"Deciphering the Antimicrobial, Antioxidant, Cytotoxic and Anti-inflammatory role of select Ethnomedicinal plants from Siriya Kalvarayan Hills, The Eastern Ghats of India conjointly with Phytochemical and Bioinformatic analyses"

Thesis submitted to Bharathidasan University
in partial fulfilment of the requirements
for the degree of
DOCTOR OF PHILOSOPHY IN BOTANY

By

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(Ref. No. 1962/Ph.D. K1/Botany/Full Time/April 2017)

Under the Guidance of

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This is to certify that the thesis, **"Deciphering the Antimicrobial, Antioxidant, Cytotoxic and Anti-inflammatory role of select Ethnomedicinal plants from Siriya Kalvarayan Hills, The Eastern Ghats of India conjointly with Phytochemical and Bioinformatic analyses"**, submitted to the Bharathidasan University, Tiruchirappalli, in partial fulfilment of the requirements for the award of the degree of **Doctor of Philosophy in Botany** is a record of the original research work done by **Mr. A. PUSHPARAJ (Ref. No. 1962 / Ph.D. K1 / Botany / Full Time / April 2017)** in the Post Graduate and Research Department of Botany, Thanthai Periyar Government Arts and Science College (Autonomous), Tiruchirappalli - 620 023, under my supervision and guidance. This is also to certify that the thesis has not formed the basis for the award of any Degree / Diploma / Associateship / Fellowship or other similar title of any candidate of any University.

Place: Tiruchirappalli - 620 023

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DECLARATION

I, **Mr. A. PUSHPARAJ**, hereby declare that the thesis entitled **"Deciphering the Antimicrobial, Antioxidant, Cytotoxic and Anti-inflammatory role of select Ethnomedicinal plants from Siriya Kalvarayan Hills, The Eastern Ghats of India conjointly with Phytochemical and Bioinformatic analyses"**, submitted to the Bharathidasan University, Tiruchirappalli, in partial fulfilment of the requirements for the award of the Degree of **Doctor of Philosophy in Botany** is a record of the original and independent research work done by me under the Guidance and Supervision of **Dr. D. KANDAVEL,** Associate Professor, PG & Research Department of Botany, Thanthai Periyar Government Arts and Science College (Autonomous), Tiruchirappalli - 620 023. I also declare that this work has not formed the basis for the award of any Degree / Diploma / Associateship / Fellowship or other similar title to any candidate in any other Universities.

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CERTIFICATE FOR PLAGIARISM CHECK

1.	Name of the Research Scholar	**A. PUSHPARAJ**
2.	Course of Study	Ph.D. Botany
3.	Title of Thesis / Dissertation	**DECIPHERING THE ANTIMICROBIAL, ANTIOXIDANT, CYTOTOXIC AND ANTI-INFLAMMATORY ROLE OF SELECT ETHNOMEDICINAL PLANTS FROM SIRIYA KALVARAYAN HILLS, THE EASTERN GHATS OF INDIA CONJOINTLY WITH PHYTOCHEMICAL AND BIOINFORMATIC ANALYSES**
4.	Name of the Research Supervisor	**Dr. D. KANDAVEL**
5.	Department / Institution / Research Centre	PG & Research Department of Botany Thanthai Periyar Government Arts and Science College (Autonomous) Tiruchirappalli - 620 023.
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ACKNOWLEDGEMENT

First and foremost, I am incredibly grateful to my Research supervisor **Dr. D. KANDAVEL, M.Sc., M.Sc., M.Phil., Ph.D.,** for the continuous support and patience during my Ph.D study. His immense knowledge and ample experience encouraged me throughout my academic research and daily life. I could not have imagined having a better advisor and mentor for my Ph.D study.

I thank my doctoral committee members, **Dr. S. ALAGUMANI** and **Dr. K. RAMAR**, for their insightful comments, encouragement and feedback, which pushed me to sharpen my thinking and brought my work to a higher level.

I like to express my sincere thanks to Honourable Principal Dr. J. SUGANTHI for the permission to use the college facilities and for her constant support and encouragement.

I also owe my deep sense of gratitude to **Dr. F. SHANTHA MARGARET** (Head of the Department) and **Dr. R. SARALA**, **Dr. P. JAYARAJU**, **Dr. K. SASIKUMAR**, **Dr. M. MANORANJITHAM**, **Dr. A. R. LAVANYA**, **Dr. S. BALAMURUGAN**, **Dr. G. SUJATHA**, **Dr. J. RUBY PRISKILLA**, **Dr. P. THERIYAPPAN** and **Dr. M. SELVARAJU**, for their influential teaching which helped to shape my career.

I thank the proprietors of Renpit Biotech Private Ltd., who gave me access to their laboratory facilities. Without their precious support, conducting part of my research would not have been possible.

I extend my sincere thanks to my friend and research collaborator **Dr. DANIEL ANDREW GIDEON, M.Sc., Ph.D.,** who provided me with an opportunity to join their team as an intern and gave constant support for my entire research and thesis. I also like to thank his family members for their unconditional support.

I greatly appreciate the support received as part of the collaborative work undertaken with **Dr. A. VINOTHKANNA** for providing suggestions. My sincere thanks also go to my friend **Mr. VIJAY NIRUSIMHAN** who always helped me learn Bioinformatic techniques and technical queries.

Words cannot express my feelings for my parents for their constant unconditional support, both emotionally and financially. I can honestly say that only their determination and continuous encouragement ultimately made it possible for me to see this research work through to the end. Special thanks to my brother **Mr. MARUTHUPANDIYAN**, Sister **Mrs. BUVANA RAJENDRAN** and Kutty's **R. DARSHINI**, **R. SAIPRADAP, R. SAYANTHIKA** for their encouragement with continuous support.

I wish to thank my wife **Mrs. P. PUSHPA**, who has stood by me through all my travails, absences, and fits of pique and impatience. She gave me support and help, discussed ideas and prevented several wrong turns. She also supported the family during much of my research studies.

The list would be incomplete without a big thanks to the People of Siriya Kalvarayan Hills for providing their ethnobotanical knowledge to my research and my friends, **Mr. VEERA**, **Mr. PRAVEEN**, **Mr. SARAVANAN**, **Mr. VIKRAM**, during the more difficult times.

I like to thank all others who have been a part of this journey and whose names might have been missed unintentionally.

Yours Sincerely,

(A. PUSHPARAJ)

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LIST OF ABBRIVATIONS

AAE	- Ascorbic acid equivalents
ABTS	- 2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid
Akt-1	- Protein kinase-B
ANOVA	- Analysis of variance
BBB	- Blood-brain barrier
BMS	- Batimastat
BTP	- Beta.-tocopherol
CYP450	- Cytochrome P450
Caco-2	- Cancer coli-2
CAT	- Computerized axial tomography
COD	- 1,2-Cyclooctanedione
CHP	- Cumene hydroperoxide
CTC	- Catechol
CTL	- Carotol
CUPRAC	- CUPric reducing antioxidant capacity
CSL	- Coprostanol
DHA	- Dihydroactinidiolide
DSP	- Dasaspidinol
DPPH	- (2,2'-diphenyl 1-picryl hydrazyl)
Da	- Dalton
DMP	- 2,6-Dimethoxyphenol
DMEM	- Dulbecco's modified eagle's medium
DNICs	- Dinitrosyl iron complexes

DEN	- Diethyl nitrosamine
DHA	- Dihydroactinidiolide
DLB	- Dactolisib
DCP	- 2,3-Dichloro-1-Propene
DFT	- Density functional theory
DSBs	- Double strand breaks
DTB	- 1,2-Di-tert-butylbenzene
ErbB2	- Receptor tyrosine-protein kinase erbB2
ECM	- Extracellular matrix
EGFR	- Estimated glomerular filtration rate
ECM	- The extracellular matrix
EDGF	- Endothelial-derived growth factor
eNOS	- Endothelial nitric oxide synthase
EBV	- Epstein-Barr virus
EDL	- Estradiol
EREs p	- Estrogen-response elements
ERβ	- Estrogen receptor
eGFP	- Enhanced green fluorescent protein
FBS	- Fetal bovine serum
FeS	- Iron sulphur
FL	- Fidelity Level
GC-MS	- Gas chromatography-mass spectrometry
GPCR	- G protein-cupled receptors
GSH	- Glutathione

GSSG	- Glutathione disulphide
GPx	- Glutathione peroxidise
GIA	- Gastrointestinal absorption
HCH	- 4-Hydroxycyclohexanone
HIV	- Human immunodeficiency virus
HBA	- H-bond acceptors
HBD	- H-bond donors
HBV	- Hepatitis B virus
HCV	- Hepatitis C virus
HER-2	- Human epidermal growth factor receptor 2
HOMO	- Highest occupied molecular orbitals
HNE	- Hydroxynonenal
IGFR	- Insulin-like growth factor
IUCN	- International union for conservation of nature
ICF	- Informant Consensus Factor
IDL	- Indole
KD	- Dissociation constant
K_i	- Inhibition constant
LC-MS	- Liquid chromatography-mass spectrometry
LOL	- Loliolide
LUMO	- Lowest unoccupied molecular orbitals
LTB	- Lapatinib
MAPK	- Mitogen-activated protein kinase
MD	- Molecular dynamics

MR	- Molecular refractivity
MMP-9	- Matrix metalloproteinase-9
MCF-7	- Michigan cancer foundation-7
MCL	- 4-Methylcatechol
MDR	- Multiple drugs resistant
MeOH	- Methanol
MERS	- Middle east respiratory syndrome
MRSA	- Methicillin resistant *Staphylococcus aureus*
mTOR	- Mammalian target of rapamycin
MlogP	- Water partition coefficient
MW	- Molecular weight
MVP	- 2-methoxy-4-vinylphenol
MTT	- [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MMS	- Marimastat
MQL	- Methaqualone
MTCC	- Microbial type culture collection
NCCS	- National centre for cell science
NCBI	- National center for biotechnology information
NOS	- Nitric oxide synthase
NO	- Nitric oxide
NSAIDs	- Nonsteroidal anti-inflammatory drugs
NrF2	- Nuclear related factor2
NfkB	- Nuclear factor kappa-light-chain-enhancer of activated B cells
NIST	- The national institute of standards and technology

NTB	- Neratinib
NPD	- Neophytadine
OATP	- Organic anion transporter proteins
OCT	- Organic cation transporter1
PI3K	- PI3kinase
PDB	- Protein data bank
pH	- Potential of hydrogen
PSA	- Prostate-specific antigen
PUFAs	- Polyunsaturated fatty acid
PVPP	- Polyvinyl polypyrrolidone
PCL	- Pyrocatechol
PTL	- Phytol
RAP	- Ferric reducing antioxidant power
RBC	- Red blood cells
RE	- Rutin equivalents
RFL	- Ridaforolimus
RMSD	- Root mean square deviation
RMSF	- Root mean square fluctuations
RMN	- Rapamycin
ROS	- Reactive oxygen species
RNS	- Reactive nitrogen species
RSS	- Reactive sulphur species
SARS	- Severe acute respiratory syndrome
SER	- Selective estrogen receptor modulators

SOD	- Sphincter of oddi dysfunction
SSBs	- Single strand breaks
STB	- Saracatinib
TAE	- Tannic acid equivalent
TCA	- Trichloroacetic acid
TBH	- Tert-butylhydroquinone
TPTZ	- 2,4,6-Trypyridyl-s-triazine
TLB	- Taselisib
TMN	- Toremifene
TNFα	- Tumor necrosis factor
TPSA	- Topology polar surface area
ULB	- Umbralisib
UV	- Use value
VD	- Volume depth
VRSA	- Vancomycin resistant *Staphyloccus aureus*
VRE	- Vancomycin resistant enterococci
w.r.t.	- With respect to
WMN	- Wortmannin
XDR	- Extensively drug resistant
ΔG	- Gibbs binding energies

1. INTRODUCTION

1. INTRODUCTION

1.1. Biodiversity

The plant kingdom is comprised of a wide array of species which differ in characteristics like growth forms, reproduction and nutrition uptake (Dawes *et al.*, 1998), from the much smaller mosses which are a few millimetres in height to close to 117 meters tall trees. The Plant kingdom covers algae, bryophytes, pteridophytes, gymnosperms (non flowering plants) and angiosperms (flowering plants). Plants are mostly autotrophs (and a few exceptions are heterotrophs) with diverse multicellular habits. The total number of plant species in the plant kingdom are around 3,91,000 species. Currently, an estimated 21% of species are under the threat of extinction. According to Kew and the Missouri Botanic Garden, which collaborated to create the world plant list (www.plantlist.org), vascular plants comprise approximately 350,700 species, of which 304,000 are flowering plant species. Gymnosperms account for 1100 species, ferns and lycopods account for 10,600 species, and 35,000 mosses and liverwort species are also known to exist (Whitton and Rajakaruna, 2001). Plants are the primary producers and they trap solar energy and convert it into chemical compounds, both primary and secondary metabolites. While primary metabolites such as carbohydrates, aminoacids, proteins, nucleic acids, and other similar substances are used by the plants for growth and nutrition, secondary metabolites are often utilised by the plants for defence against diseases. While the primary metabolites are usually similar in all plants, some secondary metabolites are often unique for each plant and hence, used as a criterion in molecular phylogeny (Chaw *et al.*, 1997).

1.2. Indian biodiversity

India is home to very rich floral and faunal biodiversity because of its huge land mass (~2.2% of the world's total land mass area) of ~329 million hectares. Also, India boasts a rich variety of climatic conditions and geographical variations which support the maintenance of various ecosystems and biomes. In the world (as per size), India is the seventh largest country and is the second major landmass in the continent of Asia and hence, is part of a subcontinent. Also, India's 7,500 km long coastline is around 15,000 km long (Venkataraman and Raghunathan, 2015). These impressive attributes have made India the possessor of several ecological habitats, which support the rich floral diversity of this country (Khandekar and Srivastava, 2014). India possesses over ten bio-geographical zones. The remarkable physiographic diversity of India includes several habitats such as low, below sea level altitudes, coastal plains, high mountain ranges (including the Himalayas to the North, which are some of the highest mountain ranges on earth); also, there are humid as well as arid regions-deserts, ice-capped and snow-laden regions, cold deserts, tropical rain forests, the massive Deccan peninsula, semi-arid regions, river plains, to the Western and also Eastern Ghats in South India. India has an estimated 10% of all angiosperms and is widely recognized to be a biodiversity-rich nation with a unique floral diversity profile. India alone possesses around 4 of the total 36 global biodiversity hotspots. The following are the 4 important hotspots: a) Indo-Burma region, b) Himalayas, c) Western Ghats and d) the Sundaland of the Andaman and Nicobar islands.

There are around 49,000 floral species in India and of those, 18,532 species are angiosperms. Among 416 known plant families, India contains 257, which in turn contain over 4,000 genera. Among the 18,000 odd plant species, 5725 species are unique and endemic to India alone. 10% of these flowering plants are threatened and over 34 plant species are known to have become extinct (Patil, 2019). The prominent plant families in India which possess the largest number of species are Acanthaceae, Asteraceae, Cyperaceae, Euphorbiaceae, Lamiaceae, Leguminosae, Orchidaceae, Poaceae, Rosaceae and Rubiaceae. When coming to gymnosperms, there are 81 species in India which belong to 46 genera from 12 families. There are over 1,293 taxa of pteridophytes which belong to 204 genera. There are 1,909 mosses in India. Besides these, India has several other floral species and hence, there is no doubt that India is biodiversity-rich (Singh, 2020). Whilst the Western Ghats have been much researched for their biodiversity, the Eastern Ghats have not been given so much attention. However, some pioneering studies have focused on the plants of this region (Muthumperumal and Parthasarathy, 2009). Some of these plants have been documented for their phytochemical profiles (Kumar *et al.*, 2018).

1.3. The Eastern Ghats

The Eastern Ghats are found in the Deccan region, in the Southern part of India. These hills are arranged like a discontinuous chain and are known to cover a length of around 1,750 km, sometimes covering even 50 miles breadth. The Eastern Ghats cover a large area between the latitudes of 11°03' and 22°03' N and the longitudes of 77°02' and 87°02' E, and they extend across several states, including Odisha, Telengana, Andhra Pradesh, Tamil Nadu, and Kerala. However, the majority of these hills are located in Odisha, Andhra Pradesh, and Tamil Nadu. In the North, the Similipal hills of Odisha are part of the Eastern Ghats. A map of the Eastern Ghats containing various kinds of forests/ecosystems is shown in (Figure 1.1). The biodiversity-rich Eastern Ghats possess many kinds of forests (Jayakumar *et al.*, 2009).

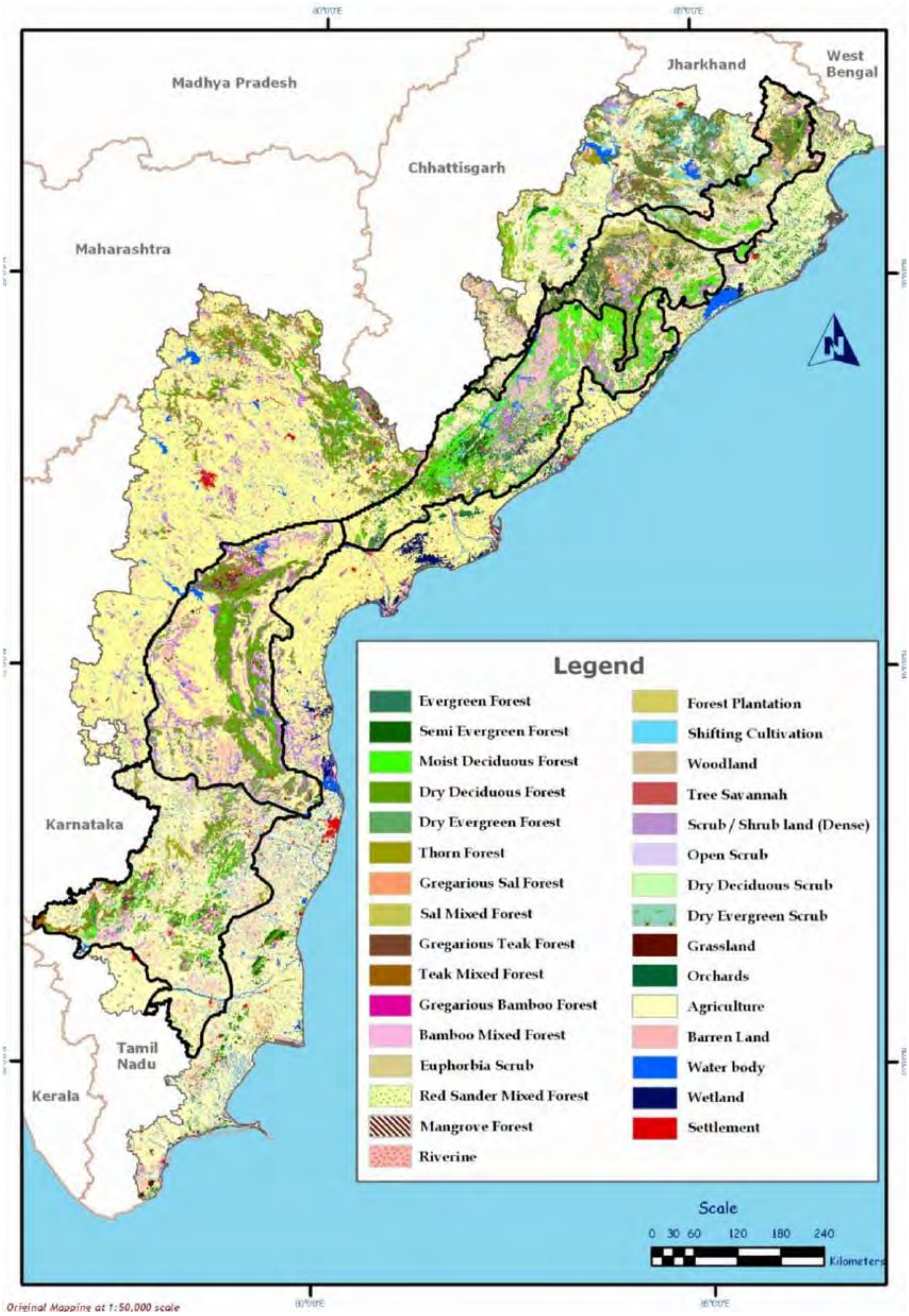

Figure 1.1. Map of the Eastern Ghats
(From https://gadgets2018blog.blogspot.com/2017/05/eastern-ghats-map.html)

The Eastern Ghats possess rich biodiversity and species endemicity. Since the regions comprising the Eastern Ghats fall in the tropical monsoon regions, these areas are rich, and are known to receive rainfall through the South-West monsoon and also the North-East retreating monsoon. Northern portions of the Eastern Ghats receive almost 1150-1660 mm rainfall (sub-humid climatic conditions), while the central and the southernmost parts of the Eastern Ghats receive annual rainfall of ~600-1050 mm, showing semi-arid climate in all other regions except in the hilly peaks (Mohapatra *et al*., 2003). The Eastern portions of the Eastern Ghats receive cyclonic rainfall and the temperature in January is anywhere between 20-40°C. During hot summer, peak mid-noon temperatures of 40-41°C are attained, while some parts of the Eastern Ghats are as cold as 2°C during winter. Monsoons display heavy rainfall (70-75%). The southern region of the Eastern Ghats, stretching from the river Krishna in Andhra Pradesh to the Tiruchirappalli district, is home to various attractive and magnificent hills and mountains. Some prominent ones are the Jawadhu hills, Periya and Siriya Kalvarayan hills, Shervarayan hills, Kolli hills and the Pachamalai hills. The Nilgiris region is a portion of the Eastern Ghats which lies near the Western Ghats.

The Shervarayan hills are impressively tall, with ~1649 m above sea level. The Kalvarayan hills are comparatively larger than the other hills of the Eastern Ghats, with a total area of 1055 sq.km. In the Kalvarayans, the climatic conditions are as follows: December-February: Winter; May-July: Summer, August-September: Spring and October-November: Monsoon. In the local region, some of the trees shed leaves during March-April, but it is uncertain whether this season can be termed as fall/autumn. Close to the study area (in this work), the annual temperature of the Siriya Kalvarayan hills is about 28°C and the region receives 1058 mm annual rainfall. The climatic condition of Kalvarayan hills, with 20 years data shows annual temperature of 28°C with 1058 mm annual rainfall (Anupama *et al*., 2000).

Kalvarayans are known to contain some unique plants that are endemic to them and it is important to protect and safeguard the forests in this region from over-exploitation and deforestation. Anthropogenic activities cause the overexploitation of medicinal plants, wood collection for fuel, cattle grazing as well as habitat destruction (Naidu and Kumar, 2016). Due to increasing population growth, the forest cover has been receding and if no preventive measures are adopted, the limited patches of lush growth and floral diversity may become lost (Young *et al*., 2005). Pragasan and Parthasarathy took an inventory of the diversity of trees in the Southern Eastern Ghats and found that there were around 272 species from ~181 genera and roughly 62 families in the entire region of their study (~60 ha area). Species richness was identified in around 169 species in the Kalvarayans (Pragasan and Parthasarathy, 2010).

1.4. Field Botany

Field Botany is an applied field of Botany in which, fieldwork is used as a means for learning several important aspects in taxonomy and ethnobotanical survey of plants. Through the field Botany, both floral survey and environmental impact is assessed. Areas covered in Field Botany are - geographical location of the plant, nutrition of the soil, morphology of the species, relative abundance of a given species, prevalent climatic conditions, vegetation mapping of entire regions/geographical areas, biotic/abiotic factors of the locale, conservation status of the identified species, influence of anthropogenic activities on the ecosystem and also ethnic/pharmacological uses of plant species (Webb *et al*., 2016).

1.5. Kalvarayan Hills

The Kalvarayans of the Eastern Ghats are a major range of hills and are found in the southern Indian state, Tamil Nadu; the key hills which comprise the Kalvarayans are – Jawadhu/Javadi, Siriya Kalvarayan, Periya Kalvarayan, Shevaroy hills, Alavaimalai and Pachaimalai (in order of North-South). These hills divide the Kaveri river basin to the south from the Palar river basin which is located in the north. The range of altitude of these hills is between 2000-3000 feet. The Kalvarayans occupy an estimated area of ~1095 sq. kms and are found to occupy two districts (Kallakurichi and Salem) of Tamil Nadu. Geographically, this is a massive land area and the Kalvarayans (Kallakurichi district) can be divided into two hills-the Siriya (small) Kalvarayan hills (altitude of 2700 feet), which are located in the northern part of Kallakurichi city and the much taller Periya Kalvarayan hills (altitude of 4000 feet), which are located to the south of the city. The Kalvarayan ranges act as a boundary between Salem and Kallakurichi districts. The soil of the Kalvarayans has been found to be conducive for plant growth and water is supplied seasonally, only in winter. The Kalvarayans contain almost ten different waterfalls (Kannan *et al*., 2015).

The water falls can be reached only by foot and some points along the hilly landscape are steep and inaccessible. The temperature is moderate with a high of 27°C and a low of 19°C. The top of the hills can be accessed only by narrow roads or by foot. The hilltops are home to a number of settlements (180 in total), some of which are inhabited by tribals known as Malayalis. The Siriya Kalvarayan hills contain over 70-80 villages and possess very rich plant biodiversity. Farming is the chief occupation of the natives and they also hunt as well as graze cattle. Several medicinal plants are found among the various plant types of the region. The floral diversity of the region is contributed by diverse algae, bryophytes, ferns, succulents, shrubs, herbs, climbers and trees (Kannan *et al*., 2015). The Kalvarayan hills are resourceful and provide food, fodder and medicine to the localites (Plate 1.1).

Plate 1.1. Usefulness of the Siriya Kalvarayan hills to the local population

1.6. Traditional medicine in India

Among the world's nations (~195), China and India are famously known for extensive dependence on traditional/herbal medicine for healthcare needs. In India, the officially recognized streams of traditional medicine are Ayurveda, Unani, Siddha, Homeopathy and Naturopathy. India has a population of ~1.38 billion people (138 crores) and an estimated 70% of the people still use some form of plant-based/herbal medicine for managing their ailments. While Ayurveda is the oldest form of medicine in India, Unani and Siddha medicine is also indigenous to India. Homeopathy came from Germany and Naturopathy involves exercise, meditation and natural foods, with the philosophy that food is medicine and medicine is food. The Indian Drugs Act (1940) does not clearly make a distinction between Westernized medicine (allopathy) and other forms of medicine (Sharma and Pundarikakshudu, 2019).

India possesses many Universities/Institutes wherein students of Siddha, Ayurveda and Homeopathic streams graduate and enter the medical/healthcare workforce. In these Institutes and in other governmental as well as private Institutions (Universities and Colleges), research on herbal medicines, medicinal plants, phytochemistry and ethnopharmacology are conducted. Herbal medicines are manufactured and used for treating many ailment categories. The herbal medicines are used for treating several diseases/ailments such as diabetes, cancer, rheumatoid arthritis, headaches, infertility, constipation, bacterial and viral infections, snake/rodent bites, fever, cough, indigestion, abdominal pain, skin diseases, respiratory problems, etc., (Williams, 2019).

The government of India has built research centres for pursuing evidence-based research on traditional medicinal streams - Ayurveda, Unani, Siddha, Homeopathy and Naturopathy in the name of AYUSH. The government hospitals and clinics administer Westernized medicine (allopathy) along with other traditional medicines (Mohiuddin, 2019). The philosophy behind this venture is that all medicine forms possess unique attributes and advantages. Traditional/herbal medicines possess antioxidants and other molecules which act as analgesics, anti-inflammatory agents, anti-emetics, sedatives, blood pressure modulators and compounds which promote wound healing, as selected/salient examples. These diverse activities and capability to bind to different receptors/biochemical targets arise from secondary metabolites of plants. The quality of food has deteriorated as a result of industrialised farming practises, as pesticide and insecticide residues have been found in vegetables, fruits, and food crops. Also, sedentary lifestyle of individuals (unlike the ways of our ancestors who laboured intensively), has led to stress, anxiety and increase in the incidence of several metabolic disorders. In these times, herbal medicine is all the more relevant and necessary and there is a need to research newer drug candidates for treating several disorders/diseases. Plant compounds and secondary metabolites are used for the treatment of malaria, cancer, dengue, liver cirrhosis, jaundice, ulcers, kidney stones and so many other ailments (Laldinsanga *et al*., 2019).

In India, almost every household use spices such as pepper, black pepper, cumin, caraway, cloves, turmeric, coriander, cinnamon, star anise, ginger, fennel and garlic. The plant compounds present in these spices are important for health. India is one of the leading producers of spices in the world. These spices are used in several forms of traditional medicine. Plant barks, roots, leaves, flowers, fruits and seeds contain varying levels of particular metabolites; in ethnomedicine, our ancestors have found empirically, the best part of a plant to use for a given ailment. India is currently the world’s leading producer of medicinal plants. Ayurvedic medical practitioners alone as per an account in as early as 2013 revealed an estimated 250,000 registered practitioners (nation-wide). Around 7000-7500 plant species are being used currently in India for treating perhaps all known possible ailments. Around 1.5 million practitioners of alternative (AYUSH) streams are estimated to be involved in the Indian healthcare system in 2013 alone (Pandey *et al*., 2013).

An estimated 80% of citizens of developing countries (especially, rural regions) rely almost solely on traditional medicines to meet their primary healthcare needs and nearly 85% of all medicines that these people use are derived from plant sources (Kamboj, 2000). As per a statistic from the year 2014, ~12% of all known angiosperms, i.e., around 50,000 out of a total count of 4,22,000 angiosperms are used in herbal medicine worldwide (Yabesh *et al.*, 2014). As of now, there are many clinical ailments which are incurable. The emergence of multi-and extensively-drug resistant bacterial pathogens and the emergence of global pandemics such as the currently raging SARS-Cov2-mediated COVID-19 disease, are threats to the existence of the entire human race. Mankind has run out of solutions and unless new metabolites/molecules can be discovered for treatment of these ailments, there is no hope for the future. Hence, many Universities and drug/pharmaceutical companies are on an intensive hunt for new candidate drugs. Exploration of as yet unreported plant species for their phytochemical profiles can lead to identification and discovery of new therapeutic molecules with potent bioactivity. Globally, countries such as Germany, Canada, Australia, France, United States and Belgium are leading consumers of herbal medicines and plant-based medicinal formulations (Zhang, 2004).

1.7. Anthropological History of Kalvarayan Hills

The history/ethnography of the Kalvarayans dates back to the Vijaya Nagar Kingdom. When Krishna Devarayar was the Emperor of Vijaya Nagar kingdom, he allowed the indigenous locals/tribals of the Kalvarayans to occupy this land and also utilize its resources, but levied many taxes on them. The warriors/indigenous natives of the area were called ‘Karalars’ and they had moved from the plains in Kanchipuram and settled in the hilly regions of the Kalvarayans. Later on, the Karalars, who were scheduled tribals in this area, were invaded by another group known as 'Vedars,' or hunters, who slaughtered the Karalar men and wedded their daughters and wives.

These generations of tribals are now called as ‘Malayalis’ or even ‘Goundars’. Three different Jagirdhars (Poligars) were known to have ruled these Kalvarayans – Sadaya Goundan, Kurumba Goundan and Arya Goundan; as per the 1901 census in India, the Sadaya Goundan ruled over 40 villages (population of 10,009), Kurumba Goundan ruled over 40 villages (7,490 populations) and finally, Arya Goundan headed 11 villages with 2,318 people. In the 2001 census, the Siriya Kalvarayan hills had a population of 20,000 and the Periya Kalvarayan hills had 15,000 people (Doss, 2011).

1.8. Ethnobotany

Ethnobotany is a branch of ethnobiology and pertains to studying past and present inter-relationships which exist between flora, fauna and mankind- and their respective relationships with their environment. Thus, it is a study of flora in the context of their habitat and geographical locale. Similar to its parent field, ethnobotany links human cultural/ethnic practices with various streams of biology. Various archaeological investigations are involved in ethnobotany, which are not restricted to a particular era; rather, they include almost everything, ranging from archaeological investigations of traditional uses of plants and their parts in ancient civilizations that have been documented in written texts to the currently researched bioengineering of new crop varieties, among other things. A side from that, ethnobotany is not only applicable in non-industrialized or non-urban societies; it is also applicable in the context of co-habitation as well as the combined adaptation of plants and human cultures, both of which have been compromised in the current era by urbanisation and globalisation. Ethnobotany is more popular in non-Westernized nations and cultures, which have rich traditions; mostly, countries in Asia (eg., China and India) and some African countries which have rich ethnobotanical heritage. Tribal groups or particular groups which study cause-effect in medicine and curative principles as well as formulations can be considered as “previously undervalued knowledge”. This knowledge of local ecology has been gained by the tribe/civilization through several centuries or even millennia of interaction with their biotic (living) environment (de Albuquerque and Hurrell, 2010). Ethnobotany has several important implications for development of sustainable agriculture and for discovery of new medicines (Rist and Dahdouh-Guebas, 2006).

1.9. Ethnomedicine

Ethnomedicine is the study of traditional medicine, which encompasses both those traditions that have been documented in relevant written sources as well as those traditions that have been passed down orally from one generation to another over several centuries. Ethnomedical studies involve the use of traditional medicines based on indigenous perception of the tribe/source; however, these studies can also lead to drug discovery and development. Since plants are the major sources of pharmaceutical medicines (owing to the rich diversity of secondary metabolites), these molecules are useful against several diseases. Salient examples of major pharmaceuticals are digoxin, morphine and atropine, which are obtained from foxglove, poppy and belladonna plants, respectively. Extensive research of phytochemical composition has led to the discovery and development of other important molecules such as reserpine for blood pressure treatment and vinblastine as well as podophyllotoxin for cancer treatment (Ramawat *et al*., 2009). Phytochemistry, plant biology, genomics, metabolomics, molecular phylogenetics and other fields are closely tied to ethnobotanical research (Bruck, 2002).

1.10. Importance of Ethnopharmacological Studies

Traditions are entities of unchanging knowledge. While traditional medicine is something we have obtained from our ancestors, it is also an evolving field, because individuals as well as communities learn new techniques and methodologies which can transform our practices. Deeper insights from phytochemical research studies have brought the wisdom of the ancestors to light. Without any knowledge of molecular biology or molecular medicine, they researched and characterized medicinal plants. This rich repository of information is useful to us even in the 21st century. Ethnopharmacology and ethnobotany fields have enriched the treatment and management of several human ailments, particularly in the "target-rich and lead-poor scenario" that we encounter. Many modern drugs originate from ethnopharmacological studies. While ethnopharmacology is an important field, pharmaceutical drug discovery process is complicated and very long. Drug regulatory processes cause many risks and increase the period of the discovery cycle. Isolation of single compounds from mixtures and the purification (as well as storage) of these compounds (to prevent their oxidation and breakdown) can lead to changes in biomolecular structure and activity. Bioavailability of these drugs, absorption-distribution-metabolism and excretion (ADME) is another important facet that must be considered. Several phases of clinical trials are mandatory before a compound or formulation can be sold on the market. Adverse drug effects and other complications such as drug-drug interactions during phase I metabolism mediated by cytochrome P450s must be addressed. In traditional medicine streams such as Ayurveda, concoctions or formulations (mixtures of drugs) are often administered and it is opined that these molecules together exert a concerted, synergistic effect on the body. One molecule balances the adverse effect of another molecule and this prevents overt allergic reactions or drug-mediated liver/organ injury. Using bioinformatics (*in silico*), live animal imaging and animal studies (*in vivo*), studies on particular tissues of the body (*in situ*) and *in vitro* evaluations, new lead molecules are being discovered for various receptors which are responsible for a disease phenotype (Patwardhan, 2005). By mid-19th century, an estimated 50% of all pharmaceutical drugs are known to have origin from plant sources. Salient examples of drugs which are used in Allopathic medicine, such as digoxin, artimesinin, ephedrine, physostigmine, colchicine, reserpine, quinine, quinidine, aspirin, morphine, atropine, taxol, vincristine and vinblastine, tubocurarine and pilocarpine are all derived from medicinal plants. Most of these drugs were identified through phytochemical studies and research on quite a few plants which are known to cure particular ailment. Our current repertoire of organic synthesis methods for modification of bioactive principles has been largely limited because not all of nature's vast array of chemical compounds can be altered or substituted. These are the limitations in our current understanding of chemistry and organic synthesis (Yuan *et al.*, 2016).

1.11. Antimicrobial Activity

Microbes such as bacteria, fungi, protozoans and viruses are able to act as intracellular pathogens in humans. They are capable of altering cellular physiology and cause adverse effects. Often, phagocytes and other immune cells secrete powerful oxidants such as hydrogen peroxide, superoxide, hydroxyl radical, peroxynitrite, hypochlorous acid and other inflammatory mediators such as TNFα, pro-inflammatory cytokines such as interleukin-6; together, these agents can destroy normal cells along with viral and bacterial infected cells as well as cancerous cells. In the process, tissue injury and multiple organ failure can result, especially during septicaemia (Darlington and Stone, 2001). Important clinically relevant pathogens are increasingly becoming resistant to our extensive repertoire of antibiotics and chemotherapeutics. Both multiple and extensively drug-resistant strains of several bacterial genera are being characterized and these are the superbugs such as MRSA and VRSA. Failure to identify novel antibiotics with activity against these pathogens can result in severe pandemics of the future, which would be either similar to COVID-19, or in effect, could even surpass this current pandemic. It is therefore necessary to identify plant-based drugs with potent antimicrobial properties. If these molecules possess antioxidant and anti-inflammatory activities also, it would be an added advantage for pursuing these molecules as lead compounds. This is because inflammation is commonly associated with microbial diseases, cancer and also heart disease. A common condition in all these problems is redox imbalance and oxidative stress. When the stress is dealt with, the symptoms of these diseases can also be managed (Myszka *et al*., 2019; Sabo and Knezevic, 2019).

1.12. Importance of Antioxidants

Redox reactions are fundamentally important in maintaining and sustaining life. Reactive oxygen species are synthesized from oxygen and can be referred to as "Electronically Modified Oxygen Derivatives" or EMODs. The ROS are produced routinely during cellular respiration *via* the electron transfer chain in mitochondria as well as certain redox enzymes and proteins found throughout the cell-example, NADPH oxidase in plasma membrane, which produces both superoxide and hydrogen peroxide, flavin-containing enzymes (FMN/FAD) such as cytochrome P450 reductase, heme containing enzymes such as catalases, peroxidases (cytochrome c peroxidase), peroxygenases, oxygenases (eg. cytochrome P450, heme oxygenase), oxidases (eg.

cytochrome oxidase, xanthine oxidase, complex IV of mitochondrial etc), epoxygenases and so on (Circu and Aw, 2010). All these enzymes produce ROS in some form or another and can shift the redox balance towards oxidative stress, if not encountered by intrinsic/cellular antioxidants, which are sometimes termed as “internal antioxidants” (Ristow *et al*., 2009). When oxidative stress is counterbalanced by reductive elements such as antioxidants, this is known as “redox homeostasis,” which is done by cellular control of gene expression. In chronic inflammation, diabetes, cancer and other diseases, experts have documented increased ROS production, with the redox balance being shifted to the oxidative side. In such cases, macromolecular damage, protein carbonylation, protein dysfunction, downregulation of certain genes and upregulation of internal antioxidants such as catalase, glutathione peroxidase, superoxide dismutase (SOD), redox shifting of the glutathione redox potential (E_{GSH}) towards the oxidative side (meaning, depletion of reduced glutathione, GSH and formation of glutathione disulfide (GSSG), oxidized glutathione), activation of peroxiredoxins and glutaredoxins, heightened increase in cellular free iron levels (Fe^{2+}) which becomes free to participate in Fenton reactions, increased hydrogen peroxide production through activation of SOD, production of the deleterious hydroxyl radical and also peroxynitrite (by combination of superoxide and nitric oxide radical) are hallmarks of free radical generation reactions in oxidative stress. Also, the hydroperoxyl radical-mediated lipid peroxidation reactions are known to occur, causing cell membrane lipid damage, generation of eicosanoids such as prostaglandins and leukotrienes which activate inflammation. Often, oxidative stress and inflammation go hand-in-hand and occur simultaneously. DNA damage products such as 8-OHdG and DNA single strand breaks are also known to occur due to ROS-mediated reactions (Pryor *et al*., 2006).

Nitrosative stress is defined by an increase in nitric oxide (NO radical, a gaseous mediator of vascular dynamics and blood vessel vasodilation), peroxynitrite (these together are termed as reactive nitrogen species/RNS). Nitric oxide-mediated signalling as well as protein nitrosylation (especially, tyrosine nitration) and the formation of dinitrosyl iron complexes (DNIC) (Thomas *et al.*, 2008). Protein tyrosine nitration is known to inactivate several proteins/enzymes. In this context, it is important to remember that ROS are important signalling agents which are involved in several physiological processes and cellular biochemical signal transductions. However, significant oxidative stress is a different thing and can be involved in pathophysiological processes (Pagliaro *et al.*, 2011). This is when external antioxidant supplementation can be helpful and necessary, to help restore cellular redox homeostasis. When the internal compensatory mechanisms of redox homeostatic balance become dysregulated, it is necessary for external influences to neutralize and quell reactive oxygen and nitrogen species and to prevent oxidative-stress mediated adverse effects such as macromolecular (DNA, protein and lipid) damage. Figure 1.2 depicts the complex interplay between ROS-mediated oxidative stress and pro-antioxidant properties of antioxidants (from both internal and external sources such as diet). While several classical antioxidants such as curcumin from turmeric and resveratrol from grape seeds are known and have been documented for their antioxidant activity, the importance of the antioxidant phytochemicals in the context of ethnopharmacology has to be realized through experimentation and exploration. That is why this present work was carried out, to highlight the important molecules which are involved in antioxidant, antimicrobial, anti-inflammatory and anti-cancer effects. While some molecules may be involved in specific activities, a few molecules may be involved in combination of the four activities or perhaps even all four activities (Minotti *et al.*, 2004).

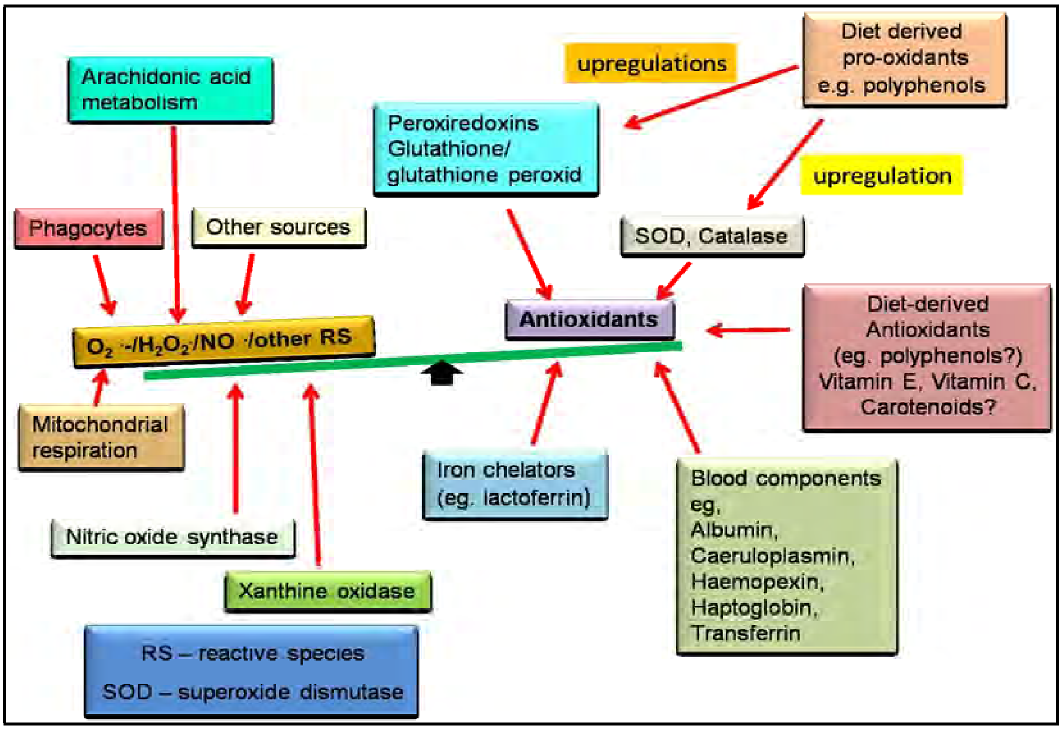

Figure 1.2. Reactive oxygen species - forms of ROS in biology (Halliwell, 2011)

1.13. Cancer and Carcinogenesis

Cancer or carcinoma refers to the unmitigated and uncontrolled division and growth of abnormal cells; while cancers can originate in any part of the body as primary tumours, some cancer cells can detach themselves from the primary site of carcinogenesis and metastasize to other parts to form secondary tumours. All tumours are not considered to be cancers, because some are benign - i.e., they do not divide rapidly or spread to other sites in the body. However, many cancers are malignant and can lead to dysfunction and failure of the affected tissues/organs. The most important cancers (based on data from the WHO and global burden of diseases) are cancers of the breast tissue, lungs, colon and brain. As per the GLOBOCAN 2018 database, 36 cancers are tied to significant mortality worldwide. The International Agency for Research on Cancer (IARC) compiled this data, which showed the incidence of 36 different cancers in 185 countries of the world. Around 18.1 million estimated new cases of cancer (and 17 million entire cases excluding non-melanoma skin cancer) were estimated and 9.6 million deaths were documented in the year 2018 alone (Ferlay *et al*., 2019). Cancer is not confined to humans alone and hence, it is found in almost all animals. Since the physiological and metabolic aspects are sex-dependent, there are differences in the types of cancer which plagues men and women. The top ten cancers worldwide in men were found to be lung/bronchial/tracheal cancer, prostate cancer, colon and rectal cancer, stomach cancer, liver cancer, urinary bladder cancer, oesophageal, non-Hogdkin's lymphoma, kidney cancer and leukemia. In women, the list is quite different. Breast cancer is the number one cancer in women; this is followed by lung/bronchial cancer, colon and rectum cancer, cervical/uterine cancer, stomach cancer, corpora luteal cancer, ovarian cancer, thyroid cancer, liver cancer and non-Hogdkin's lymphoma (Torre *et al*., 2015).

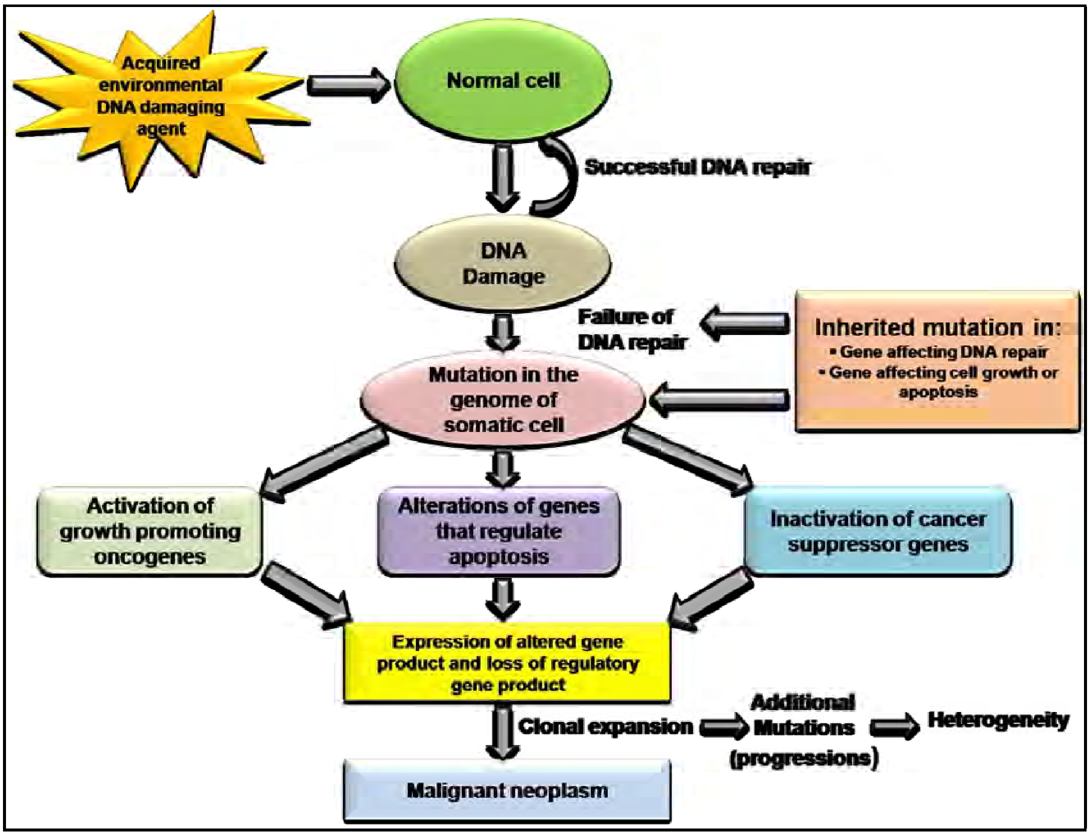

Figure 1.3. General etiology and pathogenesis of cancer

Carcinogenesis (initiation of cancer) happens when a single or multiple cells in a location undergo genetic alterations such as point mutations, deletion mutations, transversion mutations, or viral (eg., HBV, HIV, EBV) DNA insertion-mediated inactivation of tumour suppressor genes such as p53. When the aberrant gene is copied through the successive generations and the mutated cell escapes the immune system (surveillance of natural killer cells), it becomes immortalized and evades apoptosis. In Figures 1.3 and 1.4, the mechanism of carcinogenesis and the means by which cancer cells are maintained is shown. Then, the cell begins to proliferate into a mass quickly and glycolysis is activated and due to low availability of oxygen in the tumour environment, the cells undergo a glycolytic switch. Lack of ROS in the tumour environment further enables the tumour to grow unchecked. To gain access to oxygen and nutrients, cancer masses produce new blood vessels to meet the supply needs and this process is called neoangiogenesis. A malignant neoplasm is the result of these undesirable alterations (Jung *et al*., 2018).

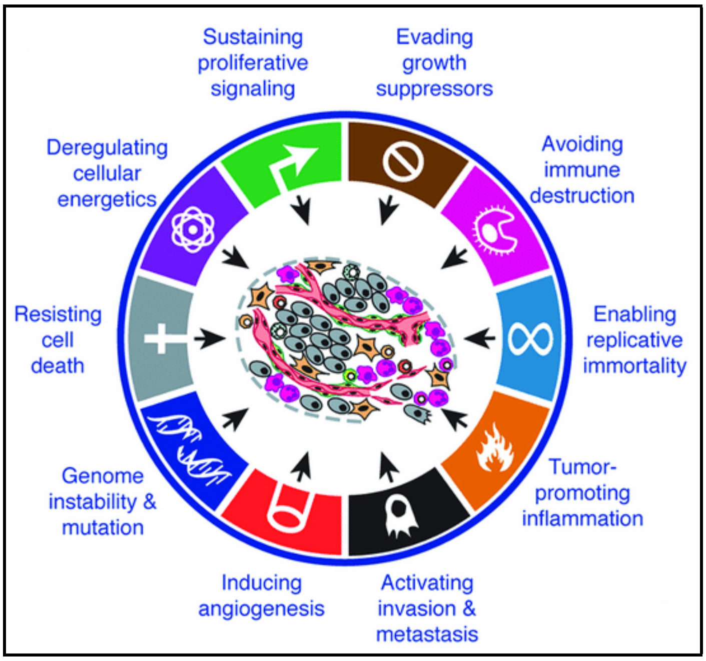

Figure 1.4. Cancer pathophysiology (Panizza, 2017)

The hallmarks of cancer pathogenesis include several factors which play key roles in progression of the tumour, evasion from the immune system and finally, cancer cell metastasis to distant sites from the original site of carcinogenesis. The key mechanisms include-evasion of growth suppressors, induction of angiogenesis/neoangiogenesis, deregulation of cellular energetics, suppression of apoptosis, activation of invasion and metastasis, sustenance of proliferative signalling, activation of replicative immortality, evasion of the immune system, activation of genome instability, changes in gene expression through epigenetic mechanisms, mutations and presence of an environment of inflammation which activates tumour proliferation. These are common features shared by different cancers in humans; despite the heterogeneity, these common mechanisms are involved in cancer pathophysiology and aberrant growth (Hanahan and Weinberg, 2011).

1.14. Importance of Anti-Inflammation

Inflammation is commonly encountered in microbial infections, cancer, heart disease, auto-immune disorders, muscular dystrophy, Alzheimer's disease, Parkinson's disease and several other pathologies. Hence, it is necessary to deal with inflammation to reduce and also restore the body to normal metabolism and homeostasis. While proinflammatory cytokines and chemokines are released during cell-mediated and protective immunity, sustained action of these agents due to underlying inflammation triggers reactions as mentioned above can lead to overdrive of the immune system and non-specific and unintended action of our own cells against our self-antigens and tissues, causing necrotic apoptosis, organ dysfunction, organ failure, and if left unaddressed, death. Removal of the stressor often results in the restoration of the body to normalcy and to normal physiological and biochemical routines. Hence, plant compounds with anti-inflammatory properties are vital for curbing as well as reducing the pathophysiological outcomes of several chronic illnesses. Inflammation is characterized by abnormally high C-reactive protein levels, tissue infiltration by innate immune cells, and also inhibition of T-cell tolerance (Li *et al*., 2019).

1.15. Pocket Identification

POCASA (POcket-CAvity Search Application) is an automatic application that implements the Roll method, which can predict binding sites by detecting pockets and cavities in proteins with known 3D structures. To begin, a 3D grid structure is built and filled with atoms from the protein molecule. Second, a probe sphere is used to roll along the protein surface in order to build a "probe surface," which is based on the inner boundary tracing algorithm used in the image processing field. The regions between the protein surface and the probe surface, as well as those surrounding the protein surface, are referred to as pockets and cavities, respectively. To eliminate noise points, two parameters were developed: the Single-Point Flag (SPF) and the Protein-Depth Flag (PDF). Furthermore, by varying the radius of the probe sphere, POCASA may forecast pockets with varying shapes and sizes. Finally, as a pocket-ranking descriptor, Volume-Depth (VD) was developed as a quantitative way of defining the volume and location information of pockets (Yu, J. *et al*., 2010b).

1.16. Molecular Docking

Molecular docking softwares were employed in science (drug discovery), and these tools provide fundamental and essential strategies for drug analysis. It can forecast the interaction of molecules that embrace both protein and ligand collectively in the bound state (Figure 1.5). Molecular docking possesses two main steps: identification/conformation of ligand molecules (small) and predicting its position and direction of protein binding site and evaluation of superiority give rise to a scoring mechanism (Figure 1.6).

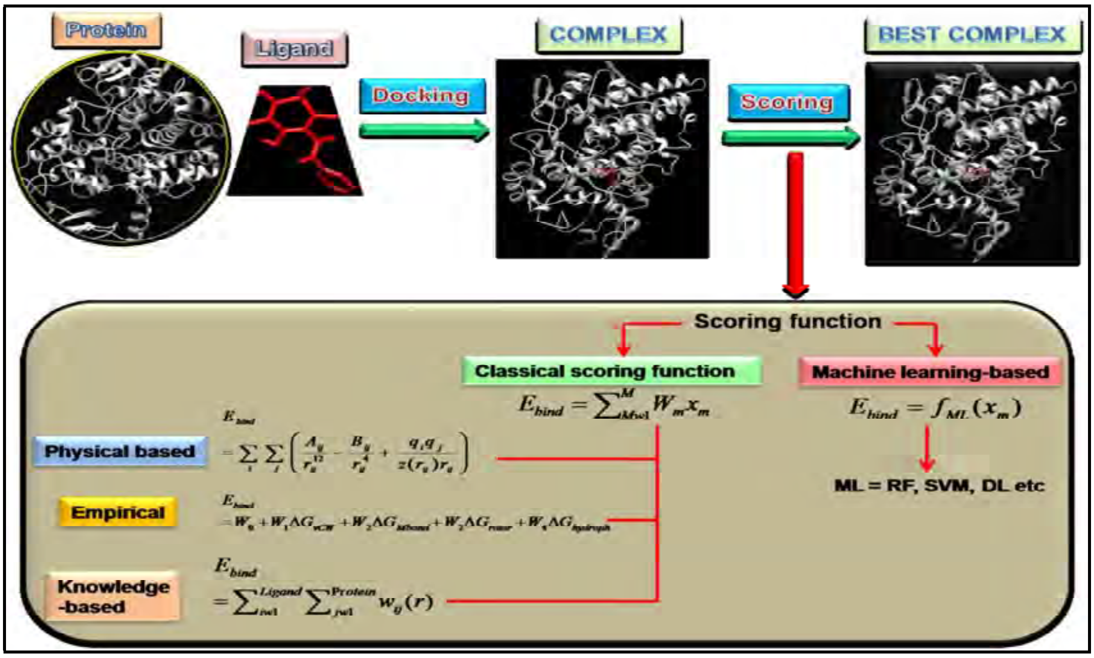

Figure 1.5. Mechanism of molecular docking studies

Molecular docking is a standard computational method of structure-based drug design, and it is used since the 1980s. This tool is used to predict the 3D (three-dimensional) structure of the micromolecules (ligand) and macromolecules (proteins). Initially, binding of ligand (small molecules) and protein (large molecules) nevertheless, in the last ten years was used for protein-protein analysis, nucleic acid-ligand analysis and nucleic acid (DNA and RNA)-ligand-protein analysis. In our study, we have done protein-ligand (phytochemicals) docking analysis on two different plants compounds (Stanzione *et al.*, 2021).

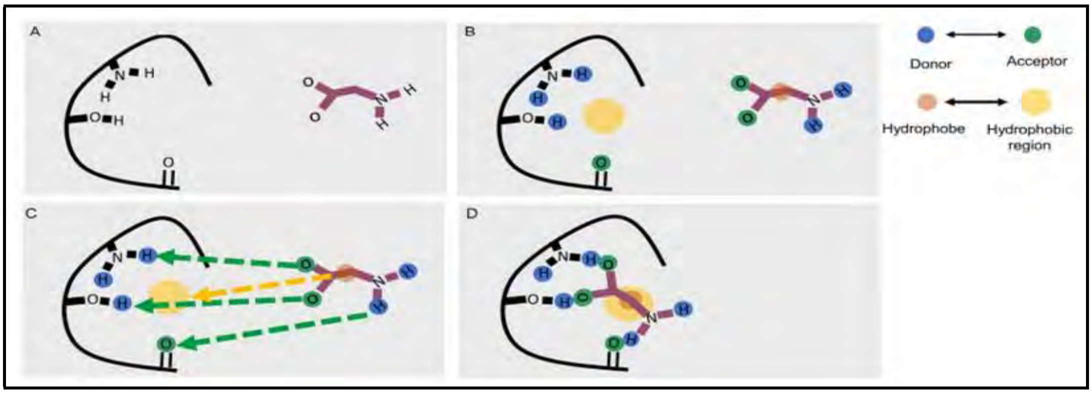

Figure 1.6. Mechanism of interaction between ligand and protein receptor (Stanzione *et al.*, 2021)

1.17. Molecular Dynamics

There are potent simulations called molecular dynamics (MD) to help scientists learn about atom-level processes that aren't possible with the tools they have right now. Molecular dynamics are computer programmes that use Newton's equations of motion to simulate N-body systems by updating particle forces and potential system energies. These are made up of interatomic/intraatomic forces and mechanical force fields. A "time step" is the amount of time between each iteration in an MD simulation. The time step is the amount of time between each iteration, which is called a "time step." In the 1950s, the idea of MD simulations first came up in theoretical physics. Later, it was used in chemical, material science, and biological molecular simulations too, as well. Later in the 1960s, the Lennard-Jones potentials for lipid simulations were added. There were limits on how large and how long simulation systems could be at that time. For the first time, a computer simulation of how a protein called bovine pancreatic trypsin inhibitor moved was done by McCammon *et al*., in 1977. Since then, more and more research has been done, and MD simulation has become a very powerful tool for studying macromolecules and how they work with each other (Fang *et al*., 2020).

1.18. Density Functional Theory (DFT)

Density-functional theory (DFT) is an excellent way to figure out how the electronic structure of atoms, molecules, and solids work. It works well. From the fundamental laws of quantum mechanics, it attempts to figure out how material properties work quantitatively. Traditional electronic structure methods try to find approximations to the Schrödinger equation for N electrons moving in an outside, electrostatic field. They try to figure out how to solve the equation (typically the Coulomb potential generated by the atomic nuclei). Even for minimal numbers of N, the problem is challenging, and the wave functions that result are very complicated. The computational effort proliferates with the number of N. This makes it impossible to describe larger systems. This is a terrible idea. Different things are done in density-functional theory. Instead of the many-body wave function, one-body density is used as the primary variable. Even though the wave function has 3N coordinates, the density n(r) only has three coordinates. This means that density-functional theory can be used even for extensive systems because it doesn't need a lot of memory (Kurth *et al*., 2005).

1.19. ADMET Properties

The Comprehensive Medical Chemistry database (CMC), the MDL Drug Data Report (MDDR), and the Derwent Worlld Drug Index are the three primary database collections of drug compounds (WDI). Currently, the CMC has 8473 drug compounds. It is updated every year with new drugs added to the United States Approved Names (USAN) list. This information is also in the CMC database. It includes information about the drug class, as well as the acid-base dissociation constant (pKa) and the octanol/water partition coefficient (log P). pKa has 1200 measured records, and log P has 120 measured and 8300 calculated records. There are 132,726 drugs in the MDDR that have been sold or are in development. They were found in the patent literature and other sources. There are about 10,000 new drugs added to the MDDR each year, and the database is updated every month. MDDR also includes information about the drug class and how it works in terms of quality. In 2005, Jónsdóttir *et al*. put together a database called the Derwent Drug File. It’s a very specific database of journal articles and conference reports on all aspects of drug development (Cheng *et al*., 2012).

