its therapeutic profile. Oxidative stress, resulting from an imbalance between reactive oxygen species (ROS) production and antioxidant defenses, is a known contributor to inflammatory processes, cellular damage, and delayed healing, all relevant to the postpartum state (Chaves et al., 2020; Agarwal et al., 2012). The demonstrated ability of *V. altissima* extracts to efficiently scavenge free radicals, as evidenced by the DPPH assay, likely contributes substantially to its overall protective and restorative effects during the postpartum period by mitigating ROS-induced damage and supporting cellular repair mechanisms. This observation is consistent with other studies that have reported notable antioxidant effects for *V. altissima* (Sridhar et al., 2005; Sundaresan et al., 2020).

The antimicrobial properties of *V. altissima* extracts, particularly VAI, provides a

strong scientific basis for the traditional use of *vethuvellam* in maintaining hygiene, preventing topical infections and promoting overall cleanliness for both mother and child.

The VAI extract showed strong, dose-dependent anti-inflammatory effects on LPS-stimulated macrophages, which is a standard model for acute inflammation. Excessive inflammation is a major problem faced during the postpartum period. The overexpression of IL-1 β and TNF- α leads to the production of COX-2 which in turn activates NF- κ B, MAPK and JAK and STAT signalling cascades resulting in chronic inflammations (Kim et al., 2010; Maity et al., 2012; Oluwamodupe et al., 2024). The over production of IFN β results in the activation of STAT 1 which also leads to inflammation (Toshchakov et al., 2002). The overexpression of CCR-5 leads to macrophage accumulation and IL-1 β production leading to chronic inflammations (Chen et al., 2021). The significant downregulation of IL-1 β , TNF α , IL-6, IFN β and the chemokine receptor CCR-5 indicates the ability of VAI to effectively reduce the overactive immune response. This capacity to reduce excessive inflammation is crucial for optimal postpartum recovery, as excessive inflammation cannot only affect wound healing (e.g., perineal tears, caesarean section incisions) and tissue repair but also contribute to systemic complications and potentially intensify conditions like postpartum depression (Ross et al., 2022; Brann et al., 2019; Kendall-Tackett, 2007). This dual action, enhancing baseline immune surveillance while simultaneously possessing the ability to control excessive inflammation indicated that plant is a good immunomodulator, capable of restoring homeostasis (Schepetkin & Quinn, 2006; Spelman et al., 2006). This aligns perfectly with the traditional therapeutic goals of post-natal care, which aim to restore balance and promote holistic recovery. The anti-inflammatory properties of various extracts *V. altissima* have been reported in other scientific investigations (Sridhar et al., 2005; Lekkala et al., 2025).

The antifungal activity against *Aspergillus niger*, though not as pronounced against *Candida albicans* in this study, also needs further investigation, as fungal infections can also pose risks in the postpartum period.

The discovery that TLC isolated compound C7 derived from VAI exhibited antibacterial activity against *Bacillus subtilis* that exceeded the potency of the standard antibiotic Amoxicillin in the tested concentration is particularly an important highlight of this research. This finding identifies a promising natural product lead for the development of novel antibacterial agents. This is of great importance in the current global health landscape, which is increasingly threatened by the rise of antibiotic-resistant microbial strains (Ahmed and Mikail, 2017; WHO, 2021). Other researchers have also reported antibacterial activity for *V. altissima*, further supporting its potential (Kannathasan et al., 2011; Irulandi et al., 2017).

This study provides a detailed *in vitro* validation for the traditional uses of these plants but, it is essential to acknowledge certain limitations of our study. The research primarily focused on the effects of individual plant extracts. However, *vethuvellam*, in its traditional application, is often a complex polyherbal preparation. The use of different plant species in this preparation could lead to synergistic, additive, or even antagonistic effects that are not captured when studying extracts in isolation (Gertsch, 2011). Future investigations should be needed to explore the bioactivity of traditional combinations, potentially employing network pharmacology approaches to study these complex interactions. Additionally, *in vitro* results, while highly indicative of potential, require careful interpretation and must be further validated through well-designed *in vivo* animal models. Such studies are necessary to assess systemic effects, bioavailability, metabolic fate, dose-response relationships, and, crucially, the safety profile (including potential effects on lactation or neonatal development if systemically absorbed) of these extracts before any consideration for clinical translation in human populations. The development of standardized extracts, ensuring consistent phytochemical profiles, will also be critical for reproducible research and therapeutic application (WHO, 1998; Kunle et al., 2012; Gaedcke and Steinhoff, 2003).

In conclusion, this work has successfully bridged the gap between traditional folk medicinal knowledge and scientific evidence. This study had documented the rich tradition of *vethuvellam* use in Palakkad, identified a wide array of phytochemicals that likely supports its therapeutic actions, and demonstrated the potent and relevant immunomodulatory, anti-inflammatory, antioxidant, and antimicrobial properties of its constituent plants, with special focus on *Vitex altissima*. The findings of the study offer a clear and scientifically grounded explanation for the traditional use of these plants in post-natal care. This research, therefore, makes a significant contribution for the preservation of invaluable folk medicinal knowledge and also laid a strong and promising foundation for future research aimed at developing standardized, safe, and effective evidence-based herbal interventions to enhance maternal and child health globally, potentially offering culturally acceptable and accessible healthcare options.

Chapter 6- Summary and Conclusion

This study aimed to document and scientifically validate the traditional use of medicinal plants in Palakkad, Kerala, for post-natal care, with a particular focus on *Vethuvellam*, a medicated water used for bathing mothers and infants after delivery. By integrating ethnobotanical surveys, phytochemical analysis, and bioactivity assessments, the research sought to bridge traditional knowledge with modern scientific validation. The findings not only preserve cultural heritage but also provide a foundation for evidence-based applications in maternal and child health.

6.1 Major Findings

The study yielded several key insights, which are summarized below:

6.1.1 Ethnobotanical Survey:

1. A total of 62 plant species were identified as being used for preparing *Vethuvellam* in Palakkad's folk medicine for post-natal care. Of these, 37 species were first reported in this study, while 25 had been previously documented. The newly reported plant species for the medicated bath were *Xylia xylocarpa*, *Vitex altissima*, *Thespesia populnea*, *Terminalia paniculata*, *Schleichera oleosa*, *Sida acuta*, *Stereospermum colais*, *Strychnos nux-vomica*, *Ricinus communis*, *Pseudarthria viscida*, *Plumbago zeylanica*, *Pongamia pinnata*, *Premna serratifolia*, *Pterocarpus marsupium*, *Coleus amboinicus*, *Piper longum*, *Ocimum gratissimum*, *Moringa oleifera*, *Mussaenda frondosa*, *Leucas aspera*, *Jatropha hernandifolia*, *Justicia gendarussa*, *Hamelia patens*, *Eucalyptus globulus*, *Chrysopogon zizanioides*, *Cymbopogon flexuosus*, *Croton persimilis*, *Coffea arabica*, *Clitoria ternatea*, *Centella asiatica*, *Causonis trifolia*, *Borassus flabellifer*, *Anethum graveolens*, *Acorus calamus*, *Achyranthes aspera*, *Acalypha fruticosa*, *Senegalia caesia*.
2. Leaves were the most commonly used plant part, with *Artocarpus heterophyllus* leaves being the most frequently cited.
3. Both single and compound preparations were employed. Single preparations included *Coleus amboinicus*, *Ocimum tenuiflorum*, *Acalypha fruticosa*, and *Vigna radiata*, while 16 combinations of different plants were used as compound preparations.
4. Quantitative ethnobotanical indices (Relative Frequency of Citation (RFC), Consensus (CI), and Use Value (UV)) indicated that *Curcuma longa* and *Artocarpus heterophyllus* were the most frequently used plants.

6.1.2 Traditional Benefits:

Vethuvellam is traditionally believed to enhance disease resistance, improve blood circulation, reduce inflammation, aid in postpartum recovery, brighten skin, and alleviate body pain in mothers. For infants, it is thought to boost immunity and facilitate umbilical cord drying. Traditional oil massaging supports maternal abdominal muscle restoration and infant development.

6.1.3 Phytochemical and Bioactivity Analysis:

1. Four plants (*Acalypha fruticosa*, *Justicia gendarussa*, *Piper longum*, and *Vitex altissima*) were selected for detailed study based on their traditional use.
2. Preliminary phytochemical analysis revealed the presence of alkaloids, flavonoids, phenolics, steroids, tannins, and other compounds in hexane, water, and isopropyl alcohol extracts.
3. Immunomodulatory studies showed that all extracts modulated the mRNA expression of IL-1 β , IFN β , TNF α , and CCR-5 in THP1-derived macrophages.
4. *Vitex altissima* (VA) was selected for further investigation due to its promising results:
 - a. **Extracts:** Hexane (VAH), water (VAW), and isopropyl alcohol (VAI) were prepared.
 - b. **Immunomodulatory Activity:** VAH and VAI upregulated IL-1 β , IFN β , TNF α , and CCR-5 in unstimulated cells, indicating immunostimulatory properties. VAW had no significant effect.
 - c. **Antioxidant Activity:** VAI exhibited the highest DPPH radical scavenging activity, with 1 mg/ml VAI equivalent to 0.025 mg/ml of ascorbic acid. The least antioxidant activity was observed with hexane extracts.
 - d. **Antibacterial Activity:** VAI showed the strongest activity, particularly against *Bacillus subtilis*.
 - e. **LCMS Analysis:** Identified 89, 55, and 55 compounds in VAW, VAH, and VAI, respectively, with phenolics, fatty acids, and flavonoids as dominant groups.
 - f. **Anti-inflammatory Activity:** VAI downregulated inflammatory genes (IL-1 β , IFN β , TNF α , IL-6, CCR-5) in LPS-stimulated macrophages, indicating anti-inflammatory potential.
 - g. **Antifungal Activity:** VAI inhibited *Aspergillus niger* but not *Candida albicans*.