2. REVIEW OF LITERATURE

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2.1. Ethnobotanical Survey

The study of the relationship between plants and animals is known as ethnobotany. This field of botany involves the study of plants/flora in their cultural/ethnic context. Hence, it consists of the deciphering of centuries/millennia old knowledge that has been especially discovered and passed onto a few people who have ancestral roots in a particular tribe/people. The word 'ethno' refers to people, and hence, the botanical knowledge possessed by specific people is ethnobotany. Since this field is not strictly confined to medicine (unlike ethnopharmacology and ethnomedicine), it is a much broader field that deals with all the potential uses of plants, such as food, fodder, medicine, particular ethnic-specific uses such as divination, cosmetics, dyeing, clothing, ritualistic applications and social life (beverages, drinks, alcohol preparation) (Newmaster and Ragupathy, 2010). Plants also serve as materials for making tools for hunting and building houses/huts. Hence, in ethnic settings, plants and humans have remarkable inter-dependence, and therefore, plants have been indispensable in the past. The buried secrets unearthed by our ancestors are at our disposal. Hence, it is imperative for those living in these times to tap into the rich heritage of our ancestors to find how they used plants for their living and sustenance. Modern medicine relies heavily on plant metabolites, and hence, the pharmaceutical industry focuses heavily on plant compounds with specific biochemical activities against various ailment categories (Heinrich, 2000).

Ethnobotany has been a critical source of medicines in the modern era. Because of the changing times due to globalization, industrial revolution and migration of populations from the rural to urban areas, ethnobotany is a changing (and also diminishing) field. Hence, attempts have been made to consider where ethnomedicine stands today (Ramakrishnan, 2001). In traditional knowledge, ethnobotanists have formulated ways to utilize plants or their specific parts in manifold ways to treat different kinds of illnesses. This is considered to be a primary stage of ethnobotany. This initial phase comprising of basic knowledge of plant uses and understanding of indigenous cultures, attempts have been made to move to a more molecular level of expertise through the advent of phytochemical research. Chemical analysis of medicinal plants in an ethnopharmacological and ethnobotanical context has led to many insights on how particular compounds present within the crude extract or herbal preparations can exert their pharmacological actions (Prance, 1991). Also, the loss of many tribes and their cultural as well as traditional knowledge has led to costly and perhaps irrecoverable loss of expertise. The depletion of forest cover and destruction of the habitat of the tribal groups due to anthropogenic activities has caused the tribal populations to migrate or dwindle in numbers (Rout *et al*., 2010).

While ancient texts such as the Rig and Atharva Veda and also the writings of the Rishis, named several plants and their potential uses in Sanskrit/Tamil, these do not effectively cover all of India's floral biodiversity (Avery, 1885). Tribal people passed on their ethnobotanical knowledge orally and taught aspirants and apprentices the trade tricks through practical demonstration. Due to the lack of systematic documentation, these secrets have become lost to humanity forever. While some of these methods and Ethnobotanical uses of some species have been documented, the practices and techniques of isolated tribal groups, unless documented in written texts, have become permanently lost. It is essential to mention that during the course of human civilization and development, ethnobotany played a critical role in culture, art, religion, medicine and literature (Mabogo, 2012). Without ethnomedicine, for example, mankind may not have overcome several diseases, and ailments, and the human race may have faced the threat of extinction. In the modern era of our time, quantitative and experimental ethnobotany includes a few basic methods such as documentation (preparation of herbarium sheets and monographs), experimental evaluation and quantitative measurement indices of Ethnobotanical uses of particular plant species such as Use Value, Fidelity Level (FL) and Informant Consensus Factor (ICF) (Abe and Ohtani, 2013; Rokaya *et al*., 2010; Srithi *et al*., 2009; Ullah *et al*., 2014). Experimental design and statistical interpretation of field surveys are done to ensure that the sampling methods and the data are statistically valid and those clear and logical conclusions are derived from the data. When sampling methods are erroneous, the data can be skewed and can lead the researcher to arrive at wrong and incorrect assumptions. Hence, ethnobotany is an evolving field in which new methods are integrated into the research to help researchers to derive meaningful and accurate data from their study. Since no information pertaining to the ethnobotany of the Siriya Kalvarayan Malayali tribals is available, this study was formulated to survey the local ethnic uses of ~100 plants that were identified to have medicinal value (Choudhary *et al*., 2008).

2.2. Herbarium Preparation

A herbarium is prepared by pressing plants on a sheet of paper; this method has occupied a central role in ethnobotanical research. Each herbarium specimen is prepared after field collection of plant specimens and is authenticated by a competent taxonomist who gives a certificate/accession number after the authentication process is completed. These plant specimens are housed in a herbarium, a library of several samples which are deposited and can be accessed upon request of researchers (Taylor *et al*., 2001). Herbaria are a rich resource of medicinally valuable plants and reveal incredible facts about the environment in which the plants grow and the ethnic aspects of the people who use these plants. While herbarium preparation and documentation has a central role in ethnopharmacology, the world is transitioning to the potential advantages of a digital herbarium over the more archaic and orthodox method of physically presenting plants on herbarium sheets (Brooks *et al*., 1977). Overrepresentation of valuable plants is one of the disadvantages, and mainly, only plants with either medicinal or commercial use are often represented in herbaria. Taxonomy has evolved from simple documentation and photography of plants to incorporate more sophisticated methods such as alpha taxonomy and molecular phylogeny; it is also plain to notice that ethnobotany and taxonomy have solid relationships with one another (Nesbitt, 2014). Typically, a group of people can be sampled at random or using other statistical approaches to identify the ethnobotanical and ethnopharmacological uses of plants. These studies involve questionnaire preparation and interviewing of participants who have reasonable knowledge of the plants, their local names and their ethnic benefits. Based on the collected data, indices such as UV, FL and ICF are calculated (Zougagh *et al*., 2019).

2.3. Secondary Metabolites

Plants possess both primary and secondary metabolites; while the primary metabolites are necessary for the growth, development and nourishment of the plants, the secondary metabolites (Namdeo, 2007) are produced in a species-specific manner (Kutchan, 2001). Plants are endowed with a vibrant repertoire of diverse organic compounds, which are called phytochemicals, and these compounds are secondary metabolites (Aharoni and Galili, 2011; Bennett and Wallsgrove, 1994; Bernhoft, 2010;

Wink, 2010). Apart from organic compounds which are termed secondary metabolites, specific primary metabolites such as lipids also possess bioactivity. An extensive review on the wide-ranging roles of the secondary metabolites against plant diseases shows the cardinal importance of these compounds in plant physiology and metabolism (Bennett and Wallsgrove, 1994). Plant secondary metabolites are used as flavours, colours/pigments which are used in the food and pharmaceutical industry and fragrances, which are used in perfumery. Since different plant parts may possess relatively varying concentrations of secondary metabolites, ethnobotany and ethnomedicine recipes of the ancients identified bioactivities of specific parts of a given species against various ailments. It is remarkable how tribals residing in sometimes uncivilized regions could possess such extensive knowledge about potential cures and medicinal attributes of specific plant parts. Today's researchers use sophisticated techniques and methods and ratify those claims and substantiate the knowledge that was available even centuries ago (Pauwels, 2010).

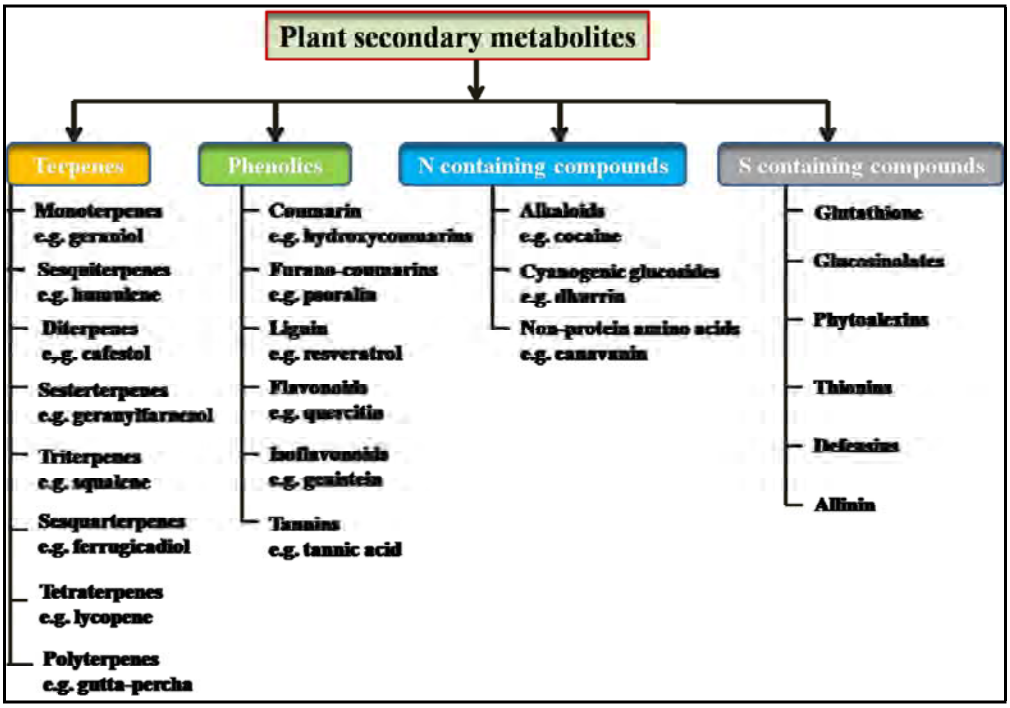


Figure 2.1. Plant secondary metabolites - an overview (Jamwal *et al*., 2018)

The chief classes of secondary metabolites, as per a recent review article (Jamwal *et al*., 2018) are - terpenes (isoprenoid compounds), phenolics (one of the most diverse classes of phytochemicals that contain phenol groups), nitrogen-containing compounds and sulphur-containing compounds (Figure 2.1). These categories are further classified into subtypes based on carbon numbers, functional groups or other considerations. Also, plants possess steroids and other lipid metabolites. To obtain particular classes of metabolites, purification strategies need to be customized based on variations in functional groups and physicochemical properties of these diverse phytochemicals. Solvent extraction is one of the main methods which can aid in separating these compound classes from the entire gamut of phytochemicals present in the whole plant or a specific part thereof such as root, leaf, stem, fruit, tuber, flower, bark or seed (Vongsak *et al*., 2013).

2.4. Phytochemical Purification and Extraction Methodologies

Throughout the centuries, scientists have investigated the phytochemical constitution of plant compounds and isolated pure compounds for commercial purposes. Several traditional methods are utilized for the extraction of plant compounds from different plant species or their specific parts. Some of the salient techniques employed for plant metabolite isolation are Soxhlet extraction, microwave-assisted extraction, supercritical fluid extraction, ultrasonication-mediated extraction and accelerated solvent extraction (Nostro *et al*., 2000). Soxhlet extraction involves packing of plant material in a powderized form into a cellulose thimble and heating of a solvent of choice in a round-bottomed flask. Perfusion of the evaporated solvent into the thimble and subsequent condensation of the evaporated solvent (after passing through the plant material) inside a water cooling jacket allows solvent to come back to the round-bottomed flask. After several rounds of extraction via this method, the solvent becomes coloured and attains a thick consistency. Upon drying inside the room or using a rotary evaporator, the powdered form of the plant extract can be obtained. This method has been followed for several decades; however, it requires enormous amounts of solvents. Solvent extraction is based on the partitioning of phytochemicals into either aqueous or hydrophobic solvents (Chua, 2013). While hydrophilic compounds are easily extracted by hydrophilic solvents, hydrophobic substances are better extracted using organic solvents-typically, alcohols (methanol, ethanol, butanol, isopropanol etc.), ketones (eg. acetone), ethers (eg. diethylether and petroleum ether), chloroform, phenol and alkanes (eg. hexane). Newer techniques such as supercritical fluid extraction and ultrasonication do not require high amounts of solvent and are less time-consuming. Each extraction technique has differential requirements such as matrix characteristics, choice of solvent, liquid-solid ratio, temperature, pressure and extraction time are all critical players in the extraction process. Solvent choice is determined mainly based on the solubility of the target compound class - eg., terpenes are incredibly hydrophobic and hence, n-hexane is the solvent of choice (Wang and Weller, 2006).

2.5. Antioxidants

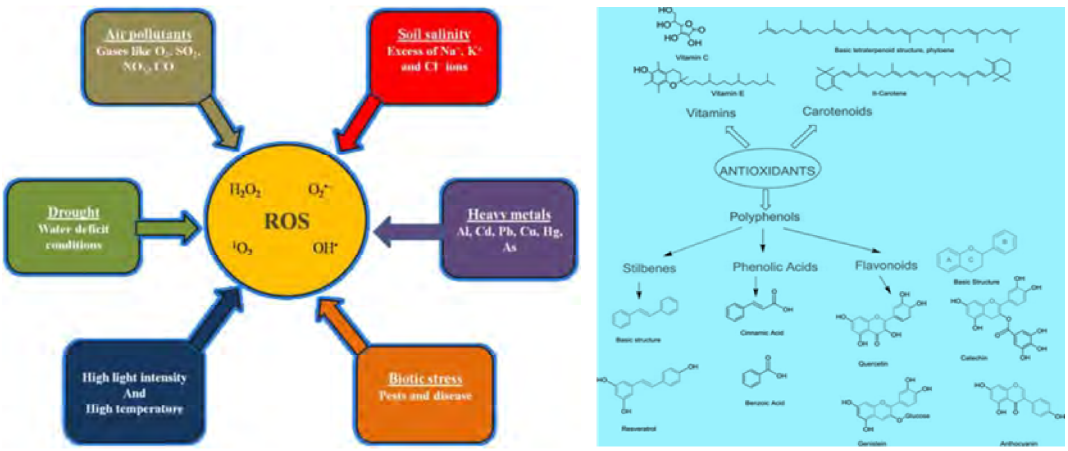


Figure 2.2. Redox homeostasis and plant antioxidants - Left: ROS in plants - When plants undergo various types of stress, they produce secondary metabolites to compensate for the pressure and to quench excess ROS; (Das and Roychoudhury, 2014). **Right:** Structure of key antioxidants from plants (García-Pérez *et al*., 2018).

In plants, redox homeostasis is maintained to achieve a delicate balance between oxidative stress and reductive stress. Cellular antioxidant enzymes such as GPx, heme and non-heme peroxidases, catalases and other such ROS-neutralizing enzymes are transcriptionally upregulated, and the protein products of these genes neutralize both radicals (superoxide, hydroxyl radical, hydroperoxyl radical etc.) and non-radical metabolites of molecular oxygen (dioxygen) metabolism (e.g. hydrogen peroxide); plants synthesize glutathione and other glutathione-related enzymes and thioredoxins to remove ROS-mediated damage and to restore cellular redox homeostatic balance (Noctor *et al*., 2018). In the process of antioxidant defence, plants also produce secondary metabolites similar to redox buffers to quell and neutralize free radical-mediated macromolecular damage. These ROS sinks can take the unpaired electron from ROS/RNS and minimize macromolecular damage mediated by excessive ROS concentrations. Figure 2.2 left panel shows how antioxidants are integral in plant defence against oxidative stress and redox homeostasis. The right panel shows the structure of some salient antioxidants derived from plants (Mittler *et al*., 2004). Aerobic metabolism involves oxygen-dependent respiration; while dioxygen is necessary for life as defined by respiration at the cellular level, dioxygen is also believed to be the reason for oxidative stress-facilitated cellular senescence, ageing, disease and ultimately, death. Hence, plants produce millimolar concentrations of antioxidant molecules to minimize, if not altogether prevent the possibility of extensive macromolecular (DNA, carbohydrates, proteins and lipids) damage that can be encountered in oxygen metabolism-mediated oxidative stress (Valentine *et al*., 1998).

There are multiple levels of organization in the antioxidant defence system- chiefly, three levels of protection are - prevention, interception and repair. Antioxidant enzymes are transcriptionally regulated by signalling mechanisms that sense the redox state of the cell-NrF2 (Loboda *et al*., 2016), NfkB (Schreck *et al*., 1992), SOD (its various isoforms) and GPx (several isoforms thereof). A hallmark paper in *Saccharomyces cerevisiae* (Thorpe *et al*., 2004) revealed the sensitivity of yeast mutants to five different oxidant chemicals such as Cumene HydroPeroxide (CHP), hydrogen peroxide, menadione, diamide and linoleic acid 13-hydroperoxide. The data revealed that each oxidant chemical caused a unique set of genes to be upregulated during the stress response. The nature of the stress response determined the type of genes expressed in yeast cells, and in this study, novel genes that were unknown heretofore in antioxidant defence were also found to be transcriptionally induced. In cells, ROS, RNS and reactive sulphur species (RSS) are known to be generated (Otasevic *et al*., 2020). While these species have specific physiological roles, increased concentrations of ROS, RNS or RSS may have severe stress-mediated repercussions such as alterations in gene expression, profound alteration of the epigenetic landscape, activation of cellular internal antioxidant gene expression and protein synthesis of SOD, GPx and thioredoxin/peroxiredoxin systems, redirection of cellular energy from routine/normal cellular functions to antioxidant defence upregulation, formation of radical-induced auto collapse products and macromolecular damage (Olson, 2020).

These redox state changes lead to altered cellular states, and in this context, antioxidants may help restore the cellular redox state and reinstate redox homeostasis. These concepts are briefly presented in Figure 2.3; plant compounds such as flavonoids, tocopherols, carotenoids, quinines, ascorbic acid (vitamin C), thiol-containing compounds etc., are involved in free radical chain termination, to prevent accentuated damage to DNA, RNA, lipids, proteins and carbohydrate macromolecules. Metal-binding proteins such as transferring, ferritin, lactoferrin are capable of capturing and thereby inactivating free redox-active metal ions and enzyme antioxidants such as SOD, CAT, GPx and caeruloplasmin act on excessive ROS and neutralize them (Sies, 1993).

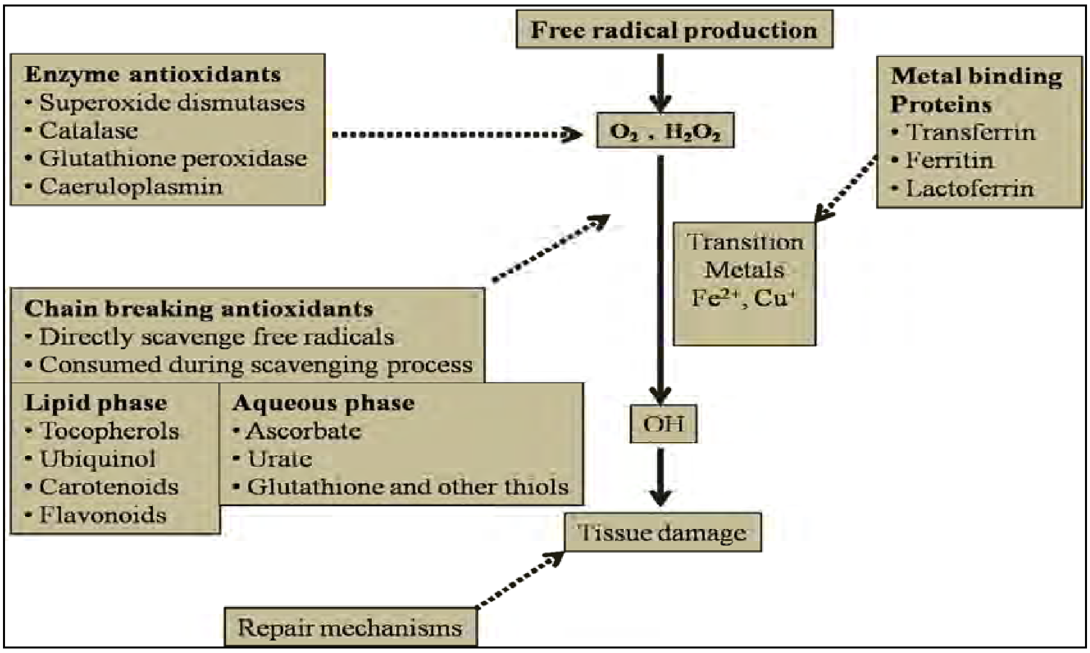


Figure 2.3. Cellular antioxidant defence systems against ROS, RNS and RSS

2.6. Antioxidant Assays

To analyze the antioxidant potential of plant isolates and extracts (or purified compounds), many different assays were performed. These assays evaluate the *in vitro* free radical scavenging ability of plants and their phytochemical constituents. The most important and routinely employed assays are - DPPH (2,2-Diphenyl-1-picryl-hydrazyl) radical scavenging activity assay (Brand-Williams *et al*., 1995; Cheung *et al*., 2003), ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical scavenging activity assay (Re *et al*., 1999), ferric ion reducing assay (Benzie and Strain, 1996), phosphomolybdenum assay (Aguilar Urbano *et al*., 2013) and nitric oxide radical scavenging assay (Rao, 1997). These methods utilize standard compounds such as ascorbic acid, trolox, gallic acid and other known antioxidants as controls, and the activity of the unknown antioxidant compounds/plant extracts is assayed against these controls.

2.6.1. DPPH Radical Scavenging Activity

In the DPPH radical scavenging assay, the stable free radical DPPH possesses an unpaired electron in its bridge nitrogen atom's valence shell. This method was first invented by Brand-Williams *et al.* (1995) and has been a method of choice for determining antioxidant activity/free radical scavenging ability of plant extracts/purified compounds. In this assay, antioxidants present in the control/test assay mixture reacts with the DPPH radical and the antioxidant molecule transfers either an electron or hydrogen atom to it, resulting in a change of the purple colour of the initial assay compound, DPPH, whose λmax is read at 517 nm, into a yellow coloured product (Figure 2.4). Hence, in this assay, the disappearance of ABTS radical is tracked. One disadvantage of the technique is the steric hindrance of the radical located at the centre of the molecule, which can be accessed by smaller molecules and not larger ones. Also, since carotenoids have λmax in the same region as the neutralized form of DPPH, assay interferences are commonplace (Holtz, 2009; Lee *et al.*, 2007).

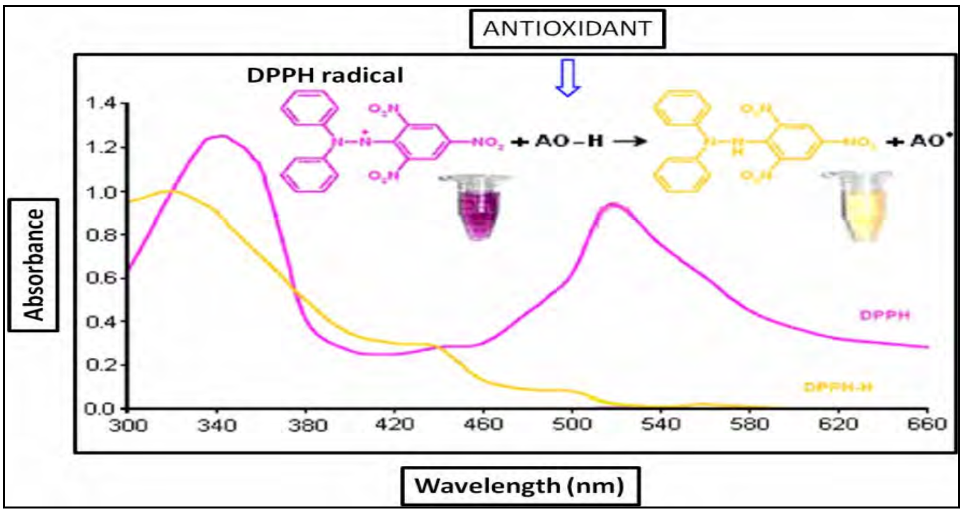


Figure 2.4. DPPH free radical scavenging assay
(https://www.researchgate.net/post/DPPH_Antioxidant_assay_Method_Development2)

2.6.2. ABTS Radical Scavenging Activity

In this method, both hydrophobic and hydrophilic antioxidants can decolourize $ABTS^{\cdot+}$, the metastable radical cation of ABTS which is generated by treatment with potassium persulphate (Ilyasov *et al*., 2020). This stable radical cation, an oxidized form of ABTS, has an unpaired electron, one of the nitrogen atoms. When an antioxidant molecule reacts with $ABTS^{\cdot+}$, it becomes reduced, and the spectrum of ABTS is restored to the original, non-radical form (Figure 2.5). In this decolourization assay, both the concentration of the antioxidant and the reaction time can lead to differences in outcomes. The radical cation is formed overnight before the assay; initially, the absorbance of the radical cation is measured at its λmax, 734 nm and later, the dark green product is diluted to a specific level/absorbance, and then the reaction is performed with a known antioxidant as a comparator, and the tests are performed separately. The % scavenging activity is reported, and by this method, the radical scavenging activity is assessed effectively for both lipophilic and hydrophilic compounds (Re *et al*., 1999).

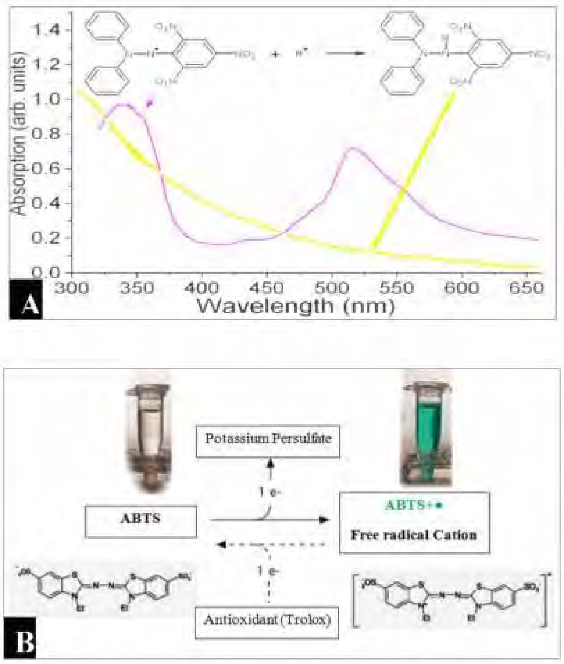


Figure 2.5. ABTS radical scavenging assay - generation of ABTS radical cation and mechanism of antioxidant action.
https://www.sciencedirect.com/science/article/pii/S1319562X20306306

2.6.3. Ferric Ion Reducing Power Assay

In the ferric reducing antioxidant power (FRAP) assay, the mechanism involves direct electron transfer from the antioxidant to the ferric (Fe^{3+}) ion to reduce it to Fe^{2+}. It does not *per se* involve hydrogen atom transfer (Huang *et al.*, 2005). Since the assay is carried out at acidic pH to keep iron soluble, this low assay pH decreases the ionization potential of hydrogen atom transfer. The low pH would mean non-dissociation of antioxidant protons, and thus, the method precludes hydrogen atom transfer and instead involves electron transfer alone. In the presence of 2,4,6-trypyridyl-s-triazine (TPTZ), the reaction proceeds to give a coloured complex of Fe^{2+}-TPTZ (λmax = 593 nm) due to reduction of iron-mediated by an antioxidant molecule. The reducing power assessed in this assay measures the degree of hydroxylation and the extent of pH-mediated conjugation. This assay can only detect compounds with redox potentials of < 700 mv, and hence, the FRAP technique is not used to see reactions which rely on radical quenching via hydrogen atom transfer which is routinely observed with redox buffer compounds such as glutathione and redox-sensitive proteins (Sarkar and Chattopadhyay, 2020). Another variation of the ferric reducing power assay is the CUPRAC assay, in which, copper ion reduction from Cu^{2+} to Cu^{+} either by known reductant control compounds (e.g. vitamin C or trolox) or unknown plant extracts/antioxidant molecules. Compounds such as bathocuprine or neocuprine are known to form chromophore complexes with reduced copper (Cu^{+}) atoms and the colour developed is monitored spectrophotometrically at 490 nm for bathocuprine-Cu complexes and 450 nm for neocuprine-Cu complexes (Cerretani and Bendini, 2010).

2.6.4. Nitric Oxide Radical Scavenging Activity

Nitric oxide radical is routinely generated during the metabolism of arginine by nitric oxide synthase enzymes. Several types of NOS such as neuronal (nNOS/NOS1), inducible (iNOS/NOS2), and endothelial NOS are present and catalyze the removal of nitric oxide from arginine (eNOS/NOS3). While nitric oxide is a crucial gaseous messenger in mammalian cells and is responsible for heart health, vasodilation of blood vessels and in the presentation of libido, excessive nitric oxide is a potent inhibitor of heme-containing systems and, therefore, can inhibit cellular respiration, energy metabolism and also affect other enzymes which possess heme as a crucial coenzyme (Mocellin *et al*., 2007). Moreover, NO radical can combine with superoxide to form peroxynitrite, a radical collapse product that further causes undesirable macromolecular damage such as protein nitrosylation. NO radicals also have the ability to bind to iron-sulphur (FeS) clusters in essential FeS proteins found in respiratory chain proteins and in enzymes such as mitochondrial aconitase to form DNICs (dinitrosyl iron complexes), which can cause FeS cluster damage. Since NO radical is considered to be a soft ligand, it is found to bind to ferrous complexes and inhibit the reaction of peroxide with the metal site, and hence, prevent ROS formation (Wink *et al*., 2001). The NO scavenging assay relies on the reduction of the amount of nitrite formed from the reaction between oxygen and nitric oxide, which is generated from sodium nitroprusside. The formation of nitrite is measured by using Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride). NO radical is found to be synthesized at higher concentrations than in physiological conditions in a range of ailments such as cancer and inflammation (Granger *et al*., 1995).

2.6.5. Phosphomolybdenum Assay

The underlying principle of the phosphomolybdenum antioxidant assay involves the reduction of molybdenum from Mo (VI) to a reduced Mo (V) by plant compounds that possess antioxidant activity (Figure 2.6). Spectrophotometric determination of the sodium sulphide-mediated reduction of phosphomolybdic acid to phosphomolybdenum blue complex has to be read at λmax of 765 nm. Further addition of nitrite oxidizes the phosphomolybdenum complex and results in decolourization of the complex; this decrease in colour is deemed to be directly proportional to the concentration of nitrite added (Khan *et al.*, 2012).

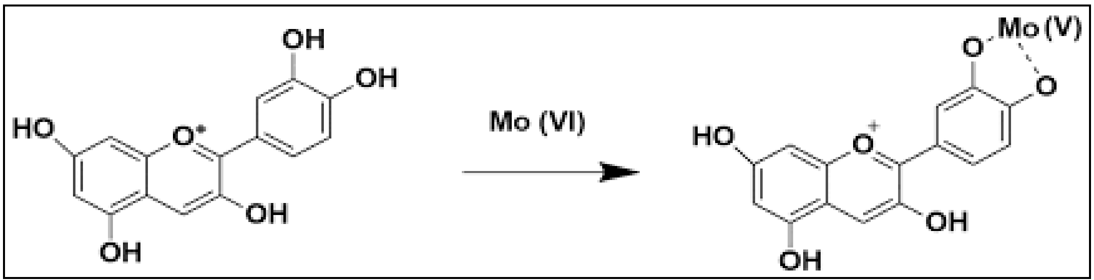


Figure 2.6. Principle of the phosphomolybdenum assay (Khan *et al.*, 2012)

Since nitrite is a known pollutant and has a propensity to react with secondary amines in biological systems, it forms carcinogenic derivatives known as nitrosamines. Diethyl nitrosamine (DEN) is a potent carcinogen. Organic carcinogens formed from proteins can cause severe health problems. Higher nitrite concentrations in blood lead to haemoglobin dysfunction by reaction with Fe(III) central metal ion of haemoglobin's porphyrin coenzyme and form methemoglobin, whose concentration elevation in the blood is termed as methemoglobinaemia (Zatar *et al.*, 1999).

2.7. Antimicrobial Activity

The human body has nine times more microbial cells than human cells. A variety of bacteria, fungi and other microbes inhabit the human body. When the immune system becomes dysfunctional, and when there is an imbalance in the ratio of beneficial vs pathogenic microbes,diseases take over the body (Lloyd-Price *et al*., 2016). Hence, consumption of plant-based diets could potentially offer similar protective and anti-microbial effects in humans similar to what these compounds do in plants. India is one of the pioneers in the spices trade, and the presence of spices with various flavours and colours is known to have caused the British to conquer our country and establish the East India Company. Herbs contain compounds that are known to kill pathogenic bacteria and thereby prolong life. Humanity has long understood the importance of medicinal plants with antimicrobial activity even before obtaining proof of their presence through advancements in microscopy (Cowan, 1999). Specific plants and their parts have been connected to some medicinal applications; for example, in various manuals of Phytotherapy, bearberry (*Arctostaphylos uva-ursi*) and cranberry juice (*Vaccinium macrocarpon*) have been provided as medicine for urinary tract infections. Lemon balm (*Melissa officinalis*), tea tree (*Melaleuca alternifolia*) and garlic (*Allium sativum*) are known to exert broad-spectrum antimicrobial activity. Essential oils of these plants have potent antimicrobial properties and have been used against pathogens of the respiratory system, gastrointestinal system, biliary system, urinary tract infections and skin-related diseases. The essential oil/tree oil of *Melaleuca alternifolia* is used to treat acne and other skin diseases (Rios and Recio, 2005).

Since antimicrobial resistance is on the rise globally, mankind is on the hunt for new antimicrobial compounds which can be used against important pathogens encountered in clinical settings, which are responsible for gangrenes and septicaemia. Overuse and indiscriminate use of antibiotics have caused microbes to become resistant against several clinically essential molecules such as ampicillin, azithromycin, linezolid, colistin, amoxicillin, methicillin and vancomycin. These resistant bacteria are known as multiple drug-resistant (MDR), and extensively drug-resistant (XDR) strains. Salient examples are Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant *Staphyloccus aureus* (VRSA), Vancomycin resistant enterococci (VRE) as well MDR/XDR *Mycobacterium tuberculosis*, whose infections are on the rise. There are other very perplexing bacterial pathogens such as *Acinetobacter baumannii*, *Clostridium perfringens*, *Clostridium difficile*, *Pneumococcus aeruginosa*, *Klebsiella pneumoniae*, *Pseudomonas* sp., *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Proteus vulgaris*, to name just a few of the most prominent pathogens which are responsible for several infection (as well as nosocomial infection)-related deaths (Goldman *et al*., 2007). These microbes/superbugs are rumoured to become global pandemics with much deadlier outcomes than the current SARS-CoV-2 mediated COVID-19 disease. In China, there is a re-emergence of the deadly bubonic plague which is caused by *Yersinia pestis*. Several viruses such as SARS, MERS, HIV, HBV, HCV, Dengue virus, Nipah virus, Ebola virus, Swine flu virus, West Nile virus, Chickenpox virus, Rabies virus and many more have been known to be inhibited by several classes of plant compounds both *in vitro* and in patient clinical trials (Ostaszewski *et al*., 2020).

With the availability of the protein sequences and some of the X-ray diffraction crystal structures of some of these important pathogens' virulent proteins, it is easier to pursue the mechanism of antibacterial action exerted by a particular biomolecule (Cushnie and Lamb, 2005). Explosion in biophysics techniques and molecular biology has brought humanity to a better position to survive against these deadly pathogens. Several fungal and protozoan diseases are also on the rise. *Giardia lamblia* (giardiasis), *Entamoeba histolytica* (amoebic dysentery), *Plasmodium vivax* (malaria), *Plasmodium* *falciparum* (malaria), *Trypanosoma cruzi* (Chaga's disease), *Trypanosoma brucei* (African sleeping sickness), *Leishmania donovani* (leishmaniasis), *Toxoplasma gondii* (toxoplasmosis) are a few protozoan organisms and the diseases they cause in humans. Many fungal pathogens are also becoming difficult to treat, and some of the major fungal pathogens in humans are - *Candida albicans*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Candida tropicalis*, *Candida glabrata*, *Trichophyton rubrum* and other *Trichophyton* sp.; these are just a few salient examples. Since mankind has almost exhausted antimicrobial and antibiotic agents against bacterial, protozoan, viral and fungal pathogens, it is vital to find new drugs or molecules which can kill as well as arrest the reproduction as well as transmission of these pathogens (Janbon *et al.*, 2019). Nature has bestowed plants with incredible molecular diversities in terms of shape, functional group, carbon atom number and through these variations, the therapeutic potential of natural plant-based compounds is high. Hence, this work recognizes the importance of ethnobotany in treating several diseases that threaten human existence and continuity (Pendleton *et al.*, 2013). In recent times, some critical pathogens have been termed as ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) and these are antibiotic-resistant bacteria commonly involved in sepsis and clinical deaths. It is therefore essential to search for new plants with novel phytochemicals which can fight these pathogens to minimize mortality rates associated with these diseases (Santajit and Indrawattana, 2016).

2.8. Cancer and Oxidative Stress

Cancer or carcinoma occurs due to mutations or epigenetic mechanisms. Cells fail to undergo normal apoptosis and gain immortality, metastasize from the site of origin, and cause organ/tissue dysfunction or failure through rapid proliferation. In the process of carcinogenesis, mutations lead to DNA and macromolecular damage, alterations in intracellular signalling cascades, change in the transcriptome, DNA base modifications, formation of apurinic and apyrimidinic base-free sites, DNA single strand or double strand breaks (SSBs/DSBs), protein-DNA crosslinking and other such potential mechanisms whereby cell cycle regulation is lost. Protooncogenes and tumour suppressor genes are often targeted in the process of mutations induced by viruses, carcinogens such as diethyl nitrosamine, acrylamide, pesticides, ionizing radiation, X-rays, etc. (Diamond and Baird, 2012; The *et al*., 2020). There are diverse causes of various kinds of cancers, and the mechanism of pathogenesis is not the same in all cancers. However, a common trait of cancers is oxidative stress. ROS such as superoxide and the powerful and harmful hydroxyl radical are implicated in mutagenesis. X-rays and ionizing radiation induce ROS and singlet oxygen production, which can lead to DNA, RNA, and protein and lipid damage (Markiewicz and Idowu, 2019). Although all of the exact mechanisms of carcinogenesis and the precise roles of ROS are not known, ROS are considered to be key players in the process of carcinogenesis. The key process of lipid peroxidation is involved in inflammation and cancer. The higher incidence of some cancers such as colon, breast and ovarian cancer is related to excessive dietary lipid intake and lipid peroxidation. Oxidative stress has long been considered to be a fundamental cause of carcinogenesis. Genetic polymorphisms and gain of function mutations/loss of function mutations of key protooncogenes, tumour suppressor genes and antioxidant defence systems have been researched at length, and the prevalence of specific mutations of critical cancer-related genes has been tracked through genetic screening (Takaki *et al*., 2019).

2.9. ROS-Induced DNA Damage Mechanisms (Cancer Initiation)

Oxidative stress is intertwined with cancer throughout various phases of cancer - such as initiation, propagation/tumour progression and dissemination/metastasis. Carcinogenesis typically begins from a single-cell and involves changes in the genetic material of single cells. Oxidative stress-mediated changes are envisaged to occur after chemical/radiation/toxic substance exposure/infection by HIV or Epstein-Barr virus (EBV) (Valavanidis, 2019). Oxidative damage has been characterized in cells due to mutations that are inheritable; subsequent replication/cell division cycles transmit these genetic alterations to daughter cells, and oxidative damage has been considered to be a key player in the overall carcinogenesis process. The essential causes of tumorigenesis via oxidative DNA damage are hydroxyl radical production from hydrogen peroxide degradation by metal ions such as Fe^{2+}/Cu^{+} (Fenton reaction and Haber-Weiss chemistry), intracellular calcium spikes calcium-mediated activation of endonucleases which can lead to DNA fragmentation. Sometimes, these processes are known to co-occur (Wise *et al*., 2019).

2.10. ROS in Tumour Promotion

ROS are known to be strongly connected to tumour growth and progression. In several studies, many promoters/causative agents of cancer have been shown to stimulate the production of endogenous oxidants and radicals and similarly, cellular metabolic functions become altered. Many tumour promoters /carcinogens are capable of inhibiting or shutting down the cellular antioxidant defence systems, which become overwhelmed by the increase in ROS/RNS and the depletion of cellular redox buffering thiol systems such as glutathione (Perillo *et al*., 2020). Altered reduced: oxidized glutathione GSH: GSSG ratio can be measured by eGFP and ratiometric reporters, which measure glutathione redox potential (E_{GSH}) through Grx1-roGFP2 expression in cells (Gutscher *et al.*, 2008). During exposure to oxidants, the E_{GSH} of cells can shift from -240 mv to lower (close to -200 or below), and this indicates oxidative stress (Acharya *et al.*, 2010). Physiological ROS concentrations would not be expected to overwhelm the glutathione buffer. When there is excessive ROS, the depletion of reduced thiol pools has been shown to trigger gene expression changes. Further ROS production stimulates the mutated cell clones to grow as a mass of cells (tumour) which then continues to grow and shift to a hypoxic state, leading to acidification and glycolytic switch, characterized by a diminished TCA cycle (Yu *et al.*, 2017). Hence, intracellular ROS production is a crucial factor in promoting carcinogenesis and oncogenesis. ROS-mediated redox switch mechanisms involve oxidation and modulation of cysteine residues of key enzymes such as protein kinase-C. NFkB is another crucial hub of antioxidant gene expression modulation. During oxidative stress, this vital transcription factor transactivates to the nucleus and recruits coactivator transcription factors and histone deacetylases and thereby controlling the expression of key antioxidant genes. NFkB is a hub that controls several facets involved in cancer pathogenesis and pathophysiology and is an essential nexus in the mediation of inflammatory responses, synthesis and release of pro-inflammatory cytokines (Figure 2.7) such as TNFα, IL-1 and other chemokines and growth factors which drive inflammation and immune system malfunction (Yang, H.-L. *et al.*, 2019). This plays a significant role in cancer cell evasion from immune surveillance and identification, targeting and cell-mediated responses (such as natural killer cells), which can selectively bind to and destroy cancerous cells. Immune system evasion and downregulation of immunity can allow cancerous cells to travel from the site of origin into the bloodstream almost undetected and finally promote metastasis and secondary tumour formation at distant places. In all these processes, ROS, antioxidants and redox biology have central roles (Zhang *et al*., 2019). NFkB is at the crossroads of key factors such as genetic and epigenetic alterations, tumour cell proliferation, enhanced tumour cell survival, immune evasion, altered cellular metabolism, angiogenesis, metastasis, immunosuppression and resistance to anticancer therapy. Activation of the NFkBnexus also promotes inhibition of apoptosis and induces the expression of oncoproteins, and simultaneously enhances genotoxic stress (Klaidman, 2011). Phytochemicals can block various receptor targets in diverse cancers and initiate transcription factors such as NFκB and NrF2. For example, gingerol inhibits NFκB, and many plant compounds block NrF2 (Surh and Na, 2008).

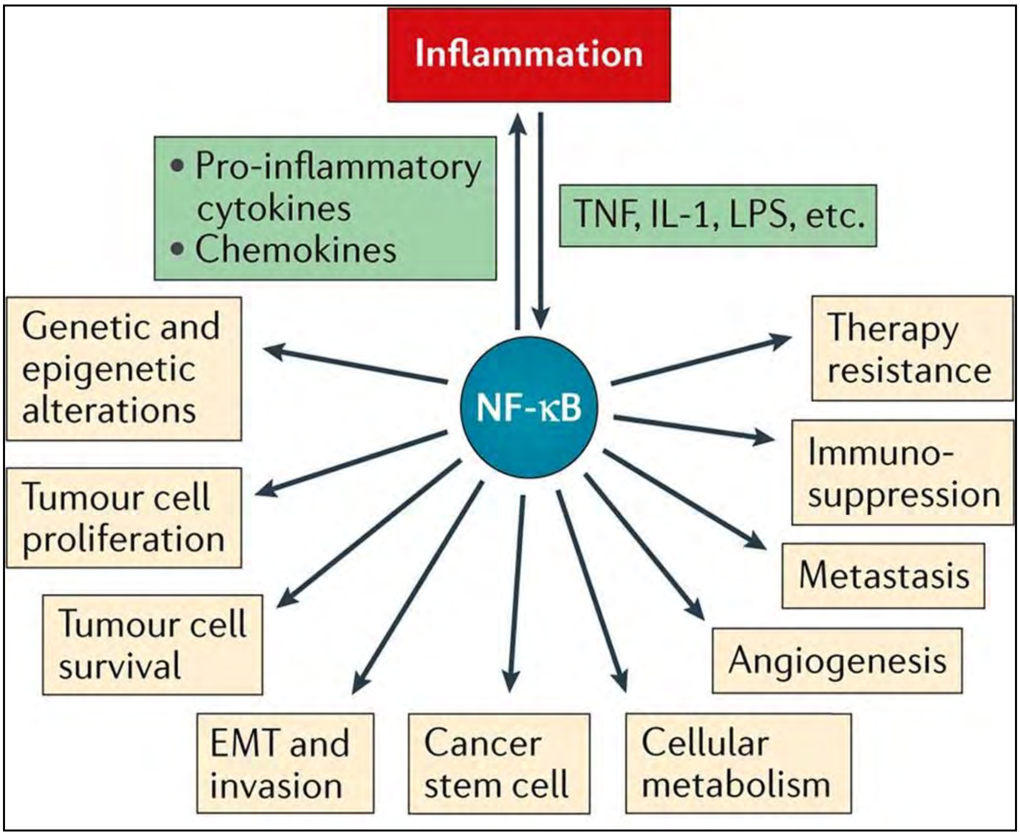


Figure 2.7. Over view of Inflammation (Taniguchi and Karin, 2018)

2.11. Breast Cancer

In 2020 alone, women's projected leading cancer worldwide is breast cancer, with an estimated 279,100 new cases and 42,170 deaths in females alone (Siegel *et al*., 2020). Among women worldwide, breast cancer is the leading cause of mortality; it is a complex and heterogenous disease, and the underlying factors are diverse in different patients. Based on histological features, breast cancer is categorized into three different types – a) hormone-receptor positive, b) human epidermal growth factor receptor-2 (HER2) overexpressing, and c) triple negative types. Treatment of breast cancer involves immunotherapy with monoclonal antibodies, radiotherapy with radionuclides and pharmacological management using selective estrogen receptor modulators (SERMs), aromatase inhibitors and selective estrogen receptor downregulators (SERDs) (Von Minckwitz *et al*., 2019). Another small molecule pharmaceutical therapeutics such as doxorubicin can also be administered. Breast cancer also involves significant inflammation, and hence, cancer and inflammation are often seen to co-exist in breast carcinoma. Tamoxifen, an ER antagonist is very widely used to block the signalling and gene expression orchestrated by ER. Estrogen receptor is a nuclear receptor that is intracellular, and since estrogen is a lipophilic steroid derivative, it crosses the membrane and binds to ER. ER then dimerizes in the presence of the ligand estrogen and can transactivate into the nucleus. The dimeric ER receptor then binds to ER response elements, recruits other transcription factors/coactivators, and facilitates gene expression of estrogen-responsive genes. Estrogen works in tandem with other growth factors such as epidermal growth factor (EDGF), which binds to EGFR/HER2 and IGFR (insulin-like growth factor), which transmutes the signalling of insulin and IGF-1/IGF-2 (Oh and Bang, 2019). Several attractive pharmacological targets of breast cancer have been pursued and researched extensively, and some of them are - EGFR/HER2, IGFR, PI3K, AKT (protein kinase-B), mTOR (mammalian target of rapamycin), which participate in ER-mediated transcriptional modulation of ER-responsive genes as well as insulin, IGF, EDGF-responsive genes (Figure 2.8). The triggering of EGFR/HER2 via ligand hormone-binding activates the SOS-Ras-Raf-MEK-MAPK pathway and leads to anti-apoptotic and cell growth responses (Modi *et al*., 2020). Similarly, IGFR signals through PKB/AKT through PI3K and finally, mTOR. The concerted effects of growth factors and estrogenpromote protein synthesis, cell growth, apoptosis inhibition, and cancer metastasis (Murthy *et al*., 2020).

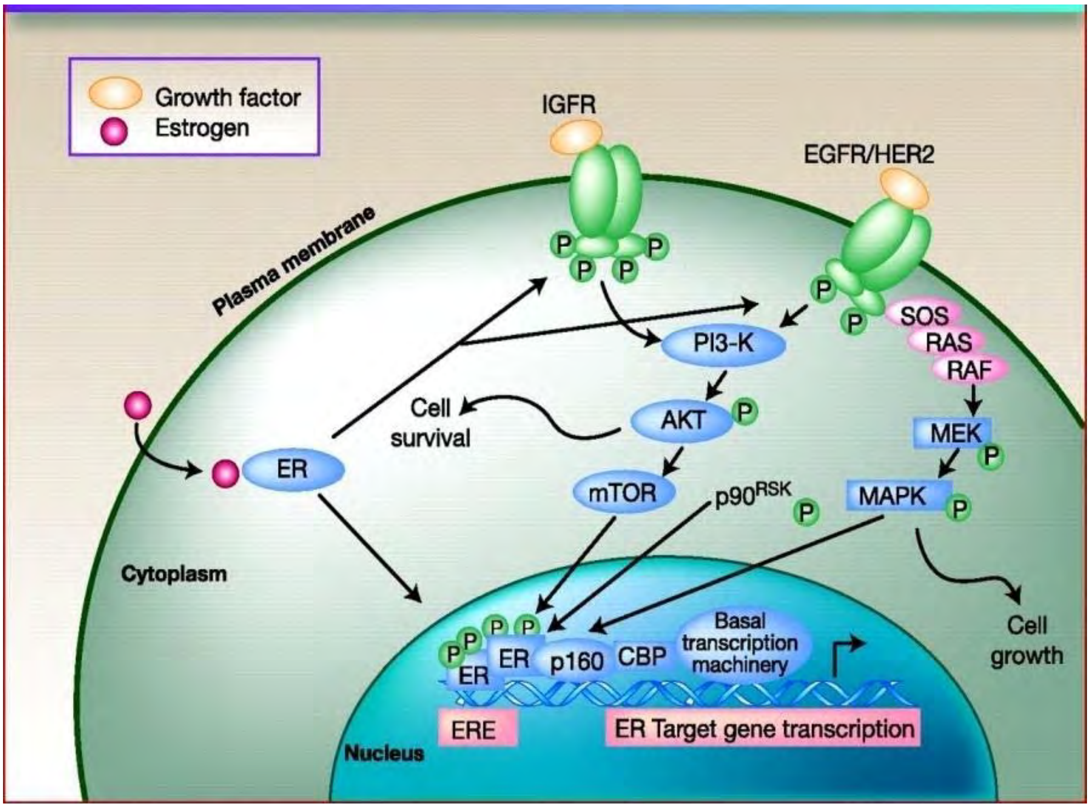


Figure 2.8. Molecular mechanisms of ER-mediated transcription and associated epigenetics of breast cancer. https://blogs.shu.edu/cancer/2014/07/03/genentech-acquires-seragon-selective-estrogen-receptor-degraders-for-breast-cancer/.

Table 2.1. Phytochemicals and their anti-breast cancer effects from published literature

Compound Name	Plant name	Cell line(s)	Receptor	Reference
Apigenin	*Matricaria chamomilla*	A431, HNSCC, SCC25	Bcl-2, TNF-R, TNF, TRAIL-R	(Chan *et al*., 2012)
Bigelovin	*Inula helianthus-aquatica*	HCT116 and HT-29	G2-M and DR-5	(Li, M. *et al*., 2017)
Linalool	*Lavandula spica*	CCD-18Co and HTC116	OR51B4,	(Yoshioka *et al*., 2014)
Curcumin	*Curcuma longa*	HeLa, HepG2, PC-3, DU-145, Caco-2, HT-29	CDK-1, COX-2, ARA70, eGFR and IGF-1R	(Chen *et al*., 2006; Ohtsu *et al*., 2002), (Patel *et al*., 2010)
Paclitaxel	*Taxus brevifolia*	HeLa, A549 and MG63	HER2, eGFR-2	(Buzdar *et al*., 2007)
Vinblastine	*Catharanthus roseus*	SK-N-MC and SK-N-AS	VEGF-2	(Klement *et al*., 2000)
Vincristine and Vinorelbine	*Catharanthus roseus*	BCap37	ERα	(Sui *et al*., 2010)
Docetaxel	*Taxus baccata*	MBC	HER-2	(Miles *et al*., 2010)
Camptothecin	*Camptotheca acuminata*	PC3	Kinase 1 and S_1P	(Akao *et al*., 2006)
Irinotecan	*Catharanthus roseus*	LS174T	PXR	(Raynal *et al*., 2010)
Etoposide	*Podophyllum peltatum*	LNCaP	TR_3 orphan	(Uemura and Chang, 1998)
Teniposide	*Podophyllum peltatum*	Walker 256	P_2X_7	(Yan *et al*., 2018)

A431 - Human epidermoid Carcinoma; HNSCC - Head and neck squamous cell carcinoma; SCC25 - Squamous Cell Carcinoma; HCT116 - Human Colorectal Carcinoma; HT-29 - Human Colorectal Adenocarcinoma; CCD-18Co - Colon Cancer Development; HeLa - Henrietta Lacks; HepG2 - Hepatoma G2; PC3 - Prostatic Carcinoma; Caco2 - Cancer coli; MG63 - Human Osteosarcoma; SK-N-MC - Neuroblastoma cell line; SK-N-AS - Neuroblastoma cell line; MBC - Cellosaurus cell line; LS174T - Cellosaurus cell line; LNCaP - Prostate Adenocarcinoma cells; Walker 256 - Breast Carcinoma cells; BCap37 - Cellosaurus cell line.

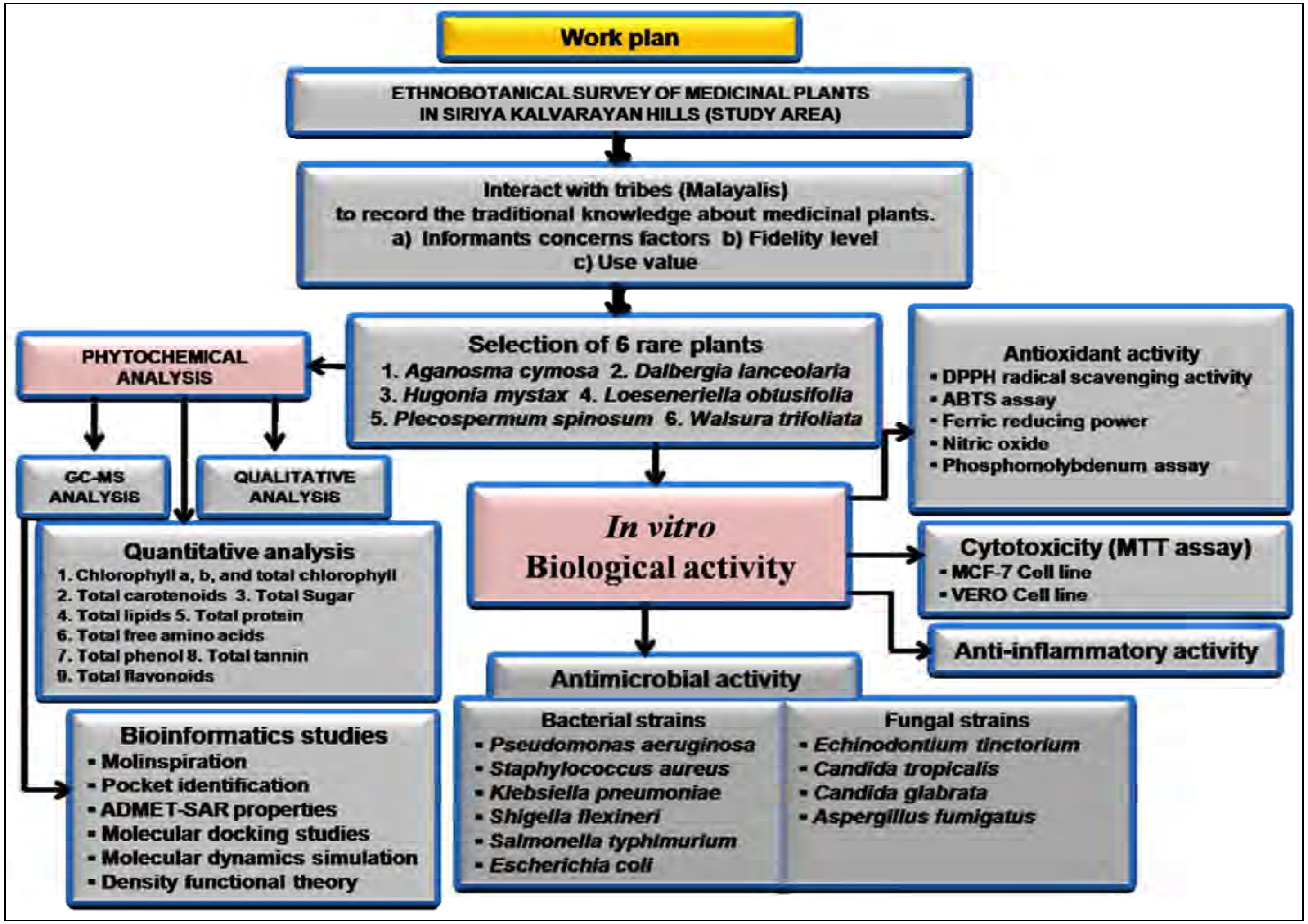
Based on literature reports, anti-cancer compounds from various plant sources were examined, and their mechanisms of cytotoxicity and ability to inhibit cancer *in vitro* as well as in animal studies are also diverse. Remarkably, the molecular targets of the plant compounds are different based on the cancer type and the associated cells/tissues. The cell lines and the receptors being blocked by the compounds are given in Table 2.1. The abbreviations for the names are presented as footnotes in the

Table. For example, vincristine and vinblastine block the cytoskeletal dynamics of tubulin, and since cancer cells divide at a much faster rate, blockage of tubulin polymerization impedes tumour growth. By binding to tubulin, vinblastine inhibits the mitotic spindle formation and elongation process and effectively causes it to become crystallized. Theresult is cell death or mitotic arrest (Steinmetz and Prota, 2018).

2.12. Biophysical Approaches in Phytochemical Structure Determination

Among the top approaches to determine the structure (and consequently, chemistry and function) of phytochemical compounds, gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) are predominantly used. These methodologies serve as the backbone for phytochemical research (Erni, 1982). A plant isolate (crude extract) is obtained through soxhlation or other methods discussed earlier. In GC-MS, the compound mixture is injected into a thin gas column loop packed with silica gel or any other competent material which is firmly bonded to the solid glass or steel column. The circular loop is long and coiled, and the injection port is connected to a carrier gas. During injection of the sample through the loop, it is carried by the inert carrier gas through the pre-heated column. Here, volatile compounds evaporate and are separated based on their physicochemical properties by partitioning between the carrier gas mobile phase and the stationary phase (chemical residues with active functional groups to which the sample components are bound). Since no two chemical compounds have the same power of adsorption, the components of the phytochemical mixture elute from the column one by one into a mass spectrometer, which fragments the molecules, travel through the apparatus and are detected by bombarding a screen. Based on their mass: charge (m/z) ratio, the molecular weight of unknown compounds can be determined. This is done by comparing the compounds to a standard, comprehensive library of compounds that are commercially available. By employing software that can predict the individual peaks obtained in the mass spectrum, the chemical structures of the compounds are deciphered. LC-MS works similarly but allows the determination of aqueous extracts also, unlike GC-MS. LC-MS is much more sophisticated, and there are LC-MS/MS instruments that give accurate mass data (Halket *et al*., 2005).

Work plan



Work plan
ETHNOBOTANICAL SURVEY OF MEDICINAL PLANTS
IN SIRIYA KALVARAYAN HILLS (STUDY AREA)
Interact with tribes (Malayalis)
to record the traditional knowledge about medicinal plants.
a) Informants concerns factors b) Fidelity level
c) Use value
Selection of 6 rare plants
1. Aganosma cymosa 2. Dalbergia lanceolaria
3. Hugonia mystax 4. Loeseneriella obtusifolia
5. Plecospermum spinosum 6. Walsura trifoliata
PHYTOCHEMICAL ANALYSIS
GC-MS ANALYSIS
QUALITATIVE ANALYSIS
Quantitative analysis
1. Chlorophyll a, b, and total chlorophyll
2. Total carotenoids 3. Total Sugar
4. Total lipids 5. Total protein
6. Total free amino acids
7. Total phenol 8. Total tannin
9. Total flavonoids
Bioinformatics studies
▪ Molinspiration
▪ Pocket identification
▪ ADMET-SAR properties
▪ Molecular docking studies
▪ Molecular dynamics simulation
▪ Density functional theory
In vitro
Biological activity
Antioxidant activity
▪ DPPH radical scavenging activity
▪ ABTS assay
▪ Ferric reducing power
▪ Nitric oxide
▪ Phosphomolybdenum assay
Cytotoxicity (MTT assay)
▪ MCF-7 Cell line
▪ VERO Cell line
Anti-inflammatory activity
Antimicrobial activity
Bacterial strains
▪ Pseudomonas aeruginosa
▪ Staphylococcus aureus
▪ Klebsiella pneumoniae
▪ Shigella flexineri
▪ Salmonella typhimurium
▪ Escherichia coli
Fungal strains
▪ Echinodontium tinctorium
▪ Candida tropicalis
▪ Candida glabrata
▪ Aspergillus fumigatus

3. MATERIALS AND METHODS

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3.1. Survey Region

The present floristic studies were carried out in the Siriya Kalvarayan hills, Eastern Ghats, Kallakurichi district. The location of the study area is shown in Figure 3.1. The survey was conducted between January 2017 and November 2019. We used a questionnaire to conduct a survey among Malayali (meaning in Tamil - mountain dwellers) tribals and other residents (total number of respondents = 105) of 8 villages in the study area – specifically, Sirukkalur, Edapattu, Vazhakuli, Athikkuzhi, Vanjikkuli, Moolakkadu, Aanaimaduvu, and Puliyankottai (in order of high-low altitude).

3.2. Study Area

The Siriya Kalvarayan hills start with a road beginning at Moolakkadu, which winds up to Serappattu, which is roughly 800 m above sea level. To reach this point, the route consists of five large hair-pin bends winding through the terrain (Figure 3.1). While this survey focuses on specific plants that have been identified in the region, it is not exhaustive. Whenever possible and when different species of flora were located, photographs were captured using a high-resolution phone camera (13 MP, Xiaomi Mi4i). The images were collated using Microsoft Office PowerPoint and refined using GIMP (ver. 2.10.6). The graphs were plotted using GraphPad Prism (v.5.02). The latitude and longitude for the study areas were given in Plate 3.1.