- h. **TLC Analysis:** Seven spots were identified in VAI, with fractions C5, C6, and C7 showing antibacterial activity against *Escherichia coli* and *B. subtilis*, surpassing Amoxicillin (15 µg).

6.2 Conclusion

This study successfully documented and validated the traditional use of medicinal plants in Palakkad for post-natal care, particularly through *Vethuvellam*. By combining ethnobotanical surveys with phytochemical and bioactivity analyses, it demonstrated the scientific basis for these practices. The findings highlight the potential for integrating traditional knowledge into modern healthcare while emphasizing the need for further research to translate these insights into practical applications. Ultimately, this research not only preserves cultural heritage but also paves the way for evidence-based herbal medicine in maternal and child care.

The study brings out the traditional knowledge about plants used for bathing mother and child in various parts of Palakkad district. The medicated bath was very common in various parts of Kerala. The traditional birth attendants took care of mother and child after delivery. They prepare the medicated bath for mother and child. This practice is very common in many rural areas of Kerala. In urban areas these treatments are provided by many Ayurvedic hospitals. From our study we documented 62 plant species used for bathing mother and child after delivery. All these plants possessed various medicinal properties. This medicated bathing helps the mothers to restore their body after delivery and increases the immunity in infants against various diseases. All the plant species cited in the study were locally available plant species. The documentation of these plant species, their methods of preparation and uses contributes a treasure for further studies on these plants leading to the production of new bioactive compounds for post-natal care.

The preliminary phytochemical analysis of hexane, water and isopropyl alcohol extracts of *A. fruticosa*, *J. gendarussa*, *P. longum* and *V. altissima* revealed the presence of many bioactive secondary metabolites like flavonoids, phenols, saponins, tannins, alkaloids etc. All these plants are used in post-natal care and the presence of these phytochemicals is attributed to its pharmacological properties.

The immunomodulatory studies in these selected plants revealed that all the selected plants possess immunostimulatory activity. The in vitro macrophage activation studies were conducted in THP1 derived macrophages treated with hexane, water and isopropyl alcohol extracts of the selected plants. The mRNA expression of IL-1 β , IFN β , TNF α and CCR-5 were

analysed with internal control gene GAPDH. We selected *V. altissima* for our further studies based on our results and literature review. Cytotoxic studies *V. altissima* hexane (VAH), water (VAW) and isopropyl alcohol (VAI) extracts on THP1 derived macrophages revealed that hexane extract is more cytotoxic and water extract is least cytotoxic. This cytotoxic property can be used to exploit the anticancer activity of *V. altissima*. The immunomodulatory studies on different concentrations of extracts revealed that VAI and VAH extract treated cells showed an immunostimulatory effect in all the treated concentrations.

Our study on LCMS analysis of *V. altissima* revealed the presence of 89, 55 and 55 compounds from water, hexane and isopropyl alcohol extracts of *V. altissima*. Majority of these compounds possessed various bioactivities. The TLC analysis of *V. altissima* isopropyl alcohol extract revealed the presence of seven bands. The presence of these phytochemicals may contribute to the various medicinal importance of *V. altissima*.

The anti-inflammatory activity studies in LPS treated THP1 derived macrophages revealed that the extract treated cells downregulates the expression of IL-1 β , IFN β , TNF α , IL-6 and CCR-5 in all treated concentrations. The immunomodulation studies confirmed the immunostimulatory and anti-inflammatory activity of *V. altissima*. VAI extract showed prominent antioxidant activity studies compared to VAW and VAH in DPPH radical scavenging assay. VAI also possessed maxim antibacterial activity than VAW and VAH. The antifungal studies on VAI revealed that VAI showed prominent antifungal activity against *A. niger*. The phytochemical studies on *V. altissima* revealed the presence of many bioactive compounds. These compounds possess many bioactivities. So, further studies on this plant can explore the production of some drug molecules from *V. altissima*.

Chapter 7- Recommendations for Future perspectives

The study highlights the importance of folk medicine of Palakkad for post-natal care. The study investigated the immunomodulatory properties and phytochemical analysis of selected plants used in folk medicine of Palakkad for post-natal care. The study aimed to validate the use of botanical remedies during the post-natal period with special focus on plants used for bathing mother and child after delivery. A systematic approach was employed with a survey on plants used for medicated baths of mother and child, followed by phytochemical and bioactivity analysis of selected plants. The recommendations suggested for future studies are:

1. Our study identified *V. altissima* as a promising candidate due to its immunostimulatory, anti-inflammatory, antioxidant, antibacterial and antifungal activity, particularly its isopropyl alcohol extract (VAI). However, the results of *in vitro* assays alone are insufficient to bridge the gap to clinical application thus limiting clinical opportunities. *In vivo* studies using animal models are needed to evaluate the safety, efficacy and pharmacokinetics of *Vitex altissima* extracts. This could involve testing VAI for its effects on postpartum inflammation or infections.
2. Advanced phytochemical analysis such as isolation and structural elucidation using techniques like NMR or mass spectroscopy will reveal the active compounds responsible for the observed bioactivities.
3. The other plants cited in the survey like *Acalypha fruticosa*, *Justicia gendarussa* and *Piper longum* should be investigated for similar bioactivities for exploiting their therapeutic potential.
4. Ethnobotanical survey on plants used for post-natal care should be extended to other regions of Kerala and India to document variations in traditional practices and plant use.
5. Studies on synergistic effects of plant combinations used in the medicated water is necessary to develop standardized formulation for consistent efficacy.
6. Comparative studies should be done with those in other regions especially with Southeast Asian countries where this type of medicated bath is common.
7. Assess the sustainability of harvesting these plants and explore cultivation methods to ensure their availability without depleting natural resources.
8. Conservation of locally available plants mentioned in the study through *in situ* or *ex situ* conservation strategies.

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Appendix
List of informants

SL.No	Name and address of informants	Job details
1.	Aalice Kazippanplackal, Munnekkar.	House wife
2.	Achamma Poullose Cheruparambil, Munnekkar.	House wife
3.	Ajeesha Kovil street, Near Victoria College Palakkad.	House wife
4.	Ajitha Near FCI, Puthuppariyaram, Palakkad.	House wife
5.	Ajitha Angadical House, Palappuram.	House wife
6.	Ambika Kannathu, Arangottukara.	House wife
7.	Ambika Sreesushil, Kottayi.	House wife
8.	Amina O Odala House, Ariyur, Mannarkad.	Folk medicine practitioner
9.	Ammini Near FCI, Puthuppariyaram, Palakkad.	House wife
10.	Ammini Sreedhanya, Panankavil, Chambaramkulam, Kottai.	House wife
11.	Ammini Evattakulambil House, Plazhy, Palakkad.	House wife
12.	Ammu Near Milma, Kottekkad, Padalikkad Rd, Kalepully.	House wife

13.	Anamma Kattumattam, Panga, Meenvallam.	House wife
14.	Anitha Angadica, Palappuram.	House wife
15.	Anju G Sree Nilayam, Mangalathanchalla, Pampampallam P O. Palakkad.	House wife
16.	Annama Maniyangandu, Kottayi	House wife
17.	Asha Near Milma, Kottekkad Padalikkad Rd, Kalepully.	House wife
18.	Aswathy Arangottukara, Pattambi.	House wife
19.	Aysha Vettiyanikkal, Panga.	House wife
20.	Aysha Nasrin Oddala House, Ariyur, Mannarkad.	House wife
21.	Bageerathi Chunkam, Tattamangalam.	House wife
22.	Bargaviamma Bargavinivas, Arangottukara.	House wife
23.	Beebathu Puthenpidika, Arangottukara.	House wife
24.	Beevathumma Pulliyangadu, Kangirampara, Munnekkar.	House wife
25.	Bhanu Vasudevan Mulakkal, Arangottukara.	House wife
26.	Bhargavi Manusmrithi, Palappuram.	House wife

27.	Bhavani Puthiyedathu, Palappuram.	House wife
28.	Bindhu Varthanagar, Palakkad.	House wife
29.	Bindhu Chakkalackal, Alamkode, Olavakkode, Palakkad.	House wife
30.	Bindhu Puthuppariyaram, Palakkad,	House wife
31.	Bindhu Bindhu Nivas, Palappuram.	House wife
32.	Chandrika Purasseriyl, Arangottukara.	House wife
33.	Chandrika Arunima, Kottayi.	House wife
34.	Chandrika Sumathi Nivas, Chambrakulam, Kottayi.	House wife
35.	Chenthamarakshi Madathil parambil house, Korenchira PO. Kizhakkenchery, Chittakalam.	House wife
36.	Chiku Kattumattam, Panga.	House wife
37.	Chinnamma Kunnel, Panga.	House wife
38.	Danalakshmi Kunnathupalayam Near Murugan Kovil Thekkaegramam Chittur	House wife
39.	Devaki Sreedhanya, Panakavil, Chambaramkulam, Kottai. Palakkad	House wife