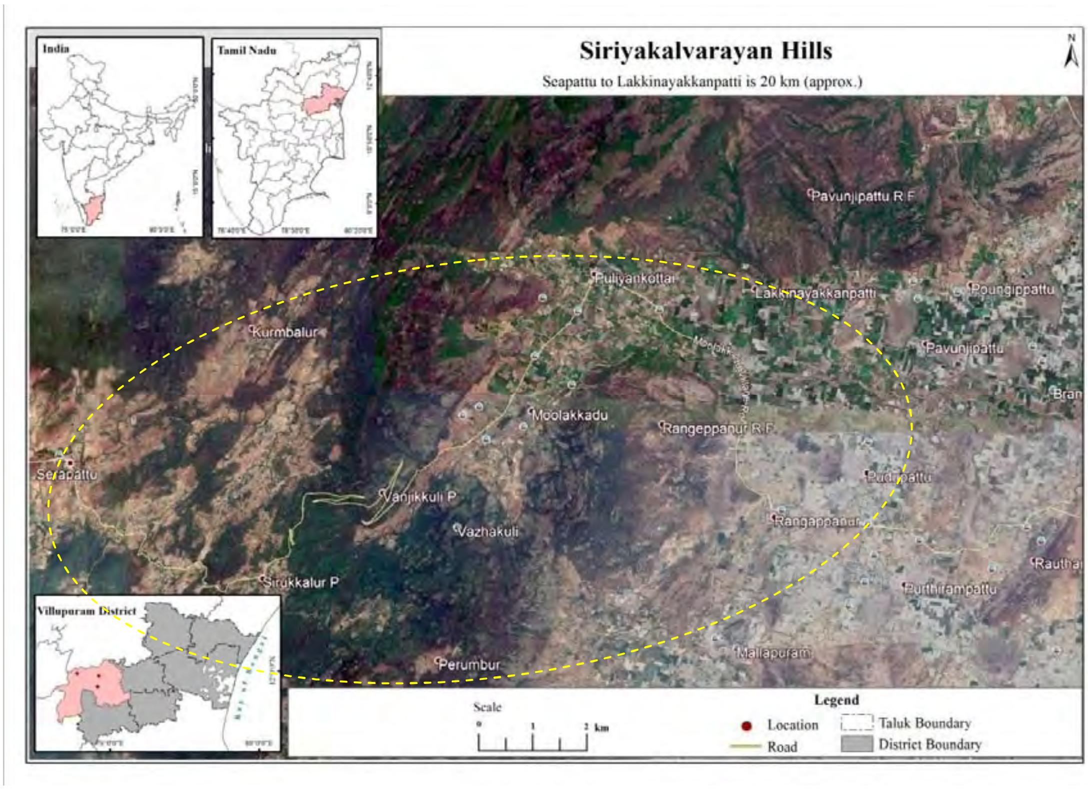

Figure 3.1. A close-up map of the study area - The study region from Serappattu to Puliyankottai is shown in this map, covering a distance of ~20 km lengthwise and a breadth of 10 km. The map indicates the study area by the dotted yellow oval marking.

Plate 3.1: Latitude and longitude view of the study area

3.3. Photography and Botanical Identification

The plants were photographed using a digital camera, and the taxonomic identification of these species was carried out at the Rapinat Herbarium, St. Joseph's College, Tiruchirappalli. The binomial names of these plants were verified by cross-checking the entire list with the names given on the plant list website (http://www.theplantlist.org).

3.4. Ethnobotanical Survey

A total of 105 subjects from eight villages (as mentioned before) which lie within the study area shown in the map (marked by a yellow dotted line, Figure3.1) Sirukkalur, Edapattu, Vazhakuli, Athikkuzhi, Vanjikkuli, Moolakkadu, Aanaimaduvu, and Puliyankottai. A questionnaire comprising seven critical questions was used to interview the residents (informants).

The survey questionnaire consisted of details like the respondent's Name; Age; Sex; Reliance on herbal medicine / naturopathy / Ayurveda / Siddha / Unani / homoeopathy / allopathy (percentage if possible); When you have fever and cough, what type of medicines do you take?; What are the few plants from this region that you use (in general) – mention specific ailments/complaints and the specific Tamil names of the plants you use and their parts (root, stem, leaf, flower, fruits) as well as the mode of administration (tea, concoction, dried powder/churanam, raw, extract, tonic etc.); Forest are the source of natural medicine, and we should use plants for treatment - a) Yes, you are 100% correct (strongly agree), b) Yes, greatly agree, c) Yes, somewhat agree, d) Somewhat disagree, e) disagree; Identify the photographs and the Tamil name given to you and talk about whether you use the plant; Do you sell or buy any preparations of any of the identified plants? – mention the specific name; How often do you visit an allopathic doctor? - a) All the time – even for the smallest health problems, b) Often, c) Sometimes, d) Very rarely, e) Never, or not at all. Using a non-probabilistic method of sampling (Kitchenham and Pfleeger, 2002), the residents of these villages who were willing to talk were interviewed. We explained the aim of the study to the residents and showed them all the photographs of the plants that had been surveyed. While some of the traditional uses of nearly 40% of the plants are known (from citations/references to those particular plants in ancient medicinal literature such as the Charaka Samhita) as well as the National Medicinal Plants Board (Government of India) website, information about the medicinal uses of the remaining 60% of the plants is either obscure or completely absent both in ancient texts and in published scientific works (when searching for publications/reports using Google Scholar). Hence, locals were asked for the medicinal uses of each of the plants, and in particular, about those plants whose medicinal properties were hitherto unknown to us.

A few medicine men (shamans) of these villages who expressed willingness to share their knowledge with us were also interviewed. Photographs of the plants (Plate 4.1-4.17) were shown to them and they were interviewed regarding the plants (and the parts thereof), their traditional medicinal uses and if applicable, ritualistic/other uses. Based on the survey findings, it is realized that the residents had an appreciable (if not thorough) extent of knowledge regarding most of the plants and their medicinal uses. The informants' responses were recorded through a questionnaire-based inquiry approach, and the responses were tabulated and counted. Using the numbers obtained through the survey, the following were calculated.

3.5. Informant Consensus Factor (ICF)

ICF was developed and first described by Trotter and Logan (1986) and then redefined by Heinrich (Heinrich, 2000; Heinrich *et al.*, 2009; Trotter and Logan, 1986). This parameter is an indicator of the homogeneity of ethnobotanical information and is defined by the formula:

$$ICF = Nur - Nt/Nur - 1$$

Where, Nur – Number of use reports present in each ailment category

Nt – Number of species which are used

ICF accurately describes each plant species' ethnopharmacological / ethnobotanical importance and high ICF values (1.0 or closer to 1.0) point to agreement/consensus among informants regarding the plant species and the specific ailment for which it is used.

3.6. Fidelity Level (FL)

The FL index is an estimate of the most preferred plant species for a particular ailment category. FL was proposed by Friedman *et al*. (1986) and it is an index which refers to the % of informants who name a specific species as ethnic medicine for a particular disease/ailment category (Friedman *et al*., 1986). The formula for FL (%) is:

$$FL(\%) = (Np/N) \times 100$$

where, Np pertains to the number of informants who claim to use a particular plant species for treating a specific illness and N describes the number of informants who rely on ethnomedicine for treating any given disease (among the list of diseases identified in this survey).

3.7. Use Value (UV)

This index was proposed in the 1980s and has been used widely to highlight the ethnobotanical importance of a given plant species among the local population (Bano *et al*., 2014; Trotter and Logan, 1986). Among a local population of informants, the Use Value (UV) is an index of the relative importance of a plant, and the formula used for UV estimation is

$$UV = \sum Ui/N$$

where Ui – number of use reports mentioned by an informant for a particular taxon and N refers to the total number of informants

3.8. Plant Collection

Plant samples (leaves) were collected from Siriya Kalvarayan hills (Kallakurichi district), situated in the Eastern Ghats of the southern Indian state of Tamil Nadu. The northern part of the Kalvarayans is known as the Siriya ("little") Kalvarayans, and the southern section is otherwise known as the Periya ("big") Kalvarayans. The geographical coordinates of the study areas are given in Plate 3.1.

3.9. Chemicals and Reagents

All chemicals, solvents and reagents used for this study were purchased from reputed vendors such as Himedia and SRL, India. DPPH (2,2'-diphenyl 1-picryl hydrazyl), ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) were purchased from Sigma Aldrich, India. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide) was purchased from Himedia, DMEM (Dulbecco's Modified Eagle's Medium) and FBS (Fetal Bovine Serum) were purchased from Gibco. Unless otherwise stated, all chemicals were of certified, analytical grade.

3.10. Plants selected for the study

Multiple criteria were followed, and the following plants were selected for further studies. *Aganosma cymosa* (Roxb.) G. Don, *Dalbergia lanceolaria* L.f., *Hugonia mystax* L., *Loeseneriella obtusifolia* (Roxb.) A. C. Sm., *Plecospermum spinosum* Trecul. and *Walsura trifoliata* (A.Juss.) Harms were the chosen plants. The parameters adopted for shortlisting is elaborated in the Results and Discussion part. Here, a brief introduction about the plant material used for the methods employed is given for enhanced clarity.

3.10.1. ***Aganosma cymosa*** (Roxb.) G. Don

Kingdom	: Plantae
Division	: Angiosperms
Family	: Apocynaceae
Genus	: *Aganosma*
Species	: *cymosa*

3.10.2. Description

Aganosma cymosa (Roxb.) G. Don is a liana that can grow up to 10 meter in length, pale brownish tomentose. Leaf stalks are 1-2 cm, leaf blade broadly ovate and orbicular, 5-16 cm, base rounded, acuminate, rarely retuse, and lateral veins 8-10 pairs. Flowers are borne in many-flowered clusters at branch ends, which are carried on stalks up to 6 cm. Bracts and bracteoles are very narrowly elliptic, about 1 cm long. Flower-stalks are about 5 mm. Flowers are borne in many-flowered clusters at branch ends, which are carried on stalks up to 6 cm. Bracts and bracteoles are very narrowly elliptic, about 1 cm long. Flower-stalks are about 5 mm. Calyx with several glands inside margin of sepals; sepals very narrowly elliptic, about 1 cm, pubescent on both surfaces. Flowers are white, minutely tomentose outside, glabrous at throat; tube shorter than sepals, 6-7 mm; lobes oblong, as long as tube. It is native to China, Bangladesh, India, Srilanka and Indochina.

Plate 3.2. Herbarium specimen of *Aganosma cymosa* (Roxb.) G. Don

3.10.3. ***Dalbergia lanceolaria*** L.f.

Kingdom : Plantae

Division : Angiosperms

Family : Fabaceae

Genus : *Dalbergia*

Species : *lanceolaria*


3.10.4. Description

Dalbergia lanceolaria L.f. is a densely foliaceous tree growing to 20 m height. The leaves have a very short stalk 2-3 cm long, imparipinnate and alternate distichous arrangements. Leaf margin is entire, obtuse apex, cuneate base and obovate shape. Flowers are axillary panicles and pale pink. Flowering peaks during April. A pod, lanceolate, faintly nerved dark green. Seeds 1-3. Fruiting May onwards. It is native to India, Srilanka, Nepal, Burma and Indo-china.

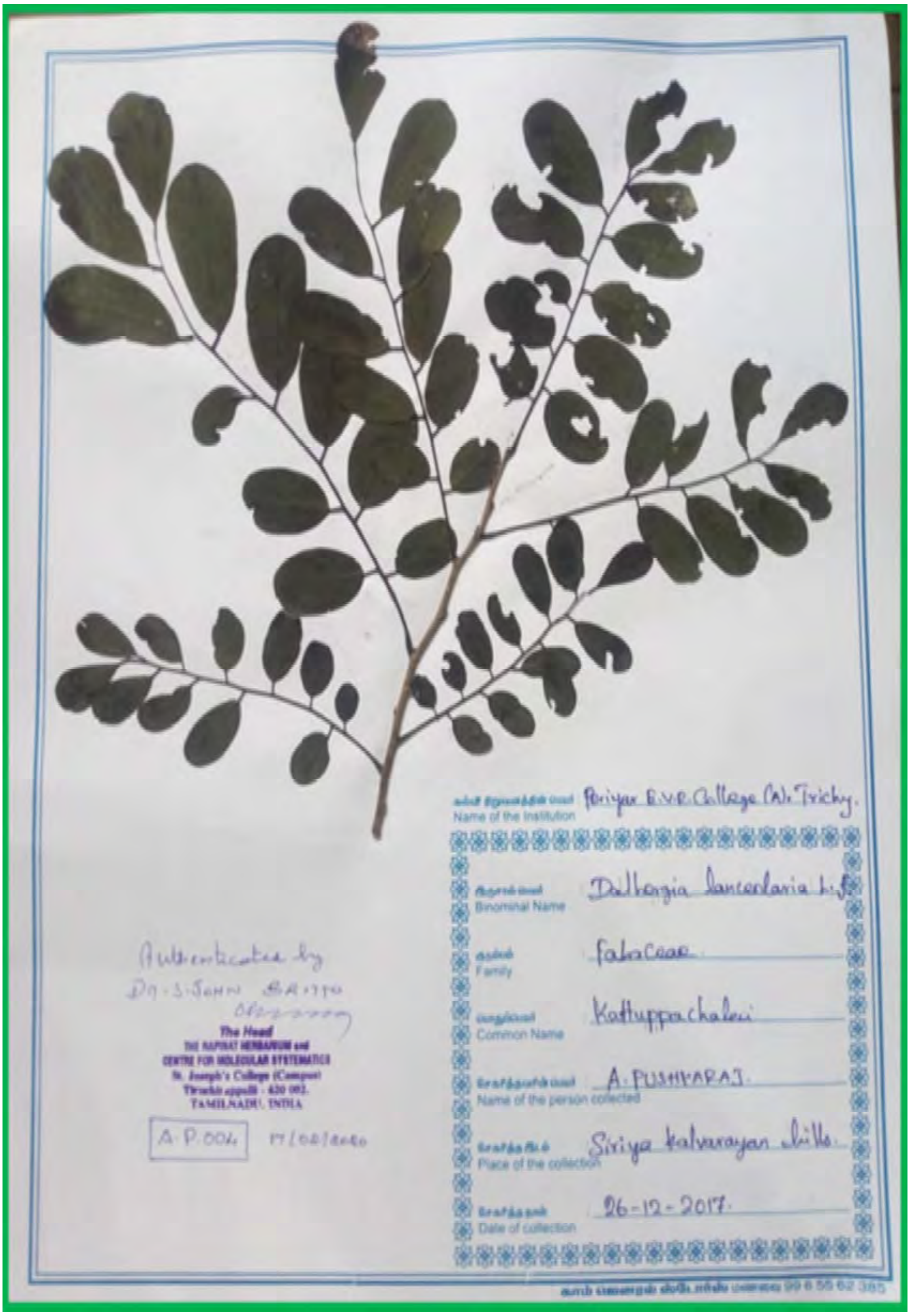

Plate 3.3. Herbarium specimen of *Dalbergia lanceolaria* L.f.

3.10.5. *Hugonia mystax* L.

Kingdom : Plantae

Division : Angiosperms

Family : Linaceae

Genus : *Hugonia*

Species : *serrata*

3.10.6. Description

Hugonia mystax L. is a scrambling shrub with spreading, yellow tomentose branches set with short, horizontal twigs leaflets below and provided. At the end, with a pair of nearly opposite, woody, reflexed, circinate, tapering, tomentose spines in the axils of the two lowest leaves or scale; leaves are 5-6 cm long and 2.5-3.5 cm in wide. Leaves are alternate, on the main branches distant on the lateral twigs crowed at their end. Leaves are oval, tapering to base, obtuse and entire with reticulate venation prominent on the side, petiole very short and stip. Flowers are large and yellow, on woody petals as long as sepals. The stem has a pair of hook-like structures for creeping on other plants. It is mainly found in the dry forests of peninsular India and Srilanka.

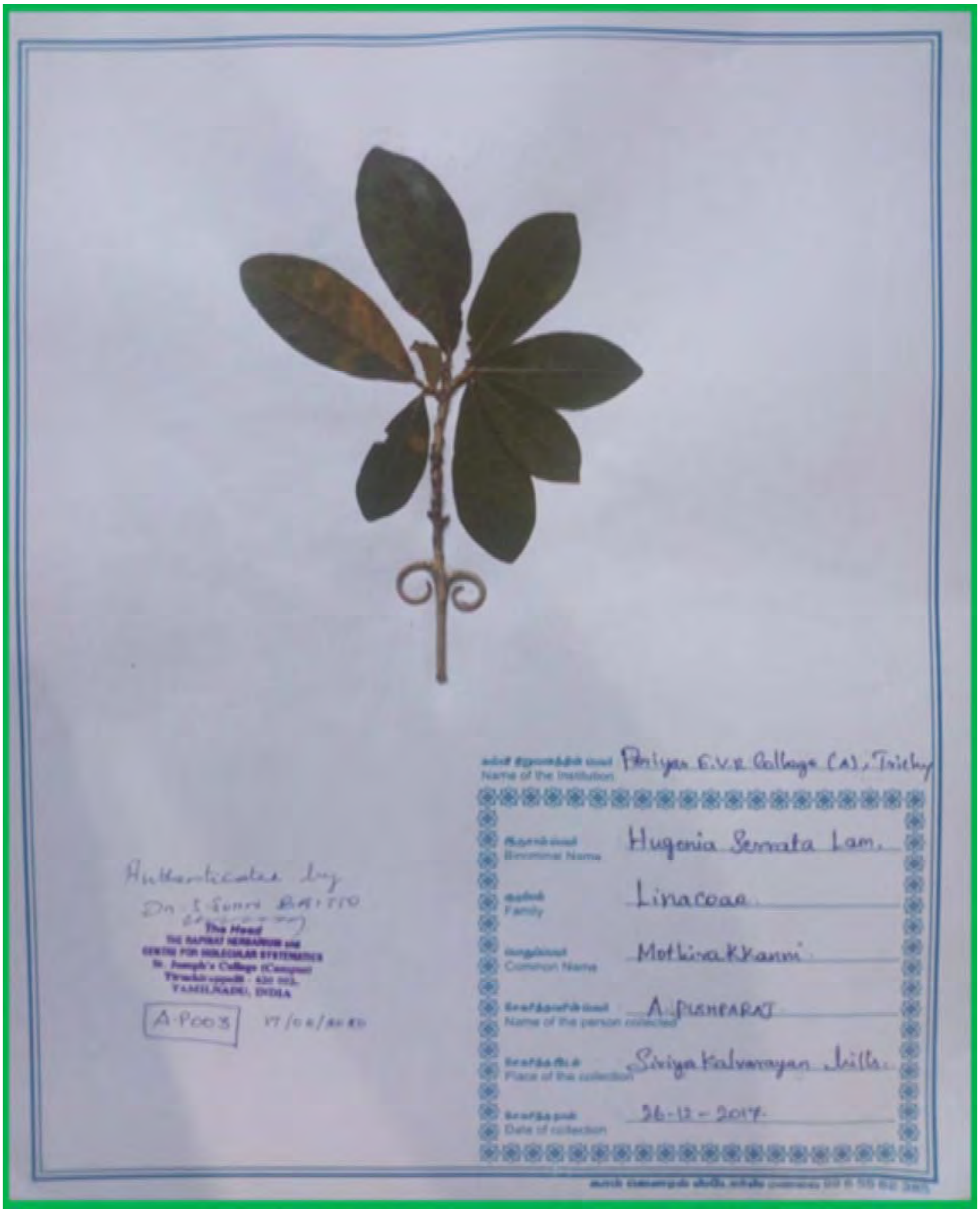

Plate 3.4. Herbarium specimen of ***Hugonia mystax*** **L.**

3.10.7. *Loeseneriella obtusifolia* (Roxb.) A. C. Sm.

Kingdom : **Plantae**

Division : **Angiosperms**

Family : ***Celastraceae***

Genus : ***Loeseneriella***

Species : ***obtusifolia***

3.10.8. Description

Loeseneriella obtusifolia (Roxb.) A. C. Sm.is an extensive wild evergreen woody climber about 8 to 10 cm long. Leaves are simple, shining, elliptic-oblong; arrangements are opposite decussate, acute-acuminate laef apex, leaf base is obtuse to the subacute and crenate leaf margin. Flowers are in axillary, extensive panicles; green. Flowering from December to March. Fruits are obovoid samaroid, obtuse-emarginate, dorsiventrally compressed; seeds 6 per cell, subfalcate, wing membranous. Fruiting February onwards. It is found in Assam, Maharashtra, Andhra Pradesh, Karnataka, Tamil Nadu and Kerala.

Plate 3.5. Herbarium specimen of *Loeseneriella obtusifolia* (Roxb.) A. C. Sm.

3.10.9. *Plecospermum spinosum* Trecul.

Kingdom **: Plantae**

Division **: Angiosperms**

Family **: Moraceae**

Genus **: *Plecospermum***

Species **: *spinosum***


3.10.10. Description

Plecospermum spinosum Trecul. is the wild large evergreen long thorny woody climber. The leaves of *Plecospermum spinosum* grow alternately on the stem (spirally arranged). The leaf is simple, bright green-coloured above and paler beneath. The leaf is coriaceous and glabrous on both sides. It is 6 to 10 cm long and 3 to 4 cm wide. The leaves have a 1.2 cm long petiole. The apex is obtuse, the base is rounded, and the margins are entire. The venation of the leaf is reticulate with a prominent midrib. It has male and female flowers separated on different individuals (dioecious). The flowers are grouped in pendulous, axillary and capitates clusters. The flowers are sessile, and the peduncle of the inflorescences is about 1 cm long. The fruits are aggregated into a subglobose syncarp. It is light green coloured turning yellow when ripe.

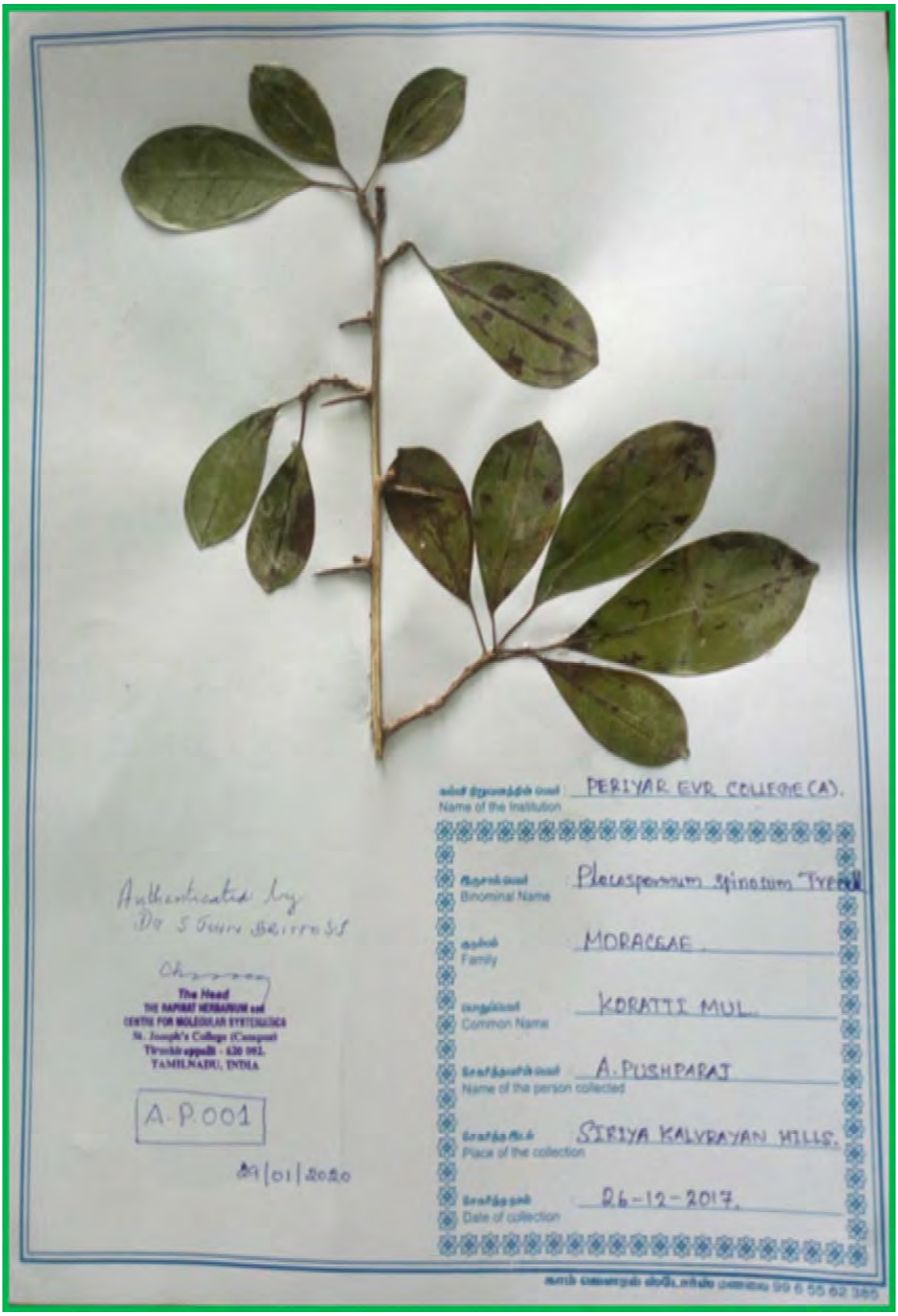

Plate 3.6. Herbarium specimen of *Plecospermum spinosum* Trecul.

3.10.11. *Walsura trifoliata* (A.Juss.) Harms

Kingdom **: Plantae**

Division **: Angiosperms**

Family **: Meliaceae**

Genus **: *Walsura***

Species **: *trifoliata***

3.10.12. Description

The leaves of *Walsura trifoliata* (A.Juss.) Harms grow alternately on the stem (spirally arranged). It has trifoliate leaves. These compound leaves are about 8 to 15 cm long and wide. The leaves have a 2.5 to 4 cm long petiole. The leaflets are leathery and coriaceous and 5 to 9 cm long, and 2.5 to 3.5 wide. They are elliptic with an obtuse-retuse apex, rounded-cuneate base and entire margins. The leaflets are glabrous, dark-green glossy above and light-green beneath with prominent midrib. The leaflets have a 4 to 5 mm long petiole. The venation of the leaflets is reticulate.

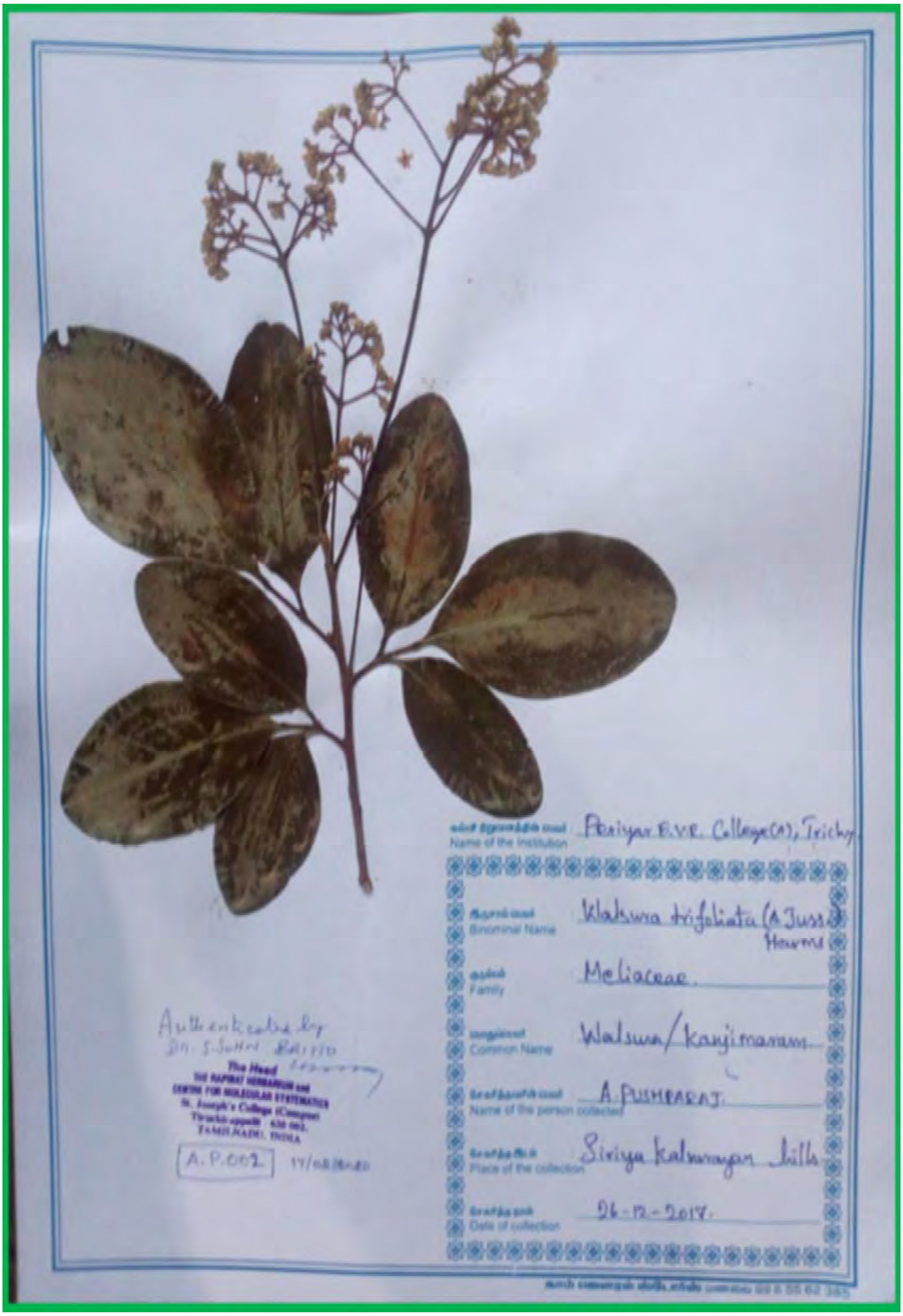

Plate 3.7. Herbarium specimen of *Walsura trifoliata* (A.Juss.) Harms

3.11. Solvent Extraction

The collected leaf samples for all the chosen plant samples were shade-dried for 3 days separately and then ground to powder using a blender-mixer. The powder was weighed and packed into a thimble made from α-cellulose cotton fibre (Whatmann, supplied by GE healthcare Ltd.). The plant powder was placed in the soxhlet apparatus and refluxed with methanol (63°C) over a heating mantle for about 8 hours until solvent concentrates were obtained. The resulting extracts were poured into glass Petri dishes and then kept for evaporation of the excess solvent (room temperature).

3.12. Antioxidant Assay

3.12.1. DPPH Radical Scavenging Assay

To determine the free radical scavenging activity of the crude plant extracts, we used a slightly modified protocol of the prevalent method of Brand-Williams *et al.*, 1995, which involves the reaction of chemical compounds in the test solution with DPPH radical (2,2'-diphenyl 1-picryl hydrazyl), which upon reaction with antioxidants, transfers the free radical to the electron-accepting antioxidant compounds, leading to a loss of the intense pinkish purple colour of the DPPH radical. 1 mL of 0.2 mM DPPH radical solution was combined with 1 mL of the methanolic leaf extracts at varying concentrations (10-100 μg/mL). We prepared corresponding blank solutions and employed L-ascorbic acid (of equal concentrations as the test solutions, in the range of 10-100 μg/mL) as a positive control antioxidant known to scavenge free radicals rapidly. We used a mixture of 1 mL methanol with 1 mL DPPH as control. The disappearance of DPPH radical was monitored at 517 nm using a spectrophotometer after incubation at room temperature in the dark. The % inhibition was calculated using the formula given below:

$$\textbf{Inhibition \% = (Ac – As/Ac)} \times \textbf{100}$$

3.12.2. ABTS$^{\bullet+}$ Radical Scavenging Assay

ABTS radical scavenging assay followed by Re *et al*., 1999 was done with few modifications. ABTS was dissolved in water to a 7 mM concentration. By reaction with 2.45 mM potassium persulfate (1:1), ABTS radical cation (ABTS$^{\bullet+}$) was produced, and this reaction was allowed to proceed in the dark at room temperature for 12-16 hours before use. After incubation, ABTS$^{\bullet+}$ solution was diluted with double distilled water to adjust the absorbance to 0.7 at 734 nm. Then, 1 mL of freshly prepared ABTS$^{\bullet+}$ solution was added with 1 mL of the sample (10-100 μg/mL). The absorbance was measured at 734 nm using a spectrophotometer. Ascorbic acid was used as a positive control.

3.12.3. Ferric Ion Reducing Power Assay

Ferric reducing power was determined with minor modifications of a method described earlier (Zhao *et al.*, 2008). Various concentrations of the plant extract (10-100 µL/mL) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide and incubated at 50°C for 20 min. After the addition of 2.5 mL of 10% TCA, the tubes were centrifuged at 10,000 rpm for 10 minutes. Then, 2.5 mL of supernatant was mixed with 2.5 mL of distilled water, and 0.5 mL 0.1% ferric chloride and the absorbance of the reaction mixture was measured at 700 nm. Ascorbic acid was used as a positive control.

3.12.4. Nitric Oxide Radical Scavenging Activity

Nitric oxide scavenging activity of plant extracts was determined using Thangaraj, 2016b method. 1 mL of different concentrations (10-100μg/mL) of the plant extract was mixed with 3 mL of 10 mM sodium nitroprusside and incubated at room temperature for 150 minutes. Then, 3 mL of Griess reagent was added to all the samples. Then, the absorbance of the chromophore was measured at 546 nm against the blank. Ascorbic acid was used as standard. The scavenging activity was calculated using the following equation:

Scavenging activity% = [(Control OD – Sample OD)/Control OD] × 100

3.12.5. Phosphomolybdenum Assay

The antioxidant activity of samples was evaluated by the phosphomolybdenum method (Thangaraj, 2016b). 1 mL of plant extract was mixed with an equal volume of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated in a water bath at 95°C for 90 minutes. Then, it was allowed to cool at room temperature, and the absorbance was measured at 765 nm against a blank. The results were reported in ascorbic acid equivalents (AAE)/g extract.

3.13. Antimicrobial Activity

The antimicrobial activity of methanolic extracts of five chosen concentrations of the obtained extracts, namely, 6 mg, 7 mg, 8 mg, 9 mg, and 10 mg per 1 mL of solvent, was treated with different fungal and bacterial strains using well diffusion method. Standard antibiotic discs such as nitrofurantoin, vancomycin, bacitracin, amoxyclav, gentamicin penicillin-g, amikacin, chloramphenicol, ampicillin, and methicillin were used as positive controls.

3.13.1. Microorganisms

The different bacterial species used were *Pseudomonas aeruginosa* MTCC 1034, *Staphylococcus aureus* MTCC 9542, *Klebsiella pneuminiae* MTCC 8911, *Shigella flexneri* MTCC 9543, *Salmonella typhimurium* MTCC 3224 and *Escherichia coli*

MTCC 584. Fungal species used include *Candida tropicalis* MTCC 2795, *Candida glabrata* MTCC 3983, *Echinodontium tinctorium* MTCC 1038 and *Aspergillus fumigatus* MTCC 2483. The bacterial and fungal species were sub-cultured on nutrient broth and incubated at 37°C for 18-24 h for reviving the organisms. Later, fresh overnight cultures were used for the experiment.

3.13.2. Well Diffusion Method

Well diffusion method was used with a few modifications to evaluate anti-microbial activity (Ćavar *et al.*, 2008). Wells were cut using sterile, autoclaved steel borers and filled with extracts (at a wide range of concentrations) prepared using different solvents (70 μL). Nutrient agar dispersion plates were swabbed with both fungal and bacterial cultures after wells were cut. Sterile antibiotic discs (positive controls) were employed to check for formation of zones of inhibition. Each extract was tested in duplicate. Control wells contained equal amounts (70 μL) of the respective solvents. Standard antibiotics such as nitrofurantoin, vancomycin, bacitracin, amoxyclav, gentamicin, penicillin-g, amikacin, chloramphenicol, ampicillin, and methicillin (Himedia) were used as references or positive controls. Agar plates containing fungal and bacteria was incubated at 37°C for 24 hours. Inhibition zones were recorded as the diameter of growth-free zones, including the radius of the disc in mm, at the end of the incubation period.

3.14. Anticancer Activity

In vitro assay for anti cancer activity (MTT assay) was performed as per the method of Mosmann, 1983. MCF-7 cell line was obtained from NCCS, Pune. The cells were maintained in DMEM (Dulbecco’s Modified Eagle’s Medium) supplemented with 10% FetalBovine Serum (FBS), penicillin (100 μg/mL), and streptomycin (100 μg/mL) in a humidified atmosphere of 50 μg/mL CO_2 at 37°C. Cells (1×10^5/well) were plated in 24-well plates and incubated at 37°C with 5% CO_2. After the cells reached confluence, various concentrations of samples were added, and the cells were incubated for 24 hrs. The excess plant extract was removed from the well by washing the wells with phosphate-buffered saline (pH 7.4) or DMEM lacking serum. 100 μL/well (5 mg/mL) of 0.5% methyl thiazol tetrazolium MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide) was added and the cells were incubated for 4 hours. After incubation, 1 mL of DMSO was added to all the wells. The absorbance at 570 nm was measured with a UV-Spectrophotometer using DMSO as the blank. Measurements were performed, and the concentration required for 50% inhibition (IC_{50}) was determined graphically. Using the following formula, the % cell viability was calculated:

% cell viability = A570 of treated cells / A570 of control cells × 100

A graph was plotted using the % of cell viability on the Y-axis and concentration of the sample in the X-axis.

3.15. Anti-Inflammatory Activity

The anti-inflammatory activity was done based on the modified method of Chaudhari *et al.*, 2013. Fresh blood was collected from healthy volunteers and mixed with an equal volume of sterilised Alsever solution, which acts as an anticoagulant [containing dextrose (2%), sodium citrate (0.8%), Citric acid (0.9%) and Sodium chloride (0.72%)]. Blood samples were centrifuged at 10,000 rpm for 15 minutes at room temperature. The supernatant was carefully removed while the packed red blood cell was washed in fresh normal saline (0.85% NaCl). Until the supernatants turned clear, we repeated the process of washing and centrifugation. This RBC suspension was used for the estimation of anti-inflammatory property. The plant sample (at various concentrations) and control were separately mixed with 2 mL of phosphate buffer, 4 mL of hypo saline and 0.9 mL of RBC suspension. All the assay mixtures were incubated at 37°C for 30 minutes. The supernatant was decanted and the haemoglobin content was estimated by spectrophotometer at 620 nm. The % haemolysis was calculated using the formula;

$$\textbf{\% Haemolysis} = \mathbf{T/C \times 100}$$

where, T-Test sample and C-Control sample

3.16. Qualitative Analysis

The method of Brindha *et al.*, 1982 was followed for analysing the phytochemical constitution of plant extracts for Triterpenoids, Sugars, Catechins, Flavonoids, Saponins, Tannins, Anthraquinones, Aminoacids, Sterols and Carbohydrates. The reagents required for this qualitative assay were prepared freshly. The plant extracts (test solutions) were also prepared freshly after drying the soxhlated extracts and then reconstituting the fine pastes in the respective solvent used for extraction. Care was taken to reconfirm all the assays, in order to rule out false-positives. Based on the results for qualitative phytochemical estimation, we proceeded with soxhlation for water (aqueous extract), methanol, ethanol, acetone, chloroform and hexane.

3.17. Quantitative Analysis

3.17.1. Chlorophyll a, b, Total Chlorophyll and Carotenoid Content

Estimation of chlorophyll and carotenoids was performed using Arnon, 1949 method. 100 mg of dried leaf powder ground in a mortar and pestle, adding 5 mL of 80% acetone. The homogenate was then centrifuged at 1000 rpm for 10 minutes. The supernatant was collected in test tube and the pellet was re-extracted with 5 mL of the same solvent and again placed in the centrifuge for agitation at 1000 rpm in 10 minutes. The supernatant was collected combined with the previous homogenate. The final volume was made up to 10 mL with 80% acetone. The absorbance was measured at 663, 645 and 480 nm against the solvent blank.

3.17.2. Total Sugar Content

Estimation of total sugars was done using Dubois *et al*., 1956 method. 100 mg of dried leaf powder was weighed and mashed with mortar and pestle. The homogenate was taken in a boiling test tube, and 5 mL of 2.5 N HCl was added to it and kept in a boiling water bath at 80°C for 1 hour. The test tube was then cooled to room temperature and neutralized with solid sodium carbonate until the effervescence stopped. The final volume was made up to 10 mL with distilled water and centrifuged at 1000 rpm for 10 minutes. The supernatant was collected, and the residue was discarded. 0.2 mL of this sample was taken in a test tube and made up to 1 mL with distilled water; 10 mg of glucose was dissolved in 100 mL of distilled water for use as standard. From this 0.2, 0.4, 0.6, 0.8 and 1.0 mL were taken in separate tubes and all were adjusted to a final volume of 1 mL with distilled water. A blank solution containing 1 mL of distilled water was also taken. To all the seven tubes, 1 mL of 5% phenol was added, and then 5 mL of H_2SO_4 was added to all the test tubes and shaken well. The set-up was left free for 10 minutes. After this, the tubes were gently shaken and placed in a water bath at 25-30°C for 20 minutes. The absorbance was measured at 490 nm, and the total sugar content was measured using the standard graph.

3.17.3. Total Lipid Content

Estimation of total lipids was done using gravimetric method (Mertens, 2002). 1 g of leaf powder sample was taken and ground in a mortar and pestle with 10 mL of chloroform: methanol mixture (2:1). Then, the homogenate was collected in a test tube and centrifuged at 1000 rpm for 5 minutes. The clear supernatant was collected in a pre-weighed boiling test tube and kept in a boiling water bath at 80°C until all the solvent evaporates. The test tube was then weighed again, and the weight of total lipids was calculated as percentage.

3.17.4. Total Protein Content

Estimation of protein content was done using Lowry *et al*., 1951 method. 1 g of leaf powder sample was taken in a mortar and ground well with pestle by adding 10 mL of phosphate buffer (pH 7.2). After centrifuging the homogenate at 1000 rpm for 10 minutes, the supernatant was collected, and an equal volume of 10% TCA was added to it. The test tube was left undisturbed for 15 minutes and then centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded, and the precipitate was dissolved in 2 mL of 0.2 N NaOH and made up to 5 mL with distilled water. BSA standard (100 μg/mL) from 0 to l mL volume with 0.2 mL of sample solution was also taken in a test tube. A blank solution with 4 mL distilled water was also taken, all the test tube were made up to a final volume of 4 mL with distilled water and 5.5 mL of reagent C (Reagent A - 2% Na_2CO_3 in 0.1 N NaOH; reagent B - 0.5% $CuSO_4$ in 1% Na K tartarate solution; reagent C - 49 mL of reagent A was mixed with 1 mL of reagent B just prior to use) was added to it and kept undisturbed for 10 minutes. After this 0.5 mL of Folin-phenol reagent was added and kept for 30 minutes and the OD was measured at 650 nm using a spectrophotometer.

3.17.5. Total Free Amino Acid Content

Estimation of total free amino acid was done using Troll and Cannan, 1953 method. 20 mg of leaf powder was weighed and taken in a mortar and pestle. The homogenization was carried out using 5 mL of 80% methanol, and the resulting homogenate was centrifuged at 1000 rpm for 10 minutes. The supernatant was collected in a test tube, and the pellet was re-extracted as above. The two supernatants were combined after centrifugation, and the volume was noted. In a separation funnel, the extract was taken and mixed with equal volumes of petroleum ether. The lower amino acid layer was taken and made up to 20 mL with 80% methanol. The sample was mixed with 0.1 mL of 80% phenol, 0.2 mL of ninhydrin and made up 10 mL with 80% methanol. Glycine was used as the aminoacid standard.

3.17.6. Total Phenol Content

Estimation of total phenolics was done by Folin-Ciocalteau method (Thangaraj, 2016a). Into a series of test tubes, a range of volumes (0.2, 0.4, 0.6, 0.8 and 1.0 mL) of a 50 μg/mL of methanolic plant extract was taken, and all the tubes were made up to 1 mL with distilled water. Another test tube was marked 'B' with 1 mL of distilled water which served as blank. The analysis was performed in triplicates. Then, 0.5 mL of 1 N Folin-Ciocalteau reagent was added to all the test tubes as well as blank. Gallic acid was used as standard. The tubes were vortexed and allowed to stand for 5 minutes at room temperature. After that, 2.5 mL of 5% sodium carbonate was added to all the test tubes, including blank. Again, all the tubes were vortexed and incubated in the dark at room temperature for 40 minutes, and the OD value was recorded at 725 nm using a spectrophotometer. A standard graph was drawn by plotting the concentration of tannic acid on X-axis and respective absorbance on Y-axis. Finally, the amount of total phenol in the sample was calculated and expressed as mg gallic acid equivalents/g sample.

3.17.7. Total Tannin Content

Took 1 mg/mL of methanolic plant extract in 2 mL eppendorf tubes. 500 μL of the stock solution was diluted with an equal volume of distilled water. Then, 100 mg of polyvinyl polypyrrolidone (PVPP) was added to the tubes. The tubes were incubated for 4 hours at 4°C. Later, the tubes were centrifuged at 3000 rpm for 10 minutes at 4°C. The supernatant contained only the non-tannin phenolics. In a series of test tubes, a stock of non-tannin phenolics was diluted to 0.2, 0.4, 0.6, 0.8 and 1.0 mL and distilled water were added to make the final volume to 1.0 mL in all the tubes. Blank was prepared by taking 1 mL of distilled water alone. Then, 0.5 mL of 1 N Folin-Ciocalteau reagent was added to all the tubes, including blank. Tannic acid was used as the standard. All the tubes were vortexed well and allowed to stand for 5 minutes at room temperature. 2.5 mL of 5% sodium carbonate was added to all the tubes, including blank. The tubes were vortexed again and incubated in the dark at room temperature for 40 minutes and the OD was recorded at 725 nm using a spectrophotometer. A standard graph was drawn by plotting the concentration of tannic acid on X-axis and respective absorbance on Y-axis. Finally, the amount of non-tannin phenolics and total phenolics in the sample was calculated as mg tannic acid equivalents/g sample (Thangaraj, 2016a).

Tannins (g) = Total phenolics (g) – Non tannin phenolics (g)

3.17.8. Flavonoid Content

The flavonoid content of the plant extract was estimated based on the aluminium chloride method (Thangaraj, 2016a). Initially, 500 µL of the extract was taken from the stock (stock contains 1 mg/mL) and then made up to 1 mL with distilled water. 1 mL of distilled water served as the blank. Then 150 µL of 5% sodium nitrite was added into the test tube, including blank. All the tubes were vortexed and incubated at room temperature for 5 minutes. After that, 150 µL of 10% aluminium chloride was added. Again, after vortexing, the tubes were incubated at room temperature for 6 minutes. Then 2 mL of 4% sodium hydroxide was added, and the final volume was made up to 5 mL with distilled water. After another round of vortexing, the tubes were incubated at room temperature for 15 minutes. The presence of flavonoid was indicated by pink colour, and the absorbance was measured at 510 nm. Rutin was used as the flavonoid standard. The results were expressed in rutin equivalents (RE).

3.18. GC-MS Profiling of the Methanolic Leaf Extract

The phytochemical evaluation of the methanolic leaf extractswas carried out using a Gas Chromatography-Mass Spectrophotometer (GC-MS) (Perkin Elmer Clarus 500, Connecticut, USA) equipped with a flame ionization detector, capillary column (30 m length × 0.25 mm ID coated with 5% phenyl 95% dimethylpolysiloxane) with a film thickness of 0.25 µm. Helium gas (mobile phase) was the carrier gas, and its flow rate was fixed at 1 mL/minutes. The temperature of the injection port was maintained at 280°C, and the volume of sample injection was 1 µL. The stationary phase (capillary column) temperature was set in the range of 60 to 300°C (raising rate of 10 minutes). Mass spectra were programmed as scan type: full scan mode and scan range: 40-450 Daltons. The peaks of the compounds were matched with the standard peaks available in NIST (The National Institute of Standards and Technology) library (Hussein *et al.*, 2016).

3.19. Computational Studies

3.19.1. Molinspiration and PASSOnline

Chemdraw Ultra 8.0 was used to draw the chemical structures from the GC-MS report. The smiles of the structures were submitted to Molinspiration Property Calculator and ACD/I-LAB server to predict the drug-likeness of the plant compounds. Properties like relative molecular mass (MW), partition coefficient (cLogP), H-bond donors (HBD), H-bond acceptors (Autschbach), number of rotatable bonds (NROTB) and topological polar surface area (TPSA) were calculated. From these results, we assessed whether the molecules obeyed Lipinski's RO5. Apart from these parameters, pKa and aqueous solubility of the compounds at a given pH (LogS) were also assessed (Tariq *et al*., 2016). PASSOnline server (http://way2drug.com/passonline/) was used to identify the phytochemicals' top biological potential, and Pa cut off value of > 0,5 was chosen. Molinspiration server also predicted the protein target types (GPCR ligand, ion channel modulator, kinase inhibitor, nuclear receptor ligand, protease inhibitor and enzyme inhibitor). By combining the results for Molinspiration and PASSOnline analysis, a few compounds that were deemed to possess anti-inflammatory and anticancer properties were shortlisted, and these were chosen for molecular docking studies.

3.19.2. Pocket Identification

POcket-CAvitySearch Application (POCASA) 1.1 server was used to identify the number and volumes of the pockets/cavities present in the selected receptor proteins using the default parameters. The size of a pocket (shape and topography) is a key factor in receptor-ligand docking as it determines whether ligands can fit into the identified pocket(s) during structure-based drug design (Yu, J. *et al*., 2010a). POCASA employs cavity search using a rolling sphere methodology to detect cavities and pockets.

3.19.3. Molecular Docking Studies

The docking studies were performed to identify the molecular interactions between the 3D model of HER2 (PDB ID: 3PP0), Akt1 (3CQW), mTOR (4JT6), MMP9 (5TH6), PI3K (5NGB) and ERβ (3OS8) and phytochemical compounds using MGL tools (AutoDock 4.2) (Morris *et al*., 1998). The receptor and small molecule ligands were converted into PDBQT format from PDB file format by following the standard AutoDock protocol. AutoGrid was adjusted and the dimensions of XYZ were set at $90 \times 90 \times 90$ Å^3, respectively. The spacing angstroms were set at 0.375 Å, and Gasteiger charges and polar hydrogen atoms were added to the target (macromolecule). In docking, the active torsions and torsional degrees of freedom for small molecules were assessed. The Genetic Algorithm (GA)-Lamarckian principle was used, and the programme was set to complete ten docking runs. The potency of the inhibitors was gauged based on binding energy (Gibbs free energy, ΔG) values (kcal/mol). PyMoL, a molecular visualization tool, was used to view the interactions between the target & small molecules and to measure the distance (bond length) for docked complexes. The best output poses were analyzed for interactions between the receptor and ligand. The 2D poses of the best hits of each of the compounds were generated using Accelrys Discovery Studio Visualizer 2.5 (Studio, 2008). Chimera 1.14 (Pettersen *et al*., 2004) was used for visualizing proteins and for checking the binding sites after completion of docking runs.

3.19.4. Molecular Dynamics Simulations

Molecular Dynamics Simulation studies were carried out to calibrate the tendency of protein and ligand molecules by following the principles of GROMACS 5.1.5 software. Ligand topology files were prepared using PRODRG (http://davapc1.bioch.dundee.ac.uk/cgi-bin/prodrg) online tool. Protein topology files were prepared by the following force field of GROMACS called GROMACS96 43a1 and the protein was solvated by Simple Point Charge water model with the help of cubic unit cell shape which helps the protein to rotate freely without any disturbances and the coordinates of X, Y & Z were set at 144 × 144 × 144 Å^3 respectively; finally, the protein was neutralized by adding sodium ions. Periodic Boundary Condition (PBC) plays a vital in avoiding the boundary effects which might lead to detachment among the protein, ligand and water molecule; it helps to maintain the connectivity among the protein, ligand and water and PBC was calibrated by the mathematical principle called, Velocity Verlet algorithm and Leap-frog algorithm. Protein Energy Minimization (EM) was performed by the steepest descent minimization algorithm with the maximum force < 10.0 kJ/mol (1000.0), and it converged by a maximum 50000 number of steps to minimize the protein, cut-off scheme to find the neighbour set of atoms/molecules and to calculate the non-bond interactions like electrostatic and Van der Waals force with the help of Verlet algorithm, Particle Mesh Ewald (Lennard Jones and Coulomb potential). Protein Restrain Topology equilibration was carried out by the following phases with the help of Parrinello-Rahman method, NVT and NPT ensemble respectively; NVT-Conjugated gradient algorithm (Leap-frog integrator) helps to minimize the complex upto 100 ps; Berendsen thermostat and V-rescale weak coupling method help to maintain the temperature coupling upto 300 K; LINCS helps to analyze the hydrogen bond interactions between the protein and ligand; Maxwell distribution assigned the velocity to minimize the complex and NPT-Isotropic Berendsen thermostat pressure coupling helps to minimize the complex upto25,000 ps (25 ns) and 50,000 ps (50 ns); time constant and pressure, tau_p&ref_p was maintained upto 2.0 ps & 1.0 atm respectively (Kumar *et al*., 2021; Langeswaran *et al*., 2019).

3.19.5. Density Functional Theory (DFT) Study

All the ground-state geometry optimizations of BTP, CTL, MQL, DHA and NPD compounds were performed in the gas phase using DFT (Becke, 1993) with Becke's three-parameter hybrid method (Lee *et al*., 1988), combined with the Lee-Yang-Parr correlation functional (Tenderholt, 2007) denoted as B3LYP functional. The molecules were optimized at B3LYP/6-311+G(d,p) basis sets wielding Gaussian 16 software suite and it has been reported to give reasonably good results for most of the organic molecules (Solomon *et al*., 2012). The coordinates were specified in the input files of the DFT calculations. The DFT calculations were performed using the hybrid B3LYP functional applying GTO (Gaussian type orbital). Frequency analysis was carried out using the functional and basis set to verify the nature of optimized molecules and to ascertain the presence of global minima (Autschbach, 2009; Dreuw and Head-Gordon, 2005). The global energy minimal structure was checked with zero imaginary frequencies.

3.19.6. ADMET-SAR for ADME profiling

Absorption, Distribution, Metabolism and Excretion (AMDE) profiling was carried out using ADMET-SAR 2.0 (Yang, H. *et al*., 2019), an online tool that predicts carcinogenicity, cytotoxicity, metabolism (CYP450 reactions as either substrates/inhibitors), P-glycoprotein binding, subcellular localization and other important *in silico* indications that aid in molecular toxicology profiling of a given compound.

4. RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

This section covers the primary findings of the work, the key takeaways from the work and the essential corollaries from literature that support the relevance of this work. The observations/results are discussed in brief, and the importance of ethnobotanical studies is overwhelmingly significant in the present scenario when there is an emergence of pathogens with resistance to clinical therapeutics. The global COVID pandemic (and other viral diseases) are also significant causes to search nature's incredible and inexhaustible arsenal of molecular 'weaponry' against unique targets in microbial cells. In the pursuit of this cause, the preliminary stage comprises of the following: identification of unique/novel plant species, phytochemical analysis, and determination of biological activities (anti-inflammatory, cytotoxic, antimicrobial, antioxidant), which will aid in the usage of these plants as sources of novel and promising experimental therapeutics. Nature has a phenomenal ability to make a variety of primary and secondary metabolites owing to species-specific variations in the genetic constitution of plants and environment-dependent epigenetic changes, which can alter the expression levels of essential genes (e.g., weather, water availability, soil nutrition, moisture content, seasonal variations in the habitat, ecological conditions and relationships with other organisms such as endophytes and temperature). These are just a few factors that may determine the species-specific production of variegated biomolecules, which exhibit biologically functional activities *in vitro* and *in vivo*. This work aimed at identification of novel species (for which no significant reports exist), novel phytochemicals, and the potential 'bioprospecting' of these molecules using *in silico* tools (molecular docking studies) in the quest to lay a solid platform for long-term research - e.g., on the molecular aspects/mechanistic chemistry of promising activities which are discovered - such as molecular targets and the mechanism of receptor-target binding. We plan to study these aspects in depth if given the opportunity to do so in the near future. Since some of these species have ethnic/ethnopharmacological applications (by the local population), a survey of the demographics of the local population (in 8 villages) of the Siriya Kalvarayan hills was done. The plants were photographed, and literature was explored to find the usefulness of these species in ancient medicinal literature to find if these species have also been used for Ayurveda and other traditional phytomedicinal streams - Siddha and naturopathy.

4.1. From "eat this root" to take this pill: A resurgence of "eat this root"

In the ages past, our ancestors used the adage, "eat this root" (Oleszek, 2002), as they pointed to specific plants or their parts, which had the potential to cure particular ailments. Then, advancements in medicine led humankind to identify, concoct and administer active principles in the form of pastes, syrups (gritham), powders (churnas), small tablets (gulikas), by taking plant parts (in proportions/ratios) which were prepared using specified methods to produce certain formulations. When administered, these substances were responsible for specific medicinal properties. *In silico* methods have broadened our understanding of how plant compounds interact with their receptors (Rollinger *et al*., 2006). With the aid of receptor-ligand docking and molecular simulation/dynamics tools, our understanding of traditional medicine has improved significantly when we find the receptor-drug interactions, which reveal deeper insights into how ancient medicine worked. One must only marvel at the fact that in ancient times when there was no knowledge about cells/tissues, receptor-drug interactions and even the knowledge that there were biomolecules, our ancestors had significant intuition as well as empirical knowledge of how to mix and match parts of plants, the proportions of the admixtures, mode of administration and the cognizance of pharmacological effects of these medicines on the whole body.

In modern times, Western medicine (referred to as allopathy in this work) has shown that each drug molecule/chemical exerts its effects through either pharmacological receptor-ligand binding (specificity), or through generic redox chemistry (either as pro-oxidant or antioxidant). Chemotherapy and pharmacological intervention using natural and synthetic molecules (as opposed to formulations containing hundreds of thousands of phytochemicals) became popular. Worldwide people have abandoned their traditional roots and embraced the western system of medicine; concomitantly, there also has been a steep rise in cases of accidental drug poisoning and dosage-related emergencies, especially among the elderly (Martins *et al*., 2015). Death due to poisoning is also one of the inevitable consequences, albeit in rare instances. Also, polymorphisms in the target genes (especially the liver microsomal CYP450s) among the populations have key implications in the pharmacokinetics and pharmacodynamics of drugs (Stingl and Viviani, 2015). While western medicine has saved countless lives, no treatment is risk-free or can be totally branded as "100% safe". Due to the high rate of drug-mediated damage to key organs, patients are turning to alternative healthcare systems, even for treatment of terminal illnesses such as cancer (Boekhout *et al*., 2016; Kessel *et al*., 2016). Isolated compounds are sometimes considered to be toxic and known to cause adverse effects in some people. Crude isolates of medicinal plants are deemed to have little or no toxicity in humans; to the contrary, isolated (pure) compounds are often presumed to exert toxicity (Chithambo *et al*., 2017). In traditional forms of medicine such as Ayurveda, certain principles were employed to discriminate between health and ill-health (disease) and drugs were prescribed to balance body humours.

Instead of just one drug molecule, mixtures of bioactive principles may have led to counterbalancing/synergistic effects. There is an urgent need to identify novel compounds/metabolites from plants - especially those which are from forest habitats. Forests are arguably nature's repertoires of medicinal compounds (Soejarto and Farnsworth, 1989), which may be used to treat illnesses that do not exhibit toxicity or cause organ dysfunction/failure that is associated with conventional chemotherapy.

Our age is characterized by the unprecedented rise of superbugs (multi- and extensively-drug resistant pathogens). Hence, the quest for finding bioactive molecules with bacteriostatic/bactericidal potential against clinically relevant pathogens (Lindsay and Holden, 2006) and diseases such as cancer, ulcer, obesity, cardiovascular disease and neurodegenerative diseases (and the entire spectrum of diseases, both communicable and non-communicable) are underway. To augment this search for newer classes of bioactive principles, we need to identify new plant genera (and species) and investigate their chemical constituents for bioactivity. Several poisonous plants are known to produce metabolites that bind to particular cellular targets and block different pathways that are responsible for the manifestation of the disease (Polya, 2003; Watt and Breyer-Brandwijk, 1962). In the recent century, unprecedented growth in ethnobotany has revolutionized our understanding of pharmacology and pharmacognosy. Through *in vitro*, *in situ*, *in vivo* and *in silico* approaches, we have researched the molecular level actions of bioactive compounds and realized, sometimes to our shock, how our ancestors understood medicine and physiology.

The Chipko movement (Shiva and Bandyopadhyay, 1986), which began in the 1970s, sparked several campaigns worldwide for forest conservation and aimed at preventing anthropogenic activities such as deforestation. In this movement, people embraced trees and prevented them from being felled by arguing that forests provide fuel, fodder, food, fibre and fertilizer. Anthropogenic activities have led to a steady decline in forest cover and have caused the destruction of endemic species, which are now rare, threatened or have gone entirely extinct (Bradshaw, 2004; Lugo and Waide, 1993). Therefore, biological conservation efforts have been proposed to save the destruction of endangered and rare species through *in vitro* propagation, and other plant tissue culture approaches. It is our earnest desire that some of the rare species that have been identified in the study area would be preserved from becoming endangered or altogether extinct through concerted efforts from Botanists who are interested in *in vitro* propagation (Sarasan *et al*., 2006).

4.2. Demographics of the Population

The villages at the foot of the Siriya Kalvarayan hills were surveyed, and mainly, those which are part of the hilly regions (confined to the study area), as shown in the map in Figure 3.1 were surveyed. The respondents answered various questions based on the questionnaire provided to them. Most of the residents who were interviewed were young (left panel, age category of 20-40). Secondly, most of them were male (centre panel), and the majority of the respondents, despite the popularity of allopathic medicine nowadays, stated that they either never visited an allopathic doctor or did so only very rarely during medical emergencies (right panel). These data are presented in (Figure 4.1). Most of the respondents stated that they had thus far never used Western medicine in their lifetime. This only reveals the population’s dependency on traditional medicines for their daily needs.