40.	Devaki Chambramkulam Colony, Kottayi.	House wife
41.	Devi Alamkode, Olavakkode, Palakkad.	House wife
42.	Dhanalekshmi Kalarikkal Parambil, Arangottukara.	House wife
43.	Dhanya Nedumani, Nenmeni P O, Kollengode.	House wife
44.	Eliamma Nadupadavil, Panga.	House wife
45.	Fathima Thekkathil, Arangottukara.	House wife
46.	Fathima, Puthenpidikayil, Arangottukara.	House wife
47.	Gayathri Palapuram angadi.	House wife
48.	Geetha Geetha Nivas, Palappuram.	House wife
49.	Geetha Mazhavil Nagar, Thekkaegram PO.	House wife
50.	Geetha Vetrivel Nilayam, Kunnathupalayam, Thekkaegramam, Chittur.	House wife
51.	Geetha Puthenparambil, Panga, Meenvallam.	House wife
52.	Girija Venkateshapuram Colony, Puthur, Palakkad.	House wife
53.	Girija Akkethaethara, Malampuzha.	House wife

54.	Girija Kottekkad, Padalikkad Road, Palakkad.	House wife
55.	Girija Oduparakunn, Arangottukara.	House wife
56.	Girija Naduvil, Arangottukara.	House wife
57.	Girija Melepurakkal, Arangottukara.	House wife
58.	Gourikutti Arangottukara, Pattambi.	House wife
59.	Greeshma Kalarikkal Parambil, Arangottukara.	House wife
60.	Hajira P Panathodi House, Munnekkar.	House wife
61.	Haritha Kizhanellor mana, Palappuram.	BAMS Student
62.	Hayarnisa Kattutheruvu, Puthunagaram.	House wife
63.	Hemalatha K B Lekshmi Nilayam, Katampazhipuram.	House wife
64.	Indhupriya Krishnakripa, Palappuram.	House wife
65.	Indira Kizhakkaepattu, Palappuram.	House wife
66.	Indira Koolath Pumb Engine Road, Olavacode.	House wife
67.	Indumathi Adiparambil, Arangottukara.	House wife

68.	Ishwari Kunnathupalayam, Thekkaegramam, Chittur.	House wife
69.	Jabeena Valiyaparambil, Odanur, Parali.	Research Scholar
70.	Jalaja Kannathu, Arangottukara.	House wife
71.	Janaki Kunnathupurakkal, Arangottukara.	House wife
72.	Jayanthi Pottakkapadam, Tattamangalam.	House wife
73.	Jishamol Kalarikkal, Kottayi.	House wife
74.	Kalamani Pottakkapadam, Tattamangalam.	House wife
75.	Kamalam Kannathil, Arangottukara.	House wife
76.	Kamalam Pottakkapadam, Tattamangalam.	House wife
77.	Kamalam Vatsalyam, Kottayi.	House wife
78.	Kamamma Alamkode, Olavakkode, Palakkad.	House wife
79.	Kamamma Near Sayi hospital, Olavacode, Palakkad.	House wife
80.	Kanakamma Kottekkad Padalikkad Road, Palakkad	House wife
81.	Karthiayini Ittiyamparambu, Arangottukara.	House wife

82.	Karthiayini Pattaruparambil, Arangottukara.	House wife
83.	Kausalya Near FCI, Puthuppariyaram, Palakkad.	House wife
84.	Kavitha Kunnathupalayam, Thekkaegramam, Chittur.	House wife
85.	Khadija Poolath house, Arangottukara.	House wife
86.	Kochamminiamma Near FCI, Puthuppariyaram, Palakkad.	House wife
87.	Komalam Kottekkad Padalikkad Road, Palakkad.	House wife
88.	Komalam Pulicaparambil, Palappuram.	House wife
89.	Krishnakumari, Tekkumpuram house, Kalappetty, Kuzhalmannam.	House wife
90.	Kumari Near Milma, Kottekkad Padalikkad Rd, Kalepully.	House wife
91.	Kunjulekshmi Chambramkulam Colony, Kottayi.	House wife
92.	Lally Patteril, Munnekkar, Meenvallam.	House wife
93.	Latha Near Milma, Kottekkad Padalikkad Rd, Kalepully.	House wife
94.	Latha Near Milma, Kottekkad Padalikkad Rd, Kalepully.	House wife
95.	Latha Thadathil, Arangottukara.	House wife

96.	Latha V Edapura House, Chanthiruthy, Koduvayoor PO.	House wife
97.	Leeja L Madathil Parambil House, Kalayani nagar, Ramanathapuram, Palakkad.	Teacher
98.	Leela Sreejesh Padariyatil, Arangottukara.	House wife
99.	Leela Vempala, Munnekkar.	House wife
100.	Leelamma Puthupillaedathu, Panga.	House wife
101.	Leelamma (Jaisy) Maruthamkadu, Munnekkar.	Folk medicine practitioner
102.	Leelavathy Cheerambathu Padinjarethu, Arangottukara.	House wife
103.	Lekshmi Near Milma, Kottekkad Padalikkad Rd, Kalepully.	House wife
104.	Lekshmi Nedumani, Nenmeni PO. Kollengode.	House wife
105.	Lekshmi Chambrakulam Colony, Kottayi.	House wife
106.	Lekshmi amma Palappuram angadii.	House wife
107.	Lekshmikutty Sneha nivas, Akathethara Engg. College PO. Malampuzha.	House wife
108.	Lekshmikuttyamma Pangilkizakkaekara, Panga.	House wife
109.	Lisy Killarikunnel, Munnekkar.	House wife