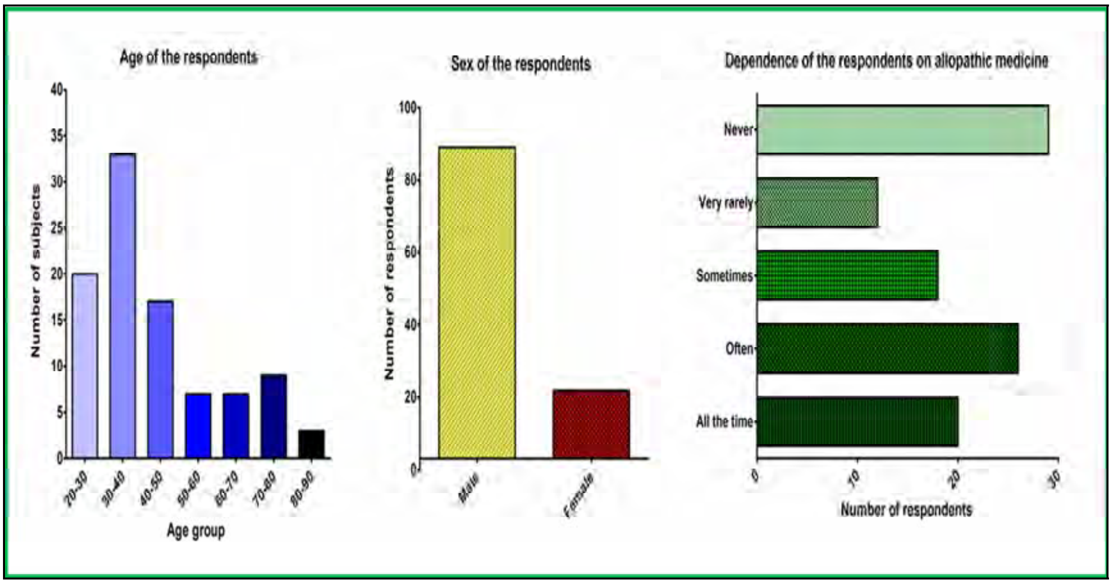

Figure 4.1. Overview of the respondents in the villages surveyed (Sirukkalur, Edapattu, Vazhakuli, Athikkuzhi, Vanjikkuli, Moolakkadu, Aanaimaduvu and Puliyankottai). **Left panel** - Age of the respondents; **Middle panel** - Sex of the respondents and **Right panel** - Dependence of the respondents on allopathic (Western) medicine.

4.3. Ethnic background of the tribal residents of Siriya Kalvarayan hills

Currently, the descendants of the Karlars (who call themselves Malayalis/Malailis) are the residents of the villages in the Periya and Siriya Kalvarayan hills (Wellman *et al*., 1997). The chief occupation of the residents are farming/agriculture, goat-herding and cattle rearing. In this survey of the residents for their reliance on traditional as well as western medicine, most of the respondents (total n = 105) exhibited strong belief in plant-based formulations and only residents of the villages which were located closer to the plain stated that they relied more on allopathy than on ethnic medicine.

4.4. Reliance of the respondents on Siddha, Ayurveda, Naturopathy and Allopathy

Information was gathered from 105 residents of 8 villages -Sirukkalur, Edapattu, Athikkuzhi, Vazhakuli, Vanjikkuli, Moolakadu, Aanaimaduvu, and Puliyankottai, which are either part of the Siriya Kalvarayan hills (the first six villages) - or, located in the vicinity of the hills (Moolakadu, Aanaimaduvu and Puliyankottai) using the aforementioned questionnaire. The number of residents of these villages who were interviewed was as follows: Sirukkalur - 16, Edapattu - 18, Athikkuzhi - 3, Vazhakuli - 2, Vanjikkuli - 30, Moolakadu - 6, Aanaimaduvu - 5 and Puliyankottai - 25. Among those who were interrogated, 86 were male, and 19 were female. The percentage of respondents in villages of the study area who follow different kinds of medicinal systems (Siddha, ayurveda, naturopathy, allopathy and combinations thereof) was assessed. The percentage of respondents (total n = 105) who came under each category is mentioned within the pertinent categories on the pie chart. These respondents were not the same as those who acted as informants (also n = 105). These data are presented in the middle and right panels of Figure 4.1.

The plant survey study led to the identification of 100 different species belonging to 46 families and 90 genera (Table 4.1 and Plates 4.1 - 4.17). The rare plants in the study (for which very little published material was found) were close to 61 species, which have not been studied hitherto (Table 4.2). Moreover, their phytochemical constituents are as yet unidentified/unknown. All the plants mentioned in Table 4.1 are numbered accordingly in the plate photographs (Plates 4.1 - 4.17), in which six plant photographs are presented per image for sake of clarity. Photoplate 17 alone contains four plant photographs.

Table 4.1. Identification of the different species and their medicinal uses (numbers given in this table correspond to the numbering for the photographs of the plants given in the photo plates, in Plate 4.1 - 4.17)

S. No.	Botanical name	Family name	Common name	Vernacular (Tamil) name	Part used	Medicinal uses
1	*Acalypha ciliata* Forssk.	Euphorbiaceae	-	-	Leaves and roots	Female sterility, antioxidant, antimicrobial, sores and Schistosomiasis
2	*Acalypha fruticosa* Forssk.	Euphorbiaceae	Birch leaved acalypha	Chinni chedi.	Leaves	Antioxidant, anti-inflammatory and antifeedant.
3	*Aganosma cymosa* (Roxb.) G.Don	Apocynaceae	-	Sellakkodi	Whole plant	Anthelmintic, emetic and bronchitis
4	*Agave sisalana* Perrine	Asparagaceae	Sisal hemp	Aanai Kathalai	Whole plant	Syphilis, antiseptic, jaundice, pulmonary tuberculosis, laxative, toothache, skin disease and lower blood pressure
5	*Allophylus cobbe* (L.) Raeusch.	Sapindaceae	Indian allophylus	Siruvalli	Whole plant	Diarrhoea, colic and bruises
6	*Anogeissus latifolia* (Roxb. ex DC.) Wall. ex Guill. & Perr.	Combretaceae	Axelwood	Vellainagai	Heart wood, exudates and bark	Wound healing, diarrhoea, bleeding piles, diabetes, scorpion bites, spider bites, skin disease and jaundice
7	*Andrographis echioides* (L.) Nees	Acanthaceae	False water willow	Gopuram thangi	Leaves and root	Hair fall, ringworm and muscular fitness
8	*Alangiumsalvii folium* (L.f.) Wangerin	Comaceae	Sage leaved alangium	Azhinjil	Leaves, fruits, bark and root	Herpes, rodent bites, dog bites, diabetes, epilepsy, pain disorder and inflammatory disease
9	*Aponogeton natans* (L.) Engl. & K.Krause	Aponogetonaceae	Floating lace plant	Kottikizhangu	Tuber and leaves	Wound healing and dandruff
10	*Atalantia monophylla* DC.	Rutaceae	Indian atalantia	Kattuelumichai	Fruits	Chronic rheumatism

S. No.	Botanical name	Family name	Common name	Vernacular (Tamil) name	Part used	Medicinal uses
11	*Azima tetracantha* Lam.	Salvadoraceae	Needle brush	Sugam cheddi	Leaves, root and milky juice	Rheumatism, toothache, dropsy and chronic diarrhoea
12	*Azadirachta indica* A. Juss.	Meliaceae	Margosa	Vembu	Bark, leaves and seeds	Wound healing, fever, asthma, sore throat, tuberculosis, jaundice, stomach ulcer, diabetes, rheumatisms, rashes, chickenpox, night blindness, ringworm and skin problems
13	*Barleria longiflora* L. f.	Acanthaceae	Long flowered barleria		Root, leaves, seeds and bark	Toothache, abscess, acid reflux and anaemia
14	*Bambusa arundinacea* Willd.	Poaceae	Bamboo	Moongil	Stem, leaves and root	Cough, wound, skin disease, nausea, digestive disorder and fever
15	*Borassus flabellifer* L.	Arecaceae	Palmyra palm	Panaimaram	Leaves, stem, male flower, root and fruit coat	Anti-oxidant, anti-inflammatory, cytotoxic and anti-diabetics
16	*Breynia vitis-idaea* (Burm.f.) C.E.C.Fisch.	Phyllanthaceae	Mountain coffee bush	-	Leaves	Postpartum remedy, boils, skin disease and bleeding
17	*Buchanania axillaris* (Desr.) Ramamoorthy	Anacardiaceae	Cuddapah almond	Mudama	Leaves	Diarrhoea, skin disease, cough, asthma and bleeding
18	*Buchanania latifolia* Roxb.	Anacardiaceae	Chirauli nut	Murala	Stem bark, nut and seed kernel	Anaemia, inflammation, oxidative stress, ulcer and diabetes
19	*Canthium coromandelicum* (Burm. f.) Alston	Rubiaceae	Coromandalcanthium	Karai	Leaves and fruits	Intestinal worms
20	*Capparis sepiaria* L.	Capparaceae	Wild caper bush	Karinthu	Fruit, bark, leaves and root	Fever, liver disorder and diarrhoea
21	*Carissa carandas* L.	Apocynaceae	Karanda	Kalakkai	Leaves, fruits, root and dried stem bark	Anaemia, acid reflux, anorexia and anxiety

S. No.	Botanical name	Family name	Common name	Vernacular (Tamil) name	Part used	Medicinal uses
22	*Cardiospermum halicacabum* L.	Sapindaceae	Balloon vein	Mudakathan	Whole plant	Constipation, cough, dyspnoea, rat bites, spider poisoning, diarrhoea and dandruff
23	*Caralluma umbellata* Haw.	Apocynaceae	Umbelled caralluma	Kallimuliyaan	Fleshy stem	Hyperglycaemia and wound healing
24	*Cassia fistula* L.	Caesalpiniaceae	Indian laburnum	Sarakkonrai	Bark and fruits	Inflammation, ulcer wounds, antiseptic and laxative
25	*Cassia occidentalis* L.	Caesalpiniaceae	Coffee senna	Nattamtakarai	Leaves and Flowers	Cough, cold, eczema and asthma
26	*Cipadessa baccifera* (Roth) Miq.	Meliaceae	Ranabili	Pulipanchedi	Leaves and root	Indigestion and cobra bites
27	*Cissus vitiginea* L.	Vitaceae	South Indian Treevine	Cembirantai	Leaves	Bone problems
28	*Chloris barbata* Sw.	Poaceae	Finger grass	Mayirkondaipul	Leaves	Rheumatism, fever, skin disease, diarrhoea and diabetes
29	*Chionanthus ramiflorus* Roxb.	Oleaceae	South indian olive	Perumsithudakki	Root, leaves and bark	Liver and gallbladder disorder
30	*Clausena dentata* (Willd.) Roem.	Rutaceae	Agbasa	Nanachedi	Leaves and root	Body ache, anorexia and burns
31	*Cleistanthus collinus* (Roxb.) Benth. ex Hook.f.	Euphorbiaceae	-	Oduvan	Leaves	Poisonous plant
32	*Clitoria ternatea* L.	Fabaceae	Butterfly pea	Sangu poo	Ieaves, flower, stem and root	Brain related health problems, chronic headache, digestive problems and respiratory problems
33	*Croton bonplandianus* Baill.	Euphorbiaceae	Ban tulsi	Rail poondu	Whole plant	Anti-tumour, Swelling, asthma and constipation
34	*Crinum asiaticum* L.	Amaryllidaceae	Poison bulb	Vishamoongil	Leaves, bulb and rhizome	Bloating, ascites and arthritis

S. No.	Botanical name	Family name	Common name	Vernacular (Tamil) name	Part used	Medicinal uses
35	*Cymbopogon citratus* (DC.) Stapf.	Poaceae	Lemongrass	Elumichaipul	Whole plant	Anti-oxidant, anti-inflammatory, digestive disorders, fever, menstrual disorders, ringworm and rheumatism
36	*Cynodon dactylon* (L.) Pers.	Poaceae	Bermuda grass	Arugam pul	Whole plant	Menstrual problems, acidity, diabetes, immunity, constipation and control obesity
37	*Dalbergia lanceolaria* L.f.	Fabaceae	Takoli	Kattupachilai	Seed and bark	Indigestion, skin disease, leprosy, rheumatism, arthritis, burns and constipation
38	*Dalbergia paniculata* Roxb.	Fabaceae	-	Pachchalanmaram	Leaf, bark and root	Bleeding piles, cough, diarrhoea, epigastria, epistaxis, gonorrhoea, leprosy, malaria, scabies, syphilis and ulcers
39	*Dioscorea oppositifolia* L.	Dioscoreaceae	-	Kavalakizhangu	Leaf, root and seed	Herbal tonic, asthma, diabetes, diarrhoea, uncontrolled urination, snake & scorpion bites and arthritis
40	*Dodonaea viscosa* Jacq.	Sapindaceae	Broad leaf hopbush	Virali	Leaves, seed, fruits, wood and bark	Bruises, aphthous and ulcers
41	*Ehretia anacua* (Teran & Berland.) I.M.Johnst.	Boraginaceae	Anacua	Kalvirasu	Unknown	Unknown
42	*Erythrina indica* Lam.	Fabaceae	Indian coral tree	Kalyanamurungai	Bark, root, leaves and Fruits	Fever, liver ailment, rheumatism, relieve joint pain, to kill tapeworm, roundworm, and threadworm
43	*Evolvulus alsinoides* (L.).	Convolvulaceae	Dwarf morning glory	Vishnukranthi	Leaves, root and stem	Fever, memory power, hair growth, reduce stress and wound healing

S. No.	Botanical name	Family name	Common name	Vernacular (Tamil) name	Part used	Medicinal uses
44	*Flacourtia indica* (Burm. f.) Merr.	Salicaceae	Indian plum	Cimaikottaikala	Leaves, root, bark	Snakebites, arthritis, cough, pneumonia, diarrhoea and throat infection
45	*Garuga pinnata* Roxb.	Burseraceae	Garuga	Karivembu	Leaves, bark, stem and fruits	Anti-bacterial, anti-cancer, anti-oxidant, anti-ulcer and wound healing activity
46	*Gloriosa superba* L.	Colchicaceae	Glory lily	Senkanthal	Rhizomes and stem	Rheumatisms and gout
47	*Grevillea robusta* A.Cunn. ex R.Br.	Proteaceae	Southern silky oak	Malaisavukku	Unknown	Unknown
48	*Grewia carpinifolia* Juss.	Tiliaceae	Bailleul	Panripputukkan	Leaves	Wounds and cuts
49	*Gymnema sylvestre* (Retz.) R. Br. ex Sm.	Apocynaceae	Cow plant	Sirukurinjan	Leaves	Diabetes, ulcer, reduce body heat, digestion problems, cough, heavy fever, liver problems and animal bites
50	*Gyrocarpus americanus* Jacq.	Hernandiaceae	Helicopter tree	Thanakku	Bark and root	Cancer, kidney pain, wounds, diarrhoea and scabies
51	*Haldina cordifolia* (Roxb.) Ridsdale	Rubiaceae	Haldu	Mannakkatambu	Root, the bark of stem and heartwood	Chronic cough, jaundice, stomach ache, diarrhoea and dysentery
52	*Helicteres isora* L.	Malvaceae	Indian screw tree	Valamburi	Root, stem, bark and fruits	Diarrhoea, dysentery, abdominal colic, intestinal parasites
53	*Hemionitis arifolia* (Burm. f.) T.Moore	Pteridaceae	Heart fern	Soikkosaniainen	Leaves	Anti-diabetes
54	*Hildegardia populifolia* Schott & Endl.	Malvaceae	-	Malaiarasu	Unknown	Unknown
55	*Hugonia mystax* L.	Linaceae	Climbing flax	Mothirakanni	Roots	Fever, viper bite, verminosis, anti-inflammation

S. No.	Botanical name	Family name	Common name	Vernacular (Tamil) name	Part used	Medicinal uses
56	*Hybanthus enneaspermus* (L.) F.Muell.	Violaceae	Spade flower	Orithalthamarai	Whole plant	Hypolipidaemic, anti-oxidant, anti-diabetes and anaemia
57	*Ichnocarpus frutescens* (L.) W.T.Aiton.	Apocynaceae	Black creeper	Udarkodi	Leaves and flower	Rheumatism, asthma, cholera, and fever
58	*Ixora pavetta* Andr.	Rubiaceae	Torchwood tree	Sulunthu.	Flower and bark	Whooping cough, anaemia and general weakness
59	*Jasminum angustifolium* (L.) Willd.	Oleaceae	Wild jasmine	Kuruvilankodi	Root and leaves	Skin disease, ulcer and eye disease
60	*Jasminum multipartitum* Hochst.	Oleaceae	Starry wild jasmine	Kattumalli	Unknown	Unknown
61	*Jatropha curcas* L.	Euphorbiaceae	Physic nut	Kattukattai	Leaves and seeds oil	Cholera, diarrhoea, skin disease and gingivitis
62	*Justicia betonica* L.	Acanthaceae	Squirrel tail	Velimoongil	Root, leaves and flower	Constipation, malaria, snake bites, vomiting, stomach ache and swelling
63	*Justicia glauca* Rottler	Acanthaceae	Gulf sandmat	Thavasimurungai	Leaves	Backache
64	*Kleinia grandiflora* (Wallich ex DC.) N.Rani	Asteraceae	Large-flower kleinia	Attukaalchedi	Root, stem, flower, leaves and fruits	Pimples and hydrophobia
65	*Lantana camara* L.	Verbenaceae	Lantana	Unnichedi	Leaves, root and flowers	Asthma, ulcers, cancers, leprosy, skin itches, rabies and chickenpox
66	*Leucas aspera* (Willd.) Link	Lamiaceae	Common leucas	Thumbai	Whole plants	Scorpion bites, insect bites, jaundice, liver disease and fever
67	*Loeseneriella obtusifolia* (Roxb.) A.C.Sm.	Celastraceae	-	Menthakkodi	Unknown	Unknown
68	*Mangifera indica* L.	Anacardiaceae	Mango tree	Maamaram	Bark, seed, kernel, flower. leaves and fruits	Digestion, skin disorders, bleeding disorder, chronic fever, eye disorder, constipation and bloating

S. No.	Botanical name	Family name	Common name	Vernacular (Tamil) name	Part used	Medicinal uses
69	*Melia azedarach* L.	Meliaceae	China berry	Malaivembu	Leaves, fruits and bark	Anti-malarial and skin disease
70	*Memecylon umbellatum* Burm.f.	Melastomataceae	Ironwood	Anjani	Leaf and root	Antimicrobial, antipyretic, anti-diabetic and anti-obesity
71	*Mimosa hamata* Willd.	Fabaceae	Hooked mimosa	Indiri	seed, stem, root and leaves	Diarrhoea, jaundice, dysentery, wounds, piles and blood purifier
72	*Oldenlandia umbellata* L.	Rubiaceae	Chay root	Saya	Root and leaves	External bleeding, snake bites, heavy menstrual bleeding and bronchitis
73	*Olax scandens* Roxb.	Olacaceae	Parrot olax	Kataliranci	Leaves and bark	Anaemia, constipation, diabetes and fever
74	*Opilia amentacea* Roxb.	Opiliaceae	Fragrant opilia	-	Root, leaves and bark	Fever, headache, Stomach problem, cough, toothache and malaria
75	*Orthosiphon thymiflorus* (Roth.) Sleesen	Lamiaceae	-	Cilannipattum	Whole leaves	Anti-inflammatory, diabetes, kidney stone, hepatitis, hypertensive, jaundice, oedema and leaf juice used by tribes as a lotion
76	*Pavetta indica* L.	Rubiaceae	White pavetta	Kattukkaranai	Root, bark and leaves	Relieving the pain of piles and haemorrhoids
77	*Pavonia zeylanica* (L.) Cav.	Malvaceae	Ceylon swamp mallow	Sevagan	Root and leaves	Haemorrhage, dysentery and inflammation
78	*Phyllanthus lawii* J. Graham	Phyllanthaceae	Laws gooseberry	-	Unknown	Unknown
79	*Psydrax dicoccos* Gaertn.	Rubiaceae	Ceylon boxwood	Nanjul	Unknown	Unknown
80	*Plecospermum spinosum* Trecul	Moraceae	-	-	Latex	Toothache

S. No.	Botanical name	Family name	Common name	Vernacular (Tamil) name	Part used	Medicinal uses
81	*Premna tomentosa* Willd.	Lamiaceae	Woolly leaved firebrand Teak	Cummotakam	Root and leaves	Anaemia, diabetes, rabies, liver disease, stomach ache and diarrhoea
82	*Pterocarpus marsupium* Roxb.	Fabaceae	Indian kino tree	Vengai	Heartwood, leaves and flower	Diabetes, inflammation and bleeding
83	*Reissantia indica* (Willd.) N.Halle	Celastraceae	Mopane Paddle-Pod	Odangod	Root bark, stem and leaves	Respiratory troubles, febrifuge, sores and wounds
84	*Sapindus emarginatus* Vahl	Sapindaceae	Soapnut	Boonthikottai	Fruit	Asthma, colic and dysentery
85	*Scutia myrtina* (Burm.f.) Kurz	Rhamnaceae	Cat-thorn	Sodali chedi	Root, bark, leaves and fruits	Fever, malaria, bilharzia, intestinal worms, ointment to hasten childbirth
86	*Sterculia urens* Roxb.	Malvaceae	Gum karaya	Kavalam	Tree gum	Laxative
87	*Symplocos cochinchinensis* (Lour.) S.Moore	Symplocaceae	Laurel sapphire berry	Kambalivettai	Bark and stem	Biliousness, haemorrhages, diarrhoea, gonorrhoea and eye disease
88	*Senna hirsuta* (L.) H.S.Irwin & Barneby	Caesalpiniaceae	Woolly cassia	Malaiyavaram poo	Leaves and roots	Kidney disorders, herpes, skin disease, and cracked nipples
89	*Tamarindus indica* L.	Fabaceae	Tamarind tree	Malaipuliyamaram	Leaves, fruits, bark, root and seeds	Scurvy, common cold, fever, dysentery, burns and sore throats
90	*Tarenna asiatica* (L.) Kuntze ex K.Schum.	Rubiaceae	Asiatic tarenna	Tharani	Leaf-bud, leaves	Antibacterial, antiviral, antioxidant, wound healing and cytotoxicity
91	*Terminalia arjuna* (Roxb. ex DC.) Wight & Arn.	Combretaceae	Arjun tree	Neermaruthu	Bark and leaves	Reliving heart disease, fever, stop bleeding, kidney stone, wound, asthma diarrhoea and dysentery
92	*Terminalia bellirica* (Gaertn.) Roxb.	Combretaceae	Behda	Thandrikai	Bark, fruit, seed, whole plant	Anaemia, hoarseness, weak eyesight and abdominal disease

S. No.	Botanical name	Family name	Common name	Vernacular (Tamil) name	Part used	Medicinal uses
93	*Terminalia chebula* Retz.	Combretaceae	Chebulic myrobalan	Kadukkai	Fruits, bark, leaves	Digestive disorder, irregular fever, ulcer, colic, haemorrhoids, ascites, piles, worms, colitis and food poisoning
94	*Terminalia paniculata* Roth	Combretaceae	Ada maruthu	Poo maruthu	Bark	Fever, inflammation, wound healing and bone fracture
95	*Tridax procumbens* (L.) L.	Asteraceae	Coat buttons	Vettukayapoondu	Stem and leaves	Wound healing and skin disease
96	*Tylophora asthmatica* (L.f.) Wight & Arn.	Asclepiadaceae	Indian ipecac	Nayppalai	Whole plants	Asthma, bronchitis and cough
97	*Vitex negundo* L.	Lamiaceae	Chaste tree	Nocchi	Dried leaf, root, fruits, flower and seed	Muscle relaxant, pain-relieving, anti-asthma, amnesia and eye diseases
98	*Walsura trifoliata* (A.Juss.) Harms	Meliaceae	Tree leaf walsura	Kanjimaram	Bark	Stimulant, expectorant, emmenagogue, emetic and also used to kill vermin in the hair
99	*Ziziphus oenoplia* (L.) Mill.	Rhamnaceae	Jackal jujube	Sooraimullu	Root, stem and leaves	Anaemia, diarrhoea, bronchitis, stomach ache and wounds
100	*Ziziphus xylopyrus* (Retz.) Willd.	Rhamnaceae	Katber, kottaielandai.	Unknown	Fruit, bark, seed and root	Diabetes, diarrhoea, urinary disorders, digestive disorders and bladder stone

Table 4.2. Families, genera and species identified in the study - The names of various families, genera, species and the plant types (based on morphology)

Name of the family	No. of genus/genera	No. of species	Herb	Shrub	Climber	Lianas	Succulents	Tree	CPs	RPs	Total no. of plants
Acanthaceae	3	4	4	-	-	-	-	-	4	-	4
Apocynaceae	5	5	-	1	3	-	1	-	1`	4	5
Aponogetonaceae	1	1	1	-	-	-	-	-	-	1	1
Amaryllidaceae	1	1	1	-	-	-	-	-	-	1	1
Anacardiaceae	2	3	-	-	-	-	-	3	1	2	3
Arecaceae	1	1	-	-	-	-	-	1	1	-	1
Asclepiadaceae	1	1	-	-	1	-	-	-	-	1	1
Asparagaceae	1	1	-	-	-	-	1	-	1	-	1
Asteraceae	2	2	2	-	-	-	-	-	1	1	2
Boraginaceae	1	1	-	-	-	-	-	1	-	1	1
Burseraceae	1	1	-	-	-	-	-	1	-	1	1
Caesalpiniaceae	2	3	2	-	-	-	-	1	2	1	3
Capparaceae	1	1	-	-	-	-	-	1	-	1	1
Celastraceae	2	2	-	-	2	-	-	-	-	2	2
Colchicaceae	1	1	1	-	-	-	-	-	1	-	1
Comaceae	1	1	-	-	-	-	-	1	-	1	1
Combretaceae	2	5	-	-	-	-	-	5	1	4	5
Convolvulaceae	1	1	1	-	-	-	-	-	1	-	1
Dioscoreaceae	1	1	1	-	-	-	-	-	-	1	1
Euphorbiaceae	4	5	3	1	-	-	-	1	2	3	5
Fabaceae	6	6	-	-	1	1	-	5	4	3	7
Hernandiaceae	1	1	-	-	-	-	-	1	-	1	1
Lamiaceae	4	4	2	1	-	-	-	1	2	2	4
Linaceae	1	1	-	-	-	1	-	-	-	1	1

Name of the family	No. of genus/genera	No. of species	Herb	Shrub	Climber	Lianas	Succulents	Tree	CPs	RPs	Total no. of plants
Malvaceae	4	4	1	1	-	-	-	2	1	3	4
Meliaceae	4	4	-	1			-	3	2	2	4
Melastomataceae	1	1	-	1	-	-	-	-	-	1	1
Moraceae	1	1	-	-	-	1	-	-	-	1	1
Olacaceae	1	1	-	1	-	-	-	-	-	1	1
Oleaceae	2	3	-	1	2	-	-	-	-	3	3
Opiliaceae	1	1	-	-	1	-	-	-	-	1	1
Phyllanthaceae	2	2	-	1	-	-	-	1	-	2	2
Poaceae	4	4	4	-	-	-	-	-	3	1	4
Proteaceae	1	1	-	-	-	-	-	1	-	1	1
Pteridaceae	1	1	1	-	-	-	-	-	-	1	1
Rhamnaceae	2	3	-	-	1	-	-	2	1	2	3
Rubiaceae	7	7	1	2	-	-	-	4	4	3	7
Rutaceae	2	2	-	1	-	-	-	1	1	1	2
Salicaceae	1	1	-	-	-	-	-	1	-	1	1
Salvadoraceae	1	1	-	1	-	-	-	-	-	1	1
Sapindaceae	4	4	1	1	1	-	-	1	2	2	4
Symplocaceae	1	1	-	-	-	-	-	1	-	1	1
Tiliaceae	1	1	-	-	-	-	-	1	1	-	1
Verbinaceae	1	1	-	1	-	-	-	-	1	-	1
Violaceae	1	1	1	-	-	-	-	-	1	-	1
Vitaceae	1	1	1	-	-	-	-	-	-	1	1
46	**90**	**99**	**28**	**15**	**12**	**3**	**2**	**40**	**39**	**61**	**100**

CPs - Common plants; RPs - Rare plants

Photoplates: Floristic Study in Siriya Kalvarayan Hills

Plate 4.1. Photographs of plants 1-6 presented in Table 4.1

Plate 4.2. Photographs of plants 7-12 presented in Table 4.1

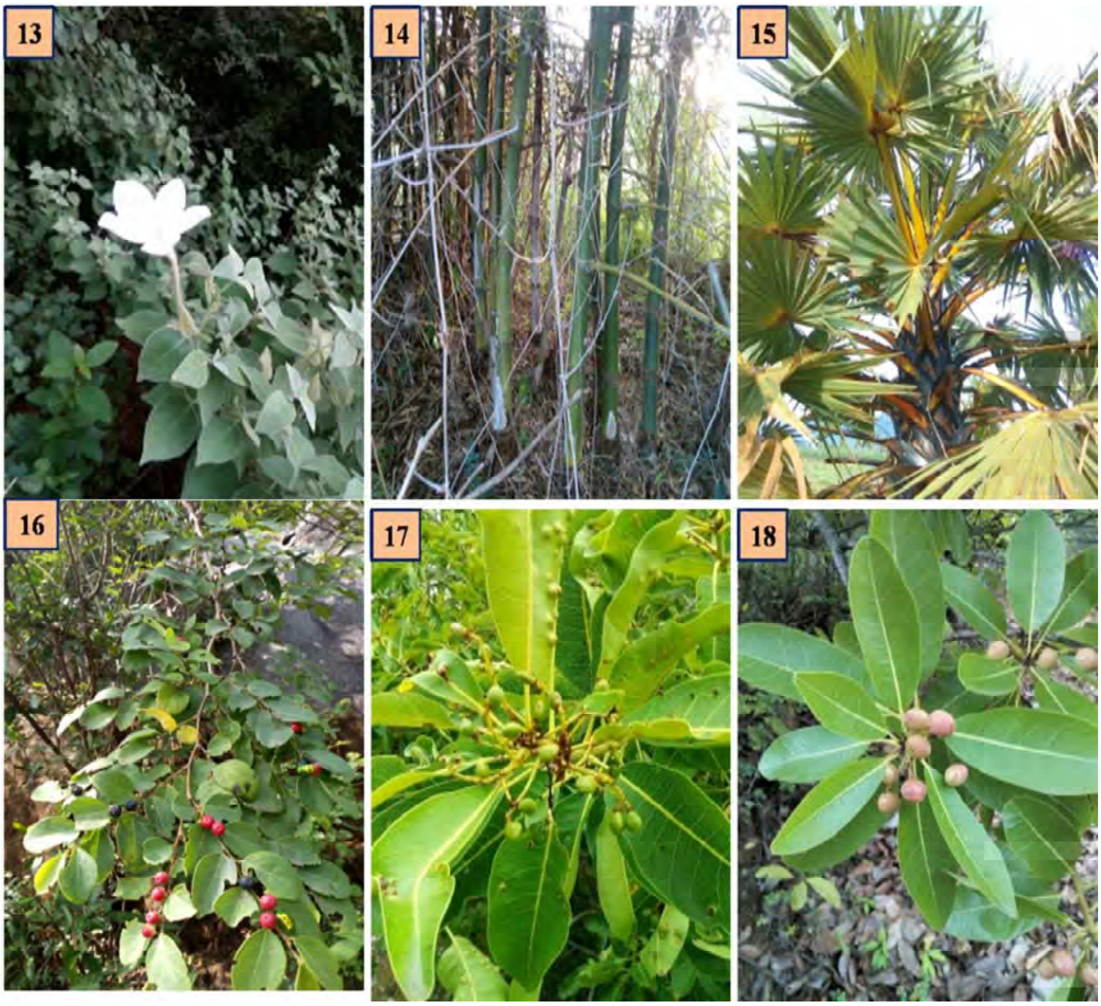

Plate 4.3. Photographs of plants 13-18 presented in Table 4.1

Plate 4.4. Photographs of plants 19-24 presented in Table 4.1

Plate 4.5. Photographs of plants 25-30 presented in Table 4.1

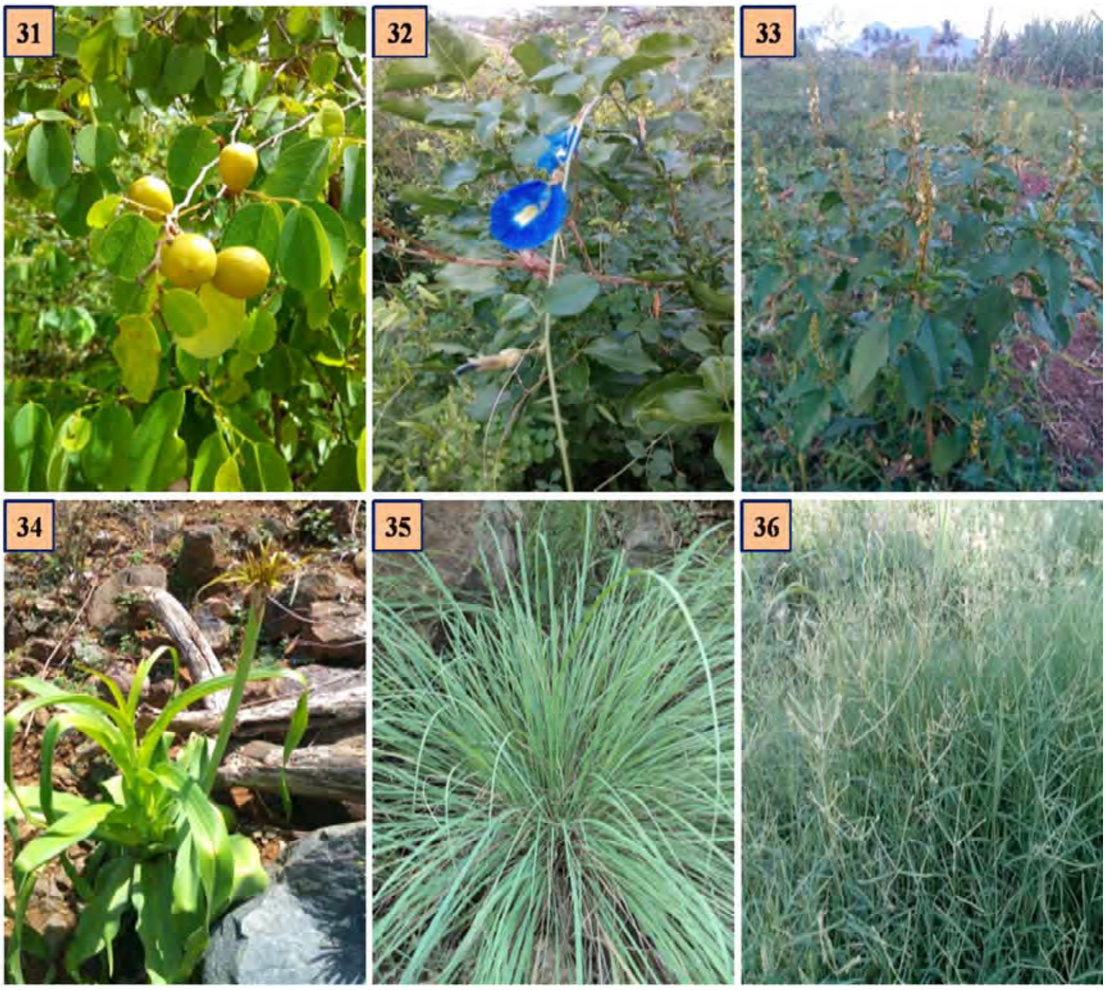

Plate 4.6. Photographs of plants 31-36 presented in Table 4.1

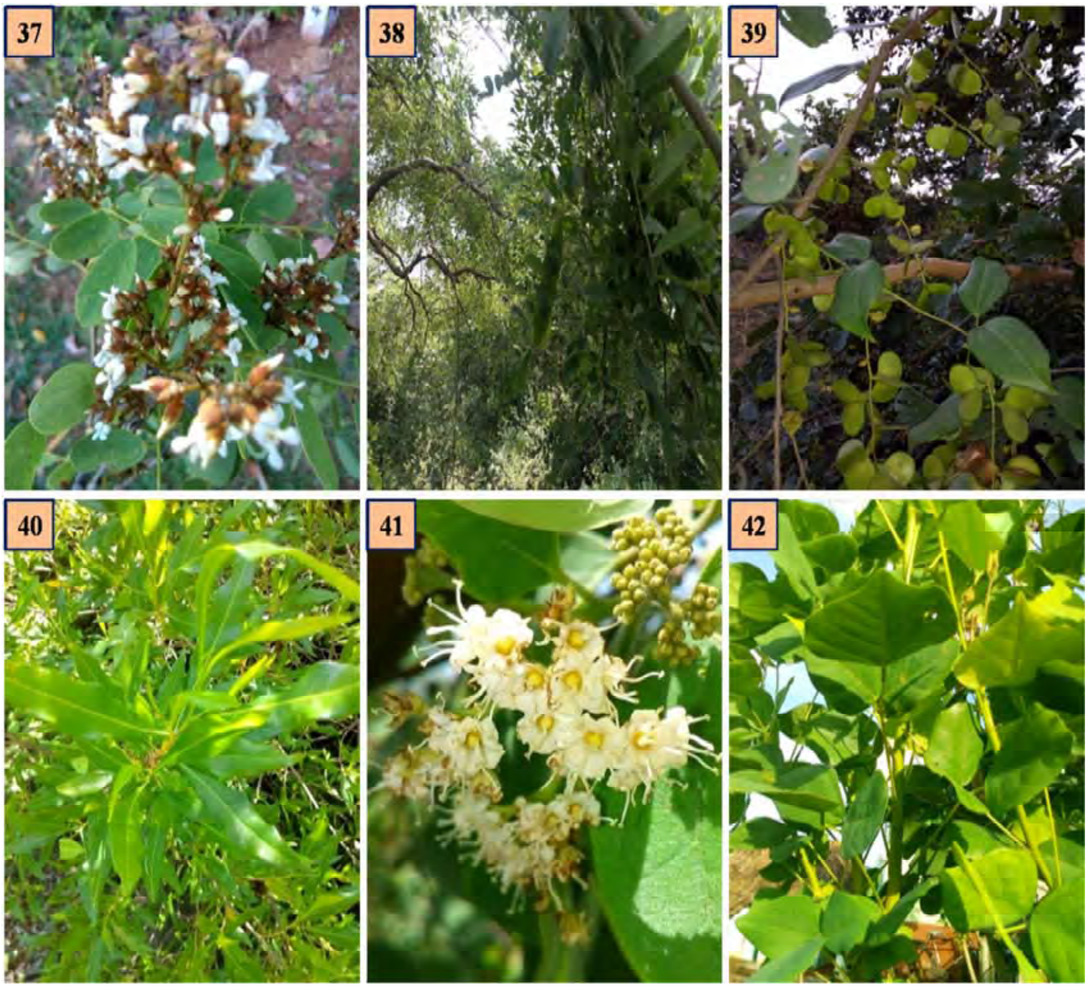

Plate 4.7. Photographs of plants 37-42 presented in Table 4.1

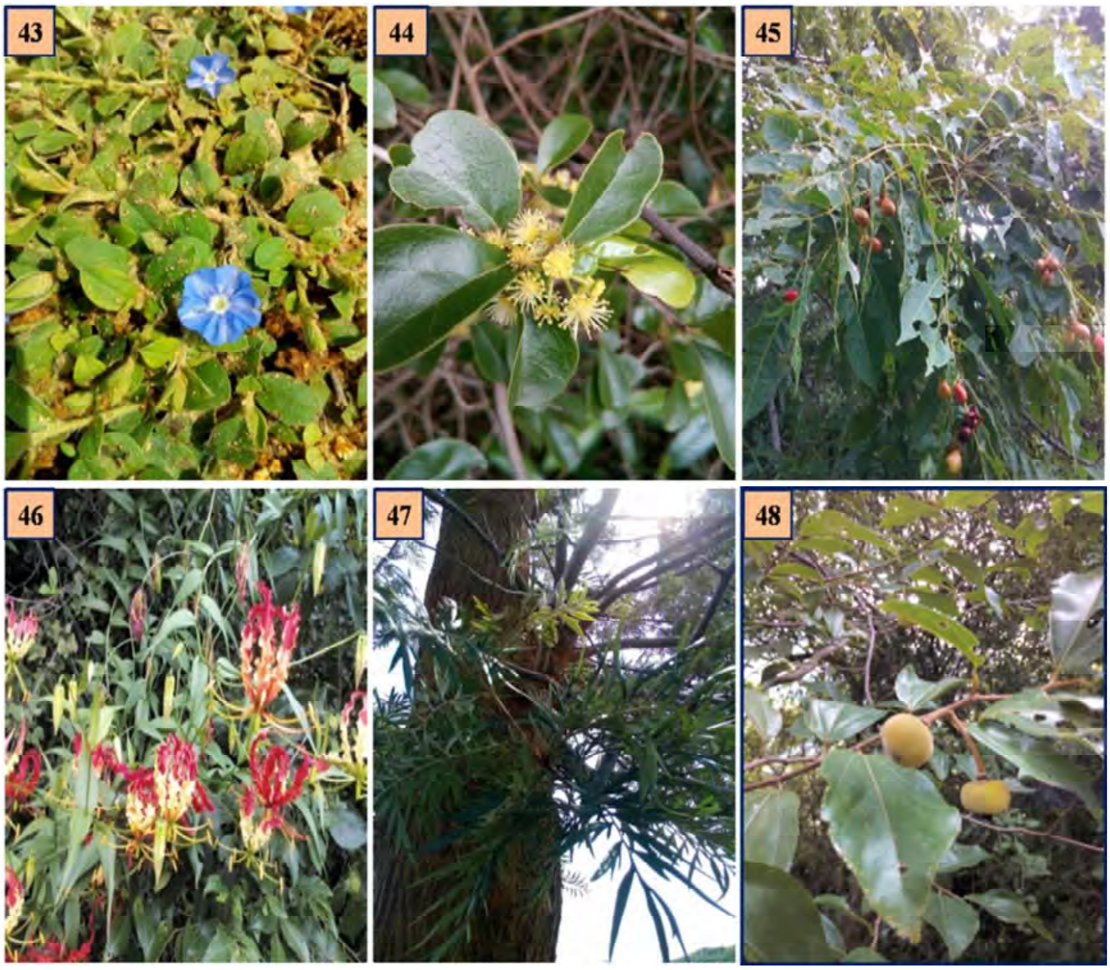

Plate 4.8. Photographs of plants 43-48 presented in Table 4.1

Plate 4.9. Photographs of plants 49-54 presented in Table 4.1

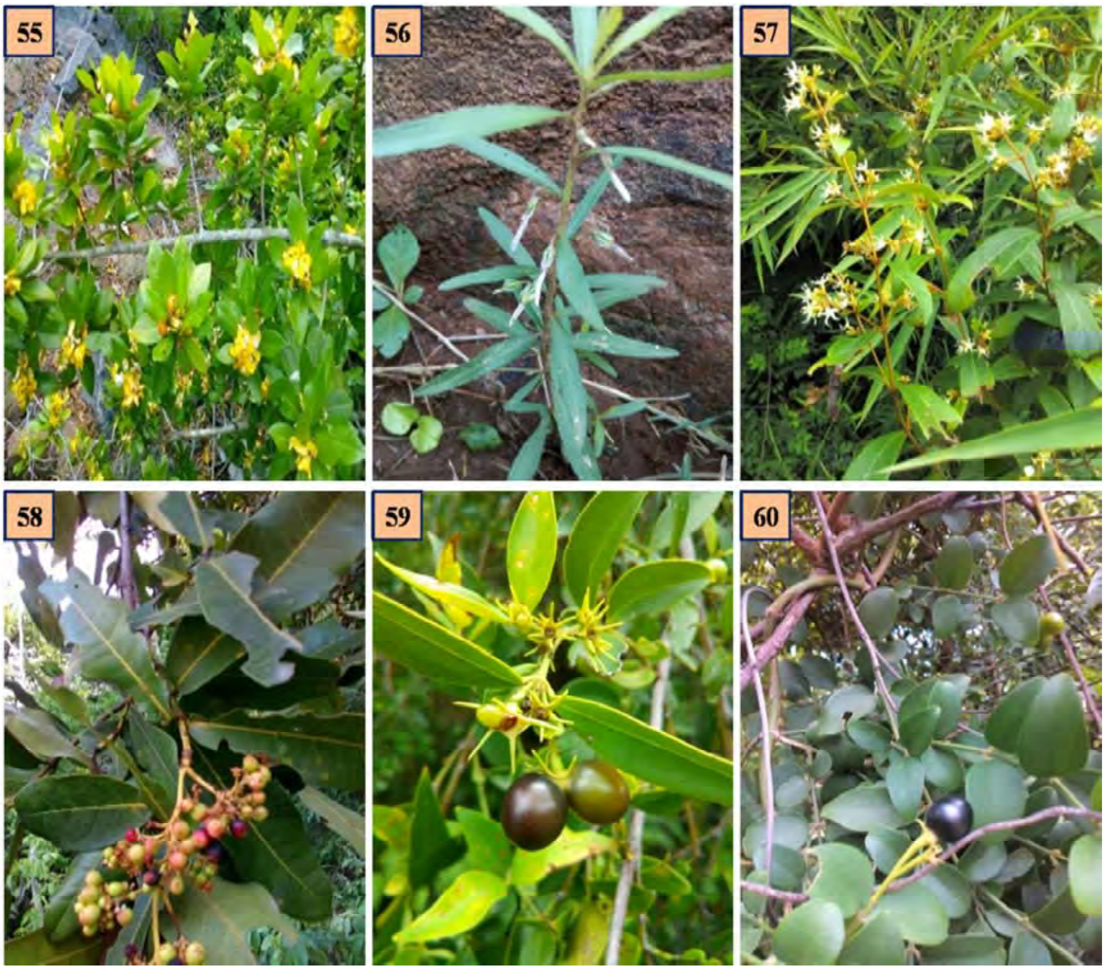

Plate 4.10. Photographs of plants 55-60 presented in Table 4.1

Plate 4.11. Photographs of plants 61-66 presented in Table 4.1

Plate 4.12. Photographs of plants 67-72 presented in Table 4.1

Plate 4.13. Photographs of plants 73-78 presented in Table 4.1

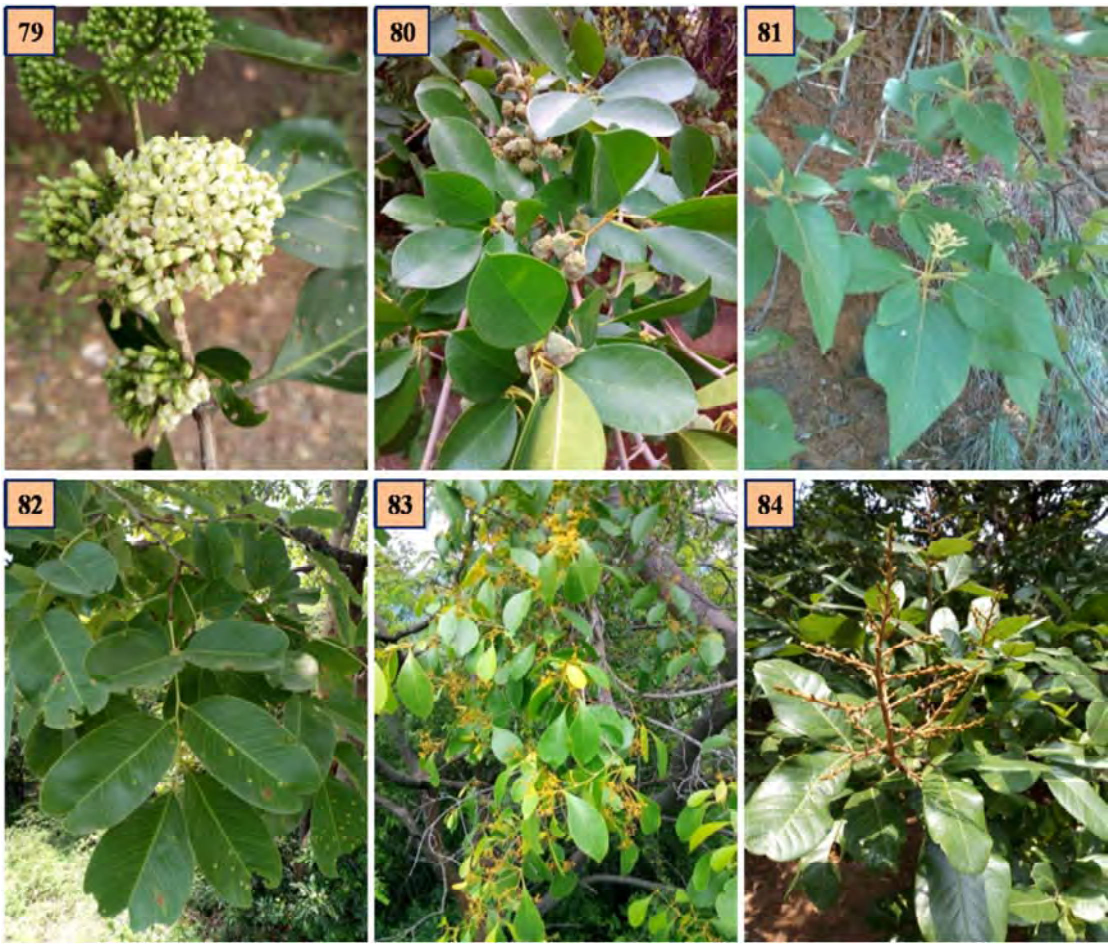

Plate 4.14. Photographs of plants 79-84 presented in Table 4.1

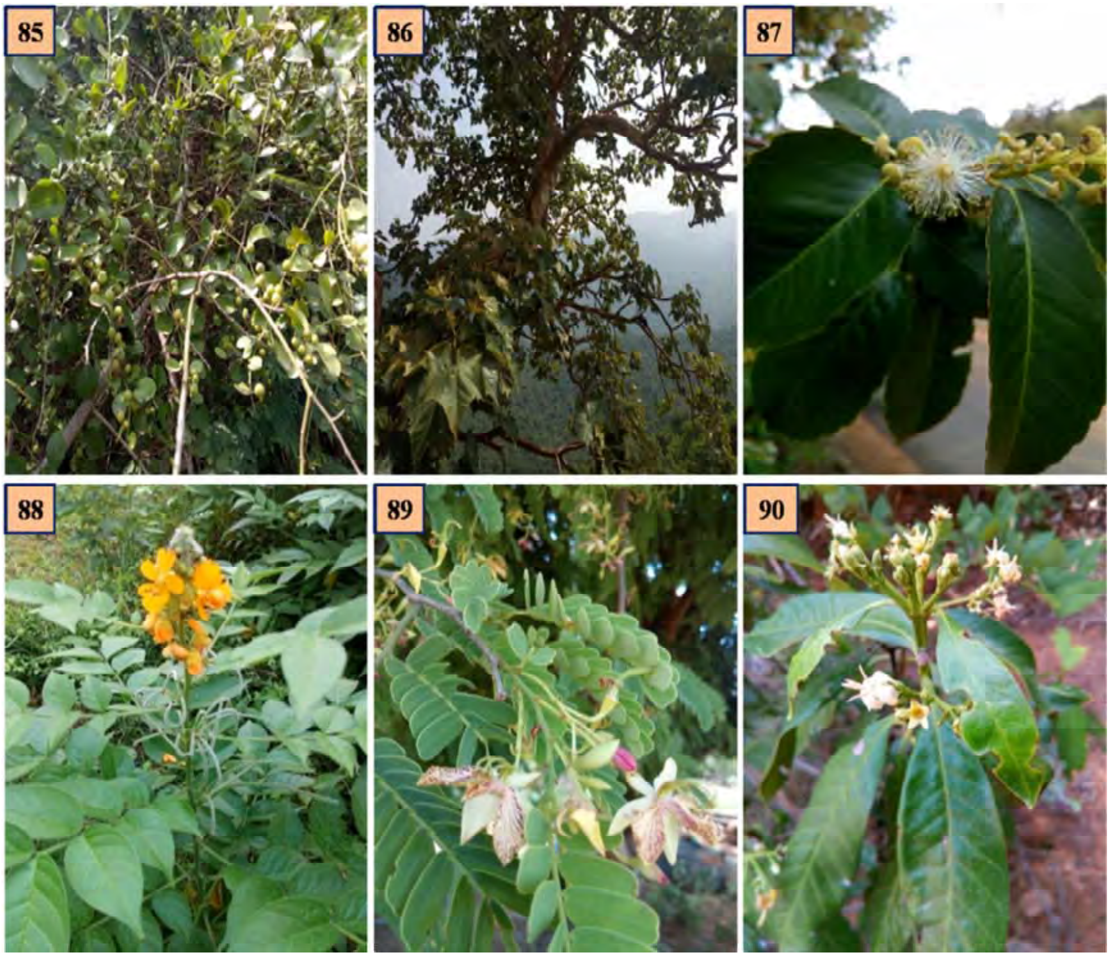

Plate 4.15. Photographs of plants 85-90 presented in Table 4.1

Plate 4.16. Photographs of plants 91-96 presented in Table 4.1

Plate 4.17. Photographs of plants 97-100 presented in Table 4.1

The total number of families (which the 100 species belonged to) covered in the survey were 46. There were 28 herbs, 15 shrubs, 12 climbers, 3 lianas, 2 succulents and 40 trees. The distribution of the various genera, species and types of flora (based on morphological characteristics) is given in (Figure 4.2 and Table 4.2). While searching the IUCN red list, we found that 18 plants were found to be of least concern (Table 4.5), while 2 were vulnerable [*Cleistanthus collinus* (Roxb.) Benth. ex Hook.f. and *Psydrax dicoccos* Gaertn.], 1 was near threatened (*Pterocarpus marsupium* Roxb.) and "data was deficient" for 1 plant (*Mangifera indica* L.). One plant was critically endangered [*Hildegardia populifolia* (Roxb.) Schott & Endl.].

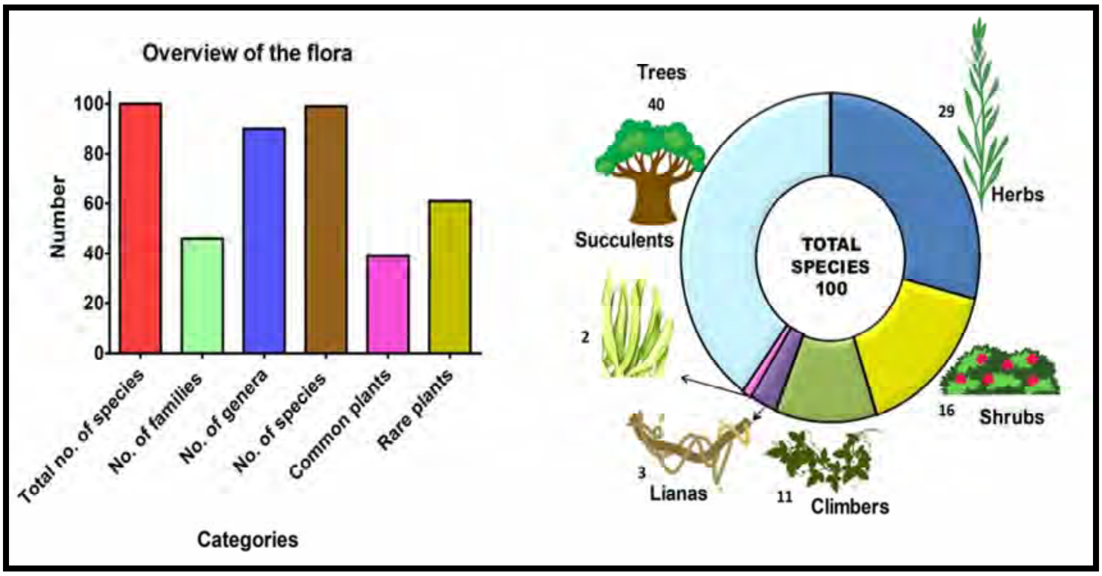

Figure 4.2. Flora of the Siriya Kalvarayan hills

4.5. Questionnaire and response of the Surveyed Residents

Most of the village respondents were young (below age 30), and some were > 70 years old. This data is presented in Figure 4.1, panels A and B. Their preferred medicine system was also enquired (Ayurveda, Siddha, Unani, homoeopathy, allopathy and naturopathy), and most of the residents of the villages in the hilly areas stated that they depended primarily on plant-based medicine (ethnic medicine, esp. Siddha and Ayurveda) and seldom opted for any allopathic medicine. Some of them were > 70 years old, and most of them stated that they had never been to an allopathic doctor (Figure 4.1, right panel). In Figure 4.3, results for the query regarding their choice of medicine system is shown, and it was found that 2.8% of the residents strictly followed Siddha medicine, while 28.5% utilized both naturopathy and Siddha medicine, 20% of the interviewed respondents strictly took allopathic medicine, while 26.6% took both siddha and allopathic medicine. Only some of residents (~22%) embraced three different forms of medicine-siddha, naturopathy and allopathy. As we went down the hill, we noticed that the residents gravitated towards modern forms of medicine such as allopathy and did not exhibit much belief in traditional medicine. But, those living on the hill were primarily dependent on conventional medicine.

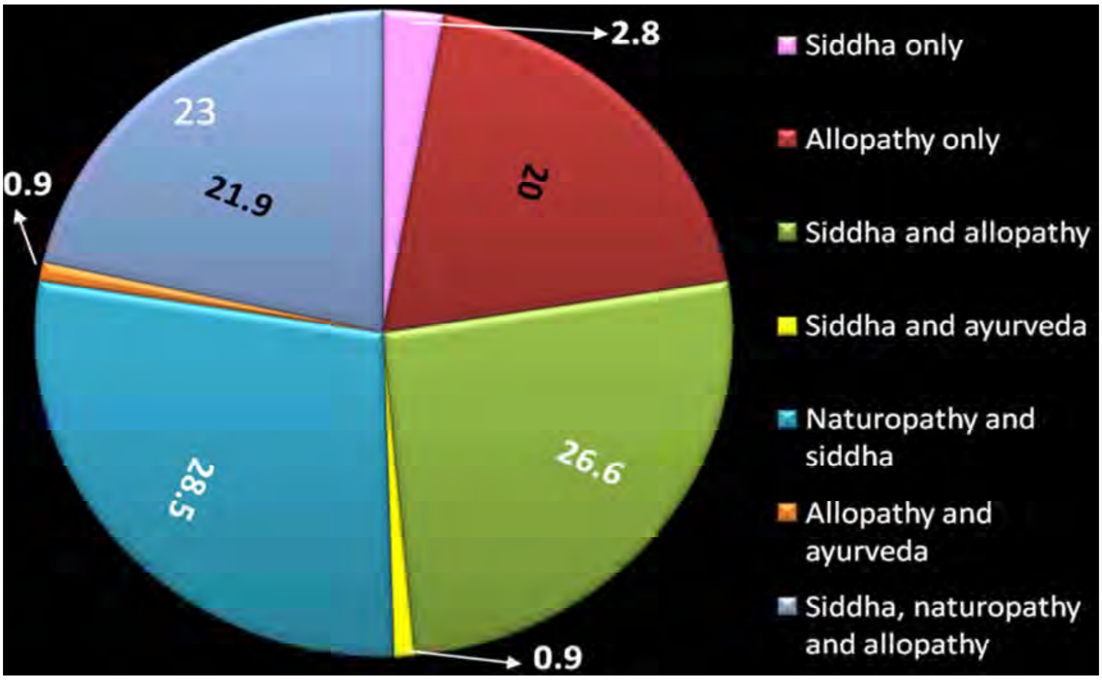

Figure 4.3. Medicine systems followed by residents of the study area

The ethnopharmacological survey revealed that among the 100 plants surveyed, the local/ethnic uses of 52 plants were identified from the population (Table 4.3). 32 unique diseases/ailments were identified as health problems - for which the residents used some of the 100 surveyed species as medicine. Quantitative indices such as ICF, FL and UV were analyzed. FL and UV values for each of the plants with known ethnic medicinal usages (confined to the study area alone) are shown in Table 4.3 and the ICF values are presented in (Table 4.4).

Table 4.3. Ethnopharmacological uses of some species identified by the local population (n = 105)

Botanical name	Local name	Part used	Ethnopharmacological uses	Fidelity level (FL%)	Use value (UV)
Acalypha fruticosa Forssk.	Oosi chedi	Leaf and root	Skin disease	21.90	0.21
Agave sisalana Perrine	Malaikathalai	Leaf	Jaundice	15.23	0.15
Anogeissus latifolia (Roxb. ex DC.) Wall. ex Guill. & Perr.	Naga maram	Leaf	Wound healing	7.61	0.07
Andrographis echioides (L.) Nees	Kopuramthangi	Leaf	Body pain	39.04	0.39
Alangium salviifolium (L.f.) Wangerin	Azhinjil	Leaf	Animal bites and ritualistic use	19.04	0.19
Atalantia monophylla DC.	Kuruthan	Leaf and root	Swelling	20	0.2
Azima tetracantha Lam.	Sokkamul	Leaf	Rheumatism	9.52	0.09
Azadirachta indica A. Juss.	Veppamaram	Leaf and fruit	Leaf - mumps, anthelminthic and antimicrobial, Fruit – edible	44.76	0.17
Bambusa arundinacea Willd.	Moongil	Seed (bamboo rice)	Diabetes	24.76	0.24
Breynia vitis-idaea (Burm.f.) C.E.C.Fisch.	Sirumani chedi	Fruit	Edible	-	-
Canthium coromandelicum (Burm. f.) Alston	Karakkai	Fruit	Edible	-	-
Carissa carandas L.	Kalakai	Fruit	Pickle	-	-
Cardiospermum halicacabum L.	Mudakuthankeerai	Fruit	Hair fall and reduce body heat	37.14	0.37
Cassia fistula L.	Konnaimaram	root	Snake bites	30.47	0.30
Cassia occidetalis L.	Peiavarai	Leaf	Cough and cold	26.66	0.26
Cipades sabaccifera (Roth) Miq.	Thamatan chedi	Leaf	Stomach pain	22.85	0.22
Cissus vitiginea L.	Cembiratai	Leaf	Bone health	11.42	0.11

Botanical name	Local name	Part used	Ethnopharmacological uses	Fidelity level (FL%)	Use value (UV)
Chloris barbata Sw.	Mayirkondaipul	Whole plant	Skin disease	40	0.11
Clausena dentata (Willd.) Roem.	Aana chedi	Fruit	Body heat	40	0.4
Cleistanthus collinus (Roxb.) Benth. ex Hook.f.	Ottanmaram	Whole plant	Poisonous; used as roofing material to protect against insects	-	-
Clitoria ternatea L.	Sangu poo kodi	Leaf	Laxative	19.04	0.19
Croton bonplandianus Baill.	Rail poondu	Leaf and root	Skin disease and jaundice	29.52	0.29
Cymbopogon citratus (DC.) Stapf.	Manjampul	Leaf	Fever	28.57	0.28
Cynodon dactylon (L.) Pers.	Arugam pul	Leaf	Laxative	40	0.4
Dodonaea viscosa Jacq.	Velleri chedi	Leaf	Throat infection	28.57	0.34
Ehretia anacua (Teran & Berland.) I.M.Johnst.	Naruni	Fruit	Edible	-	-
Erythrina indica Lam.	Kalliyanamurungai	Leaf	Fever and rheumatism	20	0.2
Evolvulus alsinoides (L.).	Echithamarai	Leaf	Wound healing	40	0.4
Gloriosa superba L.	Senkanthal	Tuber	Ulcer	22.85	0.22
Gymnema sylvestre (Retz.) R. Br. ex Sm.	Aattukodi	Leaf	Diabetes	32.38	0.32
Helicteres isora L.	Valamburi kai	Dry fruit	Digestive problems and gas trouble	24.76	0.24
Ixora pavetta Andr.	Sulunthimaram	Dry fruit	Cough and generalized weakness	18.09	0.18
Justicia betonica L.	Velichedi	Leaf and root	Constipation and stomach pain	15.23	0.15
Justicia glauca Rottler	Thavasikeerai	Leaf	Fever and back pain	15.23	0.15
Lantana camara L.	Urumpuli chedi	Fruit	Edible	-	-
Leucas aspera (Willd.) Link	Thumbai chedi	Leaf and	Cold and laxative	22.85	0.60

Botanical name	Local name	Part used	Ethnopharmacological uses	Fidelity level (FL%)	Use value (UV)
		flower			
Mangifera indica L.	Maangamaram	Leaf and fruit	Edible	-	-
Melia azedarach L.	Malaivembu	Leaf	Skin disease	25.71	0.60
Mimosa hamata Willd.	Seengaimul	Leaf	Stomach pain	26.66	-
Oldenlandia umbellata L.	Mookuthi chedi	leaf	External bleeding	7.61	0.25
Pavetta indica L.	Kattukaranai	Leaf and root	Analgesic for piles	16.19	0.26
Pavonia zeylanica (L.) Cav.	Seevagai chedi	Leaf	Inflammation	25.71	0.07
Premna tomentosa Willd.	Cumatamaram	Leaf	Stomach pain	6.66	0.16
Sapindus emarginatus Vahl	Sopukai	Dry fruit	Used like soap	26.66	0.25
Scutia myrtina (Burm.f.) Kurz	Sodali chedi	Leaf and fruit	Leaf – fever, fruit – edible	12.38	0.06
Tamarindus indica L.	Puliyamaram	Leaf, fruit and seed	Leaf – body pain, fruit – edible, seed – fracture	24.76	0.26
Terminalia bellirica (Gaertn.) Roxb.	Thandi maram	Seed	Piles and making soap	20.95	0.12
Terminalia chebula Retz.	Kadukaimaram	Seed	Diabetes and piles	18.09	0.24
Tridax procumbens (L.).	Vettukayapoondu	Leaf	Wound healing	14.28	0.20
Tylophora asthmatica (L.f.) Wight & Arn.	Kaattukodi	Leaf	Asthma and cough	28.57	0.18
Vitex negundo L.	Nochi	Leaf	Headache, cold and cough	32.38	0.14
Ziziphus oenoplia (L.) Mill.	Soorachedi	Fruit	Edible	-	-

Table 4.4. Informant Consensus Factor (ICF) values

Ailment	Nur	Nt	Nur-Nt/Nur-1
Skin disease	50	4	0.93
Jaundice	34	2	0.96
Wound healing	65	3	0.96
Body pain	41	2	0.97
Animal bites	21	1	1
Swelling	26	1	1
Rheumatism	31	2	0.96
Mumps	18	1	1
Anthelminthic	35	1	1
Hair fall	39	1	1
Diabetes	79	3	0.96
Snake bites	32	1	1
Stomach pain	59	3	0.96
Cough	94	4	0.96
Cold	86	3	0.97
Body heat	81	2	0.98
Bone health	12	1	1
Laxative	104	3	0.98
Fever	90	4	0.96
Throat infection	36	1	1
Ulcer	24	1	1
Digestive problems	26	1	1
Gastric trouble	24	1	1
Generalized weakness	19	1	1
Constipation	7	1	1
External bleeding	8	1	1
Piles	58	3	0.96
Inflammation	27	1	1
Soap	50	2	0.97
Fracture	35	1	1
Asthma	13	1	1
Headaches	34	1	1

Nur – Number of use reports present in each ailment category
Nt – Number of species which are used

Table 4.5. Surveying flora in the National Medicinal Plants Board database and traditional Indian medicinal literature

S. No.	Botanical name	Reported in traditional (ayurvedic/siddha literature) – Indian medicinal plants database of the National Medicinal Plants Board	IUCN Red list
1	*Acalypha ciliata* Frossk.	+	-
2	*Acalypha fruticosa* Forssk.	+	Least – concern
3	*Aganosma cymosa* (Roxb.) G. Don	+	-
4	*Agave sisalana* Perrine	+	-
5	*Allophylus cobbe* (L.) Raeusch.	-	-
6	*Anogeissus latifolia* (Roxb. ex DC.) Wall. ex Guill. & Perr.	+	-
7	*Andrographis echioides* (L.) Nees	+	-
8	*Alangium salviifolium* (L.f.) Wangerin	-	-
9	*Aponogeton natans* (L.) Engl. & K.Krause	+	-
10	*Atalantia monophylla* DC.	+	-
11	*Azima tetracantha* Lam.	+	-
12	*Azadirachta indica* A. Juss.	+	Least – concern
13	*Barleria longiflora* L. f.	+	-
14	*Bambusa arundinacea* Willd.	+	-
15	*Borassus flabellifer* L.	+	-
16	*Breynia vitis-idaea* (Burm.f.) C.E.C.Fisch.	+	Least – concern
17	*Buchanania axillaris* (Desr.) Ramamoorthy	+	-
18	*Buchanania latifolia* Roxb.	+	-
19	*Canthium coromandelicum* (Burm. f.) Alston	+	-
20	*Capparis sepiaria* L.	+	-
21	*Carissa carandas* L.	+	-
22	*Cardiospermum halicacabum* L.	+	-
23	*Caralluma umbellata* Haw.	+	-
24	*Cassia fistula* L.	+	Least – concern
25	*Cassia occidentalis* L.	+	-
26	*Cipadessa baccifera* (Roth) Miq.	+	Least – concern
27	*Cissus vitiginea* L.	+	-
28	*Chloris barbata* Sw.	+	-
29	*Chionanthus ramiflorus* Roxb.	-	-
30	*Clausena dentata* (Willd.) Roem.	+	-
31	*Cleistanthus collinus* (Roxb.) Benth. ex Hook.f.	+	Vulnerable
32	*Clitoria ternatea* L.	+	-

S. No.	Botanical name	Reported in traditional (ayurvedic/siddha literature) – Indian medicinal plants database of the National Medicinal Plants Board	IUCN Red list
33	*Croton bonplandianus* Baill.	+	-
34	*Crinum asiasticum* L.	-	-
35	*Cymbopogon citratus* (DC.) Stapf.	+	-
36	*Cynodon dactylon* (L.) Pers.	+	-
37	*Dalbergia lanceolaria* L.f.	+	Least – concern
38	*Dalbergia paniculata* Roxb.	+	-
39	*Dioscorea oppositifolia* L.	+	-
40	*Dodonaea viscosa* Jacq.	+	Least – concern
41	*Ehretia anacua* (Teran & Berland.) I.M.Johnst.	-	Least – concern
42	*Erythrina indica* Lam.	+	Least – concern
43	*Evolvulus alsinoides* (L.).	+	-
44	*Flacourtia indica* (Burm. f.) Merr.	+	Least – concern
45	*Garuga pinnata* Roxb.	+	-
46	*Gloriosa superba* L.	+	Least – concern
47	*Grevillea robusta* A.Cunn. ex R.Br.	+	-
48	*Grewia carpinifolia* Juss.	+	-
49	*Gymnema sylvestre* (Retz.) R. Br. ex Sm.	+	-
50	*Gyrocarpus americanus* Jacq.	+	Least – concern
51	*Haldina cordifolia* (Roxb.) Ridsdale	+	-
52	*Helicteres isora* L.	+	-
53	*Hemionitis arifolia* (Burm. f.) T.Moore	+	-
54	*Hildegardia populifolia* Schott & Endl.	+	Critically endangered
55	*Hugonia mystax* L.	+	-
56	*Hybanthus enneaspermus* (L.) F.Muell.	+	-
57	*Ichnocarpus frutescens* (L.) W.T.Aiton.	+	-
58	*Ixora pavetta* Andr.	+	-
59	*Jasminum angustifolium* (L.) Willd.	+	-
60	*Jasminum multipartitum* Hochst.	-	-
61	*Jatropha curcas* L.	+	Least – concern
62	*Justicia betonica* L.	+	-
63	*Justicia glauca* Rottler	-	-
64	*Kleinia grandiflora* (Wallich ex DC.) N.Rani	+	-
65	*Lantana camara* L.	+	-
66	*Leucas aspera* (Willd.) Link	+	-
67	*Loeseneriella obtusifolia* (Roxb.) A.C.Sm.	+	-
68	*Mangifera indica* L.	+	Data deficient
69	*Melia azedarach* L.	+	Least – concern

S. No.	Botanical name	Reported in traditional (ayurvedic/siddha literature) – Indian medicinal plants database of the National Medicinal Plants Board	IUCN Red list
70	*Memecylon umbellatum* Burm.f.	-	-
71	*Mimosa hamata* Willd.	+	-
72	*Oldenlandia umbellata* L.	+	-
73	*Olax scandens* Roxb.	+	-
74	*Opilia amentacea* Roxb.	+	-
75	*Orthosiphon thymiflorus* (Roth.) Sleesen	+	-
76	*Pavetta indica* L.	+	-
77	*Pavonia zeylanica* (L.) Cav.	+	-
78	*Phyllanthus lawii* J. Graham	-	-
79	*Psydrax dicoccos* Gaertn.	-	Vulnerable
80	*Plecospermum spinosum* Trecul	+	-
81	*Premna tomentosa* Willd.	+	Least – concern
82	*Pterocarpus marsupium* Roxb.	+	Near – threatened
83	*Reissantia indica* (Willd.) N.Halle	+	-
84	*Sapindus emarginatus* Vahl	+	-
85	*Scutia myrtina* (Burm.f.) Kurz	+	Least – concern
86	*Sterculia urens* Roxb.	+	-
87	*Symplocos cochinchinensis* (Lour.) S.Moore	+	-
88	*Senna hirsuta* (L.) H.S.Irwin & Barneby	+	-
89	*Tamarindus indica* L.	+	Least – concern
90	*Tarenna asiatica* (L.) Kuntze ex K.Schum.	+	-
91	*Terminalia arjuna* (Roxb. ex DC.) Wight & Arn.	+	-
92	*Terminalia bellirica* (Gaertn.) Roxb.	+	-
93	*Terminalia chebula* Retz.	+	-
94	*Terminalia paniculata* Roth	+	-
95	*Tridax procumbens* (L.).	+	-
96	*Tylophora asthmatica* (L.f.) Wight & Arn.	+	-
97	*Vitex negundo* L.	+	Least – concern
98	*Walsura trifoliata* (A.Juss.) Harms	+	-
99	*Ziziphus oenoplia* (L.) Mill.	+	-
100	*Ziziphus xylopyrus* (Retz.) Willd.	+	-

Least-concern - 18, critically endangered - 01, vulnerable - 02, near-threatened - 01, data-deficient - 01.

The names of the various species surveyed in this paper were searched in the National Medicinal plants database and found that some of the plants (10 species) have not been reported in that database (Table 4.5).

4.6. ICF, FL% and UV

The Western Ghats of India have been surveyed thoroughly, and several reports focusing on the medicinal plants (and their specific uses) of those regions have been published in the past few decades (Ayyanar and Ignacimuthu, 2011; Samy and Ignacimuthu, 2000). However, the number of taxonomic surveys pertaining to medicinal plants of the Eastern Ghats is limited. The Siriya Kalvarayan hills from this zone contain many rare plants (close to 60% in our survey).

The ICF values of the plants was very high (0.93-1.0) for almost all of 32 different ailments. While the UV values were considerably high (0.6) for some of the plant species (*L. aspera* and *M. azedarach*), it was low for most of the plants because a very large proportion of the population (all the 105 informants) reported that they used plant-based medicines for treating various ailments. Moreover, they opted for other forms of medicine such as allopathy only when they had medical emergencies. From the FL% values obtained, though 44.76% was the highest value (for *Azadirachta indica*), we were able to assess from the knowledge of the localites and villagers that they had sufficient confidence while citing a given species for an illness. However, since not all informants may have deep/thorough ethnobotanical knowledge, the FL levels are low. This reveals that not all of the informants knew all of the possible ethnobotanical uses of all of the plants presented to them for the survey. The photographs of the plant species were shown, and whichever plants were identified, the respondents were asked to reveal specific ethnobotanical uses. This trend indicates that only the village shamans or medicine men (who were interviewed separately), knew all of the traditional uses as they conserve their ethnomedicinal secrets. Knowledge pertaining to ethnic medicine is passed on among the tribals by word of mouth tradition; hence, it is not documented in the form of books or journal articles.

Also, the general public in the locale relies on the medicinemen/shamans for their health needs; they may not need to know everything about all the plant species and may self-medicate themselves whenever necessary (based on their limited knowledge) and relegate the problematic bits to the ‘specialist’ medicine men/shamans, whenever the need rose for them to consult them the specialists. When one of these shamans was interviewed personally, he deemed that his service to their villagers was offered free of cost. Also, he felt that certain of his ‘secrets’ should not be divulged to strangers since he thought that the information would lead to misuse of his knowledge. This first-hand experience in the survey is revealing: when these medicine-men were approached, at first, they refused to share any information. After an introduction by a friend (also a local resident), they became more confident and agreed to strike a conversation. The reported FL% values in our survey may be deemed to below, but the number of plants that most of the informants admitted to using (on a regular basis) was 52 (among the 100 plants identified earlier), a dismal 52%. This may reveal that there could be a possible decline in traditional knowledge in the population. Also, since most of these species were used for just one or two ailments, we obtained low FL% values. These data indicate that while most of the informants used traditional medicine on a daily basis, their knowledge of the medicinal plants in the Siriya Kalvarayan hills is limited and worse, it is probably declining.

The ethnobotanical survey pertaining to specific plants (by showing those photographs) was conducted separately, on another day subsequent to the questionnaire study which earlier focused on residents of 8 different villages. Hence, both these surveys (both with n = 105 participants) were conducted on separate days and involved different individuals. This random sampling of the population may actually remove underlying bias in the data. For the survey of ethnobotanical uses of 52 particular medicinal plants, the informants were carefully chosen based on their reliance on traditional forms of medicine. This was perhaps the only “inclusion criterion”. All of the informants who were interviewed were familiar with most of the plants and their uses in their own ethnic setting and locale. In this vein, Table 4.1 presents the uses of the 100 surveyed species through literature search, while Table 4.4 focuses on the ethnobotanical uses of the Malayali people dwelling in the villages of the Siriya Kalvarayan hills. We found the following six plants *Aganosma cymosa* (Roxb.) G. Don, *Dalbergia lanceolaria* L.f., *Hugonia mystax* L., *Loeseneriella obtusifolia* (Roxb.) A.C. Sm., *Plecospermum spinosum* Trecul. and *Walsura trifoliata* (A. Juss.) Harms in rich distribution in the study areas. Still, none of the respondents declared any ethnobotanical importance to them. We feel that the knowledge about the usage of these plants might have eroded over the years. The literature survey also revealed the lacunae in terms of understanding the bioactivity of these species. Hence, we chose these six plants for our further bioactivity studies.

4.7. Antioxidant Activity

Antioxidants can counterbalance the considerable damaging effects of deleterious ROS and other free radicals (RNS, RSS and RHS) which are produced during oxidative stress. Since there are both lipophilic and hydrophilic antioxidants, more research on plant-derived and natural antioxidants has been underway. This is because phenolic compounds present in plants have been shown to increase longevity, decrease cellular senescence and also promote genomic stability by reducing protein carbonylation, protein tyrosine nitration, protein oxidation (and formation of conjugated aggregates/multimers as in Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease), lipid peroxidation, oxidative macromolecular (protein, lipid, RNA and DNA damage) and also cell death. When there is a deficit in antioxidants (both lipophilic and hydrophilic free radical neutralizing reactive species), there is a protracted increase in the accumulation of damaged macromolecules, and summarily, the redox homeostasis of the cell is lost. Furthermore, unmitigated oxidative stress leads to genomic instability because of free radical-induced cellular damage, organelle dysfunction, altered cell signalling cascades and finally, cellular programming and epigenetic changes which cause carcinogenesis, heart failure/cardiovascular disease, diabetes mellitus and the incidence of many chronic illnesses. Since plants are rich in antioxidants and also utilize these molecules for cellular redox homeostasis, more consideration has been given to plant-derived natural antioxidants for several decades. Besides, most of these natural antioxidants do not usually cause untoward/lethal toxic effects in human patients because they have been encountered by the body through plant-based diets, consumption of beverages such as tea, spinaches, vegetables and fruits. Antioxidant based medicines are used to prevent as well as treat several complex diseases (Liu *et al*., 2010; Subedi *et al*., 2014).

Plants possess a repertoire of natural antioxidants, which also contains medicinal properties such as antidiabetic, anticancer, antimicrobial (antibacterial, antiviral, antifungal, antiparasitic, antiprotozoal etc.), anti-ageing, etc. (Akinmoladun *et al*., 2010). Sometimes, these activities cannot be clearly demarcated because an antioxidant compound may also have anticancer or antimicrobial properties. Hence, these compounds intrigue the experts and scientific research groups owing to the range of potential mechanisms by which they protect cells from stress and pathogenesis of diseases. Moreover, antioxidants may exert a potent effect by controlling oxidative stress in the pathophysiology of diseases. Polyphenols possess arguably the highest free radical scavenging activity among the phytochemicals. Antioxidant activity is dependent on the redox potential of these compounds; since they are reductants; they react with singlet oxygen, superoxide, nitric oxide as well as hydroxyl radicals and are also known to chelate metal ions, which accelerate in Fenton and Haber-Weiss reactions (Barabadi *et al*., 2020; Enneb *et al*., 2020). Medicinal plants possess appreciable amounts of bioactive phytochemicals such as flavonoids, phenolics, tannins, stilbenes, coumarins, lignans and lignins. These compounds include several biological and antioxidant activities (Kaur and Kapoor, 2001; Venkatachalam, 2020). DPPH is a stable free radical exhibiting violet colour in solution, which after mixing with antioxidants, changes to a long-lasting yellow colour, indicating antioxidant activity (Liu *et al*., 2010; Lu *et al*., 2010). Similarly, in the ABTS assay, ABTS radical cation can be quenched by free radicals (ROS, RNS, RSS and RHS) to regenerate ABTS, which is tracked spectrophotometrically for decolourization of green ABTS radical cation solution to colourless ABTS. Other assays such as ferric reducing power (conversion of Fe^{3+} to Fe^{2+}), nitric oxide radical scavenging activity (suppression of $NO^{\bullet}$) and phosphomolybdenum reduction assay [reduction of molybdenum (VI) to molybdenum (V)] also detect the antioxidant potential of the plant extracts/purified plant compounds. These antioxidant assays were performed for all the chosensix different plants. These five assays were conducted for each of these plants (methanolic leaf extracts taken at various concentrations-10, 20, 40, 60, 80 and 100 μg/mL), and the results are presented below:

1. *Aganosma cymosa* (Roxb.) G. Don

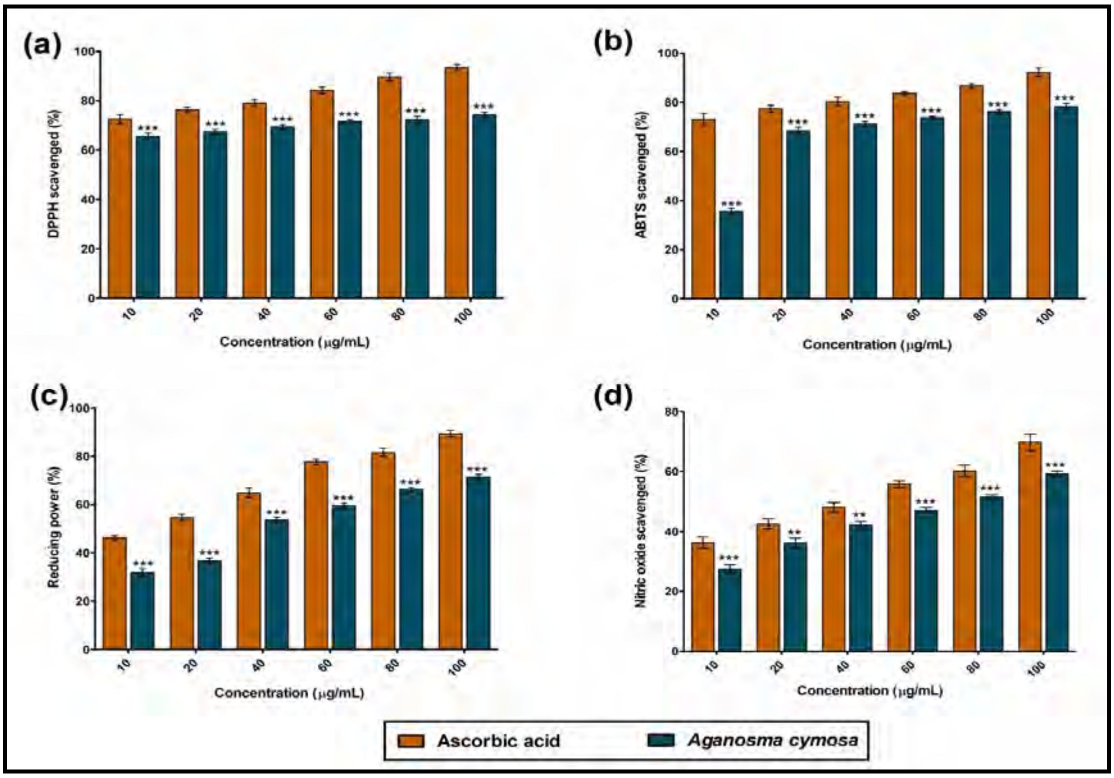

Figure 4.4. (a) DPPH radical scavenging activity of methanolic leaf extract of *Aganosma cymosa* (Roxb.) G. Don. - the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns – non significant; (b) $ABTS^{\bullet+}$ radical scavenging activity of methanolic leaf extract of *Aganosma cymosa* (Roxb.) G. Don the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns – non significant; (c) Reducing power assay of methanolic leaf extract of *Aganosma cymosa* (Roxb.) G. Don the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns – non significant; (d) Nitric oxide scavenging activity of methanolic leaf extract of *Aganosma cymosa* (Roxb.) G. Don the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns – non significant; and (e) Phosphomolybdenum assay of methanolic leaf extract of *Aganosma cymosa* (Roxb.) G. Don the values indicate mean ± standard deviation (n=3).

In the DPPH radical scavenging assay (Figure 4.4a), all the five concentrations of *A. cymosa* MeOH extract were significant w.r.t ascorbic acid control (at the same concentrations, in the range of 10-100 µg/mL). However, even 10 µg/mL was considered as effective as the higher extract concentrations. *A. cymosa* has good antioxidant properties. The MeOH extract of the plant had shown satisfactory scavenging/inhibitory activity against ROS and RNS, as shown in (Figure 4.4). The plant extract showed 60-70% inhibition (at a concentration range of 10-100 μg/mL) against DPPH radical compared to ascorbate control (70-90% inhibition in the concentration range of 10-100 μg/mL ascorbate). Similarly, for ABTS radical scavenging assays (Figure 4.4b), except for 10 μg/mL, the other concentrations were found to possess almost similar activity, irrespective of the concentrations of the plant extract. Against ABTS metastable radical cation, *A. cymosa* extract showed a considerable effect, yielding 35-70% inhibition at the concentration range of 10-100 μg/mL. The ABTS radical cation is one-electron reduced and donation of an electron by the reductants present in the plant extract essentially reversed the free radical (against 70-90% proffered by ascorbate control) and ascorbic acid exerted similar dose-dependent effects, barring the first data point (10 μg/mL). In the ferric reducing power assay (which measures the ability of chemical compounds/extracts) to convert Fe^{3+} ion to Fe^{2+}, the plant extract possessed roughly 30-65% activity, when compared to ascorbate control (~45-85%), with both control and test samples in the range of 10-100 μg/mL. An exciting find was that in both reducing power assay and $NO^{\bullet}$ scavenging activity assay, antioxidant activity (although not matching control ascorbic acid's activity at the same concentrations as those of plant extract) was found to follow a dose-dependent profile. W.r.t. ascorbic acid control (same concentrations of plant extract as control), the values derived from the standard plot indicated that it possessed considerable antioxidant activity. $NO^{\bullet}$ scavenging activity of the plant extract was almost as close to ascorbate control, with over 25->55% activity w.r.t. ascorbic acid. The phosphomolybdenum assay result revealed the antioxidant activity of 63.33% w.r.t. ascorbic acid control (standard plot slope), Figure 4.5.

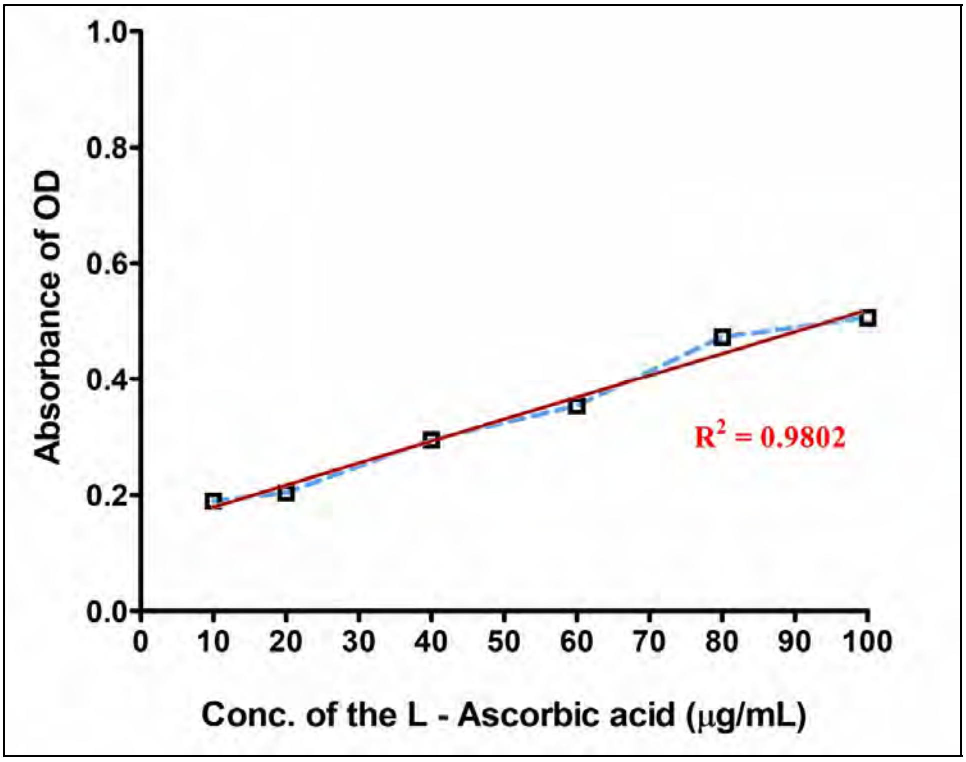

Figure 4.5. Standard graph for Phosphomolybdenum assay

2) *Dalbergia lanceolaria* L.f.

Dalbergia lanceolaria L.f. MeOH extracts were checked for a panel of antioxidant activities, and it was found that it had dose-dependent antioxidant effects in all the four antioxidant assays shown in Figure 4.6 below. The MeOH extract had the best activity against ABTS radical cation, with almost similar activity against control ascorbic acid. In the DPPH assay (panel a), it was almost 60-80% efficacious w.r.t ascorbic acid control (panel b), while this plant had a lower reducing power (only 25-50%) of activity across the concentration range of 10-100 µg/mL, when compared to ascorbic acid control at the same concentrations as plant extract. Nitric oxide showed 31-44% at the concentration of 10-100 µg/mL. Phosphomolybdenum assay yield was 75.14 ± 1.2 (AAE mg/g) in the same concentration.

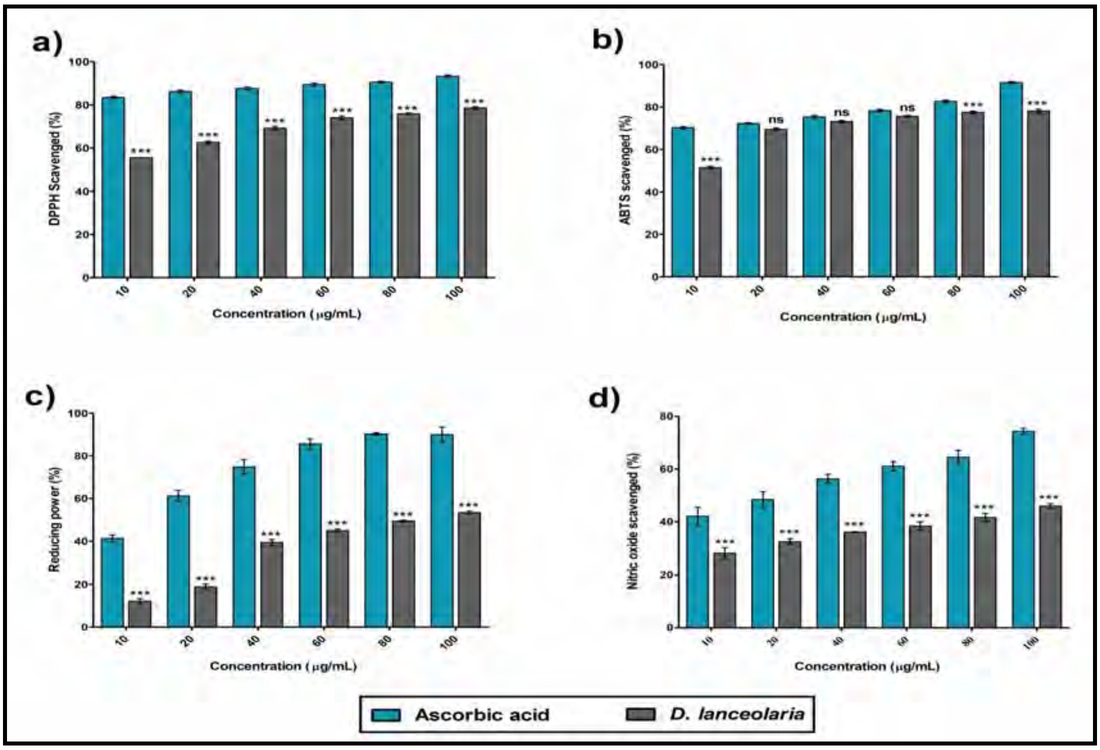

Figure 4.6. (a) DPPH radical scavenging activity of methanolic leaf extract of *Dalbergia lanceolaria* L.f. the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns – non significant; (b) $ABTS^{\bullet+}$ radical scavenging activity of methanolic leaf extract of *Dalbergia lanceolaria* L.f. the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns – non significant; (c) Reducing power assay of methanolic leaf extract of *Dalbergia lanceolaria* L.f. the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns – non significant; (d) Nitric oxide scavenging activity of methanolic leaf extract of *Dalbergia lanceolaria* L.f. the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns – non significant; (e) Phosphomolybdenum assay of methanolic leaf extract of *Dalbergia lanceolaria* L.f. the values indicate mean ± standard deviation (n=3).

3. *Hugonia mystax* L.

When comparing the antioxidant activities (ABTS radical, DPPH radical, reducing power, nitric oxide radical scavenging), the MeOH extract of *H. mystax* was the most effective against ABTS radical cations (Figure 4.7b). It had a dose-dependent profile against DPPH radicals. However, the reducing ability was weak (20 to ~35%), almost only half of the activity of ascorbic acid control (which reached a maximum of 80%). Contrastingly, nitric oxide scavenging potential of *H. mystax* extract was in the range of 20-50%, and w.r.t. ascorbic acid control (with a maximum of ~ 60%); it was

33-80% of control. These data suggest that the MeOH leaf extract of *H. mystax* was greatly effective against ABTS radicals, followed by DPPH radicals, nitric oxide radicals and finally, the least effective at ferric reducing power. Phosphomolybdenum shows 126.66 ± 1.3 (AAE mg/g).

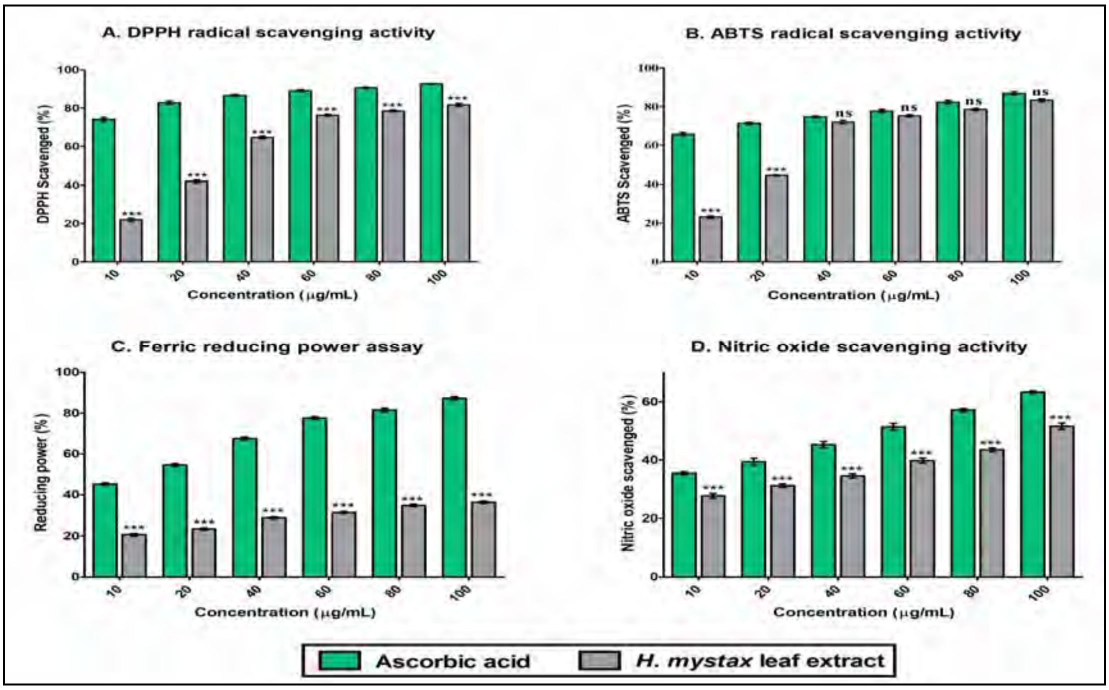

Figure 4.7. (a) DPPH radical scavenging activity of methanolic leaf extract of *Hugonia mystax* L. the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns – non significant; (b) $ABTS^{\bullet+}$ radical scavenging activity of methanolic leaf extract of *Hugonia mystax* L. the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns – non significant; (c) Reducing power assay of methanolic leaf extract of *Hugonia mystax* L. the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns – non significant; (d) Nitric oxide scavenging activity of methanolic leaf extract of *Hugonia mystax* L. the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns – non significant; (e) Phosphomolybdenum assay of methanolic leaf extract of *Hugonia mystax* L. the values indicate mean ± standard deviation (n=3).

4. *Loeseneriella obtusifolia* (Roxb.) A. C. Sm.

L. obtusifolia displayed phenomenal antoxidant activities in the concentration range of 10-100 μg/mL (40-70%) compared to ascorbic acid control (70-90%) against DPPH radical. In the ABTS radical cation scavenging assay, the plant extract displayed a good antioxidant effect in the range of 50-80% (w.r.t. ascorbic acid control, which showed 80-90%). Against ascorbic acid, control displayed Fe^{3+} reducing power of 40-80% in the concentration range of 10-100 μg/mL, while *L.obtusifolia* MeOH extract showed activity in the range of 20 - ~50%, which is roughly half of the activity of the control ascorbic acid. The nitric oxide radical scavenging activity was not dose-dependent and all concentrations of the extract gave approximately the same activity (37-40%), while control ascorbic acid gave 80-90% activity, which seemed to be dose-dependent (Figure 4.8). Phosphomolybdenum assays express better antioxidant results than other plants 370 ± 2.8 (AAE mg/g).

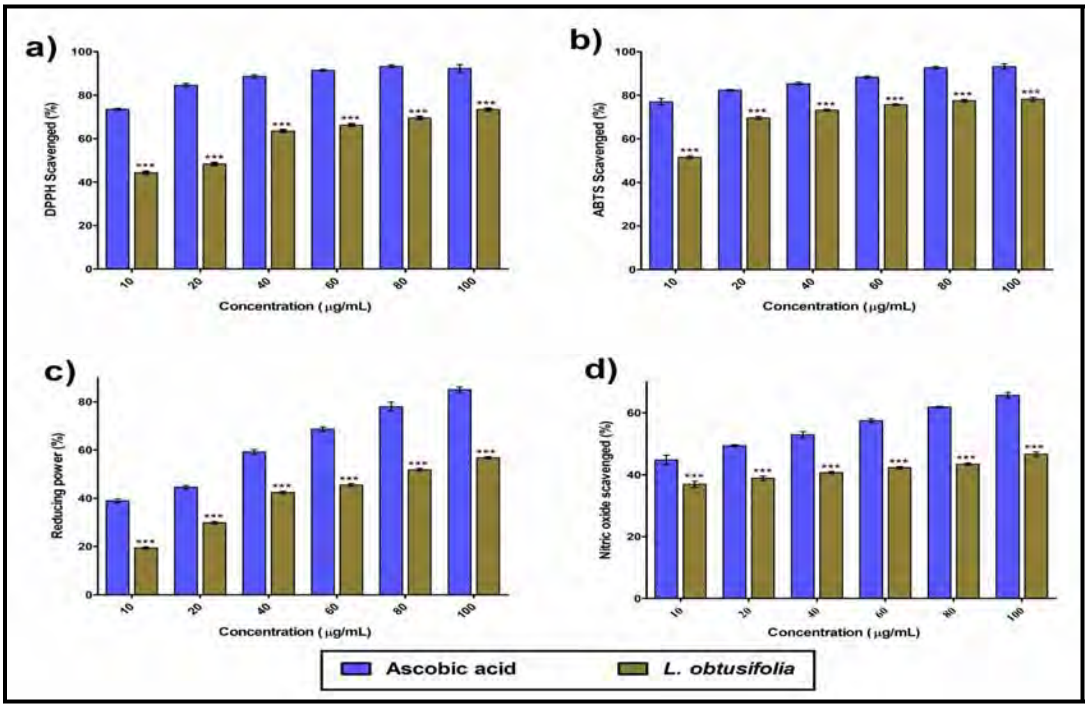

Figure 4.8. (a) DPPH radical scavenging activity of methanolic leaf extract of *Loeseneriella obtusifolia* (Roxb.) A. C. Sm. the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns - non significant; (b) $ABTS^{•+}$ radical scavenging activity of methanolic leaf extract of *Loeseneriella obtusifolia* (Roxb.) A.C. Sm. the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns - non significant; (c) Reducing power assay of methanolic leaf extract of *Loeseneriella obtusifolia* (Roxb.) A.C. Sm. the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns - non significant; (d) Nitric oxide scavenging activity of methanolic leaf extract of *Loeseneriella obtusifolia* (Roxb.) A.C. Sm. the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns - non significant; (e) Phosphomolybdenum assay of methanolic leaf extract of *Loeseneriella obtusifolia* (Roxb.) A.C. Sm. the values indicate mean ± standard deviation (n=3).

5. *Plecospermum spinosum* Trecul.

While *P. spinosum* extract (100 μg/mL) yielded 70% free radical scavenging activity, it was comparatively lower than that of L-ascorbic acid (100 μg/mL) standard as shown in Figure 4.9a. In the $ABTS^{\bullet+}$ decolourization assay, the antioxidant activity of *P. spinosum* extract (100 μg/mL) was 86.48 ± 0.8%, which was less when compared with the activity of ascorbic acid (which showed marginally more significant inhibition as shown in Figure 4.9b). Reducing power is a good indicator of antioxidant activity. It is evaluated by the transformation of Fe^{3+} to Fe^{2+} through reduction facilitated by antioxidant molecules. Nitric oxide radical was generated by the addition of sodium nitroprusside. The scavenging activity of *P. spinosum* leaf extract (100 μg/mL) exhibited 51.16 ± 1.5%, which was less compared with the ascorbic acid standard (Figure 4.9d). The reducing power of *P. spinosum* leaf extract was investigated, and the data were shown in Figure 4.9c. The leaf extract exhibited 32.45 ± 1.2% at the maximum concentration, which was low compared with the standard ascorbic acid. In the phosphomolybdenum assay, molybdenum VI was reduced into molybdenum V in the presence of antioxidant molecules of the leaf extract (Rana *et al*., 2020). A linear profile was observed for the standard concentrations of ascorbic acid (10-100 μg/mL) with $R^2 = 0.9802$ (and a straight line equation of $y = 0.003\ x - 1.44$) and the significantly higher antioxidant activity obtained (reduction of molybdenum VI to V) was 93.33 ± 1.7 mg AAE/g. Ascorbic acid was the standard, and the results are shown in (Figure 4.5) (Annadurai *et al*., 2021).

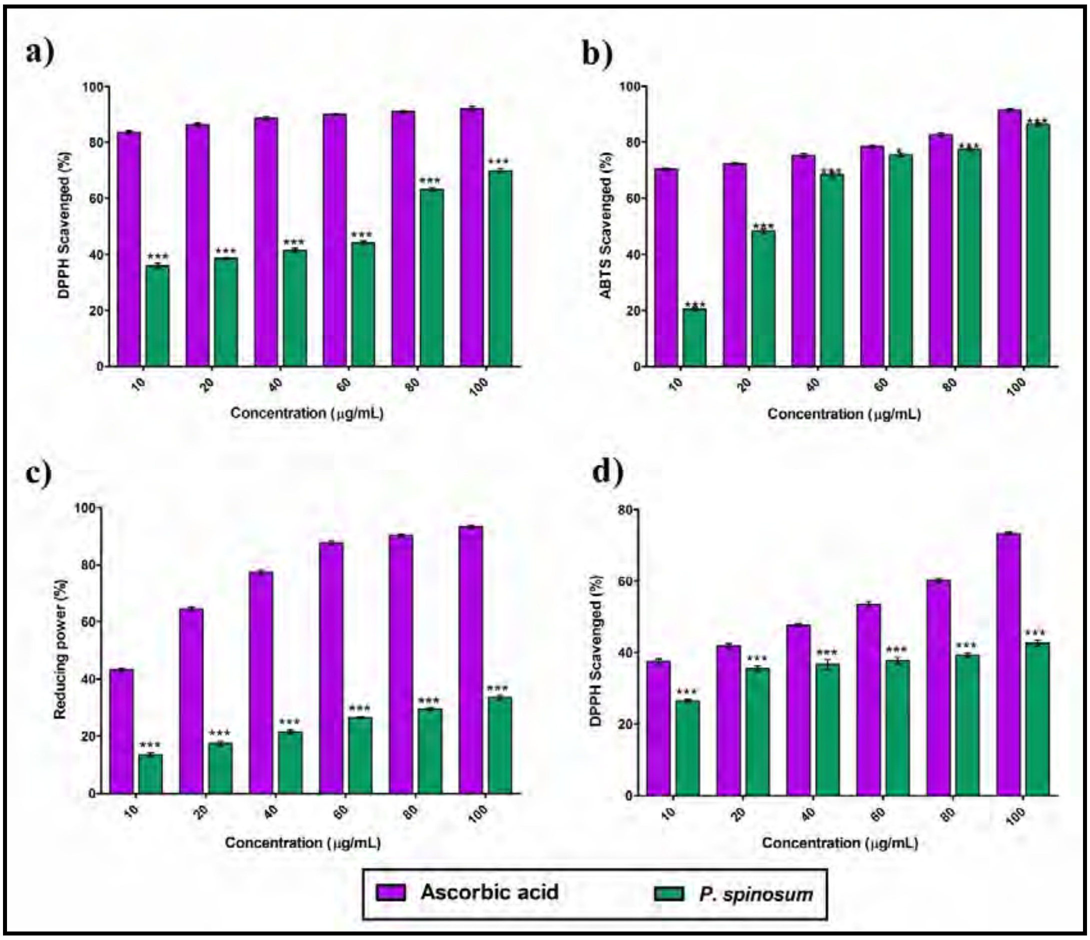

Figure 4.9. (a) DPPH radical scavenging activity of methanolic leaf extract of *Plecospermum spinosum* Trecul. the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns - non significant; (b) $ABTS^{\bullet+}$ radical scavenging activity of methanolic leaf extract of *Plecospermum spinosum* Trecul. the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns - non significant; (c) Reducing power assay of methanolic leaf extract of *Plecospermum spinosum* Trecul. the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns - non significant; (d) Nitric oxide scavenging activity of methanolic leaf extract of *Plecospermum spinosum* Trecul. the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns - non significant; (e) Phosphomolybdenum assay of methanolic leaf extract of *Plecospermum spinosum* Trecul. the values indicate mean ± standard deviation (n=3).

6. *Walsura trifoliata* (A. Juss.) Harms

The various antioxidant assays for MeOH leaf extract of *W. trifoliata* revealed that the plant possessed appreciable antioxidant/free radical scavenging activity against DPPH and ABTS radicals (almost 80-90% of the activity of control ascorbic acid)

(Figure 4.10 a, b). Ferric reducing power was much poorer than other plants (comparatively), in the range of 10-40% (while ascorbic acid yielded 40-85% activity) at the same concentration ranges. NO radical scavenging activity was roughly 15-50% against ascorbic acid control, which showed nearly 40-75% activity. Phosphomolybdenum assay shows 174.29 ± 1.4 (AAE mg/g) in the leaf extrct of *W. trifoliata*.

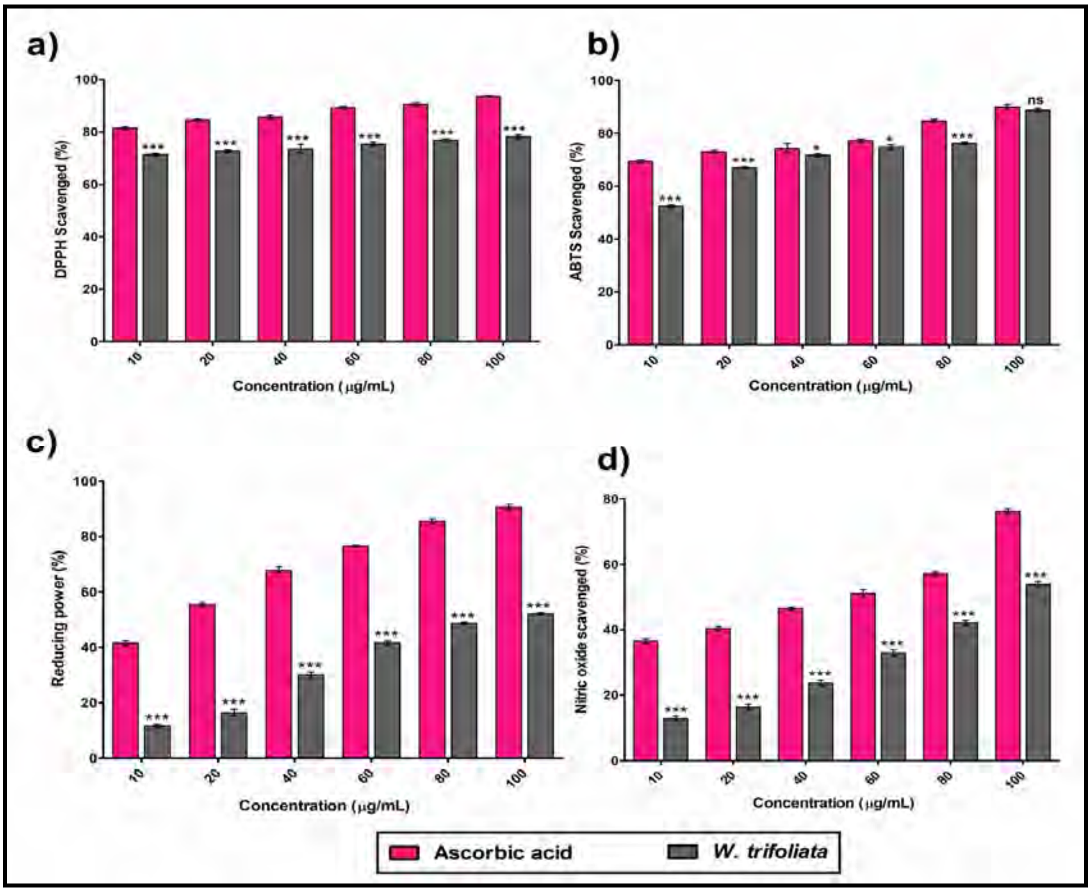

Figure 4.10. (a) DPPH radical scavenging activity of methanolic leaf extract of *Walsura trifoliata* (A. Juss.) Harmsthe values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns – non significant; (b) $ABTS^{\bullet+}$ radical scavenging activity of methanolic leaf extract of *Walsura trifoliata* (A. Juss.) Harms the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns – non significant; (c) Reducing power assay of methanolic leaf extract of *Walsura trifoliata* (A. Juss.) Harms the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns – non significant; (d) Nitric oxide scavenging activity of methanolic leaf extract of *Walsura trifoliata* (A. Juss.) Harms the values indicate mean ± standard deviation (n=3). The mean difference is significant at the levels of *p<0.05, **P<0.01 and ***P<0.001 Vs standard. ns – non significant; (e) Phosphomolybdenum assay of methanolic leaf extract of *Walsura trifoliata* (A. Juss.) Harms the values indicate mean ± standard deviation (n=3).

Using two-way ANOVA, the results were significant when the data points were compared between each ascorbate control point (same concentration of ascorbate as the test) and Bonferroni post-tests were performed. The P-value for all the four assays (between control and test points in all cases) was significant (*** at P <0.001), and in isolated instances, the level of significance was ** (P < 0.01), *p<0.05 and ns- non-significant. We are currently assessing the antioxidant properties of the aqueous extract of the plant.

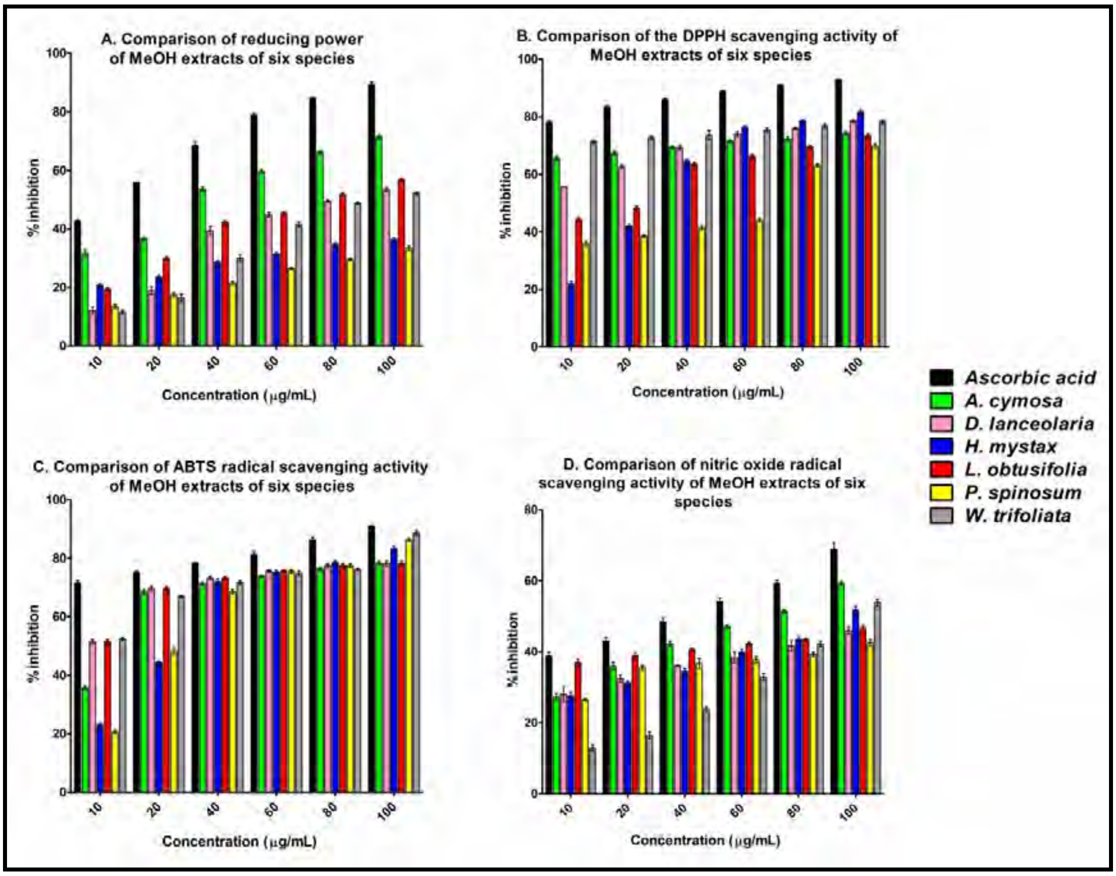

Figure 4.11. Summary of findings for antioxidant activity of the six plant species – The antioxidant activity (A. reducing power, B. DPPH activity, C. ABTS radical scavenging activity and D. nitric oxide radical scavenging activity) for six plants which were explored in this study are summarized in this graph.

In Figure 4.11 above, the antioxidant activities of the MeOH extracts of six different plant species are compared in one graph at several concentrations (10, 20, 40, 60, 80 and 100 μg/mL). When comparing the antioxidant data for the plant species, at the same concentrations (side by side for each concentration, different plants), the following key findings were gleaned from the data:

A. Reducing power - When comparing reducing power, amongst all the species, the plant species closest (~80%) to ascorbic acid control (across all concentrations compared) was *A. cymosa*. The order of reducing power is in the order - *A. cymosa* > *L. obtusifolia* > *D. lanceolaria* > *W. trifoliata* > *H. mystax* > *P. spinosum.*

B. DPPH scavenging activity - The order of DPPH scavenging ability is in the order **–** *W. trifoliata* > *A. cymosa* > *D. lanceolaria* > *H. mystax* > *L. obtusifolia* > *P. spinosum.*

C. ABTS radical scavenging activity - The order of ABTS radical cation scavenging ability at lower concentrations of the extracts is in the range of *D. lanceolaria / L. obtusifolia / A. cymosa* (these three have almost similar activities) > *W. trifoliate* > *H. mystax / P. spinosum* (which have similar activities). However, at much higher concentrations (40 µg/mL and above), this trend is lost and all the plant extracts have almost equal anti-ABTS radical activity.

D. **$NO^{\bullet}$ radical scavenging activity -** Based on the comparative profiles obtained (comparing concentration for concentration), it can be surmised that *A. cymosa* > *L. obtusifolia* > *D. lanceolaria* > *P. spinosum* > *H. mystax* > *W. trifoliata.*

Hence, in all these studies, *A. cymosa* seems to be power packed with antioxidant molecules when compared to the other plant extracts. Since equal concentrations of crude extracts were taken (and the same amounts of plant powder and MeOH were used to obtain extracts), this kind of comparison can be made.

4.8. Antimicrobial Activity

Natural plant sources have been utilized for the treatment of several humans as well as animal diseases. Plants contain various types of chemical compounds which possess numerous pharmacological applications. The incidence of infectious diseases increases the mortality rate; about 70% of hospital deaths are primarily due to diverse contagious diseases caused by bacteria, fungi and viruses (Al-Dhabi *et al*., 2018; Gnanamani *et al*., 2003). Natural drugs have been to possess efficacy against antibiotic-resistant microorganisms; plant compounds have been shown to act as natural antibiotics (Farjana *et al*., 2014). Botanists, ethnopharmacologists, and natural chemists use medicinal plants for isolating a plethora of phytochemicals with potent activity against several clinically relevant pathogens, some of which are multidrug-resistant (MDR) and extensively drug-resistant (XDR) (Appapalam and Panchamoorthy, 2017). The potential antimicrobial activity of plants and herbs were already documented against different pathogens (Arulmozhi *et al*., 2018; Pendota *et al*., 2017). Plant extracts contain several compounds which have potent antibacterial and antifungal activity because plants contain secondary metabolites which aid in defence against potential pathogens which may attack plants. Several classes of metabolites/phytochemicals have been reported to elicit antimicrobial activity - such as essential oils, terpenoids, alkaloids, phenolics - flavonoids and polyphenols (Cowan, 1999). These substances may either act directly by binding to biochemical targets inside the microbial cells or may indirectly inhibit their growth (static activity) or alternatively, cause microbial cell death (cidal activity). When mixtures/crude extracts of plants are utilized, the combinations of effects sponsored by the various compounds can be mediated by a host of receptor-drug interactions in permutations and combinations. ROS are known to be responsible for the action of antibiotics. James Collins' group has shown that ROS which is released from antibiotics, complement the primary target-binding effects of antibiotics (e.g. aminoglycosides binding to the 30S ribosomal subunit or fluoroquinolone binding to DNA gyrase) (Dharmaraja, 2017). Some plant compounds may increase ROS production by interacting with cellular components and thereby exert antimicrobial effects. These effects may be confirmed through docking (*in silico*) studies, which are underway; however, *in vitro* studies ought to be performed to back up any *in silico* predictions. Some of the plant compounds may have prooxidants activities (despite being strong antioxidants); the cellular redox state determines the difference in the action of these compounds - as either prooxidant or antioxidant (Kristinova *et al*., 2009). Only further investigations with purified compounds *in vitro* and *in vivo* can ascertain theoretical assumptions about some of the identified compounds (through GC-MS). Hence, in these preliminary studies, we may not envisage the mechanisms of action but can only speculate the antimicrobial mechanisms of certain compounds. When compared to standard antibiotic positive controls, which are routinely used against bacteria in clinical scenarios, the plant extracts do not exhibit significant inhibition zones. This is because there are mixtures of antimicrobial as well as nontoxic compounds (which would perhaps enhance the growth of the microbes by acting as carbon and nitrogen sources). Therefore, only purified compounds, when tested individually against clinical isolates as well as lab strains, may yield noteworthy antimicrobial activity results.

1. *A. cymosa*

Standard antibiotic discs (at low μg levels) were several orders of times more effective than the crude extracts (at mg/mL concentrations). The aim of this part of the study was to identify even modest zones of inhibition with the crude extracts. Care was taken to include MeOH as a control in order to compensate for MeOH as solvent in which crude extracts are suspended, so that if MeOH were to independently exert any antimicrobial effects, the inhibition zone measurements for MeOH can be substracted from those of the plant extracts. *A. cymosa* extract showed modest activity against

E. coli and *P. aeruginosa*, while standard antibiotics gentamicin (10 μg), Amoxyclav (30 μg) and the other antibiotics also were highly effective against *S. aureus* and *S. typhimurium*. The crude extract did not inhibit any of the other strains studied. In the antimicrobial studies, the efficacy of the plant extracts was explored against clinically relevant pathogens - both bacteria and fungi. Among the bacteria, both gram positive (*S. aureus*) and gram negative (*E. coli, S. flexneri, P. aeruginosa, K. pneumoniae* and *S. typhimurium*) strains were chosen.

Amongst the bacterial strains which were studied (Table 4.6 and Plate 4.18), the plant extract was efficacious against *Escherichia coli* (8 mm diameter) and *Pseudomonas aeruginosa* (6 mm). Still, it did not show any effect (even at high concentrations of 10 mg/mL) against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Shigella flexneri* and *Salmonella typhimurium* at any of the concentrations studied in the concentration range of 6-10 mg/mL. The standard antibiotics were efficacious against the bacterial species at much lower (μg) amounts; for example, gentamicin had profound inhibitory action against almost all the bacterial species used. About antifungal activity of the plant extract, it was effective against *E. tinctorium*, a plant pathogen; however, the extract did not inhibit any other fungi cultured in this study (*Candida tropicalis*, *Candida glabrata* and *Aspergillus fumigatus*), as observed from (Table 4.7 and Plate 4.19). Antimicrobial efficacy also largely depends on the rate of drug uptake and solubility of the compounds, and since we did not use any purified compounds for our antimicrobial assays, we do not know the rate of drug uptake. Since this is a methanolic extract (and the plant extract was dissolved in methanol), the effect of methanol on microbial growth was checked using a solvent control (methanol alone) and no zone of inhibition was observed in the solvent control wells.

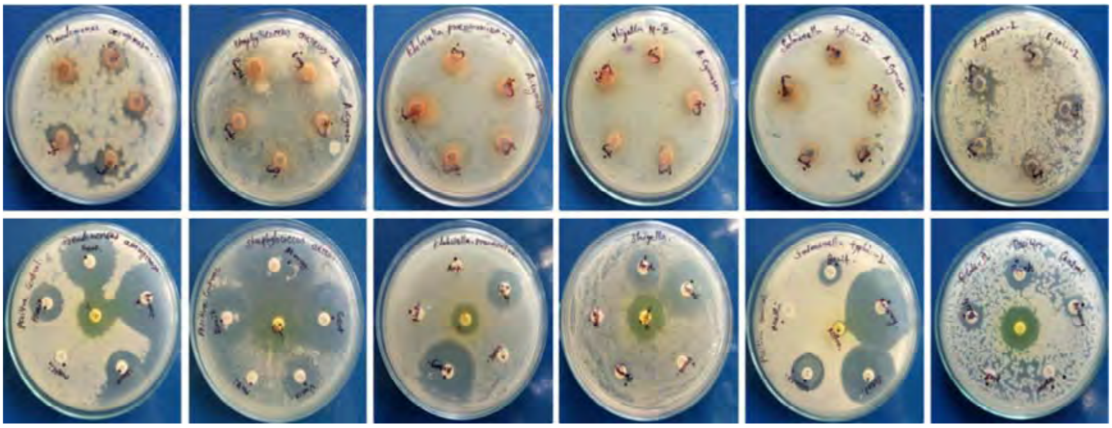

Plate 4.18. Antibacterial activity of *A. cymosa* - Left to right: The photographs for test and control antibiotic disc diffusion results are presented above and below (top and bottom row) from left to right in the following order - *P. aeruginosa, S. aureus, K. pneumoniae, S. flexneri, S. typhimurium* and *E. coli.* The respective test and control combinations are arranged above and below, for the corresponding test organisms as per the given order.