110.	Madhavi Near Milma, Kottekkad Padalikkad Rd. Kalepully.	House wife
111.	Madhaviamma Kizhupulli, Panga.	House wife
112.	Madhavikutty Payiazhi, Palappuram.	House wife
113.	Madhavikutty Palappuram angadi.	House wife
114.	Manikam Kunnathupalayam, Thekkaegramam, Chittur.	House wife
115.	Maniyamma Ayyapuram, Palakkad.	House wife
116.	Mariumma Thorapalayam, Palakkad.	Folk medicine practitioner
117.	Mariya Vengilaparambil, Arangottukara.	House wife
118.	Mariyam Thorapalayam, Palakkad.	House wife
119.	Mary Near Milma, Kottekkad Padalikkad Rd, Kalepully.	House wife
120.	Mary Kunnel, Panga.	House wife
121.	Maya Kurunkulathil, Chorottur, Ottapalam.	Research Scholar
122.	Meena Kumari Sreelakshmi house, Arangottukara.	House wife
123.	Meenakshi Thavalathil, Palappuram.	House wife

124.	Meharin Near Milma, Kottekkad Padalikkad Rd, Kalepully.	House wife
125.	Mini Memana, Munnekkar.	House wife
126.	Molamma Munnekar, Meenvallam.	Employed
127.	Mumthas Kallekkulangara, Akathethara, Palakkad.	House wife
128.	Mumthas Valavathu, Arangottukara.	House wife
129.	Nabeesa Kovil street Near Victoria College, Palakkad.	House wife
130.	Nabhisa Padikkaparambil, Arangottukara.	House wife
131.	Nabhisa Keelathuvalappil, Arangottukara.	House wife
132.	Nabhisa Muzhavankodu, Kottayi.	House wife
133.	Nanikutty Sayoojyam, Punnassery House, Katampazhipuram.	House wife
134.	Naseema Parakunnam, Victoria College Road.	Employed
135.	Neeli Cherukunnathu House, Arangottukara.	House wife
136.	Nirmala Arangottukara.	House wife
137.	Nirmala Ambadi Parambil, Arangottukara.	House wife

138.	Nisha Kootupilak, Arangottukara.	House wife
139.	Nisha Onganattu, Arangottukara.	House wife
140.	Noorjahan Keelathuvalappil, Arangottukara.	House wife
141.	Noushitha Poolath house, Arangottukara.	House wife
142.	Nurjahan Puthanpeedikayil, Arangottukara.	House wife
143.	Omana Thorapalayam, Palakkad.	House wife
144.	Omana Parithikattilvalapil, Arangottukara.	House wife
145.	Omana Chambramkulam Colony, Kottayi.	House wife
146.	Omana Kizhakkaekara, Munnekkar.	House wife
147.	Padmavathi Kurupath House, Alampallam, Kallepully P O.	House wife
148.	Padmavathi Kunnathupalayam, Thekkaegramam, Chittur.	House wife
149.	Padmini K Thekkekara House, Kottayi, Palakkad.	House wife
150.	Panchali Chambramkulam Colony, Kottayi.	House wife
151.	Panjali Kurupath House, Alampallam, Kallepully P O.	House wife

152.	Pankajakshi Kottekkad Padalikkad Road, Palakkad.	House wife
153.	Parameshwari Kunnathupalayam, Thekkaegramam, Chittur.	House wife
154.	Parvathi Kurupath House, Alampallam, Kallepully P O.	House wife
155.	Parvathi Udayam, Kunnathupalayam, Thekkaegramam, Chittur.	House wife
156.	Parvathy Thekaeparambil, Arangottukara.	House wife
157.	Pathmavathi amma Near Milma, Kottekkad Padalikkad Rd, Kalepully.	House wife
158.	Pazhaniammal Kunnathupalayam, Thekkaegramam, Chittur.	House wife
159.	Prabhavathi Kunnathupalayam Thekkaegramam Chittur	House wife
160.	Prasana Sreeshayilam, Palapuram.	House wife
161.	Prasana 16/144 (10), Mazhavil Nagar, Thekkegramam PO, Palakkad.	House wife
162.	Preetha Cheruvallikunnathu, Arangottukara.	House wife
163.	Preetha Chambramkulam Colony, Kottayi.	House wife
164.	Prema Near Milma, Kottekkad Padalikkad Rd, Kalepully.	House wife
165.	Prema Kalluvattiparambu, Chambrakulam, Kottayi.	House wife