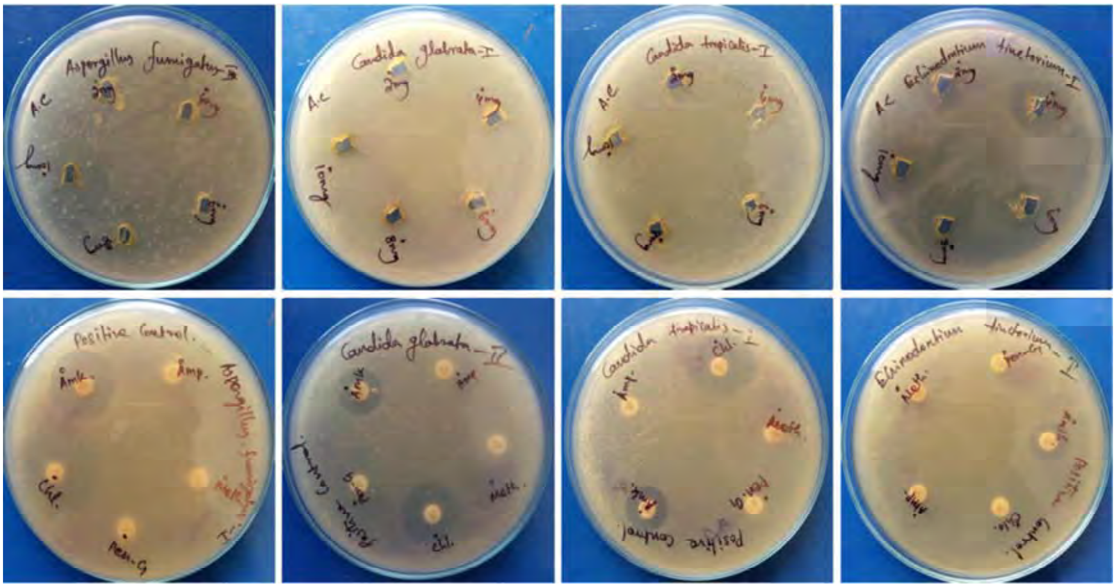

Plate 4.19. Antifungal activity of *A. cymosa* - MeOH leaf extract against antibiotic disc controls – From left to right: *A. fumigatus*, *C. glabrata*, *C. tropicalis* and *E. tinctorium* (test plates on the top row and correspondingly, the control rows at the bottom row) with test and control aligned one over another, correspondingly.

Table 4.6. Antibacterial activity of *A. cymosa*

Antibiotic discs (µg)	**Plant extract (mg/mL)**	***Pseudomonas aeruginosa*** **(mm)**	***Staphylococcus aureus*** **(mm)**	***Klebsiella pneumoniae*** **(mm)**	***Shigella flexneri*** **(mm)**	***Salmonella typhimurium*** **(mm)**	***Escherichia coli*** **(mm)**
-	6	4 ± 0.2	N.E.	N.E.	N.E.	N.E.	3 ± 0.2
-	7	4 ± 0.2	N.E.	N.E.	N.E.	N.E.	4 ± 0.2
-	8	5 ± 0.5	N.E.	N.E.	N.E.	N.E.	4 ± 0.5
-	9	7 ± 0.6	N.E.	N.E.	N.E.	N.E.	5 ± 0.7
-	10	8 ± 0.5	N.E.	N.E.	N.E.	N.E.	6 ± 0.8
Nitrofurantoin (10)	-	8 ± 1.7	13± 1.3	9 ± 1.1	8± 1.2	3± 0.8	11± 0.7
Vancomycin (10)	-	10 ± 1.3	11 ± 1.4	NE	NE	9 ± 1.5	NE
Gentamicin (10)	-	15 ± 1.3	15 ± 1.2	13 ± 1.8	9 ± 0.8	16 ± 1.8	6 ± 1.0
Bacitracin (10)	-	7 ± 1.4	11 ± 1.1	NE	NE	6 ± 1.4	9 ± 1.2
Amoxyclav (30)	-	17 ± 1.9	20 ± 1.0	15 ± 1.6	3 ± 0.7	21 ± 1.3	14 ± 2.0
Methicillin (5)	-	NE	NE	NE	NE	NE	NE

N.E. - no effect detected; zones are represented as radius (mm).

Table 4.7. Antifungal activity of *A. cymosa*

Antibiotics (µg)	Plant extract (mg/mL)	*Aspergillus fumigatus* (mm)	*Candida glabrata* (mm)	*Candida tropicalis* (mm)	*Echinodontium tinctorium* (mm)
-	2	N.E.	N.E.	N.E.	6 ± 1.4
-	4	N.E.	N.E.	N.E.	12 ± 0.1
-	6	N.E.	N.E.	N.E.	11 ± 0.1
-	8	N.E.	N.E.	N.E.	12 ± 0.2
-	10	N.E.	N.E.	N.E.	14 ± 0.4
Penicillin-G (10)	-	N.E.	N.E.	N.E.	N.E.
Methicillin (5)	-	NE	NE	NE	NE
Chloramphenicol (30)	-	9 ± 1.2	10 ± 0.8	10 ± 0.9	9 ± 0.7
Amikacin (30)	-	8 ± 0.7	9 ± 1.2	7 ± 0.5	5 ± 0.4
Ampicillin (10)	-	NE	NE	NE	NE

N.E. - no effect detected; zones are represented as radius (mm).

2. *D. lanceolaria*

Against the laboratory strains of various bacteria, *D. lanceolaria* crude extract was found to inhibit *P. aeruginosa*, *S. aureus*, *S. typhimurium* and *E. coli*. Still, it did not show any inhibitory effects at any concentrations (6-10 mg/mL) against *K. pneumoniae* or *S. flexneri* (Table 4.8 and Plate 4.20). Against the fungal species, the extract was most effective against *E. tinctorium*, but did not at all inhibit any of the other four test species (Table 4.9 and Plate 4.21). The apparent ambiguities/discrepancies of the extract for these compounds in different strains are not evident and there is no perceived mechanism of action that explicates these observed differences in efficacy.

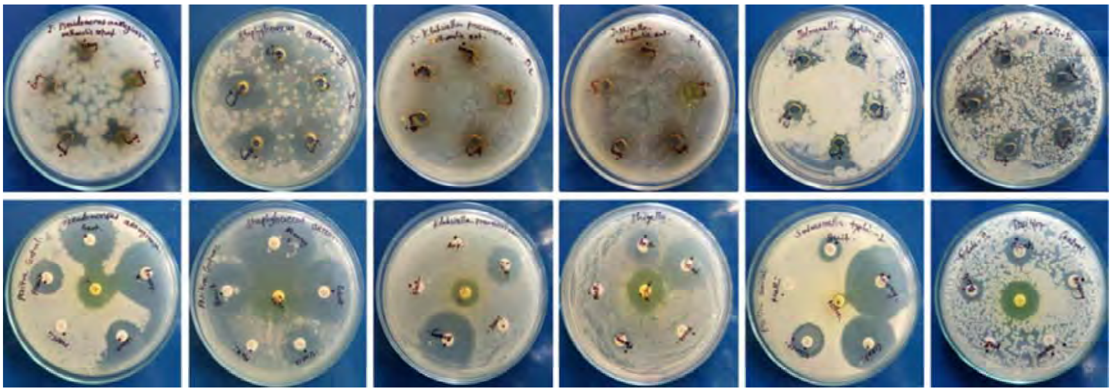

Plate 4.20. Antibacterial activity of *D. lanceolaria* - Left to right: The photographs for test and control antibiotic disc diffusion results are presented above and below (top and bottom row) from left to right in the following order - *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, *S. flexneri*, *S. typhimurium* and *E. coli*. The respective test and control combinations are arranged above and below, for the corresponding test organisms as per the given order.

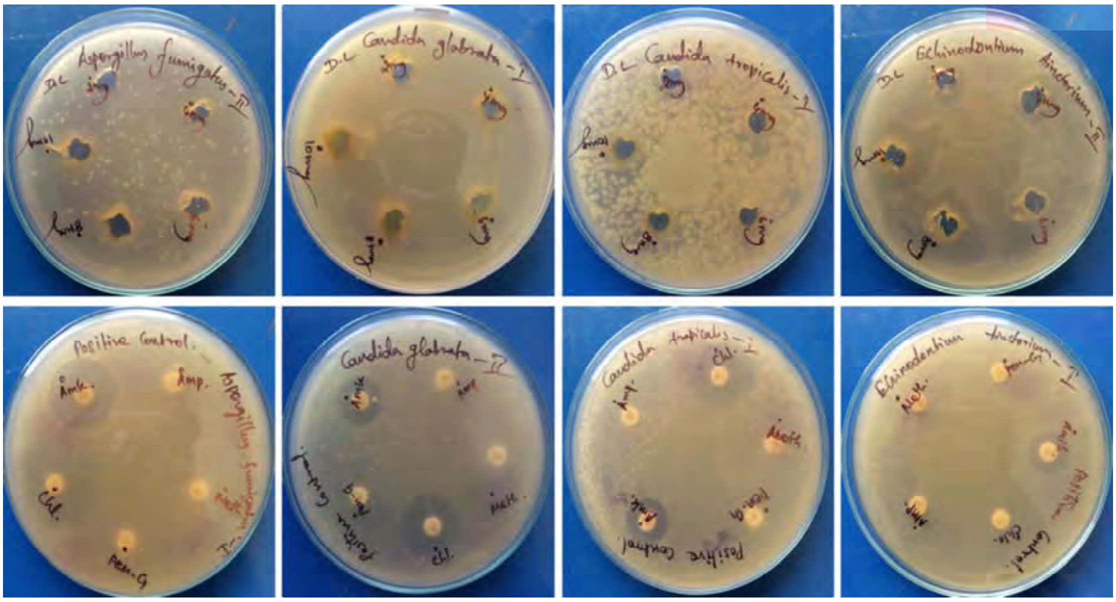

Plate 4.21. Antifungal activity of *D. lanceolaria* – The petridishes shown above indicate zones of inhibitions for *D. lanceolaria* extract at different concentrations (and their respective controls/standard antibiotics) at the bottom of the respective test plates.

Table 4.8. Antibacterial activity of *D. lanceolaria*

Antibiotic discs (µg)	Plant extract (mg/mL)	*Pseudomonas aeruginosa* (mm)	*Staphylococcus aureus* (mm)	*Klebsiella pneumoniae* (mm)	*Shigella flexneri* (mm)	*Salmonella typhimurium* (mm)	*Escherichia coli* (mm)
-	6	5 ± 0.1	6 ± 0.1	NE	NE	6 ± 0.3	5 ± 0.2
-	7	6 ± 0.1	7 ± 0.1	NE	NE	7 ± 0.4	6 ± 0.5
-	8	7 ± 0.4	7 ± 0.4	NE	NE	7 ± 0.4	7 ± 0.6
-	9	7 ± 0.6	8 ± 0.6	NE	NE	8 ± 0.3	7 ± 0.6
-	10	7 ± 0.6	8 ± 0.5	NE	NE	8 ± 0.3	7 ± 0.6
Nitrofurantoin (10)	-	8 ± 1.2	14 ± 1.3	8 ± 1.4	9 ± 1.9	2 ± 1.0	12 ± 0.9
Vancomycin (10)	-	9 ± 1.3	11 ± 1.4	NE	NE	10 ± 1.5	NE
Gentamicin (10)	-	16 ± 1.4	17 ± 1.6	12 ± 1.5	11 ± 0.9	17 ± 1.6	5 ± 1.2
Bacitracin (10)	-	7 ± 1.3	13 ± 1.6	NE	NE	5 ± 1.0	7 ± 1.0
Amoxyclav (30)	-	18 ± 1.4	22 ± 1.7	17 ± 1.4	2 ± 0.5	22 ± 1.7	13 ± 2.1
Methicillin (5)	-	NE	NE	NE	NE	NE	NE

N.E. - no effect detected; zones are represented as radius (mm).

Table 4.9. Antifungal activity of *D. lanceolaria*

Antibiotics (µg)	Plant extract (mg/mL)	*Aspergillus fumigatus* (mm)	*Candida glabrata* (mm)	*Candida tropicalis* (mm)	*Echinodontium tinctorium* (mm)
-	2	N.E.	N.E.	N.E.	6 ± 0.2
-	4	N.E.	N.E.	N.E.	7 ± 0.2
-	6	N.E.	N.E.	N.E.	8 ± 0.2
-	8	N.E.	N.E.	N.E.	8 ± 0.2
-	10	N.E.	N.E.	N.E.	10 ± 1.0
Penicillin-G (10)	-	N.E.	N.E.	N.E.	N.E.
Methicillin (5)	-	NE	NE	NE	NE
Chloramphenicol (30)	-	9 ± 0.2	11 ± 0.1	11 ± 0.0	7 ± 0.1
Amikacin (30)	-	7 ± 0.1	8 ± 0.0	8 ± 0.0	6 ± 0.0
Ampicillin (10)	-	NE	NE	NE	NE

N.E. - no effect detected; zones are represented as radius (mm).

3. *H. mystax*

Against the different bacterial strains chosen for the well diffusion experiments, *H. mystax* crude extract was found to inhibit *P. aeruginosa*, *S. aureus*, *S. typhimurium* and *E. coli*, *K. pneumoniae* and *S. flexneri*atthe concentrations (6-10 mg/mL). The highest measured zone was 8 mm (i.e., 0.8 cm) with 10 mg/mL of the extract against *E. coli* (Plate 4.22 and Table 4.10). Against the fungal species, the extract was most effective against *E. tinctorium* (10 mm zone), but did not inhibit any of the other four test species (Plate 4.23 and Table 4.11). The apparent ambiguities in this discrepancy of the extract for these compounds in different strains are not evident, and there is no perceived mechanism of action that explicates these observed differences in efficacy.

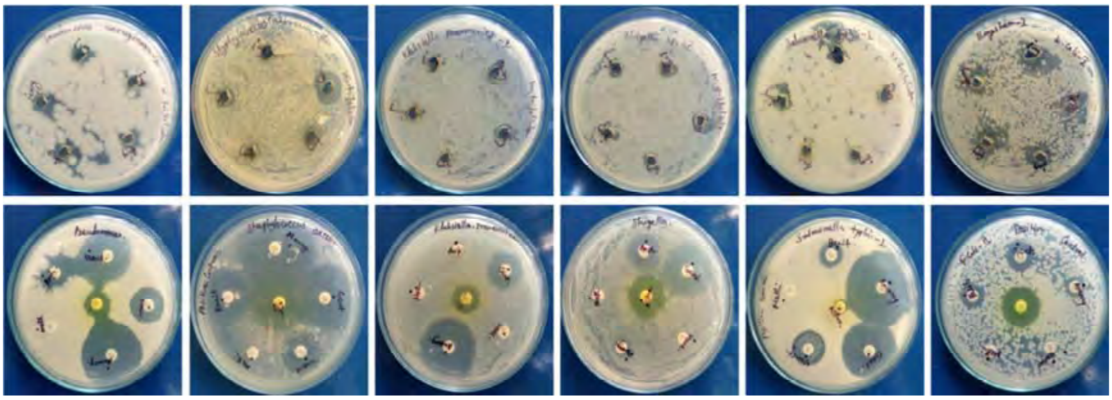

Plate 4.22. Antibacterial activity of *H. mystax* - Left to right: The photographs for test and control antibiotic disc diffusion results are presented above and below (top and bottom row) from left to right in the following order **-** *P. aeruginosa, S. aureus, K. pneumoniae, S. flexneri, S. typhimurium* and *E. coli.* The respective test and control combinations are arranged above and below, for the corresponding tests organisms as per the given order.

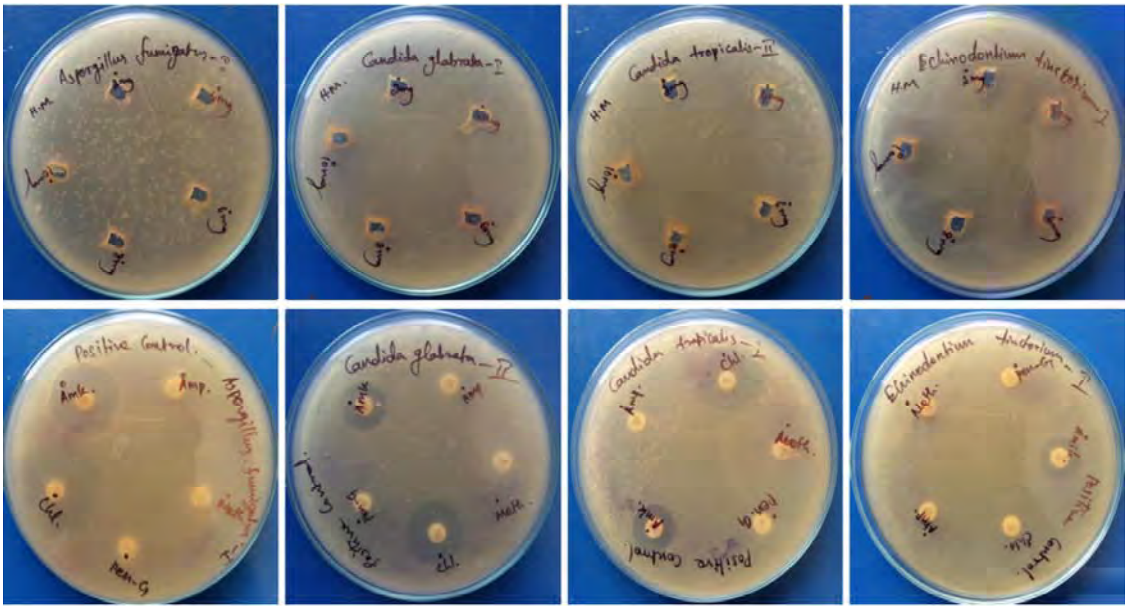

Plate 4.23. Antifungal activity of *H. mystax* - The petridishes shown above indicate zones of inhibitions for *H. mystax* extract at different concentrations (and their respective controls/standard antibiotics) at the bottom of the respective test plates.

Table 4.10. Antibacterial activity of *H. mystax*

Antibiotic discs (µg)	Plant extract (mg/mL)	*Pseudomonas aeruginosa* (mm)	*Staphylococcus aureus* (mm)	*Klebsiella pneumoniae* (mm)	*Shigella flexneri* (mm)	*Salmonella typhimurium* (mm)	*Escherichia coli* (mm)
-	6	5 ± 0.1	3 ± 0.3	2 ± 0.2	3 ± 0.2	2 ± 0.1	6 ± 0.2
-	7	5 ± 0.2	4 ± 0.3	2 ± 0.2	4 ± 0.2	3 ± 0.1	8 ± 0.2
-	8	6 ± 0.4	4 ± 0.2	3 ± 0.3	4 ± 0.2	4 ± 0.3	8 ± 0.5
-	9	7 ± 0.5	4 ± 0.3	4 ± 0.3	5 ± 0.3	5 ± 0.4	8 ± 0.7
-	10	7 ± 0.5	5 ± 0.3	4 ± 0.4	5 ± 0.3	5 ± 0.4	8 ± 0.7
Nitrofurantoin (10)	-	8 ± 1.2	14 ± 1.3	8 ± 1.4	9 ± 1.9	2 ± 1.0	12 ± 0.9
Vancomycin (10)	-	9 ± 1.3	11 ± 1.4	NE	NE	10 ± 1.5	NE
Gentamicin (10)	-	16 ± 1.4	17 ± 1.6	12 ± 1.5	11 ± 0.9	17 ± 1.6	5 ± 1.2
Bacitracin (10)	-	7 ± 1.3	13 ± 1.6	NE	NE	5 ± 1.0	7 ± 1.0
Amoxyclav (30)	-	18 ± 1.4	22 ± 1.7	17 ± 1.4	2 ± 0.5	22 ± 1.7	13 ± 2.1
Methicillin (5)	-	NE	NE	NE	NE	NE	NE

N.E. - no effect detected; zones are represented as radius (mm).

Table 4.11. Antifungal activity of *H. mystax*

Antibiotics (µg)	Plant extract (mg/mL)	*Aspergillus fumigatus* (mm)	*Candida glabrata* (mm)	*Candida tropicalis* (mm)	*Echinodontium tinctorium* (mm)
-	2	N.E.	N.E.	N.E.	7 ± 0.3
-	4	N.E.	N.E.	N.E.	8 ± 0.3
-	6	N.E.	N.E.	N.E.	8 ± 0.3
-	8	N.E.	N.E.	N.E.	9 ± 0.2
-	10	N.E.	N.E.	N.E.	10 ± 1.0
Penicillin-G (10)	-	N.E.	N.E.	N.E.	N.E.
Methicillin (5)	-	NE	NE	NE	NE
Chloramphenicol (30)	-	9 ± 0.2	11 ± 0.1	11 ± 0.0	7 ± 0.1
Amikacin (30)	-	7 ± 0.1	8 ± 0.0	8 ± 0.0	6 ± 0.0
Ampicillin (10)	-	NE	NE	NE	NE

N.E. - no effect detected; zones are represented as radius (mm).

4. *L. obtusifolia*

Amongst the bacterial strains which were studied to compare the efficacy of the crude MeOH extract of *L. obtusifolia* using well diffusion assay, the extract was found to inhibit *P. aeruginosa*, *S. aureus*, *S. typhimurium* and *E. coli*, at the concentrations of 6-10 mg/mL. However, no inhibitory effects were observed whatsoever against *K. pneumoniae* and *S. flexneri* at any of the concentrations used. The highest measured zone was 8 mm (i.e., 0.8 cm) with 10 mg/mL of the extract against *E. coli* (Plate 4.24 and Table 4.12). Against the fungal species, the extract was most effective against *E. tinctorium* (11 mm zone), but did not at all inhibit any of the other four test species (Plate 4.25 and Table 4.13). Once again, we can surmise that the apparent ambiguities in this discrepancy of the extract for these compounds in different strains are not evident and there is no perceived mechanism of action which explicates these observed differences in efficacy.

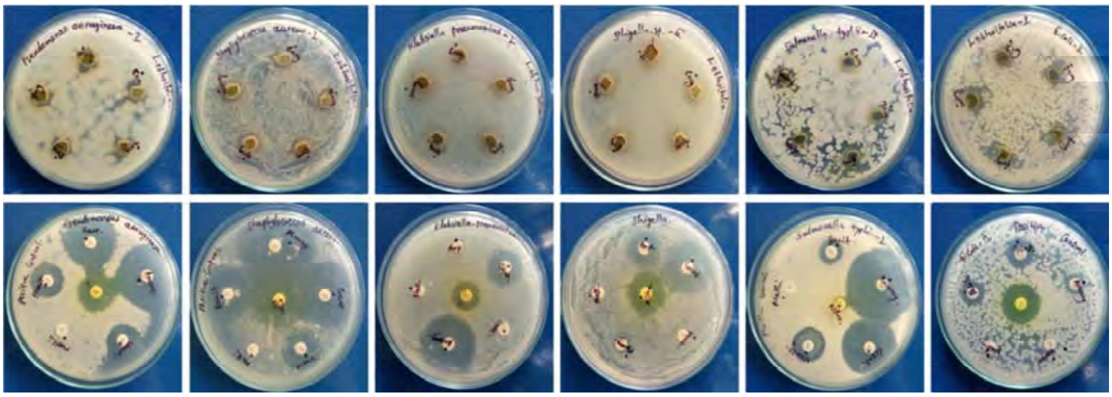

Plate 4.24. Antibacterial activity of *L. obtusifolia* - Left to right: The photographs for test and control antibiotic disc diffusion results are presented above and below (top and bottom row) from left to right in the following order - *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, *S. flexneri*, *S. typhimurium* and *E. coli.* The respective test and control combinations are arranged above and below, for the corresponding tests organisms as per the given order.

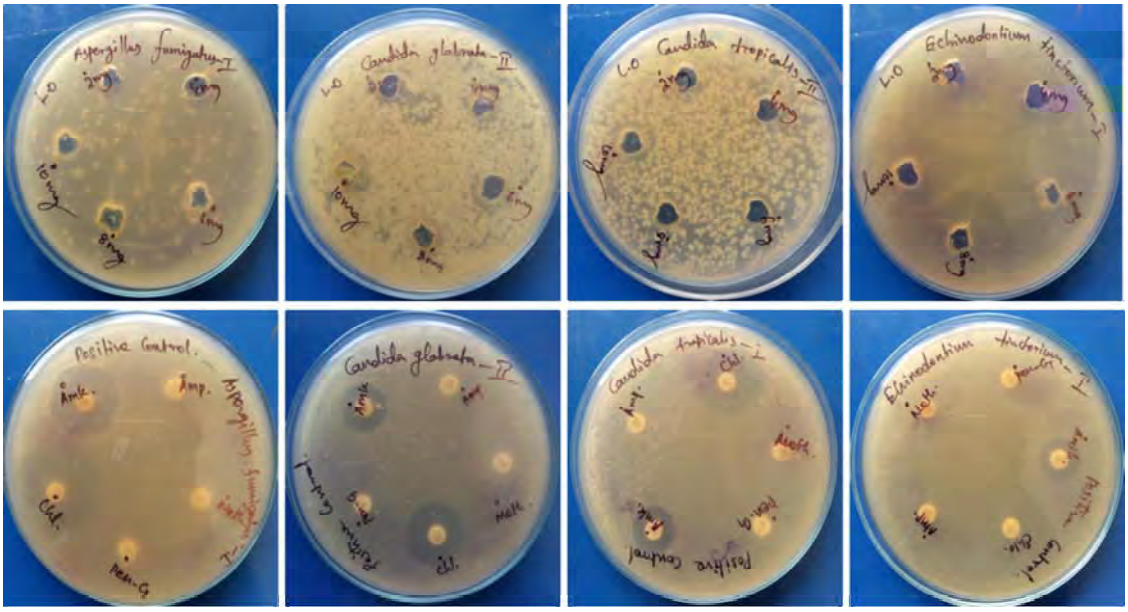

Plate 4.25. Antifungal activity of *L. obtusifolia* - The petridishes shown above indicate zones of inhibitions for *L. obtusifolia* extract at different concentrations (and their respective controls/standard antibiotics) at the bottom of the respective test plates.

Table 4.12. Antibacterial activity of *L. obtusifolia*

Antibiotic discs (µg)	Plant extract (mg/mL)	*Pseudomonas aeruginosa* (mm)	*Staphylococcus aureus* (mm)	*Klebsiella pneumoniae* (mm)	*Shigella flexneri* (mm)	*Salmonella typhimurium* (mm)	*Escherichia coli* (mm)
-	6	2 ± 0.1	2 ± 0.1	NE	NE	2 ± 0.1	5 ± 0.2
-	7	3 ± 0.1	3 ± 0.1	NE	NE	2 ± 0.1	6 ± 0.1
-	8	4 ± 0.4	4 ± 0.4	NE	NE	4 ± 0.3	7 ± 0.4
-	9	4 ± 0.6	5 ± 0.5	NE	NE	6 ± 0.5	7 ± 0.6
-	10	5 ± 0.6	5 ± 0.6	NE	NE	6 ± 0.5	8 ± 0.6
Nitrofurantoin (10)	-	8 ± 1.2	14 ± 1.3	8 ± 1.4	9 ± 1.9	2 ± 1.0	12 ± 0.9
Vancomycin (10)	-	9 ± 1.3	11 ± 1.4	NE	NE	10 ± 1.5	NE
Gentamicin (10)	-	16 ± 1.4	17 ± 1.6	12 ± 1.5	11 ± 0.9	17 ± 1.6	5 ± 1.2
Bacitracin (10)	-	7 ± 1.3	13 ± 1.6	NE	NE	5 ± 1.0	7 ± 1.0
Amoxyclav (30)	-	18 ± 1.4	22 ± 1.7	17 ± 1.4	2 ± 0.5	22 ± 1.7	13 ± 2.1
Methicillin (5)	-	NE	NE	NE	NE	NE	NE

N.E. - no effect detected; zones are represented as radius (mm).

Table 4.13. Antifungal activity of *L. obtusifolia*

Antibiotics (µg)	Plant extract (mg/mL)	*Aspergillus fumigatus* (mm)	*Candida glabrata* (mm)	*Candida tropicalis* (mm)	*Echinodontium tinctorium* (mm)
-	2	N.E.	N.E.	N.E.	7 ± 0.3
-	4	N.E.	N.E.	N.E.	8 ± 0.3
-	6	N.E.	N.E.	N.E.	8 ± 0.3
-	8	N.E.	N.E.	N.E.	9 ± 0.2
-	10	N.E.	N.E.	N.E.	11 ± 0.9
Penicillin-G (10)	-	N.E.	N.E.	N.E.	N.E.
Methicillin (5)	-	NE	NE	NE	NE
Chloramphenicol (30)	-	9 ± 0.2	11 ± 0.1	11 ± 0.0	7 ± 0.1
Amikacin (30)	-	7 ± 0.1	8 ± 0.0	8 ± 0.0	6 ± 0.0
Ampicillin (10)	-	NE	NE	NE	NE

N.E. - no effect detected; zones are represented as radius (mm).

5. *P. spinosum*

P. spinosum leaf extract was tested for inhibitory action against six bacterial strains and four fungal strains, and some commercially available antibiotic discs were used as positive controls, respectively. The highest zone of inhibition (9 ± 0.7 mm) was obtained against *P. aeruginosa* at the concentration of 10 mg/mL, which was equal to the zone obtained with 10 µg of Vancomycin (9 ± 1.3 mm). The other pertinent details of zones of inhibition against various strains (*P. aeruginosa*, *S. aureus*, *K. pneumoniae*, *S. flexneri*, *S. typhimurium* and *E. coli*) are provided in Table 4.14, and the petriplate photographs are displayed in Plate 4.26. Against the fungal strain of *E. tinctorium*, the extract (2 mg/mL) exhibited 8 ± 0.2 mm zone of inhibition (Plate 4.27 and Table 4.15). However, the zones obtained were several orders of magnitude lesser than those which were yielded by the standard antibiotics. It is important to note that the purified antimicrobial phytochemicals would generally possess substantial antimicrobial activity compared with the crude extracts.

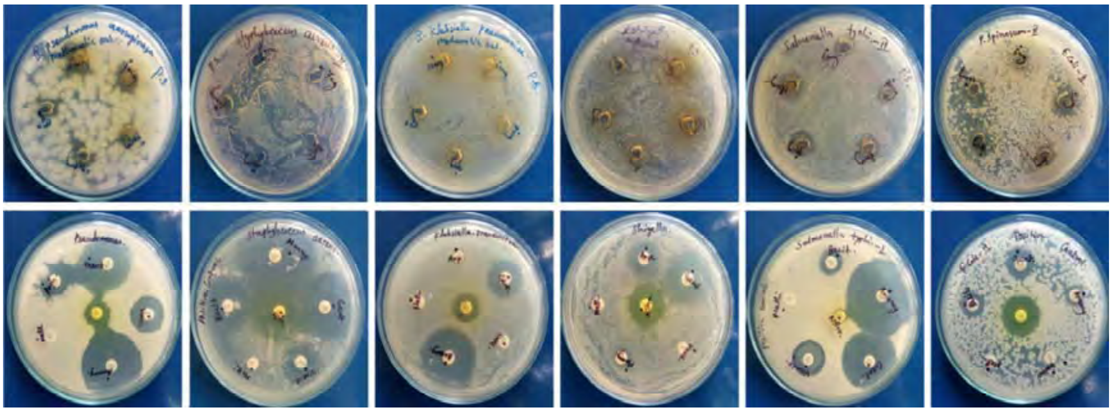

Plate 4.26. Antibacterial activity of *P. spinosum* - Left to right: The photographs for test and control antibiotic disc diffusion results are presented above and below (top and bottom row) from left to right in the following order - *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, *S. flexneri*, *S. typhimurium* and *E. coli.* The respective test and control combinations are arranged above and below, for the corresponding tests organisms as per the given order.

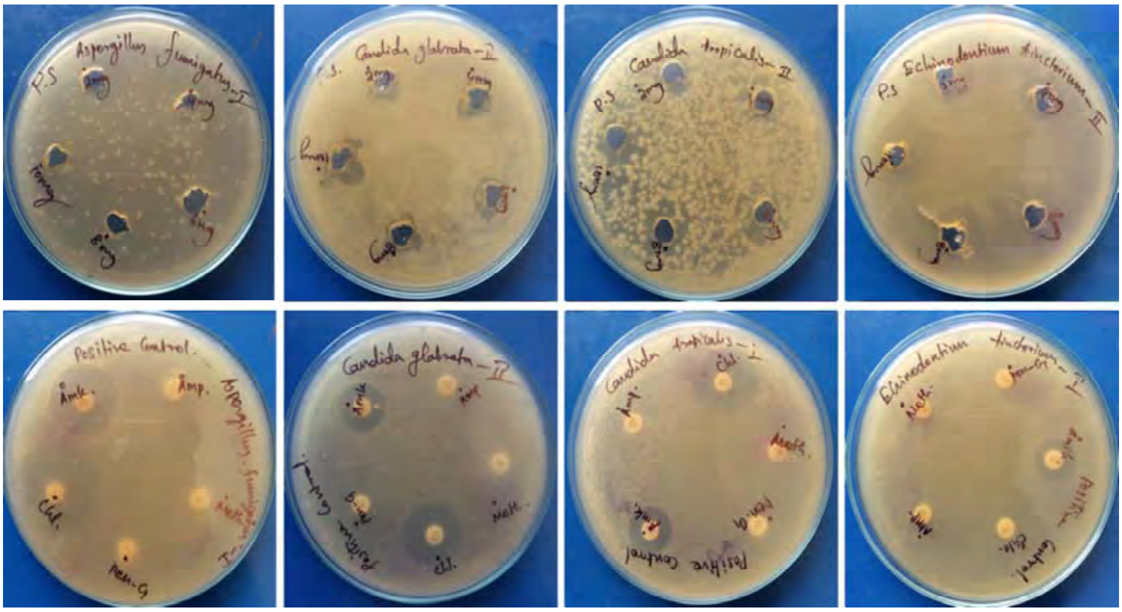

Plate 4.27. Antifungal activity of *P. spinosum* - The petridishes shown above indicate zones of inhibitions for *P. spinosum* extract at different concentrations (and their respective controls/standard antibiotics) at the bottom of the respective test plates.

Table 4.14. Antibacterial activity of *P. spinosum*

Antibiotic discs (µg)	Plant extract (mg/mL)	*Pseudomonas aeruginosa* (mm)	*Staphylococcus aureus* (mm)	*Klebsiella pneumoniae* (mm)	*Shigella flexneri* (mm)	*Salmonella typhimurium* (mm)	*Escherichia coli* (mm)
-	6	6 ± 2.3	3 ± 2.3	NE	NE	6 ± 0.7	2 ± 1.9
-	7	7 ± 1.0	4 ± 1.1	NE	NE	6 ± 0.4	3 ± 1.1
-	8	8 ± 0.5	4 ± 1.5	NE	NE	7 ± 0.9	4 ± 0.5
-	9	8 ± 0.7	5 ± 2.4	NE	NE	8 ± 1.6	5 ± 1.7
-	10	9 ± 0.7	6 ± 0.7	NE	NE	8 ± 0.5	7 ± 0.6
Nitrofurantoin (10)	-	8 ± 1.2	14 ± 1.3	8 ± 1.4	9 ± 1.9	2 ± 1.0	12 ± 0.9
Vancomycin (10)	-	9 ± 1.3	11 ± 1.4	NE	NE	10 ± 1.5	9 ± 2.0
Gentamicin (10)	-	16 ± 1.4	17 ± 1.6	12 ± 1.5	11 ± 0.9	17 ± 1.6	5 ± 1.2
Bacitracin (10)	-	7 ± 1.3	13 ± 1.6	NE	NE	5 ± 1.0	7 ± 1.0
Amoxyclav (30)	-	18 ± 1.4	22 ± 1.7	17 ± 1.4	2 ± 0.5	22 ± 1.7	13 ± 2.1
Methicillin (5)	-	NE	NE	NE	NE	NE	NE

N.E. - no effect detected; zones are represented as radius (mm).

Table 4.15. Antifungal activity of *P. spinosum*

Antibiotics (µg)	Plant extract (mg/mL)	*Aspergillus fumigatus* (mm)	*Candida glabrata* (mm)	*Candida tropicalis* (mm)	*Echinodontium tinctorium* (mm)
-	2	N.E.	N.E.	N.E.	8 ± 0.2
-	4	N.E.	N.E.	N.E.	8 ± 0.3
-	6	N.E.	N.E.	N.E.	9 ± 0.3
-	8	N.E.	N.E.	N.E.	10 ± 0.3
-	10	N.E.	N.E.	N.E.	12 ± 0.9
Penicillin-G (10)	-	N.E.	N.E.	N.E.	N.E.
Methicillin (5)	-	NE	NE	NE	NE
Chloramphenicol (30)	-	9 ± 0.2	11 ± 0.1	11 ± 0.4	7 ± 0.1
Amikacin (30)	-	7 ± 0.1	8 ± 0.3	8 ± 0.3	6 ± 0.2
Ampicillin (10)	-	NE	NE	NE	NE

N.E. - no effect detected; zones are represented as radius (mm).

6. *W. trifoliata*

Amongst the bacterial strains, the *W. trifoliata* extract was found to inhibit all of the six bacterial strains, viz., *P. aeruginosa*, *S. aureus*, *S. typhimurium* and *E. coli*, *K. pneumoniae* and *S. flexneri* at a concentration range of 6-10 mg/mL. The highest measured zone was 10 mm (i.e., 1.0 cm) with 10 mg/mL of the extract against *E. coli* (Plate 4.28 and Table 4.16 above). Against the fungal species, the extract was most effective against *E. tinctorium*, but did not inhibit any of the other fungi. Comparatively, the control antibiotic chloramphenicol was effective against - *C. tropicalis* and *C. glabrata* (11 mm zone each), and inhibited the other two fungal species, i.e., *E. tinctorium* and *A. fumigatus* (Plate 4.29 and Table 4.17). Once again, we can surmise that the apparent ambiguities in this discrepancy of the extract for these compounds in different strains are not evident and there is no perceived mechanism of action that explicates these observed differences in efficacy.

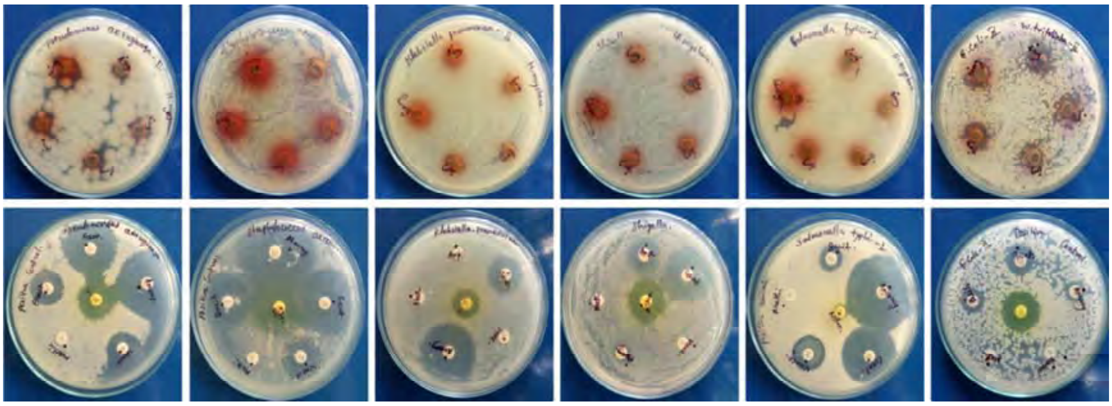

Plate 4.28. Antibacterial activity of *W. trifoliata* - Left to right: The photographs for test and control antibiotic disc diffusion results are presented above and below (top and bottom row) from left to right in the following order **-** *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, *S. flexneri*, *S. typhimurium* and *E. coli.* The respective test and control combinations are arranged above and below, for the corresponding tests organisms as per the given order.

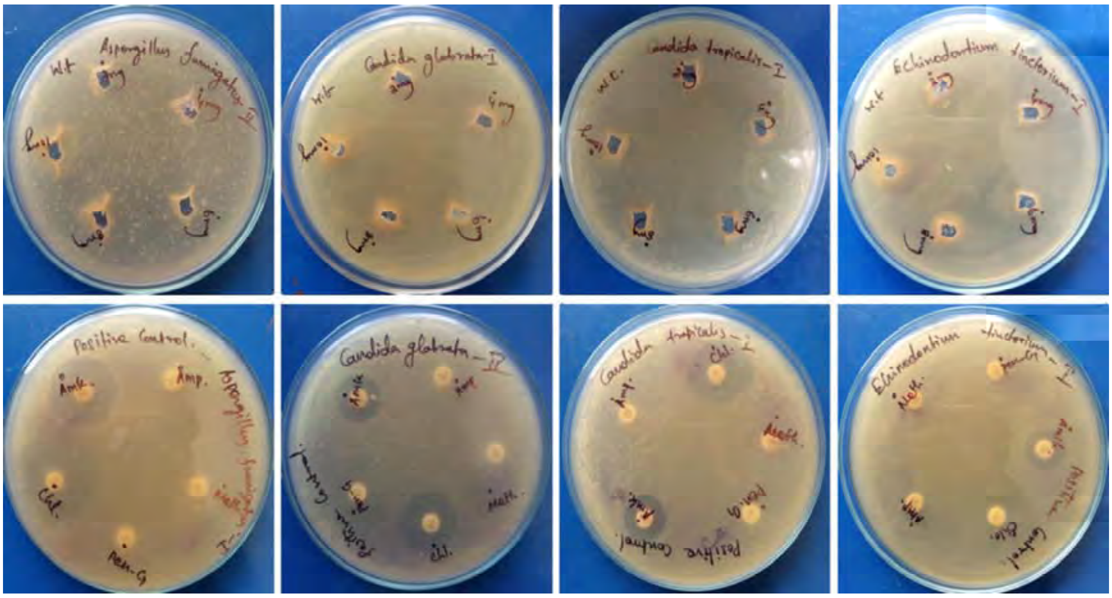

Plate 4.29. Antifungal activity of *W. trifoliata* - The petridishes shown above indicate zones of inhibitions for *W. trifoliata* extract at different concentrations (and their respective controls/standard antibiotics) at the bottom of the respective test plates.

Table 4.16. Antibacterial activity of *W. trifoliata*

Antibiotic discs (µg)	Plant extract (mg/mL)	*Pseudomonas aeruginosa* (mm)	*Staphylococcus aureus* (mm)	*Klebsiella pneumoniae* (mm)	*Shigella flexneri* (mm)	*Salmonella typhimurium* (mm)	*Escherichia coli* (mm)
-	6	5 ± 0.2	4 ± 0.1	5 ± 0.2	4 ± 0.1	4 ± 0.1	8 ± 0.2
-	7	6 ± 0.2	5 ± 0.1	5 ± 0.2	5 ± 0.1	4 ± 0.1	8 ± 0.3
-	8	6 ± 0.4	6 ± 0.4	5 ± 0.3	5 ± 0.3	4 ± 0.3	10 ± 0.6
-	9	7 ± 0.6	7 ± 0.5	6 ± 0.4	6 ± 0.5	5 ± 0.4	10 ± 0.8
-	10	8 ± 0.6	7 ± 0.5	7 ± 0.5	7 ± 0.5	6 ± 0.5	10 ± 0.8
Nitrofurantoin (10)	-	8 ± 1.2	14 ± 1.3	8 ± 1.4	9 ± 1.9	2 ± 1.0	12 ± 0.9
Vancomycin (10)	-	9 ± 1.3	11 ± 1.4	NE	NE	10 ± 1.5	NE
Gentamicin (10)	-	16 ± 1.4	17 ± 1.6	12 ± 1.5	11 ± 0.9	17 ± 1.6	5 ± 1.2
Bacitracin (10)	-	7 ± 1.3	13 ± 1.6	NE	NE	5 ± 1.0	7 ± 1.0
Amoxyclav (30)	-	18 ± 1.4	22 ± 1.7	17 ± 1.4	2 ± 0.5	22 ± 1.7	13 ± 2.1
Methicillin (5)	-	NE	NE	NE	NE	NE	NE

N.E. - no effect detected; zones are represented as radius (mm).

Table 4.17. Antifungal activity of *W. trifoliata*

Antibiotics (µg)	Plant extract (mg/mL)	*Aspergillus fumigatus* (mm)	*Candida glabrata* (mm)	*Candida tropicalis* (mm)	*Echinodontium tinctorium* (mm)
-	2	N.E.	N.E.	N.E.	6 ± 0.8
-	4	N.E.	N.E.	N.E.	7 ± 0.9
-	6	N.E.	N.E.	N.E.	7 ± 0.1
-	8	N.E.	N.E.	N.E.	8 ± 0.2
-	10	N.E.	N.E.	N.E.	9 ± 0.5
Penicillin-G (10)	-	N.E.	N.E.	N.E.	N.E.
Methicillin (5)	-	NE	NE	NE	NE
Chloramphenicol (30)	-	9 ± 0.2	11 ± 0.1	11 ± 0.0	7 ± 0.1
Amikacin (30)	-	7 ± 0.1	8 ± 0.0	8 ± 0.0	6 ± 0.0
Ampicillin (10)	-	NE	NE	NE	NE

N.E. - no effect detected; zones are represented as radius (mm).

4.9. Cytotoxicity

Cancer is becoming an increasingly significant burden to governmental healthcare agencies worldwide. While cancer treatment depends on the type and stage of cancer, radiation and chemotherapeutic regimen (and chemoradiotherapy, a combination of both) is the usual treatment modalities for both early and later stages of several cancers. Although monoclonal antibodies and another novel therapeutic regimen (alternative medicines) have been explored for cancer chemotherapy, radiation therapy still serves as an important avenue for treating several kinds of cancers. Plant-based compounds such as vinca alkaloids, etoposide and paclitaxel have been used for cancer chemotherapy, and the major classes of plant compounds that are known to possess anticancer activity are flavonoids polyphenols and brassinosteroids (Greenwell and Rahman, 2015). Plant compounds with anticancer activities (and their mechanism of action) have been reported in the literature (Li, W. *et al.*, 2017). Tumour heterogeneity, drug resistance, and the high cost of current medicinal approaches are some of the latest problems related to the successful management of breast cancer (Chakraborty *et al*., 2012). The importance of plant-derived compounds has been recognized by regulating cellular proliferation and separation. However, the phytopharmacological and physiological processes of cell death proffered by phytochemicals have recently been elucidated (Atanasov *et al*., 2015; Chen *et al*., 2019). Plants have been explored as major sources of clinically critical bioactive compounds such as taxol, camptothecin, vinblastine, vincristine, vindesine and vinflunine etc., which have been used against a variety of cancers (Akram *et al*., 2017; Eswaraiah *et al*., 2020b). Undesirable side effects of chemotherapy, drug efflux and high costs of breast cancer treatment have enhanced/increased the need to develop novel, safe, effective and cost affordable natural medicines. The results for cytotoxicity (cell culture images, MTT assay/IC_{50} graphs), are presented in the forthcoming pages in a plant-wise manner. The findings are discussed together for all the plant species after the data are presented.

1. *A. cymosa*

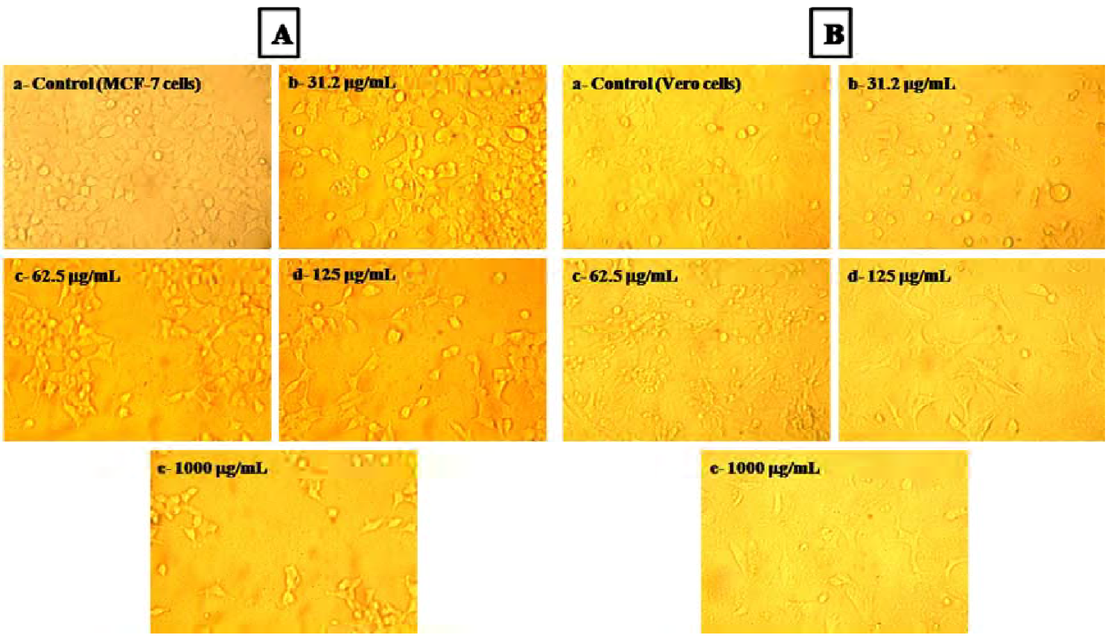

Plate 4.30. Cytotoxicity of *A. cymosa* on A. MCF-7 and B. Vero cells

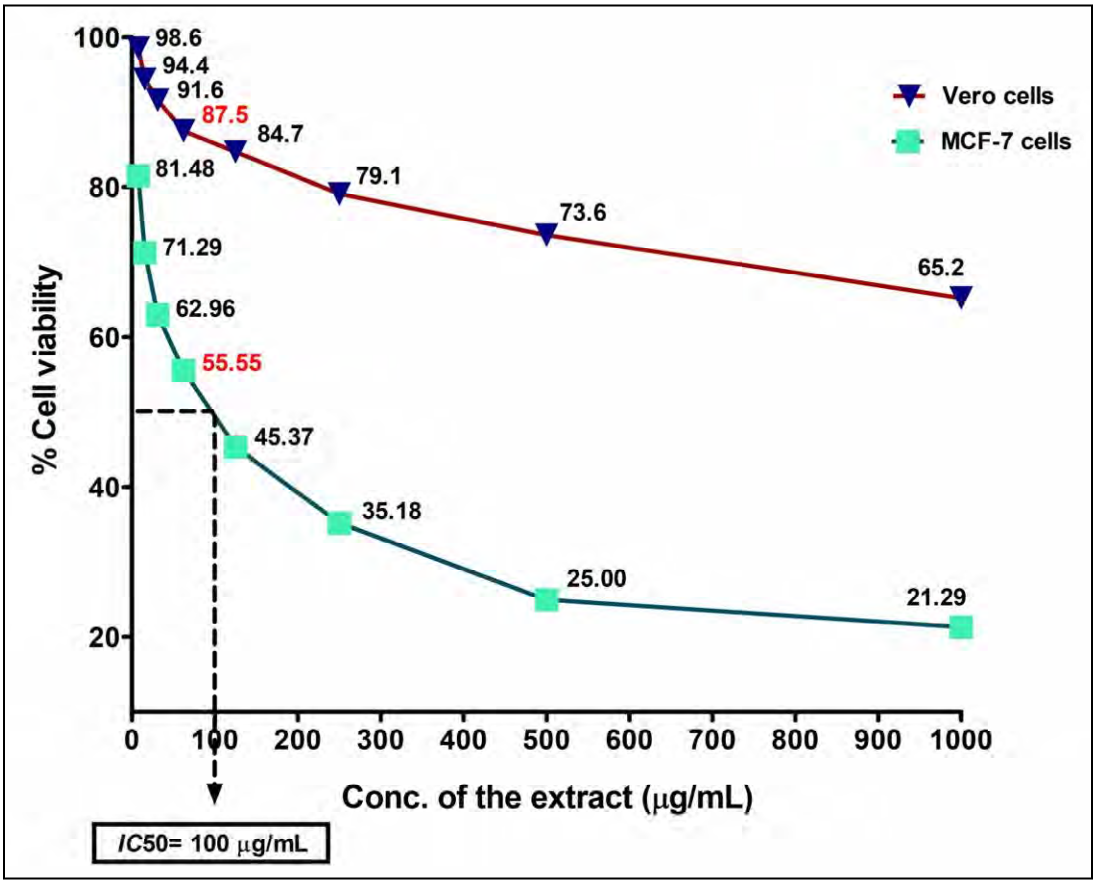

Figure 4.12. Cytotoxicity of *A. cymosa* on MCF-7 and Vero cell line

Table 4.18. Cytotoxic effects of *A. cymosa* Vero cells vs. MCF-7 cells

S. No.	Plant extract (µg/mL)	Dilutions	Absorbance (O.D)	Cell Viability (%)	
				MCF-7 cell line	Vero cell line
1	1000	Neat	0.23	21.29	65.2
2	500	1:1	0.27	25.00	73.6
3	250	1:2	0.38	35.18	79.1
4	125	1:4	0.49	45.37	84.7
5	62.5	1:8	0.60	55.55	87.5
6	31.2	1:16	0.68	62.96	91.6
7	15.6	1:32	0.77	71.29	94.4
8	7.8	1:64	0.88	81.48	98.6
9	Cell control	-	1.08	100	100

2. *D. lanceolaria*

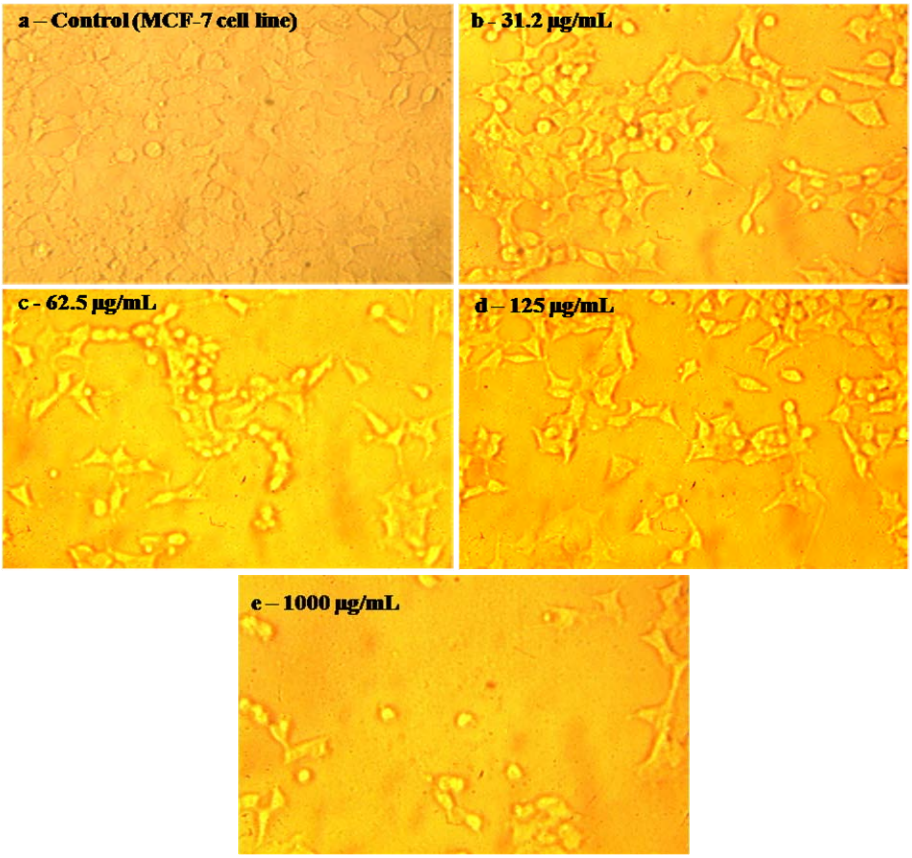

Plate 4.31. Cytotoxicity of *D. lanceolaria on* MCF-7 cells

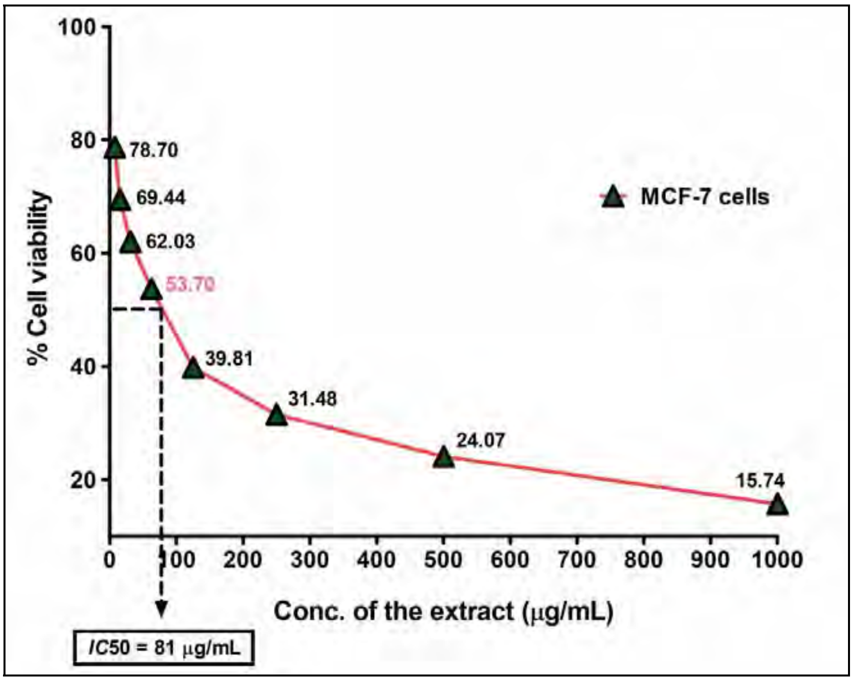

Figure 4.13. Cytotoxicity of *D. lanceolaria* on MCF-7 cell line

Table 4.19. Cytotoxic effects of *D. lanceolaria* MCF-7 cells

S. No.	Plant extract (µg/mL)	Dilutions	Absorbance (O.D)	Cell Viability (%)
1	1000	Neat	0.17	15.74
2	500	1:1	0.26	24.07
3	250	1:2	0.34	31.48
4	125	1:4	0.43	39.81
5	62.5	1:8	0.58	53.70
6	31.2	1:16	0.67	62.03
7	15.6	1:32	0.75	69.44
8	7.8	1:64	0.85	78.70
9	Cell control	-	1.08	100

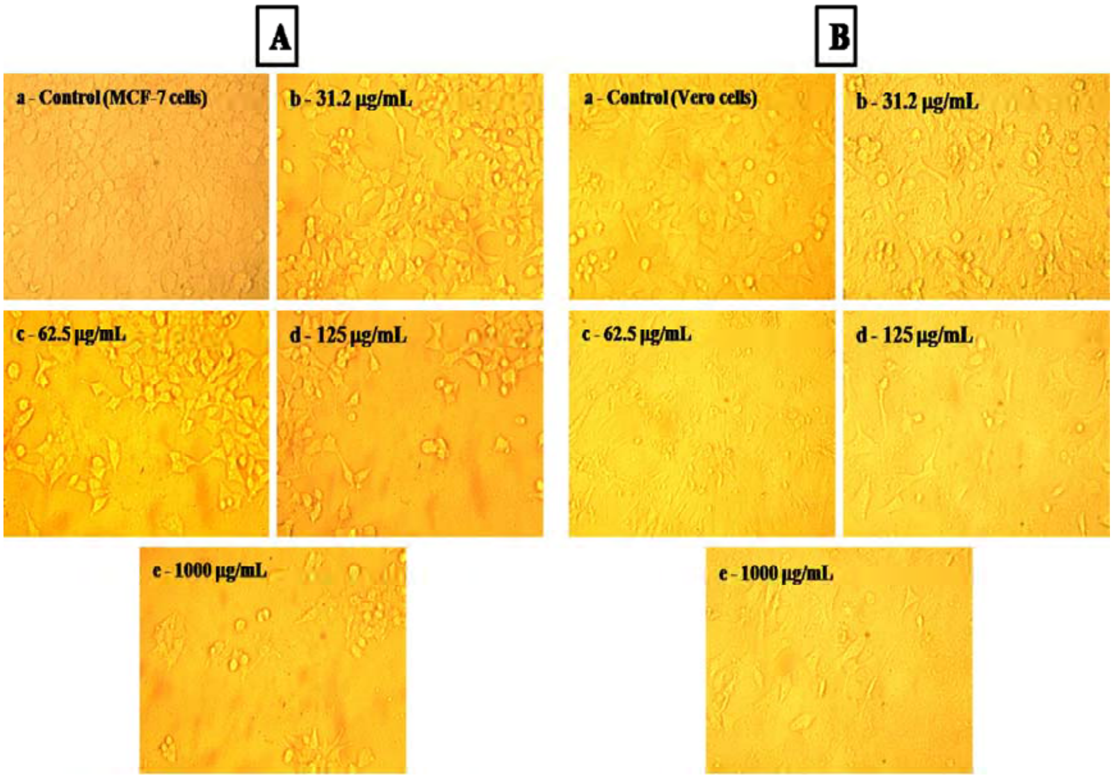

Plate 4.32. Cytotoxicity of *H. mystax* on A. MCF-7 and B. Vero cells

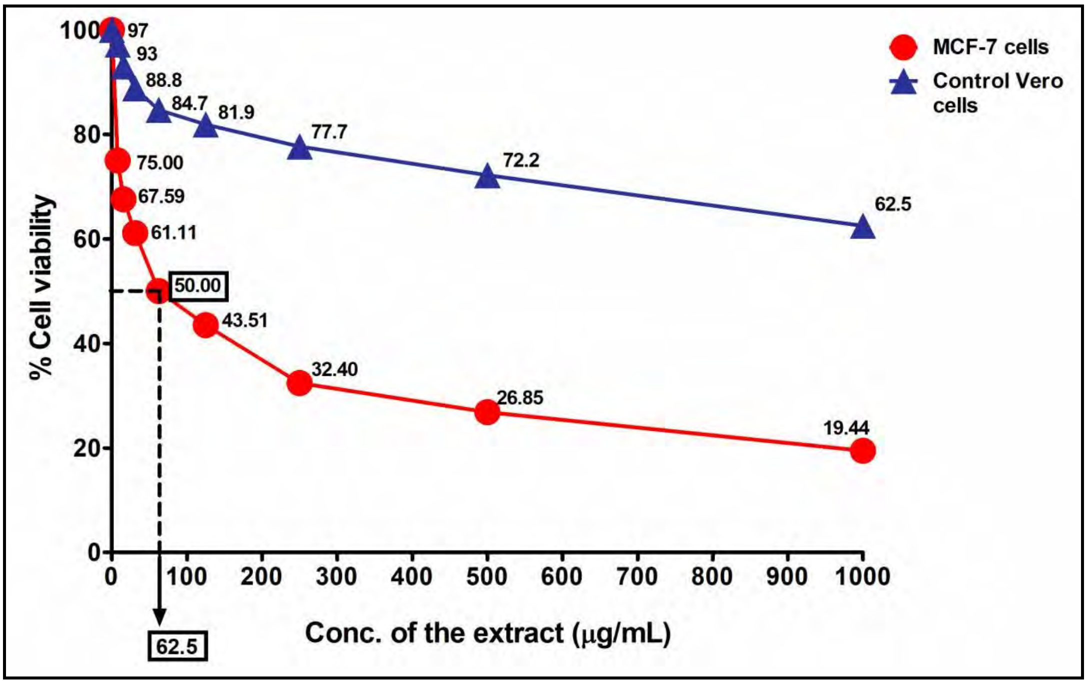

Figure 4.14. Cytotoxicity of *H. mystax* on MCF-7 and Vero cells

Table 4.20. Cytotoxic effects of *H. mystax* Vero cells vs. MCF-7 cells

S. No.	Plant extract (µg/mL)	Dilutions	Absorbance (O.D)	Cell Viability (%)	
				MCF-7 cell line	Vero cell line
1	1000	Neat	0.21	19.44	62.5
2	500	1:1	0.29	26.85	72.2
3	250	1:2	0.35	32.40	77.7
4	125	1:4	0.47	43.51	81.9
5	62.5	1:8	0.54	50.00	84.7
6	31.2	1:16	0.66	61.11	88.8
7	15.6	1:32	0.73	67.59	93.0
8	7.8	1:64	0.81	75.00	97.2
9	Cell control	-	1.08	100	100