166.	Princy Near electricity office, Pudhupariyaram, Palakkad.	House wife
167.	Pushpa Near Milma, Kottekkad Padalikkad Rd, Kalepully.	House wife
168.	Radha Ashirvadam, Palappuram.	House wife
169.	Radha Kuriyallur, Nenmara.	House wife
170.	Radha Chambramkulam Colony, Kottayi.	House wife
171.	Rahmath M Kunningal House, Pudupariyaram.	House wife
172.	Rahumath Madakkati, Vadakathil, Arangottukara.	House wife
173.	Rajalekshmi Kunnathupalayam, Near Murugan Kovil, Thekkaegramam.	House wife
174.	Rajamani Adattukunnu colony, Arangottukara.	House wife
175.	Rajamma Aadi Nivas, Thekkaegramam, Chittur.	House wife
176.	Rajani P K Aiswarya, Punnassery House, Katampazhipuram.	House wife
177.	Rajeshwari Kunnathupalayam, Near Murugan Kovil, Thekkaegramam. Chittur.	House wife
178.	Rajeshwari, Palappuram angadi.	House wife
179.	Raji Kalapathy, Palakkad.	House wife

180.	Rajitha Kalarikkal Parambil, Arangottukara.	House wife
181.	Rajitha Chambramkulam Colony, Kottayi.	House wife
182.	Rakki Chambrakulam Colony, Kottayi.	Folk medicine practitioner
183.	Ramadevi Kizhakkaepattu variyam, Palappuram.	House wife
184.	Ramla Kunnathupaedikayil, Arangottukara.	House wife
185.	Rasheeda Pulikkal, Arangottukara.	House wife
186.	Raziya Kakkadath, Arangottukara.	House wife
187.	Rema Near FCI, Puthuppariyaram, Palakkad.	House wife
188.	Renuka Pulavar House, Palappuram.	House wife
189.	Rosemary Thudikode, Munnekkar.	House wife
190.	Rugmani Pothundy, Nemara.	House wife
191.	Sajini Thadathil, Arangottukara.	House wife
192.	Sajini K P Kalathilpadi, Kizhumuri P O, Pullaserry.	Research Scholar
193.	Sajitha Ayyapuram, Palakkad.	House wife

194.	Sajitha Adattukunnu colony, Arangottukara.	House wife
195.	Sajitha Vishwavihar Palace Corner, Kollengode.	Teacher
196.	Sajitha Puthenpura House, Thakkaegramam, Chittur.	House wife
197.	Sakira Pilakodu, Arangottukara.	House wife
198.	Sandhya Palapuram angadi.	House wife
199.	Sangeetha Chandrodayam, Kottayi.	House wife
200.	Santha Near Milma, Kottekkad Padalikkad Rd, Kalepully.	House wife
201.	Santha Erakkattil House, Punchappadam, Palakkad.	House wife
202.	Santhini Kalavarapadi, Thirumittakode, Kootanad.	House wife
203.	Saranya Chambrakulam Colony, Kottayi.	House wife
204.	Saraswathi Palappuram theruvu.	House wife
205.	Saraswathi Saraswathi Nivas, Palappuram.	House wife
206.	Saraswathi Sreelakshmi Nilayam, Arangottukara.	House wife
207.	Saraswathi Kunnathupalayam, Thekkaegramam, Chittur.	House wife

208.	Saraswathi R Athira House, Althara, Kodumbu, Palakkad.	House wife
209.	Sarojini Kunnatupurakkal, Arangottukara.	House wife
210.	Sarojini Moothedathu, Estatepadi, Arangottukara.	House wife
211.	Sarojini Kurupath House, Alampallam, Kallepully P O.	House wife
212.	Sarojini Kunnathupalayam, Thekkaegramam, Chittur.	House wife
213.	Sarojini Kuthanur, Mannanur, Palakkad.	House wife
214.	Sarojinidevi Palappuram theruvu.	House wife
215.	Sathyabhama Muzhavankodau, Kottayi.	House wife
216.	Savithri Pulavar House, Palappuram.	House wife
217.	Savithri Kunnathupalayam, Near Murugan Kovil, Thekkaegramam.	House wife
218.	Sebunisa Kovil street, Near Victoria College, Palakkad.	House wife
219.	Selvi Kunnathupalayam, Thekkaegramam, Chittur.	House wife
220.	Shamila Ismail Ameena Manzil, Thottapura, Malampuzha.	Research Scholar
221.	Sheela Kottekkad Padalikkad Road, Palakkad.	House wife

222.	Shoba Chelathuparambil, Arangottukara.	House wife
223.	Shobana Shobanam, Palappuram.	House wife
224.	Shobhana Pulari House, Arangottukara.	House wife
225.	Shyama Shyama Nivas, Palappuram.	House wife
226.	Shyla Pottamplakkal, Panga.	House wife
227.	Sindhu House No 4(1), Near Parassala High School, Chittur Palakkad.	House wife
228.	Sindhu Chambrakulam Colony, Kottayi.	House wife
229.	Smitha Cheruvalli, Arangottukara.	House wife
230.	Soumya Peruvallikunnathu, Arangottukara.	House wife
231.	Sreedevi Tharekkad Vinayaka colony, Palakkad.	House wife
232.	Sreedevi Sankarathu Padinjaraethil, Arangottukara.	House wife
233.	Sreedevi Kalarikkal Parambil, Arangottukara.	House wife
234.	Sreeja Kanjirampara, Munnekkar.	House wife
235.	Sreelakshmi Thorapalayam, Near Victoria College, Palakkad.	House wife

236.	Sreelatha Kovil street, Near Victoria College, Palakkad.	House wife
237.	Sreelatha Puthan Nivas, Arangottukara.	House wife
238.	Sudha Arangottukara.	House wife
239.	Sukanya Near FCI, Puthuppariyaram, Palakkad.	House wife
240.	Sumathi Sreenilayam, Thekkaegramam, Chittur.	House wife
241.	Sumitra Near Milma, Kottekkad Padalikkad Rd, Kalepully.	House wife
242.	Sumitra Sumitra Bhavan, Arangottukara.	House wife
243.	Sundari House No 265, Near Parassala High School, Chittur.	House wife
244.	Suni Maruthamalayi, Panga.	House wife
245.	Sunira Kunnumpurath, Peruvallikunn, Arangottukara.	House wife
246.	Sunitha Chirakkandathu, Mankara.	Self employed
247.	Sunitha Elavallipadi, Arangottukara.	House wife
248.	Sunitha Chembakulam, Munnekkar, Meenvallam, Mannarkad.	House wife
249.	Sunitha Kaliyathu, Munnekkar.	House wife

250.	Sushama Kizhakoottu, Arangottukara.	House wife
251.	Syama P S Pragathi, Chettiamkunnath Thazhathethil, Punchappadam.	Research Scholar
252.	Thanka Chambramkulam Colony, Kottayi.	House wife
253.	Thankam Kunnathupalayam, Near Murugan Kovil, Thekkaegramam.	House wife
254.	Thankamani Near Milma, Kottekkad Padalikkad Rd, Kalepully.	House wife
255.	Thankamani Haritham, Arangottukara.	House wife
256.	Thankamani Chambrakulam Colony, Kottayi.	House wife
257.	Thankamma Near Milma, Kottekkad Padalikkad Rd, Kalepully.	House wife
258.	Thankamma Mannath, Valmutti, Thekkaegramam, Chittur.	House wife
259.	Theresiamma Malamel, Panga.	House wife
260.	Thithu Kurinjimayil, Arangottukara.	House wife
261.	Unniamma Kinassery, Palakkad.	Folk medicine practitioner
262.	Usha Chambramkulam Colony, Kottayi.	House wife
263.	Usha Puthenveedu Kunnathupalayam, Thekkaegramam, Chittur.	House wife

264.	Usha Surendaran Arukattu, Panga	House wife
265.	Ushadevi Kurunkulathil, Chorottur, Ottapalam.	House wife
266.	Ushakumari Pangaekizakkaekara, Panga.	House wife
267.	Valsala Anand Nivas, Arangottukara.	House wife
268.	Vanaja Adichera, Puthunagaram.	House wife
269.	Vasanth Near Milma, Kottekkad Padalikkad Rd, Kalepully.	House wife
270.	Vasanthi A K Akshya Villa, Nambullipura P O, Mundur.	House wife
271.	Vatsala Kovil street, Near Victoria College, Palakkad.	Rtd. Government employee
272.	Vidhya Variyathu Valappil, Palappuram.	House wife
273.	Vijayakumari Vinod Nivas, Kottayi.	House wife
274.	Vijayalakshmi Mankara, Palakkad.	House wife
275.	Vijayalekshmi Mattummel Kanjirampara, Meenvalam.	House wife
276.	Vijayalekshmi Chemanaparambil, Munnaekkar.	House wife
277.	Vijayamma Near FCI, Puthuppariyaram, Palakkad.	House wife

278.	Vimala Kottekkad, Padalikkad Road, Palakkad.	House wife
279.	Vimala Bhavani Nivas, Arangottukara.	House wife
280.	Vimaladevi Sekharipuram, Kalpathy, Palakkad.	House wife
281.	Vinodini Kadavathu, Koduvayur, Palakkad.	House wife
282.	Vishalakshi Kulamprumpurath, Arangottukara.	House wife

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