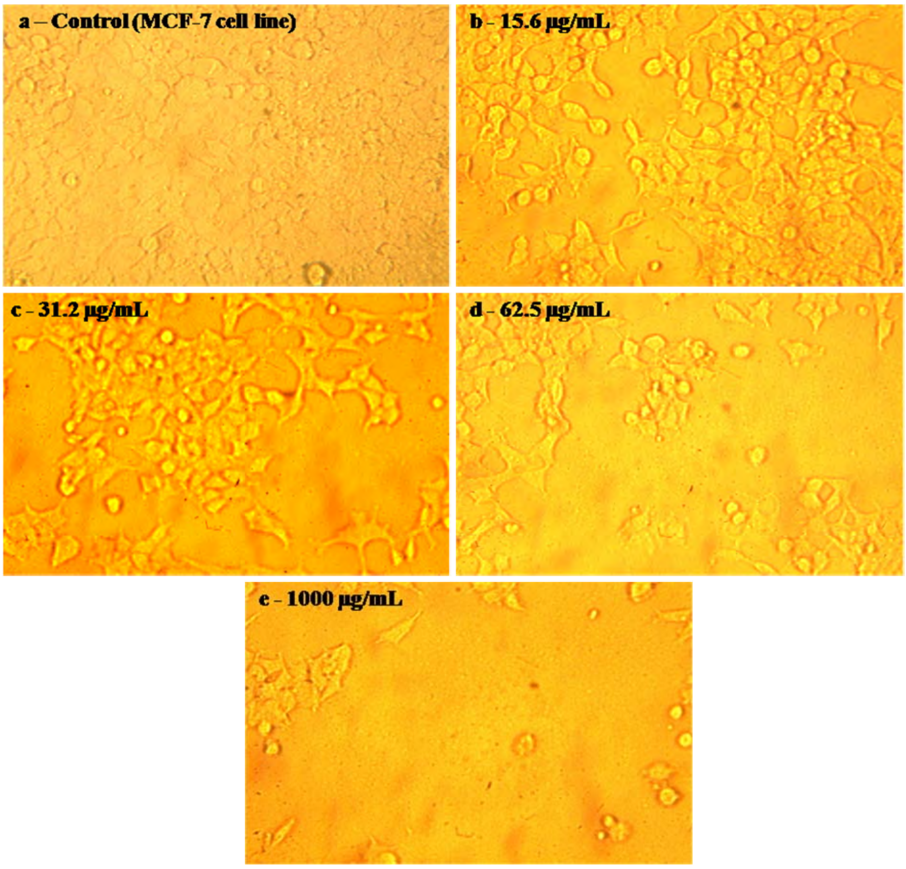

Plate 4.33. Cytotoxicity of *L. obtusifolia* on MCF-7 cells

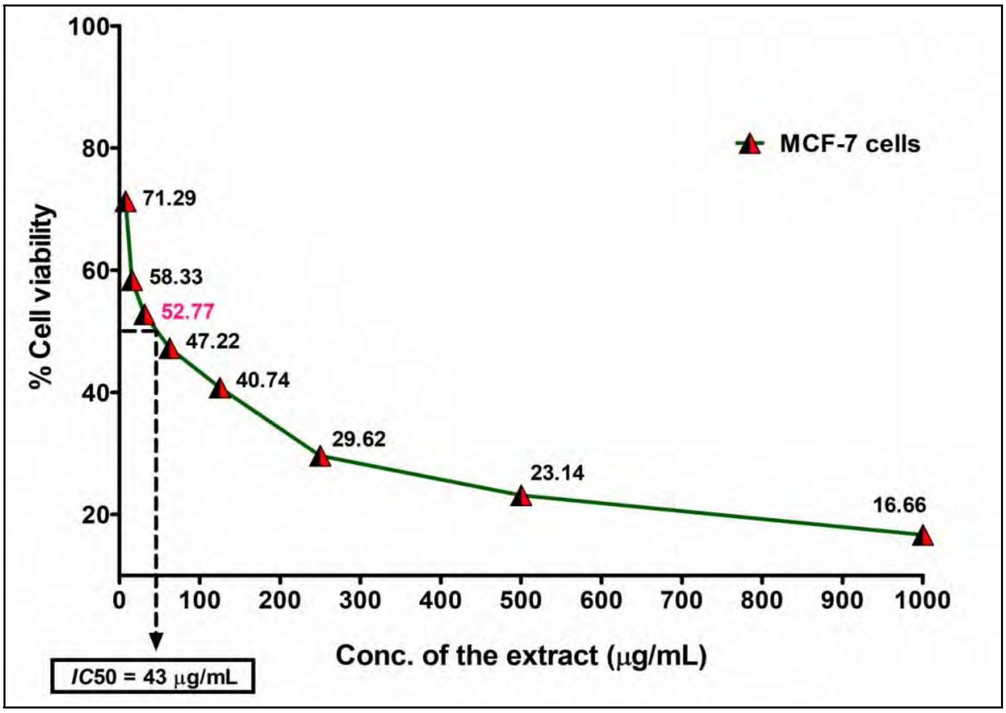

Figure 4.15. Cytotoxicity of *L. obtusifolia* on MCF-7 cells

Table 4.21.Cytotoxic effects of *L. obtusifolia* MCF-7 cells

S. No.	Plant extract (µg/mL)	Dilutions	Absorbance (O.D)	Cell Viability (%)
1	1000	Neat	0.18	16.66
2	500	1:1	0.25	23.14
3	250	1:2	0.32	29.62
4	125	1:4	0.44	40.74
5	62.5	1:8	0.51	47.22
6	31.2	1:16	0.57	52.77
7	15.6	1:32	0.63	58.33
8	7.8	1:64	0.77	71.29
9	Cell control	-	1.08	100

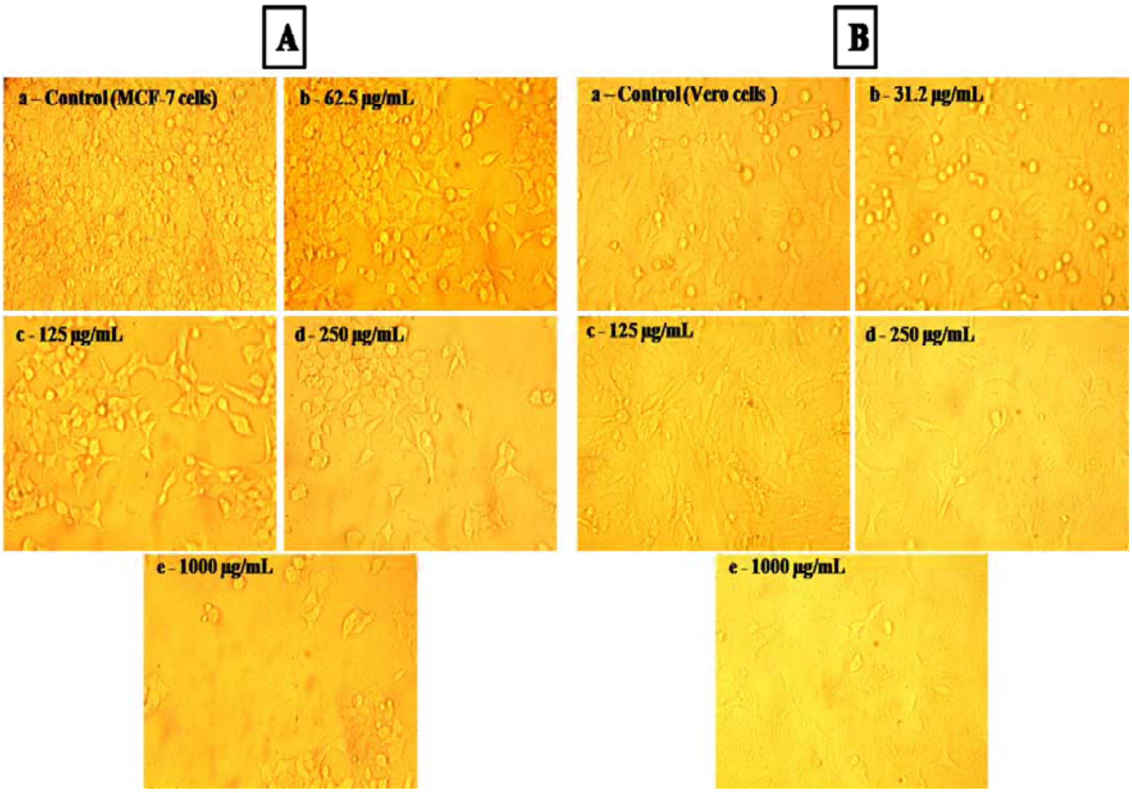

Plate 4.34. Cytotoxicity of *P. spinosum* on A. MCF-7 and B. Vero cells

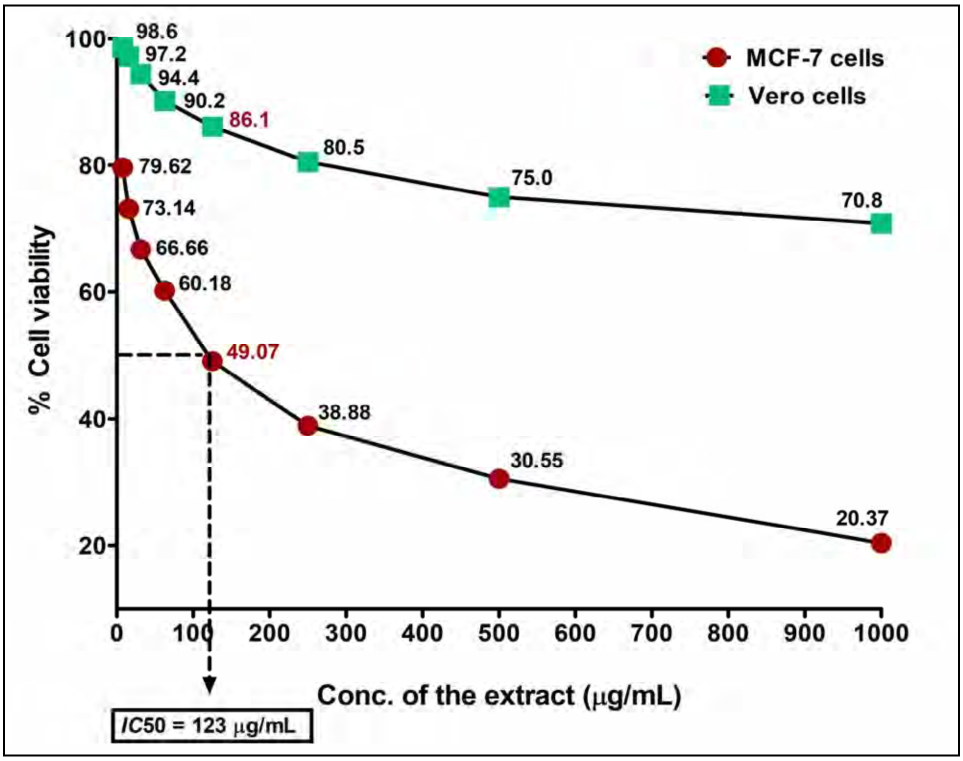

Figure 4.16. Cytotoxicity of *P. spinosum* on MCF-7 and Vero cells

Table 4.22.Cytotoxic effects of *P. spinosum* Vero cells vs. MCF-7 cells

S. No.	Plant extract (µg/mL)	Dilutions	Absorbance (O.D)	Cell Viability (%)	
				MCF-7 cell line	Vero cell line
1	1000	Neat	0.22	20.37	70.8
2	500	1:1	0.33	30.55	75.0
3	250	1:2	0.42	38.88	80.5
4	125	1:4	0.53	49.07	86.1
5	62.5	1:8	0.65	60.18	90.2
6	31.2	1:16	0.72	66.66	94.4
7	15.6	1:32	0.79	73.14	97.2
8	7.8	1:64	0.86	79.62	98.6
9	Cell control	-	1.08	100	100

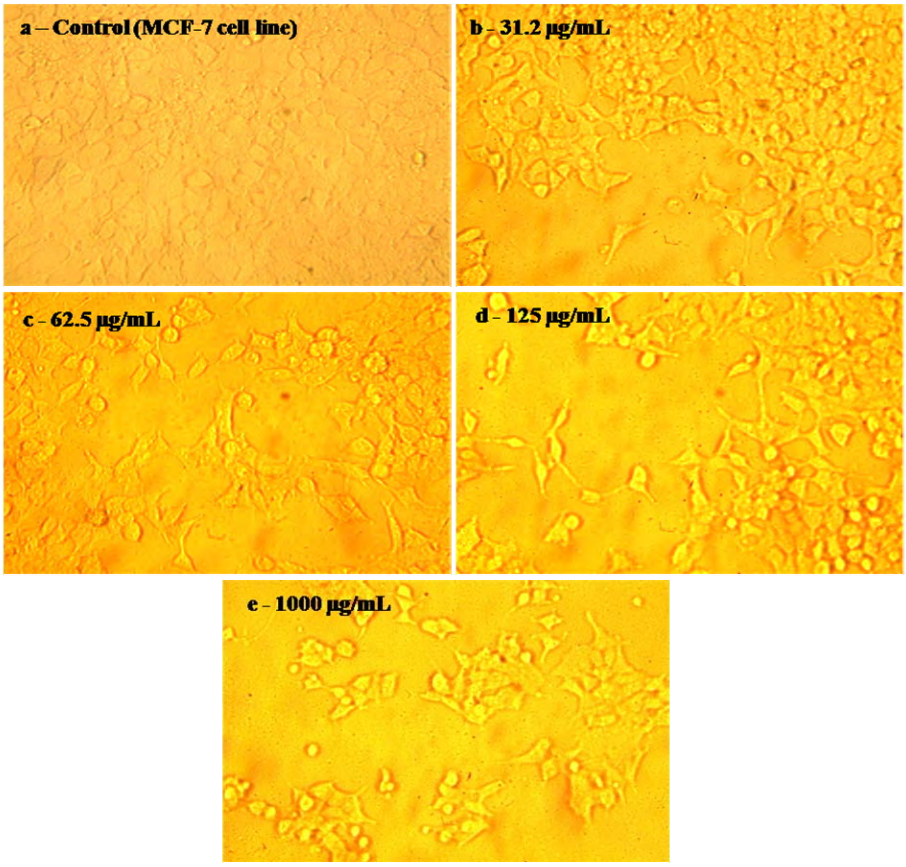

Plate 4.35. Cytotoxicity of *W. trifoliata* on MCF-7 cells

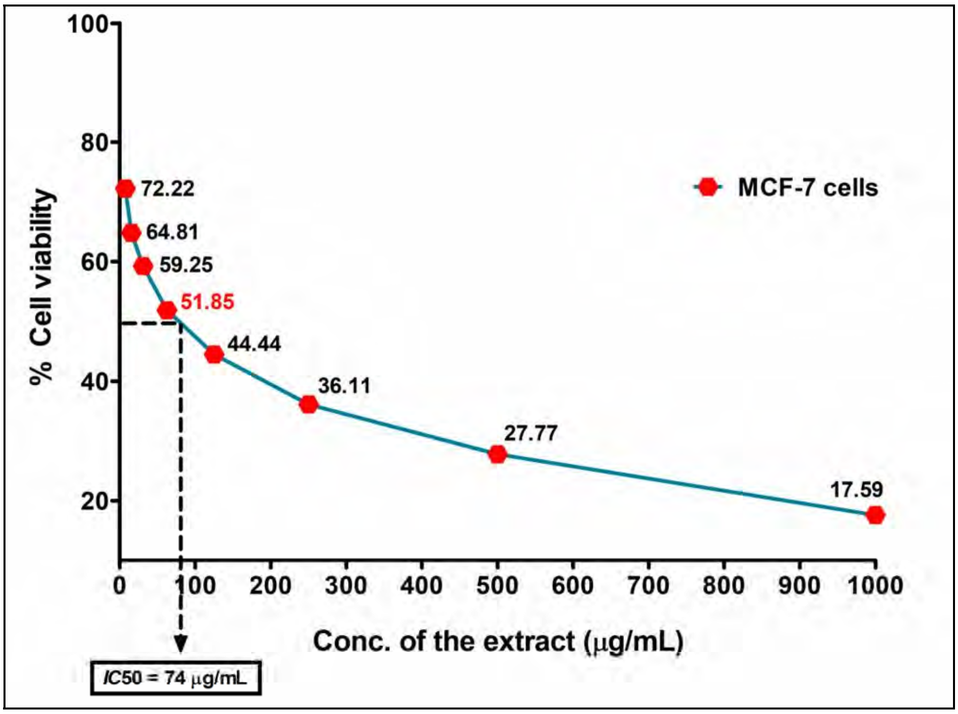

Figure 4.17. Cytotoxicity of *W. trifoliata* on MCF-7 cells

Table 4.23.Cytotoxic effects of *W. trifoliata* MCF-7 cells

S. No.	Plant extract (µg/mL)	Dilutions	Absorbance (O.D)	Cell Viability (%)
1	1000	Neat	0.19	17.59
2	500	1:1	0.30	27.77
3	250	1:2	0.39	36.11
4	125	1:4	0.48	44.44
5	62.5	1:8	0.56	**51.85**
6	31.2	1:16	0.64	59.25
7	15.6	1:32	0.70	64.81
8	7.8	1:64	0.78	72.22
9	Cell control	-	1.08	100

In this study, MTT assay was performed (Figure 4.12 and Table 4.18) to evaluate the anticancer potential (can be determined by cytotoxicity) of the crude MeOH leaf extract of *A. cymosa* and the IC_{50}values was found to be 100 µg/mL. The role of plant extract in MTT assay is given in the Figure 4.18. MCF-7 is a breast cancer cell line, and cancer cells are usually immortal; the plant extract was able to induce cell death, which is confirmed by the formation of insoluble purple formazan by metabolically active cells. Any cell death leads to lower product formation.

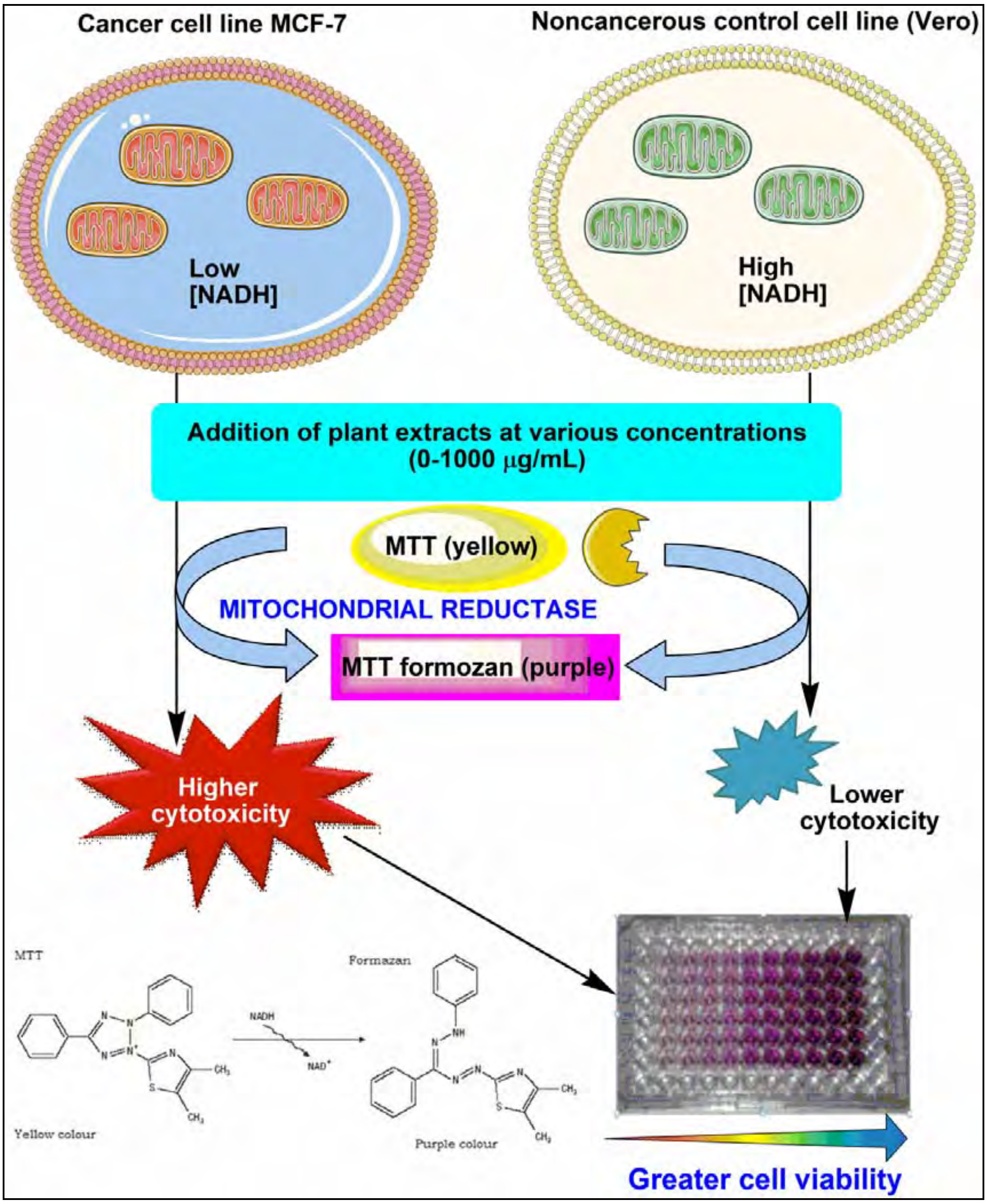

Figure 4.18. Role of plant extract in MTT assay to determine cytotoxicity: Contribution of cellular NADH and mitochondrial reductase

With a much lesser concentration of *A. cymosa* crude extract (62.5 μg/mL), around 55.5% cell viability was observed (Plate 4.30) and the highest cell death was observed with 1000 μg/mL (21.29%). Considering the other plants (*H. mystax*), a very similar cytotoxicity profile can be observed (Figure 4.14 and Table 4.20). The given image affords a simultaneous comparison of the cytotoxicity of all the plant extracts studied in this work. For very effective anticancer activity, much higher concentrations are required; however, considering that these are crude extracts, we may not have sufficient amounts of the anticancer compounds. The aim (in the near future) is to study each of the compounds identified through GC-MS to find novel or as yet novel anticancer compounds. Only after using these isolated compounds in cell lines and by comparing these compounds with known anticancer compounds we may arrive at a more meaningful conclusion to sufficiently claim that a said compound or compounds possess cancer-inhibiting properties (Kharwar *et al*., 2011).

The *H. mystax* leaf extract (dissolved in DMSO) was taken at a range of concentrations, and MTT assay was performed. In control (Vero cells), the cytotoxicity was much lower; with a very high concentration of 1000 μg/mL of the MeOH extract, the viability was still 62.5% (cell death of 37.5%) as shown in Plate 4.32. But, against the MCF-7 cells, the extract showed 50% killing (IC_{50}) at a much lower concentration of 62.5 μg/mL (Figure 4.14). For a range of concentrations (100-1000 μg/mL), the data have been compared, and at the same concentrations, the extract displayed differences in cytotoxicity in the two cell lines. This shows that the extract acts selectively against cancer cells. However, more studies are required to identify the potential mechanisms by which the compounds exert their toxic effects. Since 50% cell death was not attained against Vero cells even with 1000 μg/mL of plant extract, the IC_{50} may go well beyond this value for non-cancerous/control cells.

As of now, we find that these plants show anticancer activity because of the appreciable cytotoxic activities observed *in vitro*. There are still no clear indications of whether the observed anticancer activity is caused by a few or many compounds (synergistic effect) and among the host of possible permutations and combinations, the exact combinations and the relative concentration regimes thereof are elusive. Further investigations are needed to identify the same compound(s) and the mechanisms by which this effect is caused. Using MTT assay, the cytotoxic effect of the crude extracts of *P. spinosum* were evaluated by supplying the extract at various concentrations to MCF-7 cells, and an IC_{50} value of 123 μg/mL was obtained (Figure 4.16 and Table 4.22). However, other plant extracts yielded much lesser (and therefore, more promising) cytotoxic effects against the cancerous cell line. MCF-7 cells treated with an adequate concentration of *P. spinosum* leaf extract were found to cause shrinkages and chromatin condensation, indicating cytotoxic activity (Plate 4.34). Plant extract in MCF-7 cells induced cell death, which were resistant to apoptosis. While the exact mechanisms of cell death mediated by the plant extract were unclear, free radical formation sponsored by the bioactive compounds present in the extract could be the most probable cause. This is where the prooxidant capabilities of antioxidant phytochemicals become relevant (Halliwell, 2000). The phytochemical compounds detected using GC-MS are being explored for cellular biochemical targets (Eswaraiah *et al*., 2020a). While the plant extract yielded an IC_{50} of 125 μg/mL in MCF-7 cells, it exerted 14% cytotoxicity (cell viability of 86%) in Vero cells (Figure 4.16) at the same concentration (125 μg/mL). The cytotoxic effects of the other plant extracts and the relative differences in effects of these extracts *in vitro* are compared in Figures 4.13, 4.15 and 4.17; Plates 4.31, 4.33 and 4.35; Tables 4.19, 4.21 and 4.23.

Table 4.24. summarizes the exact IC_{50} values for each of the plant extracts and shows the comparison of the effects of a few of the plant extracts in control Vero cells vs MCF-7 cells. The toxicity obtained in Vero cells can be further minimized by

identifying and removing the active principle responsible for this cytotoxic effect if that compound has no relevant anti-cancer effects in MCF-7 cells. Also, because other phytochemical constituents may also contribute to the cytotoxicity observed in Vero cells, care must be taken to identify exactly which compounds mediate this effect. These results prove that the methanolic leaf extracts of the study plants are much more cytotoxic to MCF-7 cells when compared to control (Vero cell line). However, the degree of cytotoxicity can vary from plant to plant (Figure 4.19). While *A. cymosa* extract was > 10 times more effective against MCF-7 cells (w.r.t. control Vero cells), this fold difference in activity was lower (>8.13 times) when *P. spinosum* extract was studied and also much higher when *H. mystax* was tested in the two cell lines.

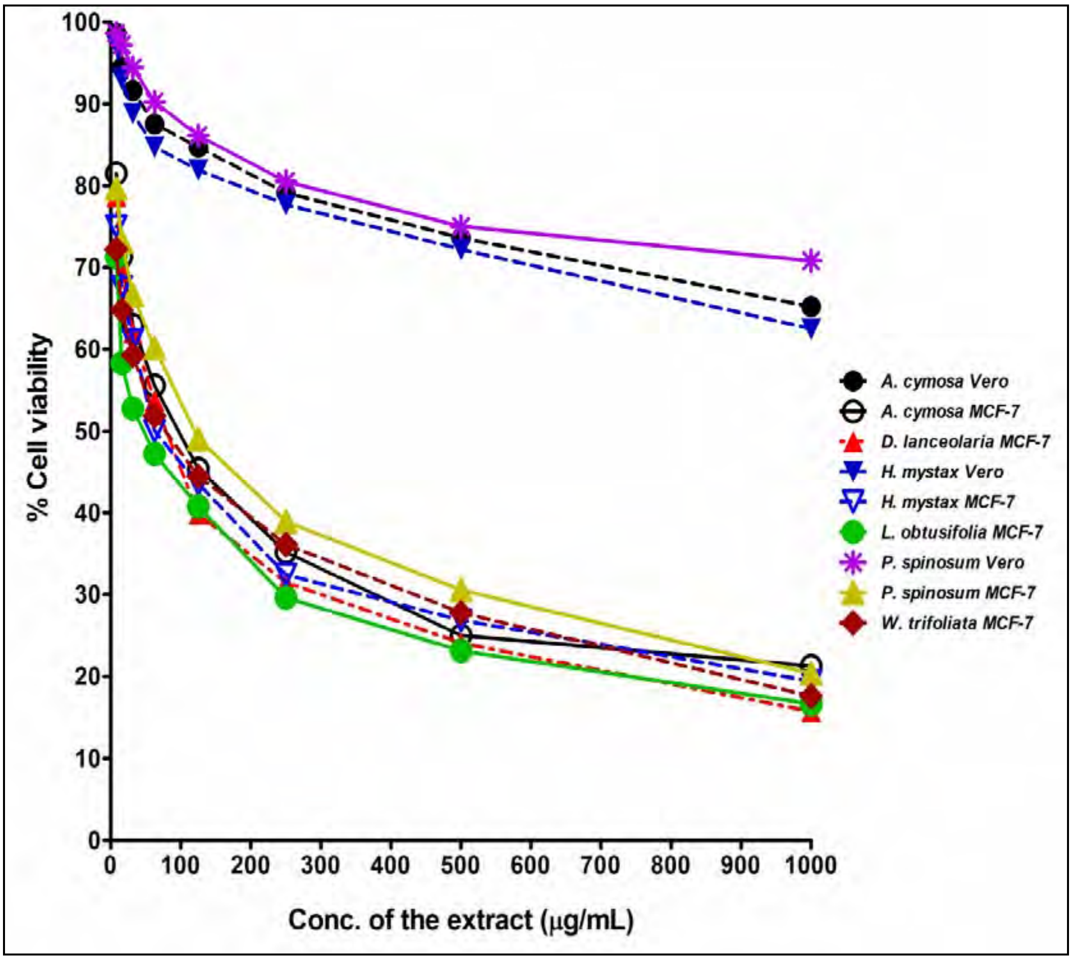

Figure 4.19. Comparision of cell viability studies (control Vero vs. MCF-7)

Table 4.24. Evaluation of the IC_{50} values for the six plant species in control vs. MCF-7 cells

Plant species	IC_{50} value (μg/mL) in MCF-7 cells	IC_{50} value (μg/mL) in control Vero cells	Fold difference in cytotoxicity between control vs. MCF-7 cells
A. cymosa	100	> 1000	>10
D. lanceolaria	81	n.d.	n.d.
H. mystax	61.5	> 1000	> 16.2
L. obtusifolia	43	n.d.	n.d.
P. spinosum	123	> 1000	> 8.13
W. trifoliata	74	n.d.	n.d.

4.10. Anti-Inflammatory Activity

Inflammation is characterized by phagocyte activation, leading to the discharge of chemical mediators such as prostaglandins, TNF-α and interleukins (Checker *et al.*, 2012). The leaky gut syndrome is known to be a leading cause of inflammation. Astonishingly, inflammation itself is the underlying cause of several diseases such as cardiovascular disease, cancer and arthritis. Escalation of oxidative stress-mediated tissue damage leads to vascular changes (Matés *et al.*, 1999). Oxidative stress causes inflammation, and thereby, several diseases take root. Oxidative stress causes macromolecular (DNA/RNA/protein/lipid) damage.

4.10.1. Lipid Peroxidation and Macromolecular Damage through Oxidative Stress

Damage to lipids directly causes membrane structure and disruption of transmembrane potentials of plasma membranes of cells and that of the endomembranes of cellular organelles. Lipid peroxidation is another key hallmark of inflammation and lipids are damaged by ROS to form lipid peroxidation products (lipid hydroperoxides) which are then cleaved to form secondary lipid peroxidation products such as malondialdehyde (MDA) and 2-hydroxynonenal (2-HNE), which are known to act in a dose dependent manner, to cause a range of effects. When MDA and HNE concentrations are low (physiological), these compounds cause anticancer effects (Pizzimenti *et al*., 2013). When the concentrations are slightly higher, there are changes in the cellular transcriptome and induction of the antioxidant stress response. With 'medium' levels of these secondary lipid peroxidation products, processes such as autophagy, senescence and cell cycle arrest are known to happen owing to contribution from protein adduction by lipid peroxidation products and hence, cells subsist under these conditions. Much higher lipid peroxide and secondary product concentrations stimulate adducts of DNA, RNA and proteins, effectively programming cells towards the pathogenesis of diseases; these concentrations of toxic aldehydes derived from polyunsaturated fatty acids (especially ω-6 PUFAs) are maintained in cancer progression, cancer signalling, angiogenesis, tumour development/growth and finally, metastasis. Even higher or abnormal concentrations of these products can trigger cell death (apoptosis) and cause tissue destruction through necrosis (D'Arcy, 2019). The combination of macromolecular damage (DNA base adductions, RNA base adduction, formation of protein adducts through Michael additions, depurination of DNA, etc.) can drive pathogenesis of cancer, diabetes and chronic inflammatory diseases. Hence, cancer and inflammation go together often and the two interconnected processes must be managed effectively. Therefore, it is necessary to search for the best molecules/drug candidates which can effectively block both cancer signalling events (and the associated epigenetic alterations as evidence by the altered cellular transcriptome), and block the primary "fire burning within" process of inflammation, which is the single most important process which downgrades the immune system (and causes greater production of pro-inflammatory cytokines) and thereby, reduces cell mediated immunity through dysregulation of cytotoxic T cells which are known to bind to and kill cancer cells. In traditional medicinal formulations, the primary goal is to lower inflammation in the gut and the critical organs. When inflammation is reduced, mental health and physiological processes are amended, causing significant improvement in overall physiology. Ayurveda mainly addresses inflammation as the primary root cause of diseases and pathophysiological manifestations (Chopra and Doiphode, 2002).

Plant formulations and hydroalcoholic extracts have the potential to improve digestive health and increase memory power. Antioxidants with anti-inflammatory activity were also reported (Bag *et al.*, 2013; Torres-Martínez *et al.*, 2017). Inflammation is a phenomenon in which circulating WBCs are recruited to a site of infection/ higher ROS levels, leading to oedema and erythema. It is interesting to note that most of the anti-inflammatory plant compounds are phenolics; phytochemicals can lower inflammation by exerting antioxidant properties (Zhu *et al.*, 2015). These compounds are essential for the alleviation of rheumatic diseases (and other chronic inflammatory diseases). In tandem with promising antioxidant and anticancer/cytotoxicity, compounds that also exert anti-inflammatory activity can be a big bonus in the race to discover promising therapeutics.

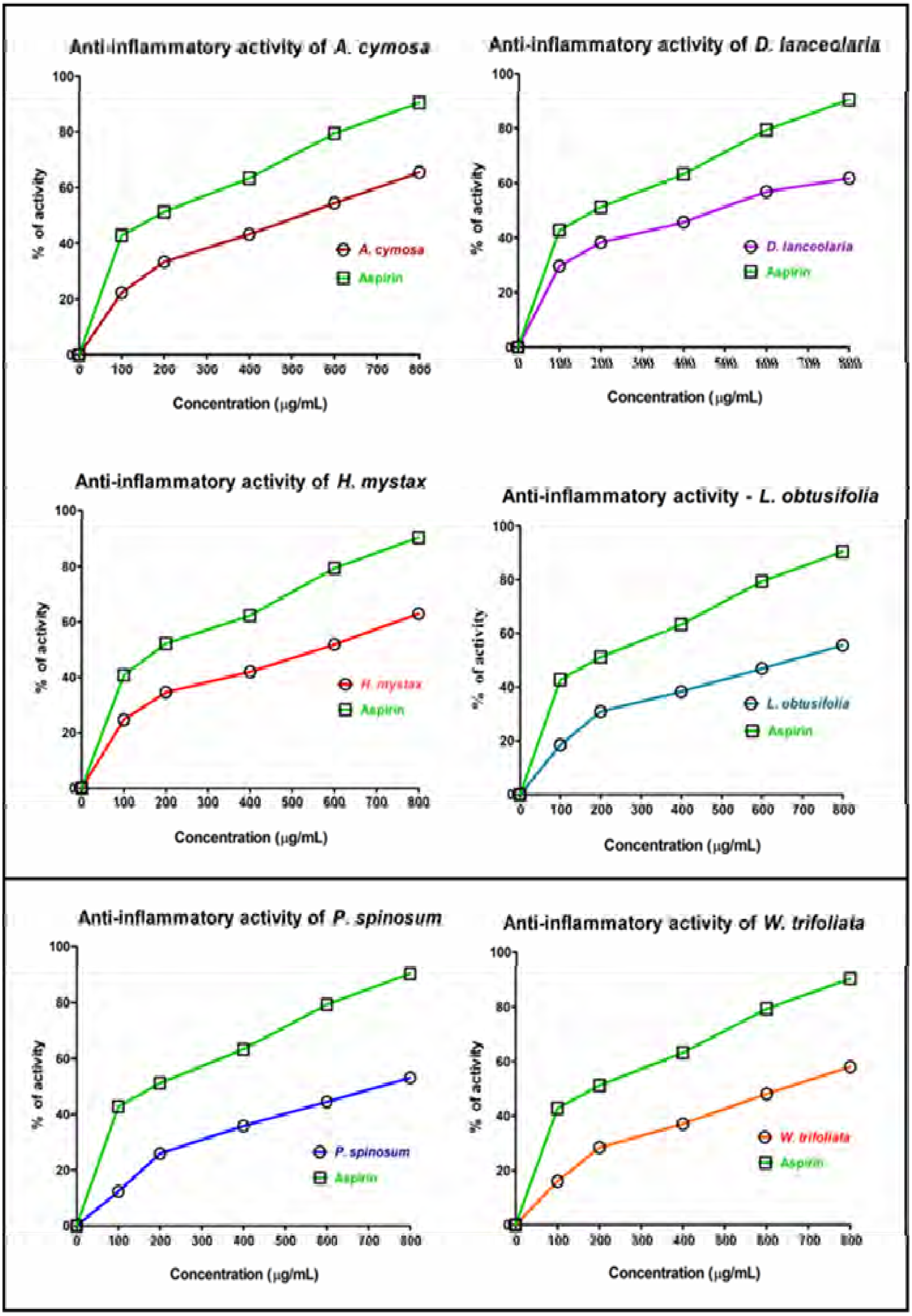

Figure 4.20. Comparison studies of anti-inflammatory activities

The ability of the plant extract to prevent RBC lysis was considered to be its anti-inflammatory activity (Figure 4.20). High levels of oxidative stress can lead to membrane lysis due to excessive lipid damage. When compared against aspirin control (which yielded 40-90% inhibition), the plant extract exhibited considerable anti-inflammatory activity in the range of 20-60% (both aspirin and plant extract samples were used at a concentration range of 100-800 μg/mL). A summary of the anti-inflammatory activities of the plant species studied in this work is provided based on the comparison of effects observed in Figure 4.21 below:

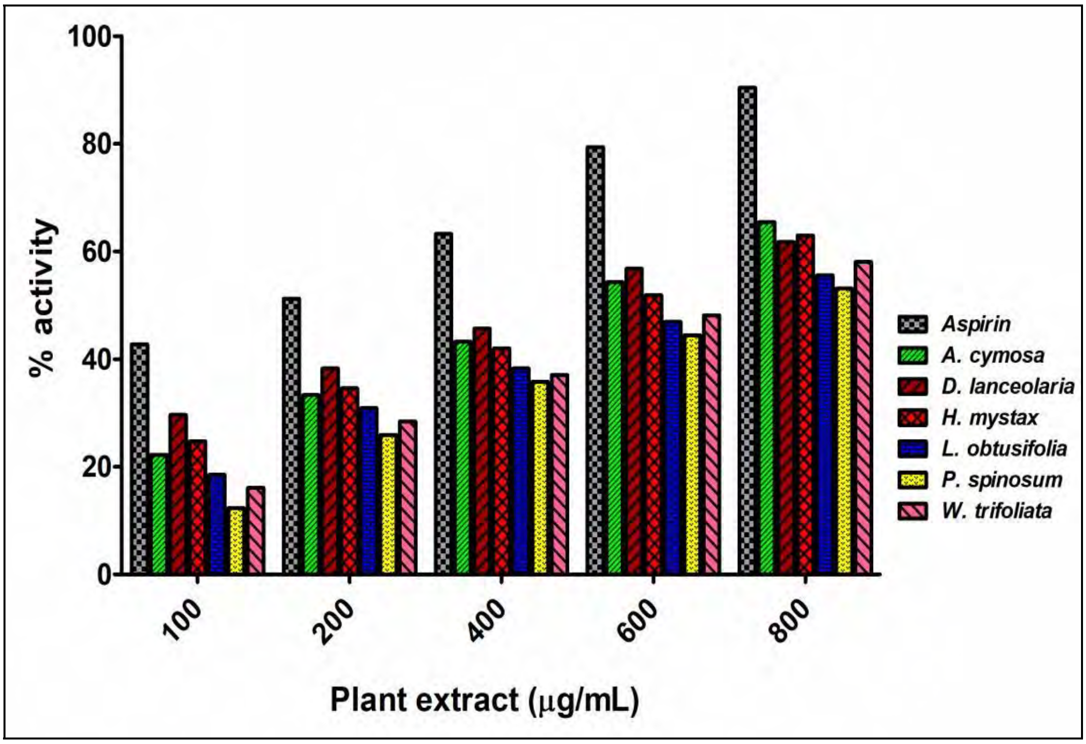

Figure 4.21. Summary of anti-inflammatory effects of six medicinal plants w.r.t aspirin control

Control aspirin was more effective against the plant extracts at all concentrations studied in this work (100, 200, 400, 600 and 800 μg/mL). At the same concentrations, the plant extracts were capable of emulating 50-60% of the activity (against each concentration of aspirin studied). The leaf extract of *P. spinosum* was studied for the anti-inflammatory effect through HRBCs membrane stabilization (inhibition of hemolysis) in comparison to aspirin (positive control) (Figure 4.20). The inhibition (%) was obtained in a dose-dependent manner. At high concentrations, the plant extract (800 μg/mL), 53.08 ± 1.5% inhibited membrane lysis. However, aspirin control exhibited very high (90.41 ± 1.3%) activity at the same concentration (800 μg/mL). There is a clear dose-response for the plant extracts (and aspirin control). The two best plant species (judging from the graph) could be *D. lanceolaria* and *A. cymosa*. However, there are no statistically significant differences in the effects (as evidenced by the absence of error bars in the experiments). Experiments with isolated and purified compounds are necessary to prove which compounds exert anti-inflammatory effects. The data shows that aspirin is a far better anti-hemolytic (and anti-inflammatory) agent than the plant extract. This is probably through the production of resolvins and other anti-inflammatory compounds through the oxidation of ω-6 fatty acids (Groeger *et al*., 2010). If the active principles responsible for this activity are purified and used in the assay, better efficacies can be obtained.

4.11. Qualitative and quantitative phytochemical analysis

It was evident that the methanolic extract possessed the most incredible variety of phytochemical classes (triterpenoids, sugars, saponins, tannins, sterols and carbohydrates) followed by ethanolic extracts (catechins), aqueous and chloroform extracts (anthraquinones) and chloroform (carbohydrates) (Figures 4.36 - 4.41 and Table 4.25). The other solvents were effective at extracting other phytochemical types. This was based on the relative polarities of the solvents and the affinities of the respective solvents for different plant compounds. Plant secondary metabolites are often unique and possess specific biological activities. Phenolic compounds and tannins possess antimicrobial properties (Appapalam and Panchamoorthy, 2017), and these compounds also are known to have bioactive principles such as triterpenoids which exhibit anticancer properties (Saleem, 2009). There is a close relationship between the phenolic content and the antioxidant activity of plants, and phenolics alone have diverse biological activities (Bag *et al*., 2013; Lü *et al*., 2010). Catechins are a group of flavonoids exhibiting several therapeutic activities against Parkinson's disease, Alzheimer's disease, type II diabetes and prion diseases. These molecules also possess anti-inflammatory, anti-allergic, anticancer, antiplatelet, antiviral and antioxidant activities etc (Koirala *et al*., 2016; Yang *et al*., 2018). Crozier *et al*. reported that the plant-derived saponins showed antimicrobial, anti-inflammatory and anticancer activities (Crozier *et al*., 2008). Previous studies demonstrated that the tannins exhibited antimicrobial, antimutagenic, anticarcinogenic, antidiarrhoeal and antiseptic properties (Bertram *et al*., 2013; Graça *et al*., 2016). Anthraquinone glycosides were found to be minimally distributed among the plant species. Phytosterols are helpful in lowering blood cholesterol levels and are relatively underused. Among the phytochemicals, phenols are a significant group of antioxidants due to their therapeutic potential and ability to inhibit free radical generation.

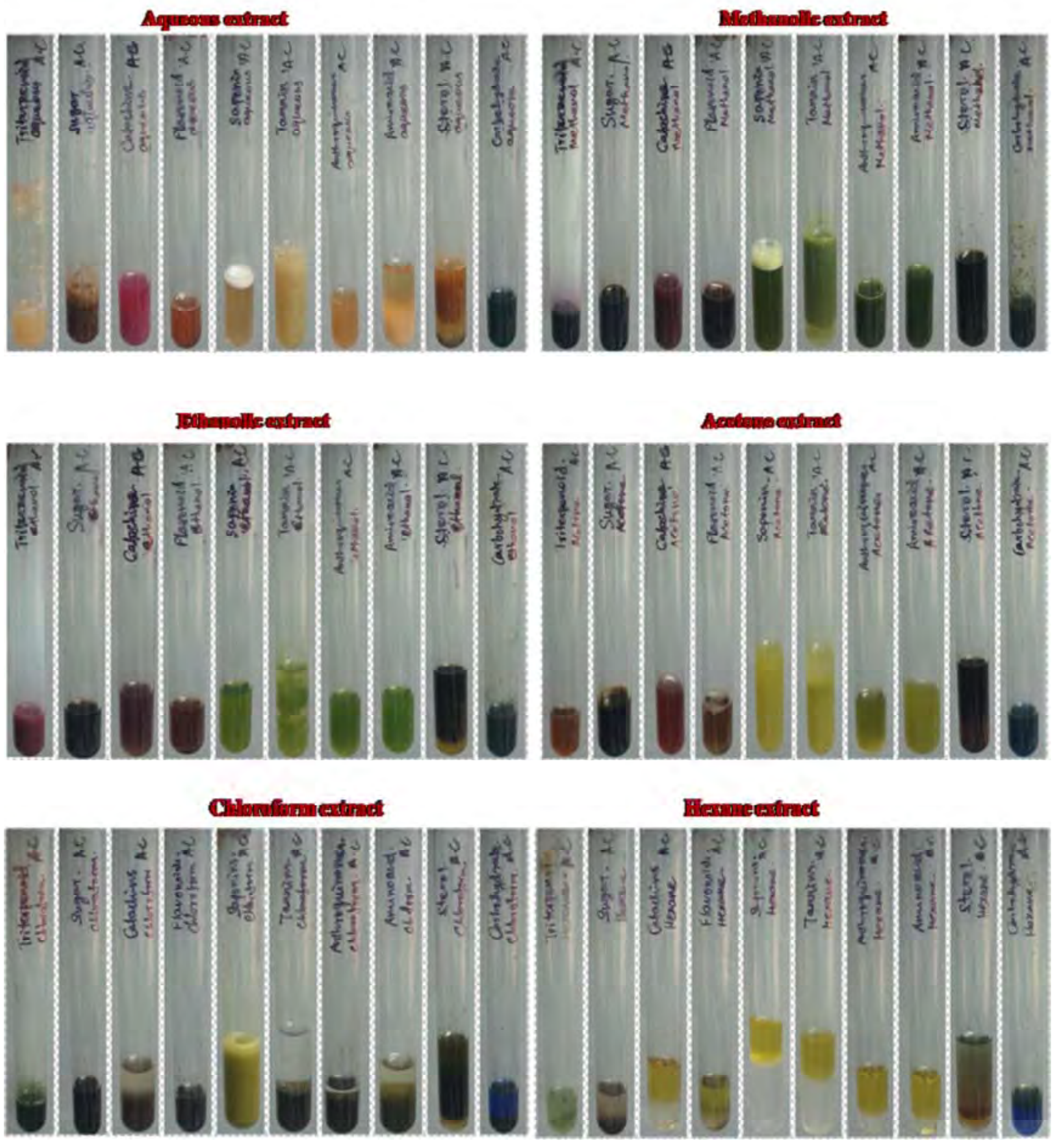

Plate 4.36. Qualitative analysis of *A. cymosa* leaf extract; from left to right: Triterpenoids, sugars, catechins, flavonoids, saponins, tannins, anthraquinones, aminoacids, steroids and carbohydrates.

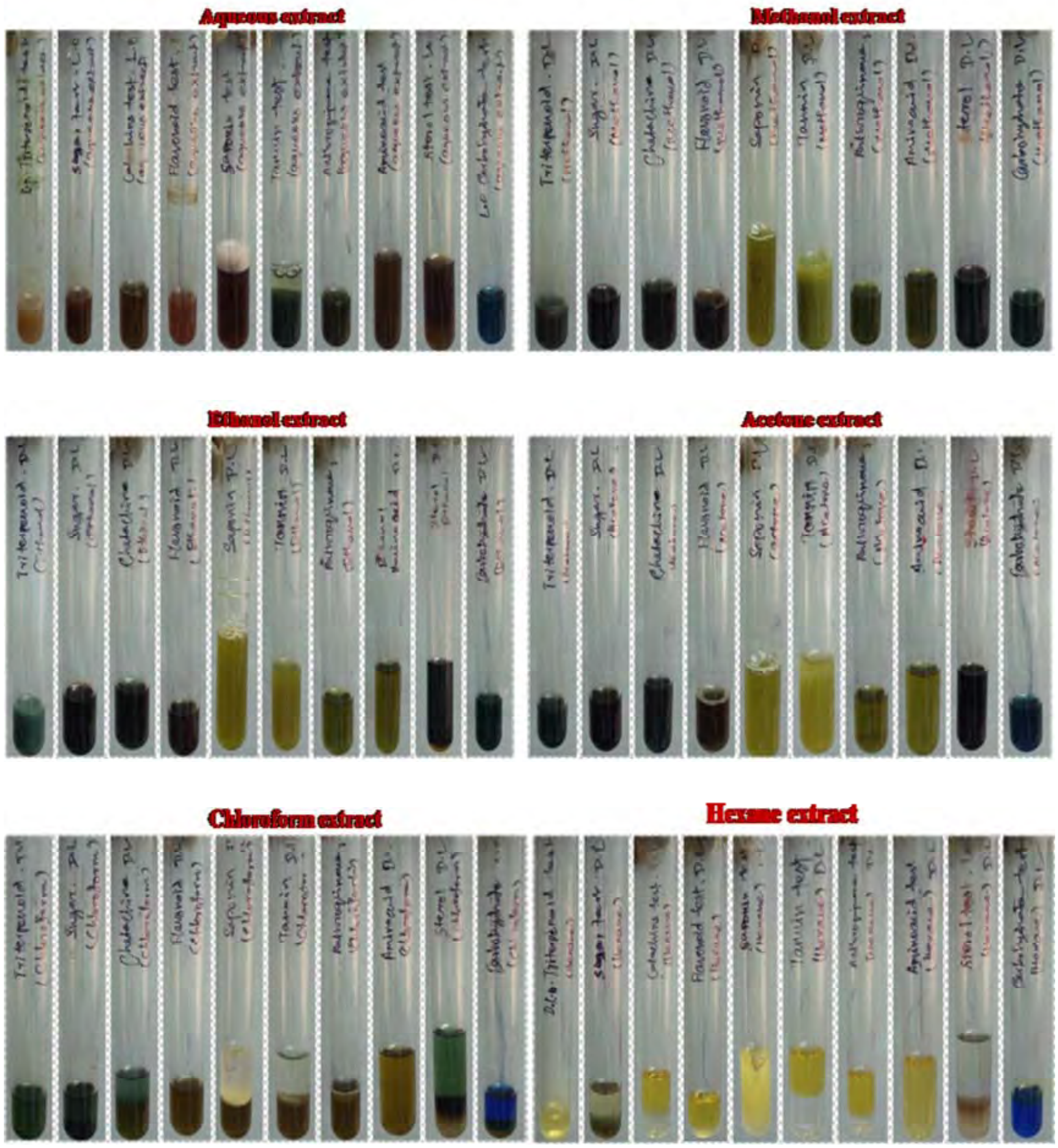

Plate 4.37. Qualitative analysis of *D. lanceolaria* leaf extract; from left to right: Triterpenoids, sugars, catechins, flavonoids, saponins, tannins, anthraquinones, aminoacids, steroids and carbohydrates.

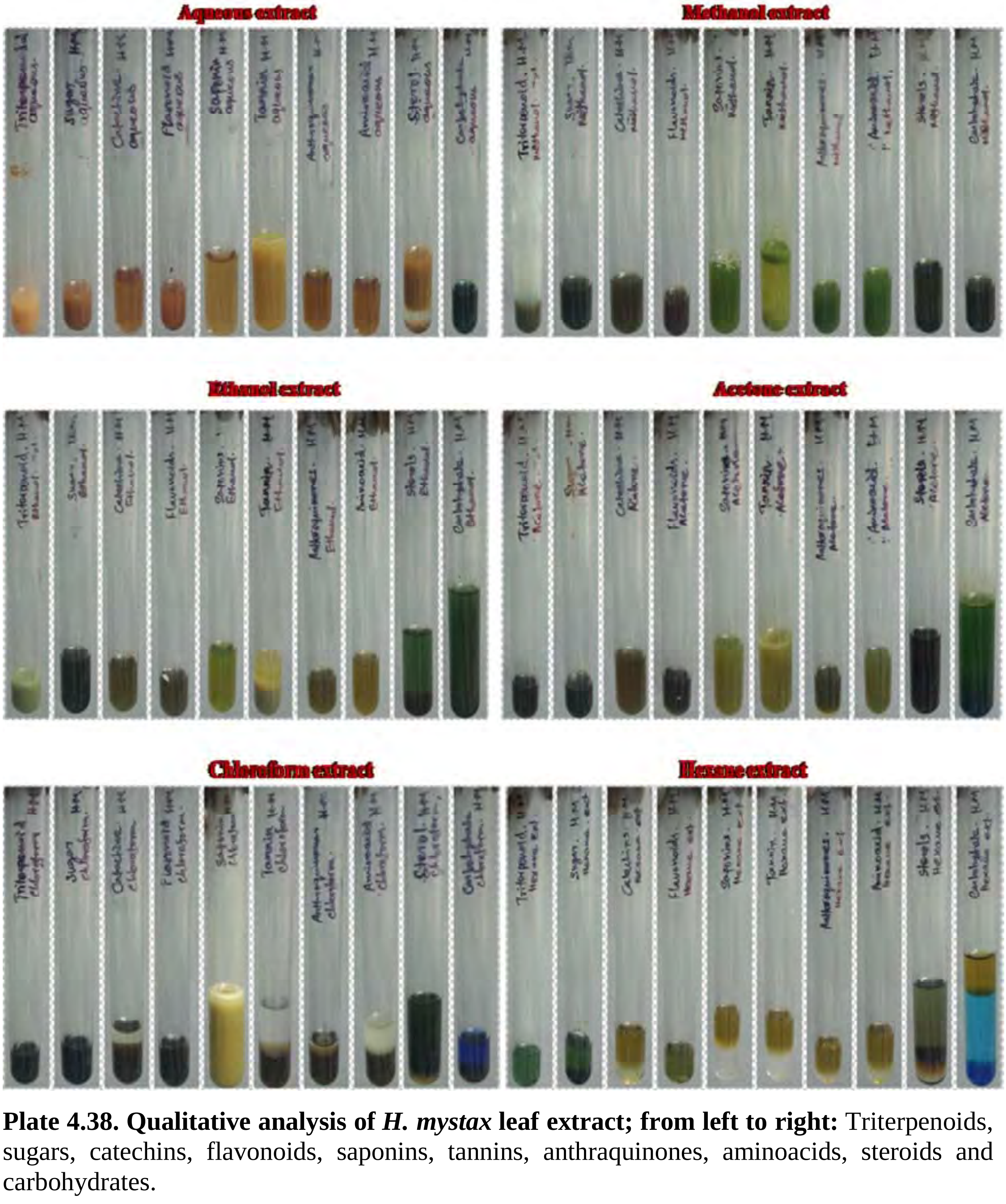

Plate 4.38. Qualitative analysis of *H. mystax* leaf extract; from left to right: Triterpenoids, sugars, catechins, flavonoids, saponins, tannins, anthraquinones, aminoacids, steroids and carbohydrates.

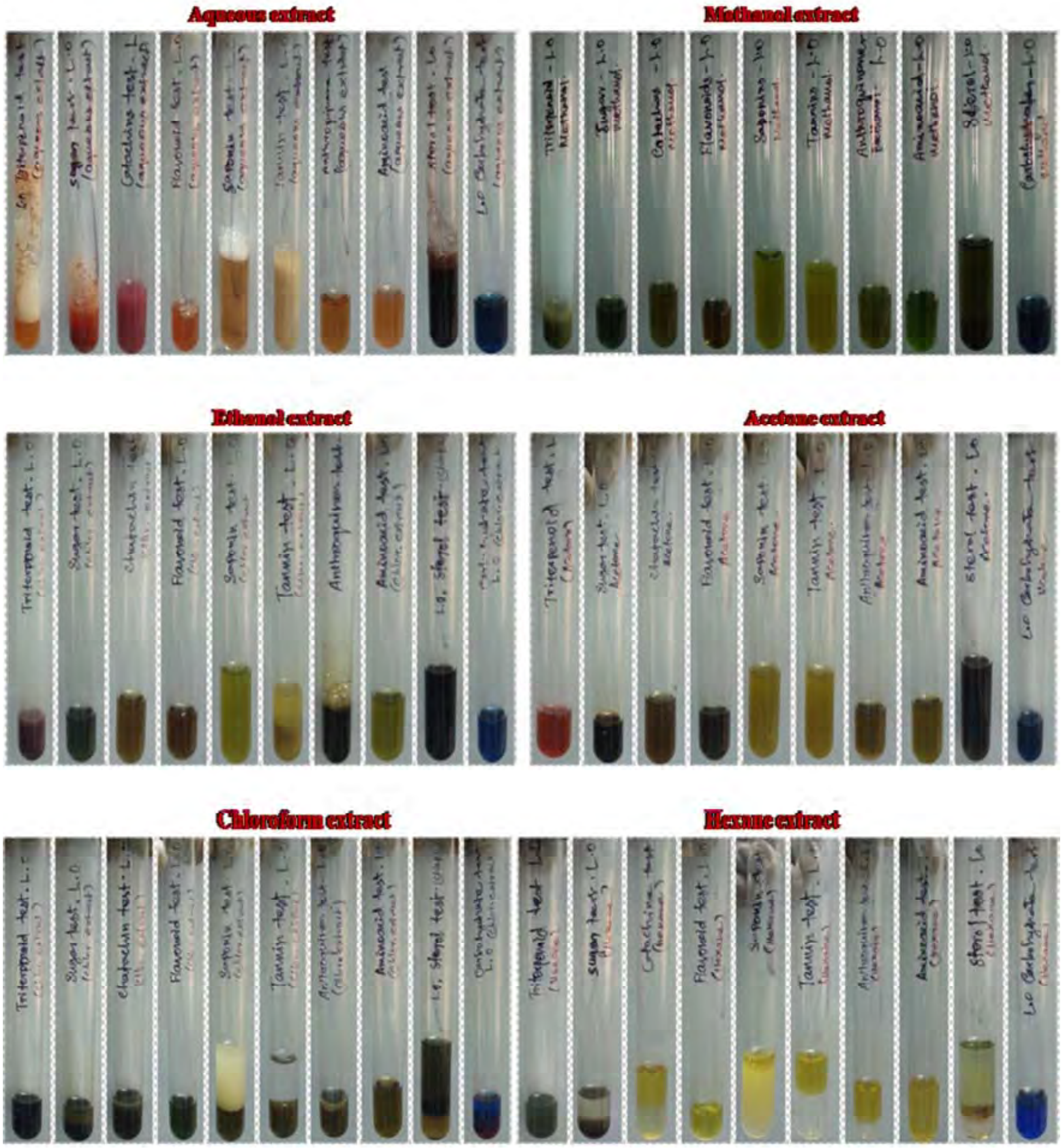

Plate 4.39. Qualitative analysis of *L. obtusifolia* leaf extract; from left to right: Triterpenoids, sugars, catechins, flavonoids, saponins, tannins, anthraquinones, aminoacids, steroids and carbohydrates.

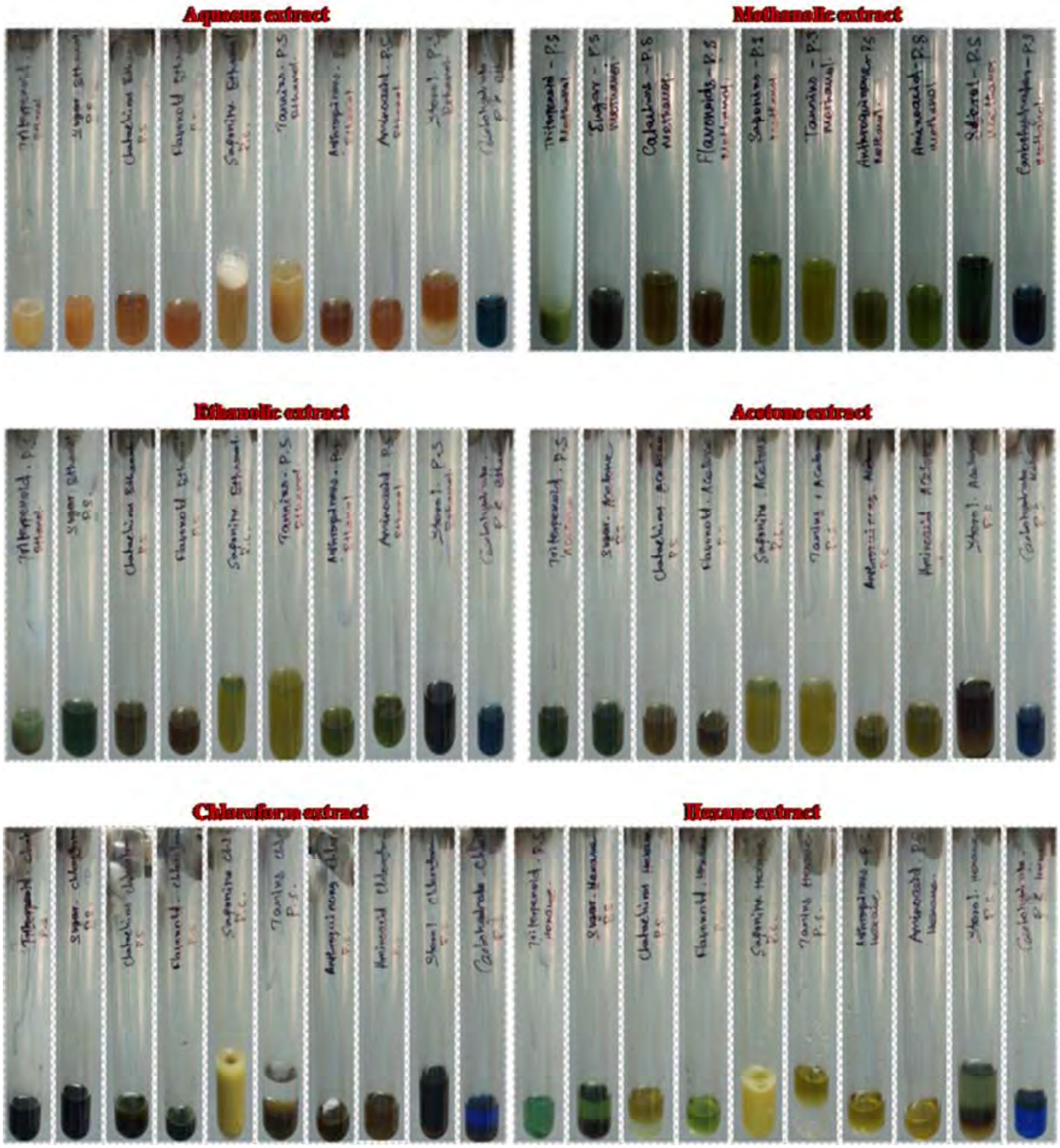

Plate 4.40. Qualitative analysis of *P. spinosum* leaf extract; from left to right: Triterpenoids, sugars, catechins, flavonoids, saponins, tannins, anthraquinones, aminoacids, steroids and carbohydrates.

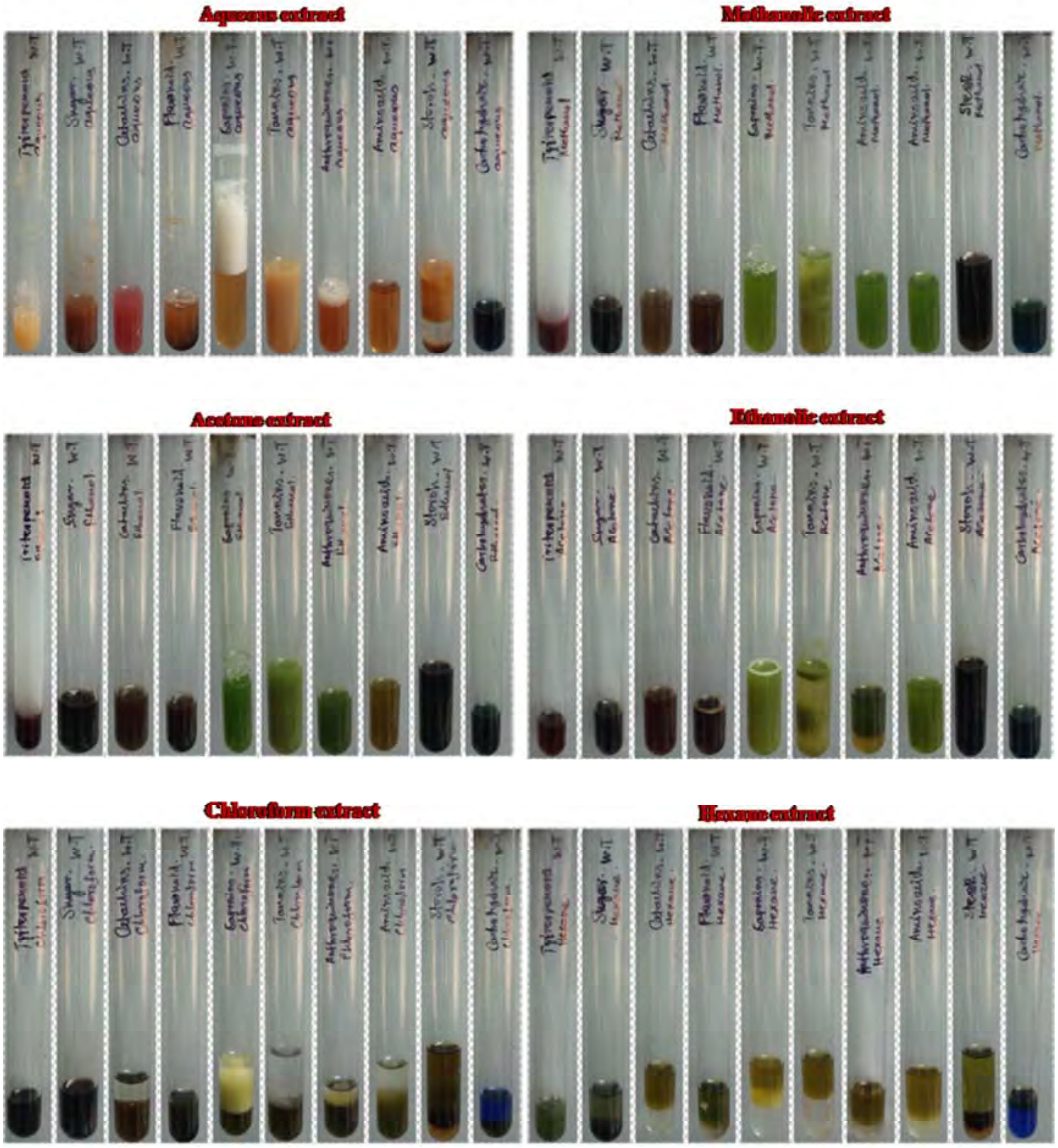

Plate 4.41. Qualitative analysis of *W. trifoliata* leaf extract; from left to right: Triterpenoids, sugars, catechins, flavonoids, saponins, tannins, anthraquinones, aminoacids, steroids and carbohydrates.

Table 4.25. Comparison of qualitative test forsix selected plant extracts

Plants name	Test	Aqueous	Methanol	Ethanol	Acetone	Chloroform	Hexane
***Aganosma cymosa* (Roxb.) G. Don**	Triterpenoids	-	+	+	-	-	-
	Sugars	+	+	+	+	+	+
	Catechins	+	+	+	+	-	-
	Flavonoids	+	+	+	-	-	-
	Saponins	+	+	+	-	+	+
	Tannins	+	+	+	+	-	-
	Anthraquinones	-	-	-	-	-	-
	Aminoacids	-	+	+	-	-	-
	Sterols	-	-	+	+	+	+
	Carbohydrates	-	-	-	-	+	-
***Dalbergia lanceolaria* L.f.**	Triterpenoids	-	+	+	+	-	-
	Sugars	+	+	+	+	+	+
	Catechins	-	+	-	-	-	-
	Flavonoids	+	+	-	+	+	-
	Saponins	+	+	+	+	+	+
	Tannins	+	+	+	+	-	-
	Anthraquinones	-	-	-	-	-	-
	Aminoacids	-	-	-	-	-	-
	Sterols	+	-	-	-	+	+
	Carbohydrates	-	-	-	-	+	-
***Hugonia mystax* L.**	Triterpenoids	-	-	+	-	-	+
	Sugars	-	+	+	+	+	+
	Catechins	-	+	-	+	+	-
	Flavonoids	+	+	-	-	-	-
	Saponins	+	+	+	-	+	+
	Tannins	+	+	+	+	-	-
	Anthraquinones	-	-	-	-	-	-
	Aminoacids	-	-	-	-	-	-
	Sterols	-	+	+	-	+	+
	Carbohydrates	-	+	-	+	+	-

Plants name	Test	Aqueous	Methanol	Ethanol	Acetone	Chloroform	Hexane
***Loeseneriella obtusifolia* (Roxb.) A.C. Sm**	Triterpenoids	-	+	+	+	-	+
	Sugars	-	+	+	+	+	+
	Catechins	+	-	+	-	-	-
	Flavonoids	+	+	+	-	-	-
	Saponins	+	-	-	-	+	+
	Tannins	+	+	+	+	-	-
	Anthraquinones	-	-	-	-	-	-
	Aminoacids	-	-	-	-	-	-
	Sterols	-	+	-	-	+	+
	Carbohydrates	-	+	-	-	+	-
***Plecospermum spinosum* Trecul.**	Triterpenoids	-	+	+	-	-	+
	Sugars	-	+	+	+	+	+
	Catechins	-	-	+	-	-	-
	Flavonoids	+	+	+	-	-	-
	Saponins	+	+	-	-	+	+
	Tannins	+	+	+	+	-	-
	Anthraquinones	+	-	-	-	+	-
	Aminoacids	-	+	-	-	-	-
	Sterols	+	+	-	-	+	+
	Carbohydrates	-	-	-	-	+	-
***Walsura trifoliata* (A. Juss.) Harms**	Triterpenoids	-	+	+	-	-	-
	Sugars	+	+	+	+	+	+
	Catechins	+	+	+	+	-	-
	Flavonoids	+	+	+	+	-	-
	Saponins	+	+	+	-	-	-
	Tannins	+	+	+	+	-	-
	Anthraquinones	-	-	-	+	-	-
	Aminoacids	-	-	-	-	-	-
	Sterols	+	+	-	-	+	+
	Carbohydrates	-	-	-	-	+	-

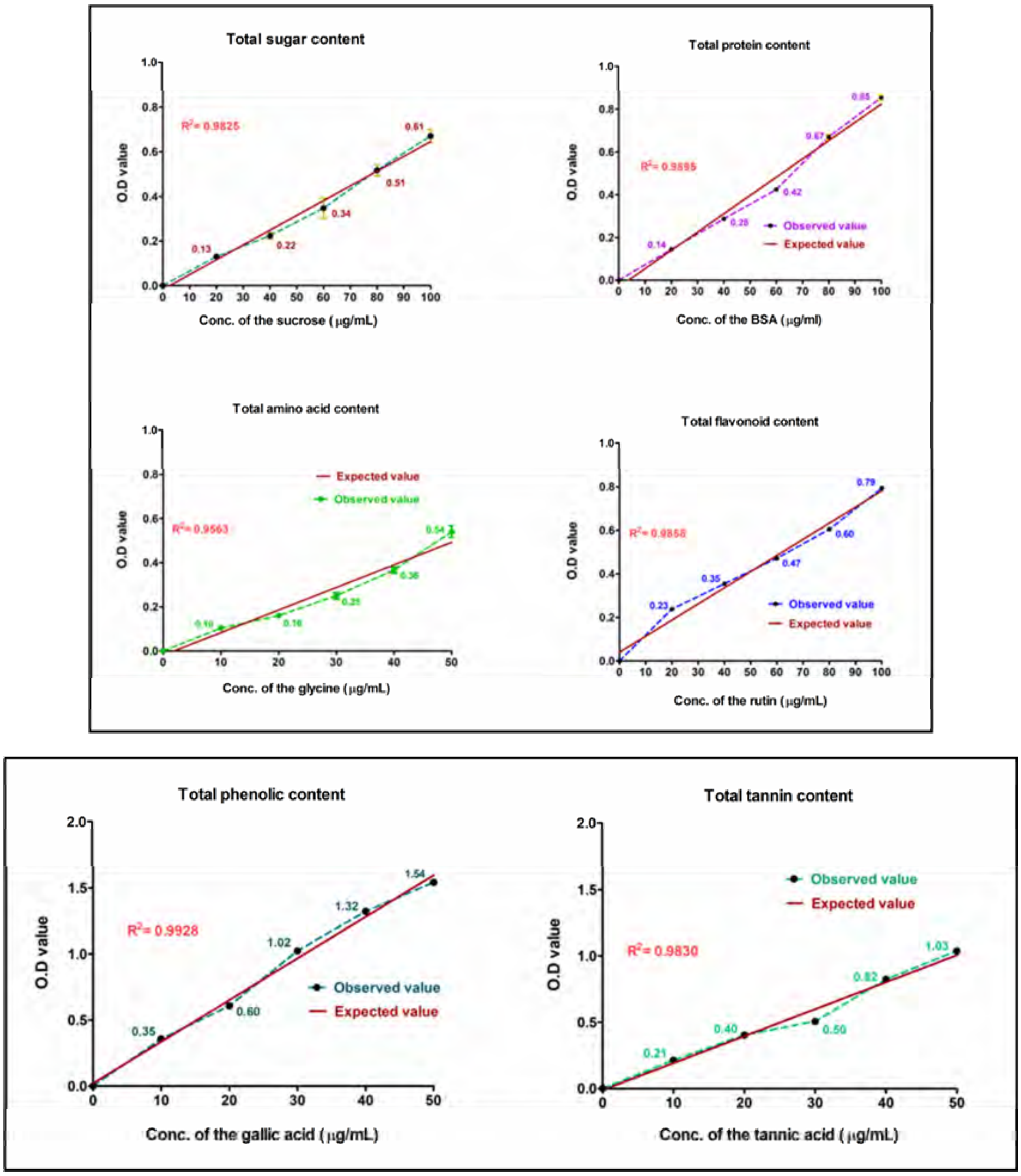

Figure 4.22. Standard graph for quantitative analysis

Table 4.26. Comparison of quantitative test for six selected plant extracts

Name of the test	*A. cymosa*	*D. lanceolaria*	*H. mystax*	*L. obtusifolia*	*P. spinosum*	*W. trifoliata*
Chlorophyll ‘a’ (mg/g) dwt.	8.70 ± 0.4	8.99 ± 0.3	6.37 ± 1.1	6.87 ± 1.2	6.64 ± 0.1	7.28 ± 0.9
Chlorophyll ‘b’. (mg/g) dwt.	16.41 ± 1.1	17.86 ± 1.5	11.18 ± 1.2	12.94 ± 1.6	11.55 ± 0.3	13.35 ± 1.0
Total Chlorophyll (mg/g) dwt.	17.15 ± 0.5	18.53 ± 0.1	11.80 ± 0.2	13.53 ± 1.0	12.22 ± 0.5	14.01 ± 0.9
Total Carotenoid (mg/g) dwt.	1.94 ± 0.1	2.14 ± 0.6	1.22 ± 1.1	1.62 ± 1.7	1.34 ± 0.4	1.50 ± 1.5
Total sugar (mg/g) dwt.	43 ± 1.6	17.5 ± 2.1	10 ± 1.5	16.5 ± 0.8	360 ± 1.7	26 ± 0.4
Total protein (mg/g) dwt.	2.1 ± 1.1	1.95 ± 0.5	1.07 ± 2.3	0.45 ± 1.7	0.8 ± 0.1	0.95 ± 1.7
Total lipids (mg/g)	9 ± 0.6	170 ± 0.6	70 ± 0.7	80 ± 1.8	160 ± 1.0	110 ± 0.9
Total free amino acid (mg/g) dwt.	2.4 ± 0.4	2.7 ± 0.3	0.8 ± 0.4	1.4 ± 0.6	0.2 ± 0.2	5.07 ± 0.3
Total phenolics (mg/g)	97.93 ± 0.3	173.79 ± 0.8	100.68 ± 1.2	105.57 ± 2.8	70.34 ± 2.1	284.13 ± 2.1
Total tannin (mg/g)	30.93 ± 0.4	46.79 ± 1.8	23.68 ± 0.6	48.51 ± 1.1	43.34 ± 1.2	67.13 ± 0.9
Total flavonoid (mg/g)	40.84 ± 1.8	20.85 ± 1.2	18.6 ± 2.0	36.57 ± 1.4	164.28 ± 1.1	75.71 ± 1.7

The presence of many secondary metabolites shows the promising nature of this medicinal plant. *A. cymosa* quantitative analyses were performed to identify the concentrations of chlorophylls, carotenoids, sugars, protein, lipids, free amino acids, phenolics and tannins. Substantial amounts of phenolics (97 mg/g of leaf powder) and flavonoids (40 mg/g) were found, and the other phytochemical classes - sugars (43 mg/g), tannins (30 mg/g) and lipids (9 mg/g), were not very abundant (Figure 4.22; Table 4.26).

The presence of plant phytocompounds in *H. mystax* leaf extract the total chlorophyll content of the MeOH leaf extract of *H. mystax* was found to be 11.80 mg/g of the dried leaf powder. Similarly, total carotenoids content was 1.22 mg/g. Total sugar content was found to be 10 mg/g, total lipids content was 70 mg/g (showing a high fatty acid content). Phenolic content was found to be 100 mg/g, total tannin content was ~24 mg/g and total flavonoids content was 18.6 mg/g of the dried leaf powder. Other pertinent details are given in Table 4.26.

In *P. spinosum* leaf extract shows Chlorophyll a (6.64 ± 0.1 mg/g), chlorophyll b (11.55 ± 0.3 mg/g), total chlorophyll (12.22 ± 0.5 mg/g) and total carotenoids (1.34 ± 0.4 mg/g) were estimated. The plant hadrich pigments contributing to its photosynthetic potential. Carbohydrate content in the plant extract was 360 ± 1.7 mg/g of total sugar. The total protein was 0.8 ± 0.1 mg/g protein as bovine serum albumin (BSA) equivalent. Total free amino acids could not be detected and might have been present in minute quantities. Phenolic content was found to be 70.34 ± 2.1 mg TAE/g extract. Tannin content was 43.34 ± 1.2 mg TAE/g extract. Total flavonoid content was 164.28 ± 1.1 mg/g. In this study, the *P. spinosum* leaf extracts were found to contain a wide range of potential phytochemicals, which would possess multiple therapeutic efficacies and may be relative to synthetic drugs. Further studies are required to prove the therapeutic potential of purified active principles.The remaining plants' quantitative results were found in abow (Table 4.26).

4.12. GC-MS Profiling of Methanolic Leaf Extract

GC-MS analysis of the methanolic extract was performed since it contained diverse classes of phytochemicals. The GC-MS spectrum (Figure 4.23 - 4.28) revealed the presence of over 192 compounds. The peaks were integrated, and the area under the curve (AUC) was calculated (in %) to find the overall abundance of the phytochemical. After peak identification using a compound library, the compounds for which literature reports exist were screened by searching the names of each of the compounds using Google Scholar as well as PubChem (Table 4.27). *H. mystax* is a relatively poorly studied plant species and since it is usually found in forests and hilly regions, it is not very accessible. There are very scanty reports of the bioactivity of this species in various traditional ayurvedic/siddha literature. Hence, the plant was studied in sufficient detail to justify its anti-inflammatory and anthelmintic activities (Duarte Galhardo de Albuquerque *et al*., 2020). The GC-MS results showed that compounds (of different phytochemical classes) were present in the MeOH leaf extract. Among the nearly 65 peaks which were obtained (and 58 other compounds), based on area under the curve (AUC) data, the most prominent compounds present in the MeOH leaf extract were - a) Bis[(2-nitrophenyl)methyl] pentanedioate (Table 4.27 and Figure 4.25), with 23.06%;

b) palmitic acid, 14.9%; c) 4-vinyl phenol, 8.64%; d) 1,3,4,5-Tetrahydroxycyclohexane-1-carboxylic acid and 3.24%. Most of the other detected compounds were far lesser in relative abundance, with < 1%. Among these four abundant compounds, the first seems unknown and maybe a new compound with no literature reports of structure/activity. Palmitic acid is a well-known, saturated 16C fatty acid. In a study, palmitic acid-rich diet (9.5% palmitic acid and 0.5% linoleic acid) was found to decrease the incidence of breast cancer when compared to a 10% linoleic acid diet (Senzaki *et al*., 1998). However, in another study, dietary palmitic acid intake raised the risk for breast cancer (Saadatian-Elahi *et al*., 2004). 4-vinyl phenol was reported to target breast cancer stem-like cells and also possesses anti-angiogenic, anti-tumour activities and inhibits metastasis (Leung *et al*., 2018). Moreover, we have identified several compounds (though with lower abundance based on area under the curve in GC-MS profiling) which had a greater propensity for binding to the chosen receptors. Naturally occurring vitamin E consists of a mixture of α, β, γ and δ tocopherol; these forms of vitamin E differs in the position and extent of methylation. While MQL is known to be toxic because of its sedative and hypnotic qualities (Gerald and Schwirian, 1973), the other drugs - β-tocopherol, carotol, dihydroactinidiolideand methyl-m-hydroxycinnamate are safe for human consumption (as per ADME results, Table 4.48).

The major bioactive phytochemicals present in the methanolic leaf extract of *P. spinosum* were identified using GC-MS. There were 35 different phytochemical compounds obtained from the leaf extract, and their retention time, names, % of peak area, molecular weight, molecular formula and therapeutic uses are represented (In the publications). Another study has suggested that 4-hydroxy benzyl alcohol (naturally occurring phenolic compound) was a potent inhibitor of lipid peroxidation and protein oxidation, indicating it could be considered as a better free radical scavenger (Dhiman *et al*., 2009). Further, 4-hydroxy benzyl alcohol was the potent antiangiogenic agent for treating different angiogenesis-related diseases including arthritis, diabetic retinopathy, cancer and endometriosis (Laschke *et al*., 2011). The 4-hydroxy benzyl alcohol ameliorates cerebral ischaemic injury by the suppression of apoptotic pathways and upregulation of protein disulphide isomerase without causing any toxicity (Yu, F. *et al*., 2010). 4-hydroxy benzyl alcohol showed antidepressant activity in rat by reducing monoamine metabolism and modulated cytoskeleton protein expression in the pathway of Slit-Robo. A previous study has also demonstrated that isonicotinic acid was active against the pathogenic *Mycobacterium* sp. (Bruckner *et al*., 1983). The Compound 2-pyridinecarboxaldehyde conjugated with metals such as cobalt, manganese, nickel, copper, and other chemical derivatives exhibited antimicrobial and antineoplastic activities (Chandra *et al*., 2008; Esmadi *et al*., 2016; Loh and Law, 1980). 5-Bromo-2-hydroxy-4-methyl-benzaldehyde suppressed lipopolysaccharide-stimulated inflammatory mediators by the inactivation of NF-κB, ERK and p38 in RAW 264.7 macrophages (Jang *et al*., 2016). The compound 2-(4-alkoxybenzylidine)-2,3-dihydro-5,6-dimethoxy-1H-inden-1-one derivatives exhibited significant inhibition of human butyrylcholinesterase and acetylcholinesterase enzymes when compared with the standard donepezil (Mozaffarnia *et al*., 2019). Research findings demonstrated that palmitic acid was a potential therapeutic agent because it specifically targeted multiple myeloma cells (Nagata *et al*., 2015).

Further, this compound suppressed HIV-1 infection by binding to CD4 receptor and efficiently blocked gp120-to-CD4 attachment and by this mechanism is known to prevent transmission of HIV infection through sexual intercourse. Phytol (diterpene) and its derivatives exhibited many therapeutic applications in pharma and biotechnological industries. Phytol exerts phytopharmacological activity on the central nervous system, and causes anxiolytic and antidepressant effects (Islam *et al*., 2018). Moreover, linolenyl alcohol showed considerable antibacterial effect and was particularly effective against dental caries and periodontal disease (Crout *et al*., 1982). Conjugation of two compounds with γ-linolenyl alcohol exhibited potential antiproliferative effects against colon, breast and prostate cancer in *in vitro* as reported (Higgins and Stearns, 2009). The GC-MS spectrum *A. cymosa* revealed the presence of over 44 compounds. The peaks were integrated, and the area under the curve (AUC) was calculated (in %) to find the overall abundance of the phytochemical which is Undecanoic acid 12.55%. After peak identification using a compound library, each of the compounds was searched for earlier reports of bioactivity using Google Scholar and PubChem. Pyrocatechol has anti-inflammatory activity (Funakoshi-Tago *et al*., 2020), present in 1.26 percent. Some of the compounds present in meagre amount but possess immense anticancer activity are Palmitic Acid (1.85%), Indole (0.06%), 2-Methoxy-4-vinylphenol (0.07%), Tyrosol (0.11%), Citral diethyl acetal (0.05%), Phytol (0.14%), Glyceryl Monostearate (0.15%) (Bailly, 2020; Bhatt *et al*., 2018; Fuggetta *et al*., 2012; Harada *et al*., 2002; Kim *et al*., 2019; Shariare *et al*., 2021; Xu and Xu, 2020).

In *D. lanceolaria* methanolic 17 secondary metabolites were through GC-MS analysis. Methylthio-acetonitrile is present in more significant amount (50.87%) in that methanolic extract. Tridecanoic acid from *Bacillus* sphas been known to possess antimicrobial activity (Chowdhury *et al*., 2021), and this compound presents 3.17% in theextract. Phytol (3.23%) int the extract is an anticancer compound (Shariare *et al*., 2021). *L. obtusifolia* showed 15 compounds and Squalene from the list was shown to have antioxidant (Yu *et al.,* 2012) and anticancer activity (Kotelevets *et al*., 2017). *W. trifoliata* showed around 50 different compounds. Carotol occupied a larger area (12.66%) and it is known to offergood anticancer activity (Sieniawska *et al*., 2016). This compound is an essential oil isolated initially from carrots. Loliolide (0.47%) found in the extract can inhibit the activation of TNF-α and IL-6 in pro-inflammatory cytokines (Silva *et al*., 2021). Neophytadiene (0.56%) have anti-inflammatory effect (Bhardwaj *et al*., 2020), Glyceryl monostearate (1.24%) have anti-breast cancer activity (Bhatt *et al*., 2018) and Palmitic Acid (4.92%) and Stearic Acid (2.21%) also possess anticancer potential (Harada *et al*., 2002; Lerata *et al*., 2020).

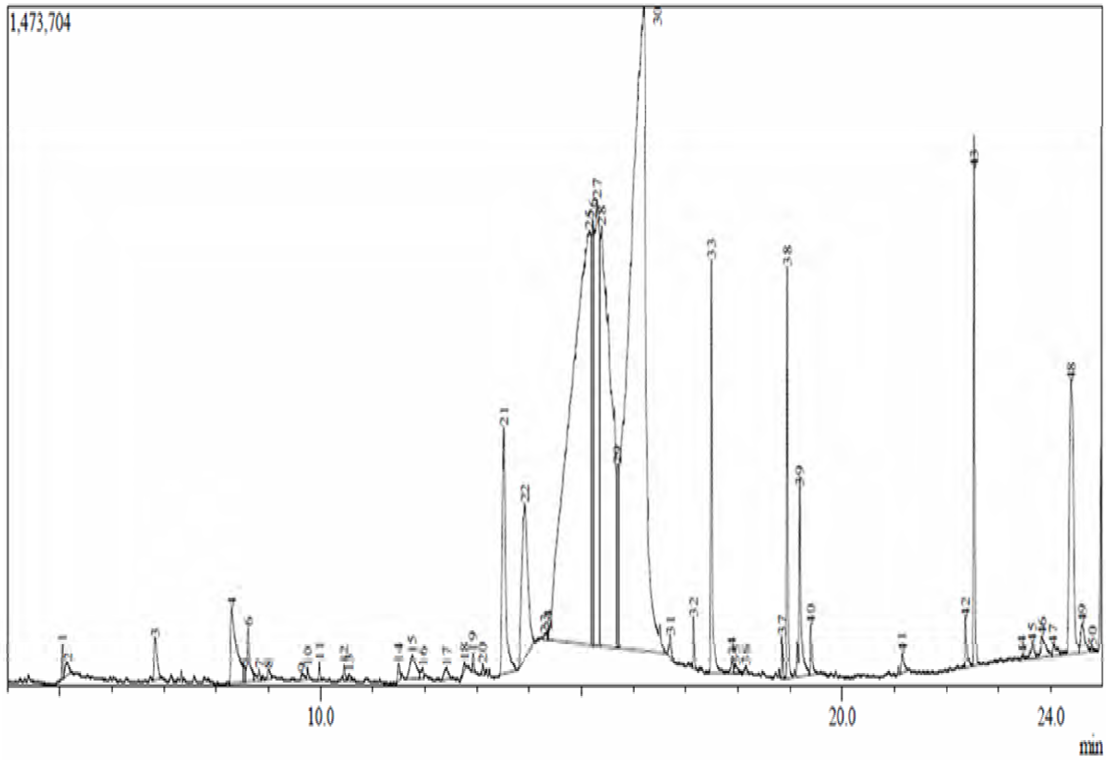

Figure 4.23. GC-MS chromatogram of methanolic leaf extract of *A. cymosa*

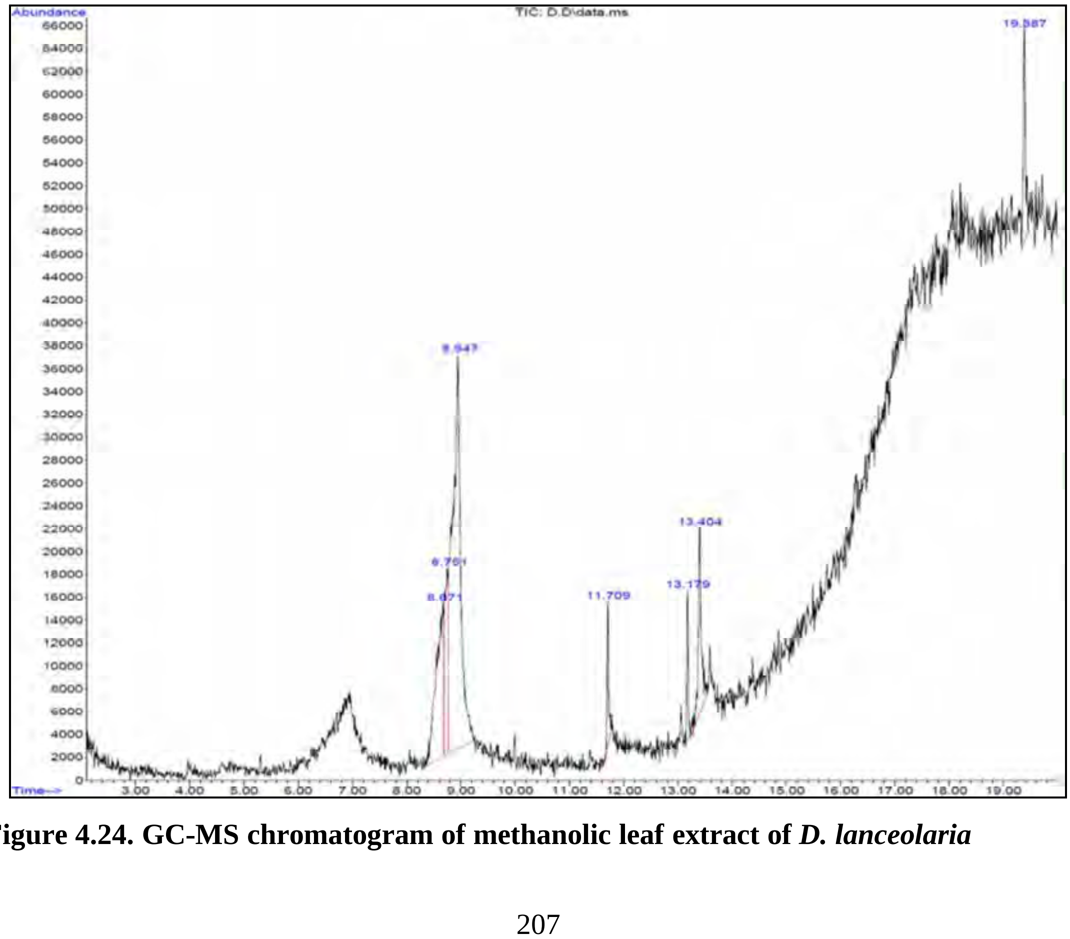

Figure 4.24. GC-MS chromatogram of methanolic leaf extract of *D. lanceolaria*

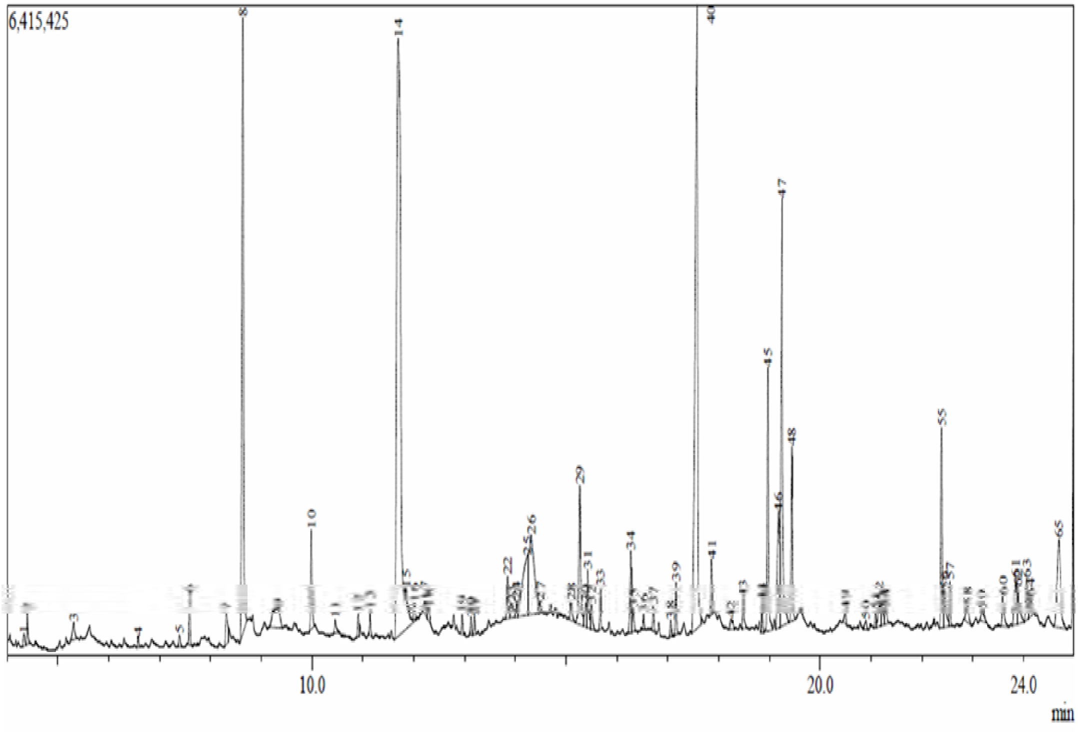

Figure 4.25. GC-MS chromatogram of methanolic leaf extract of *H. mystax*

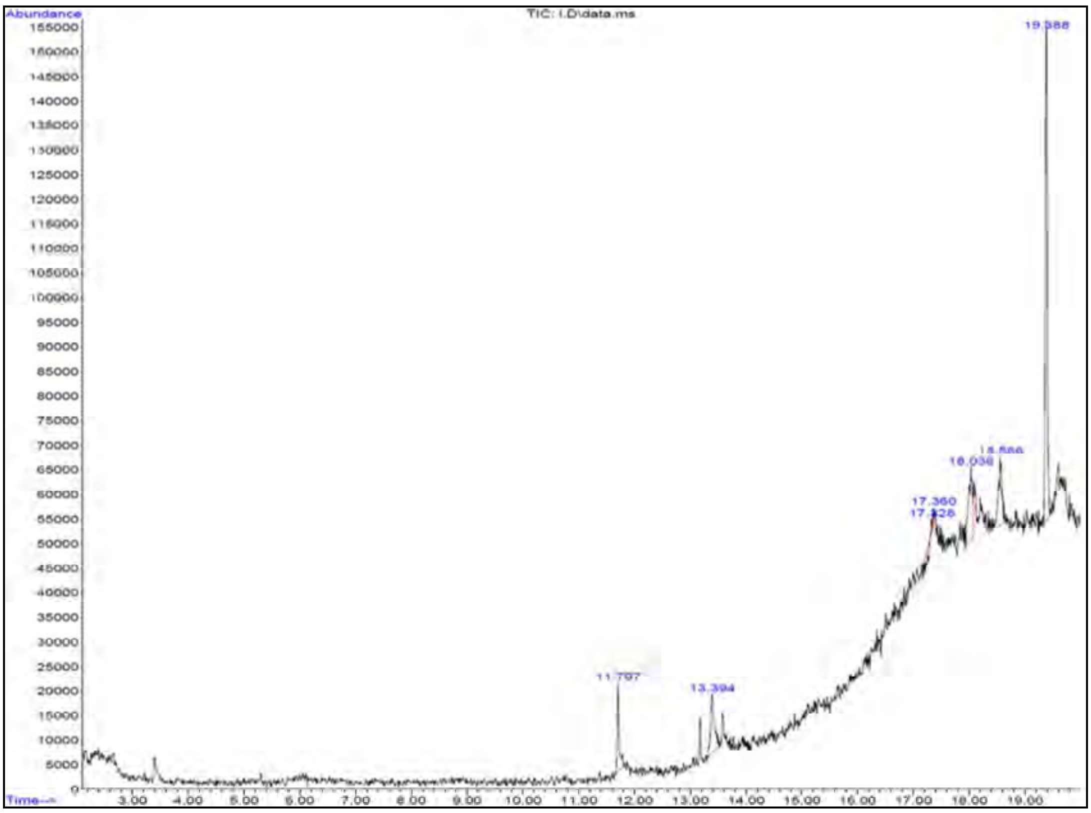

Figure 4.26. GC-MS chromatogram of methanolic leaf extract of *L. obtusifolia*

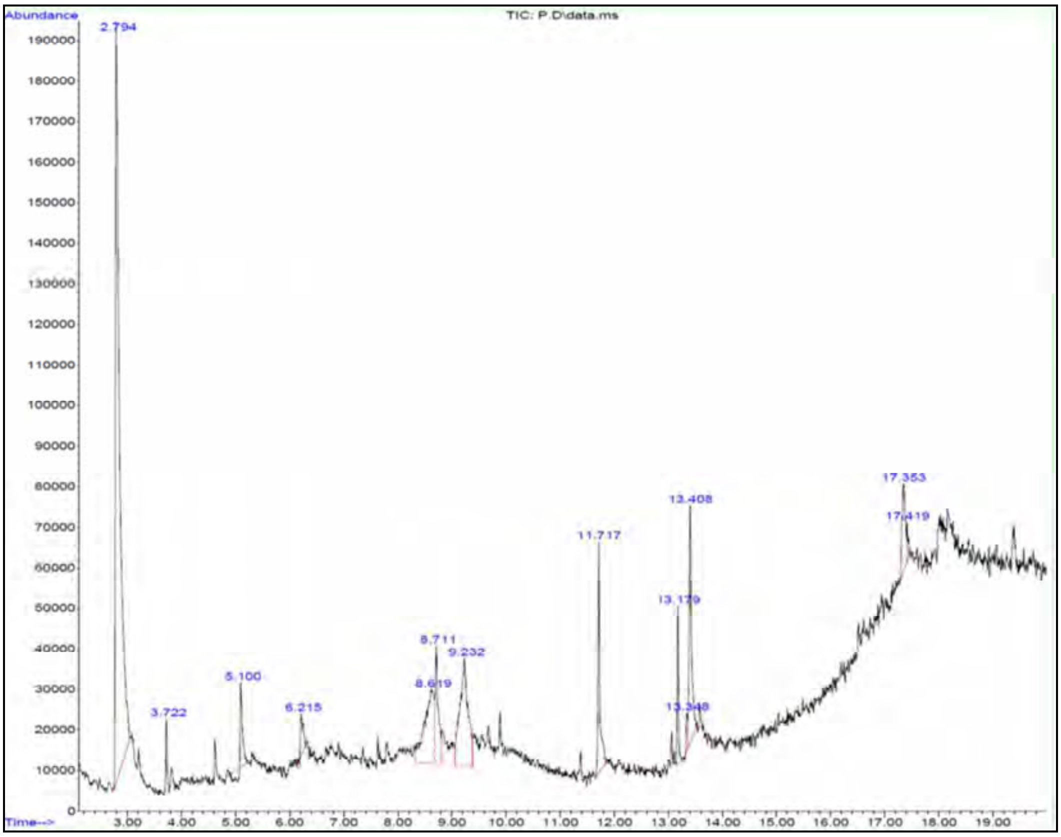

Figure 4.27. GC-MS chromatogram of methanolic leaf extract of *P. spinosum*

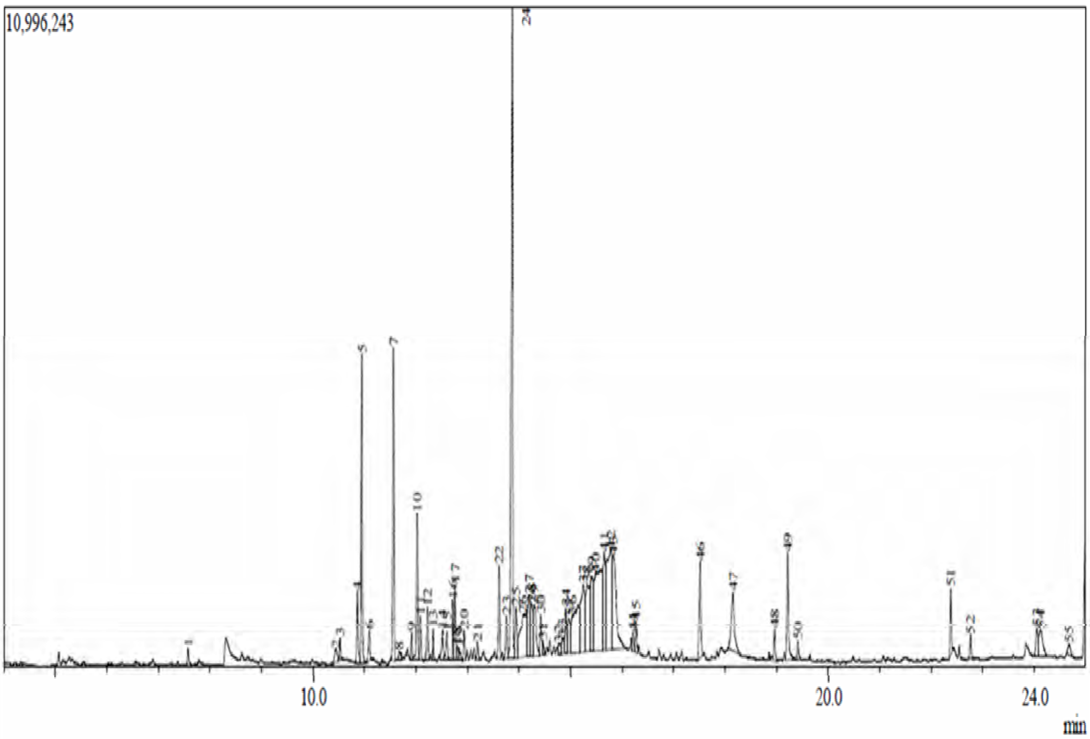

Figure 4.28. GC-MS chromatogram of methanolic leaf extract of *W. trifoliata*

Table 4.27. GC-MS analysis of selected six plant methanolic leaf extracts

S. No.	Compound Name	*A. cymosa*	*D. lanceolaria*	*H. mystax*	*L. btusifolia*	*P. spinosum*	*W. trifoliata*
1	Cyclomethicone	+	-	-	-	-	-
2	1,3-Difluoroacetone	+	-	-	-	-	-
3	Isoamyl formate	+	-	-	-	-	-
4	Pyrocatechol	+	-	-	-	-	-
5	2,3-dichloro-1-propene,	+	-	-	-	-	-
6	4-Vinylphenol	+	-	+	-	-	-
7	3-Ethyl-4-methyl-1H-pyrrole-2,5-dione	+	-	-	-	-	-
8	Phenylacetic acid	+	-	-	-	-	-
9	4-Methylcatechol	+	-	-	-	-	-
10	Indole	+	-	-	-	-	-
11	2-Methoxy-4-vinylphenol	+	-	+	-	-	-
12	2,6-Dimethoxyphenol	+	-	+	-	-	-
13	4-(1H-Pyrrol-3-yl)butanoic acid	+	-	-	-	-	-
14	Tyrosol	+	-	-	-	-	-
15	Tris(Hydroxymethyl)Nitromethane	+	-	-	-	-	-
16	[Butoxy bis(trimethylsilyloxy)silyl] tris(trimethylsilyl) silicate	+	-	-	-	-	-
17	beta.-D-glucopyranose, 1,6-anhydro-	+	-	-	-	-	-
18	2-Oxovaleric acid, TBDMS derivative	+	-	-	-	-	-
19	Dihydroactinidiolide	+	-	+	-	-	-

S. No.	Compound Name	*A. cymosa*	*D. lanceolaria*	*H. mystax*	*L. btusifolia*	*P. spinosum*	*W. trifoliata*
20	Capric acid	+	-		-	-	-
21	Mome inositol	+	-	-	-	-	+
22	Citral diethyl acetal	+	-	-	-	-	-
23	1,3,4,5-Tetrahydroxycyclohexane-1-carboxylic acid	+	-	+	-	-	-
23	Diethylene glycol diacetate	+	-	-	-	-	-
25	Undecanoic acid	+	-	-	-	-	-
26	Ethyl-1-thio-.beta.-d-glucopyranoside	+	-	-	-	-	-
27	Phytol	+	+	+	-	+	+
28	Methyl palmitate	+	-	+	-	-	-
29	Palmitic acid	+	-	+	+	+	+
30	4-Decenal, (4z)-	+	-	-	-	-	-
31	2,3,4-Trimethyl-3-pentanol	+	-	-	-	-	-
32	Cyclohexyl laurate	+	-	-	-	-	-
33	Methyl hexadeca-9,12-dienoate	+	-	-	-	-	-
34	9-Tetradecenal, (9Z)-	+	-	-	-	-	-
35	Stearic acid	+	-	+	-	-	+
36	1,3-O-Benzylidene glyceryl-2-myristate	+	-		-	-	
37	Glyceryl 2-palmitate	+	-	+	-	-	+
38	Nonadecanoic acid, dimethyl(isopropyl)silyl ester	+	-	-	-	-	-
39	22,23-Dibromostigmasterol acetate	+	-	-	-	-	-
40	Glyceryl trielaidate	+	-	-	-	-	-

S. No.	Compound Name	*A. cymosa*	*D. lanceolaria*	*H. mystax*	*L. btusifolia*	*P. spinosum*	*W. trifoliata*
41	Glyceryl monostearate	+	-	+	-		+
42	Cholest-5-en-3-ol (3beta)-	+	-	-	-	-	-
43	Coprostanol	+	-	-	-	-	-
44	5-Chlorovaleric acid, tridec-2-ynyl ester	+	-	-	-	-	-
45	Silane, ethyltrimethyl-	-	+	-	-	+	-
46	Dimethyl-cyano-phosphine	-	+	-	-	+	-
47	2-O-Methyl-D-mannopyranosa	-	+	-	-	-	-
48	Isopropyl valerate	-	+	-	-	-	-
49	3,4-Di-O-methyl-L-arabinopyranose	-	+	-	-	-	-
50	(Methylthio)-acetonitrile	-	+	-	-	-	-
51	2-[2-[2-[2-(2-Butoxyethoxy)ethoxy]ethoxy]ethyl acetate	-	+	-	-	-	-
52	Tridecanoic acid	-	+	-	-	+	-
53	Myristic acid	-	+	+	-	-	+
54	1,2-Dihexylcyclopropene	-	+	-	-	-	-
55	3-vinylcyclohexanone	-	+	-	-	-	-
56	7-Heptadecyne, 17-chloro-	-	+	-	-	-	-
57	Exo-Tetrahydrodicyclopentadiene	-	+	-	-	-	-
58	1,1,1,3,5,5,5-Heptamethyltrisiloxane	-	+	-	-	-	-
59	1,4-Bis(trimethylsilyl)benzene	-	+	-	-	-	-
60	Cyclopentene-1-carboxylic acid, 4-[2-(diphenylmethyl)-2-propen-1-yl]-, methyl ester	-	+	-	-	-	-

S. No.	Compound Name	*A. cymosa*	*D. lanceolaria*	*H. mystax*	*L. btusifolia*	*P. spinosum*	*W. trifoliata*
61	Diisoamyl ether	-	-	+	-	-	-
62	1,2-Cyclooctanedione	-	-	+	-	-	-
63	4-Hydroxycyclohexanone	-	-	+	-	-	-
64	2,4-Dihydro-2,4,5-trimethyl-3H-pyrazol-3-one	-	-	+	-	-	-
65	N-Nitrosodiethylamine	-	-	+	-	-	-
66	2,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	-	-	+	-	-	-
67	Catechol	-	-	+	-	-	-
68	1-Methylpyrrolidine-2-carboxylic acid	-	-	+	-	-	-
69	4-Ethylresorcinol	-	-	+	-	-	-
70	1-(3,6,6-Trimethyl-1,6,7,7a-tetrahydrocyclopenta(c)pyran-1-yl)-ethanone	-	-	+	-	-	-
71	Bis[(2-nitrophenyl)methyl] pentanedioate	-	-	+	-	-	-
72	Vanillyl Alcohol	-	-	+	-	-	-
73	Methaqualone	-	-	+	-	-	-
74	1,2-Di-tert-butylbenzene	-	-	+	-	-	-
75	Tert-butylhydroquinone	-	-	+	-	-	-
76	Lauric Acid	-	-	+	-	-	-
77	2,5-Dimethoxy-4-ethylamphetamine	-	-	+	-	-	-
78	Carotol	-	-	+	-	-	+
79	Acetamide, N,N'-[1,3-cyclohexanediyl-1,3-D2-bis(methylene)]bis-, cis-	-	-	+	-	-	-
80	Megastigmatrienone A	-	-	+	-	-	-

S. No.	Compound Name	*A. cymosa*	*D. lanceolaria*	*H. mystax*	*L. btusifolia*	*P. spinosum*	*W. trifoliata*
81	Coniferyl Alcohol	-	-	+	-	-	-
82	2-Propanone, 1-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-	-	-	+	-	-	-
83	Methyl-m-hydroxycinnamate	-	-	+	-	-	-
84	Desaspidinol	-	-	+	-	-	-
85	Loliolide	-	-	+	-	-	+
86	Neophytadiene	-	-	+	-	-	+
87	6,10,14-Trimethylpentadecan-2-one	-	-	+	-	-	-
88	Farnesyl acetone, (5E,9E)-	-	-	+	-	-	-
89	Sinapyl Alcohol	-	-	+	-	-	-
90	Nonadecanoic acid	-	-	+	-	-	-
91	Margaric Acid	-	-	+	-	-	-
92	Methyl dihomo-gamma-linolenate	-	-	+	-	-	-
93	Linoleic Acid, TMS	-	-	+	-	-	-
94	7-Tetradecenal, (7Z)-	-	-	+	-	-	-
95	N-Demethylcarbachol	-	-	+	-	-	-
96	Henicosanal	-	-	+	-	-	-
97	4,8,12,16-Tetramethylheptadecan-4-olide	-	-	+	-	-	-
98	Heptatriacotan-1-ol	-	-	+	-	-	-
99	1-[(Phenylsulfonyl) methyl]-7-oxabicyclo[4.1.0]heptane	-	-	+	-	-	-
100	1,6,10,14,18,22-Tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl-, (all-E)-	-	-	+	-	-	-

S. No.	Compound Name	*A. cymosa*	*D. lanceolaria*	*H. mystax*	*L. btusifolia*	*P. spinosum*	*W. trifoliata*
101	Phthalic Acid	-	-	+	-	-	-
102	Beta.-Tocopherol	-	-	+	-	-	-
103	4-Methoxy-4',5'-methylenedioxybiphenyl-2-carboxylic Acid	-	-	+	-	-	-
104	Glyceryl monooleate	-	-	+	-	-	-
105	n-Propyl 9,12,15-octadecatrienoate	-	-	+	-	-	-
106	Phytosterols	-	-	+	-	-	-
107	Alpha-Tocopherol	-	-	+	-	-	-
108	Cis,cis,cis-7,10,13-Hexadecatrienal	-	-	-	+	+	-
109	Linolenic acid	-	-	-	+	-	-
110	Methyl linolenate	-	-	-	+	-	-
111	2-Ethylacridine	-	-	-	+	+	-
112	Cyclotrisiloxane, hexamethyl-	-	-	-	+	-	-
113	Arsenous acid, tris(trimethylsily) ester	-	-	-	+	+	-
114	1-methyl-2-phenylindole	-	-	-	+	-	-
115	N-methyl-1-adamantaneacetamide	-	-	-	+	-	-
116	Benzo[h]quinoline, 2,4-dimethyl-	-	-	-	+	-	-
117	1,2-Benzisothiazol-3-amine tbdms	-	-	-	+	-	-
118	Trimethyl(4-tert-butylphenoxy)silane	-	-	-	+	-	-
119	Benz[b]-1,4-oxazepine-4(5H)-thione, 2,3-dihydro-2,8-dimethyl	-	-	-	+	-	-
120	2,6,10,14,18-Pentamethyl-2,6,10,14,18-	-	-	-	+	-	-

S. No.	Compound Name	*A. cymosa*	*D. lanceolaria*	*H. mystax*	*L. btusifolia*	*P. spinosum*	*W. trifoliata*
	eicosapentaene						
121	Supraene				+	-	-
122	Squalene				+	-	-
123	1-Pentanol	-	-	-	-	+	-
124	L-Serine, N-methyl-	-	-	-	-	+	-
125	Diethylhydroxylamine	-	-	-	-	+	-
126	2-Butanethiol, 2-methyl-	-	-	-	-	+	-
127	4-Cyanobenzoic acid, 2-phenylethyl ester	-	-	-	-	+	-
128	2-phenylethyl pivalate	-	-	-	-	+	-
129	4-hydroxybenzyl alcohol	-	-	-	-	+	-
130	4-Pyridinecarboxylic acid	-	-	-	-	+	-
131	2-Pyridinecarboxaldehyde, N-oxide	-	-	-	-	+	-
132	N,N-dimethyl-m-phenylenediamine	-	-	-	-	+	-
133	Benzaldehyde, 2-hydroxy-4-methyl-	-	-	-	-	+	-
134	1H-Inden-1-one, 2,3,4,5,6,7-hexahydro	-	-	-	-	+	-
135	Decanoic acid, 3-methyl-	-	-	-	-	+	-
136	2-Butenoic acid, 4-hydroxy-, methyl ester	-	-	-	-	+	-
137	.beta.-d-Mannofuranoside, methyl-	-	-	-	-	+	-
138	Butanoic acid, 3-methyl-, 1-methylethyl ester	-	-	-	-	+	-
139	Butanoic acid, 3-methyl-, 3,7-dimetyl-6-octenyl ester	-	-	-	-	+	-
140	11,13-Dimethyl-12-tetradecen-1-ol acetate	-	-	-	-	+	-

S. No.	Compound Name	*A. cymosa*	*D. lanceolaria*	*H. mystax*	*L. btusifolia*	*P. spinosum*	*W. trifoliata*
141	7-Pentadecyne	-	-	-	-	+	-
142	Z,Z-3,13-Octadecedien-1-ol	-	-	-	-	+	-
143	E-11,13-Tetradecadienal	-	-	-	-	+	-
144	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	-	-	-	-	+	-
145	Methyl (Z)-5,11,14,17-eicosatetraenoate	-	-	-	-	+	-
146	Silane, trimethyl[5-methyl-2-(1-methylethyl)phenoxy]-	-	-	-	-	+	-
147	Propanamide, N-(4-methoxyphenyl)-2,2-dimethyl	-	-	-	-	+	-
148	2-Methyl-7-phenylindole	-	-	-	-	+	-
149	Brallobarbital	-	-	-	-	+	-
150	3,5-Dihydroxy-6-methyl-2,3-dihydropyran-4-one	-	-	-	-	-	+
151	Syringol	-	-	-	-	-	+
152	alpha-cubebene	-	-	-	-	-	+
153	Ylangene	-	-	-	-	-	+
154	Copaene	-	-	-	-	-	+
155	gamma.-murolene	-	-	-	-	-	+
156	Caryophyllene	-	-	-	-	-	+
157	3,5-Dimethylanisole	-	-	-	-	-	+
158	alpha gurjunene	-	-	-	-	-	+
159	1,5,9,9-Tetramethyl-1,4.7-cycloundecatriene	-	-	-	-	-	+
160	Alloaromadendrene	-	-	-	-	-	+
161	Germadrene D	-	-	-	-	-	+

S. No.	Compound Name	*A. cymosa*	*D. lanceolaria*	*H. mystax*	*L. btusifolia*	*P. spinosum*	*W. trifoliata*
162	alpha muurolene	-	-	-	-	-	+
163	Isodaucene	-	-	-	-	-	+
164	gamma-Cadinene, (+)-	-	-	-	-	-	+
165	Cadina-1(10),4-diene	-	-	-	-	-	+
166	Calamenene	-	-	-	-	-	+
167	Epiglobulol	-	-	-	-	-	+
168	Selin-6-en-4.alpha.-ol	-	-	-	-	-	+
169	10-Methyl-2,5:3,10-diepoxybicyclo[4.3.1]decane	-	-	-	-	-	+
170	(-)-5-Oxatricyclo[8.2.0.0(4,6)]Dodecane,12-trimethyl-9-methylene-[1R-(1R*,4R*,6R*,10S*)]-	-	-	-	-	-	+
171	Globulol	-	-	-	-	-	+
172	Humulene epoxide II	-	-	-	-	-	+
173	Sclereodiol	-	-	-	-	-	+
174	11,11-Dimethyl-4,8-dimethylenebicyclo[7.2.0]undecan-3-ol	-	-	-	-	-	+
175	Daucol	-	-	-	-	-	+
176	.alpha.-Cadinol	-	-	-	-	-	+
177	2,2,8,8,9-Pentamethyl-2,3,4,6,7,8,9,10-octahydro-5,9-methanobenzo[8]annulen-5(1H)-ol	-	-	-	-	-	+
178	(1R,7S,E)-7-Isopropyl-4,10-dimethylenecyclodec-5-enol	-	-	-	-	-	+
179	7-Methyl-4-(1-methylethylidene)bicycle[5,3,1]undec-1-en-8-ol	-	-	-	-	-	+

S. No.	Compound Name	*A. cymosa*	*D. lanceolaria*	*H. mystax*	*L. btusifolia*	*P. spinosum*	*W. trifoliata*
180	Cyclohexanol, 3-ethenyl-3-methyl-2-(1-methylethenyl)-6-(1-methylethyl)-, [1R-(1.alpha.,2.alpha.,3.beta.,6.alpha.)]-	-	-	-	-	-	+
181	Tetracosa-2,6,14,18,22-pentaene-10,11-diol, 2,6,10,15,19,23-hexamethyl-	-	-	-	-	-	+
182	9-Isopropyl-1-methyl-2-methylene-5-oxatricyclo[5.4.0.0(3,8)]undecane	-	-	-	-	-	+
183	(E)-4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenol	-	-	-	-	-	+
184	1-((1S,3aR,4R,7S,7aS)-4-Hydroxy-7-isopropyl-4-methyloctahydro-1H-inden-1-yl)ethanone	-	-	-	-	-	+
185	(2-Ethoxyphenoxy)-trimethylsilane	-	-	-	-	-	+
186	Strophanthin K	-	-	-	-	-	+
187	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	-	-	-	-	-	+
188	2-(Hydroxymethyl)-3,4-dihydro-2H-pyran-3,4-diol	-	-	-	-	-	+
189	Tridec-2ynyl 2,2-dichloroacetate	-	-	-	-	-	+
190	16-Hydroxykauran-18-oic acid	-	-	-	-	-	+
191	gamma-Sitosterol	-	-	-	-	-	+
192	alpha-Tocopherol acetate	-	-	-	-	-	+

4.13. Bioinformatic studies

The results presented in this work are promising, and we wanted to increase our understanding of the chosen six plants through the bioinformatics approach. However, in-depth analysis of the molecular interactions for all the plants is beyond the scope of this work when seen in entierty. Hence, we have attempted these studies with two of our chosen plants: *Aganosma cymosa* (Roxb.) G. Don and *Hugonia mystax* L.

4.13.1. Molinspiration study on *A. cymosa* compounds

The biochemical/biological activities of therapeutic drugs mainly depend on the structure of the compounds and their chemicophysical properties. Molinspiration tool is a convenient online tool that is widely used for the prediction of drug-likeness and bioactivity of molecules such as secondary phytochemical metabolites and synthetic organics (Rajan *et al*., 2019). This tool measures the $_{mi}$logP value (Octanol-water partition coefficient logP) and TPSA (Topological polar surface area) values of the compounds using Bayesian statistics. Among the major drugs known to man and in clinical use, the key classes of drugs target these protein receptor classes: G protein-coupled receptors (GPCR), ion channels (e.g. Na^+/K^+, Cl^-etc.), kinases, nuclear hormone receptors (e.g. vitamin D receptor, steroid and xenobiotic receptor, aryl hydrocarbon receptor etc.), proteases, and other critical enzymes (Rosenbaum *et al*., 2009). Moreover, prediction of *in silico* activity of the compounds identified through GC-MS yields valuable information for commencing experimental studies, as the type of target which the drug/test molecule could exhibit the most significant efficacy/activity can be predicted. In this context, among the five (2,3-dichloro-1-Propene (DCP), 4-Methylcatechol (MCL), Coprostanol (CSL), Indole (IDL) and

Pyrocatechol (PCL), indole and coprostanolwere the best and most potent inhibitors since they possessed lower negative or positive values for the different receptor types (Table 4. 28; Table 4.29).

Table 4.28. SAR properties of *A. cymosa* compounds

Ligands	Molecular Weight	Volume	n Violations	nrotb	nON (HBA)	nOHNH (HBD)	n Atoms	TPSA	miLogP
2,3-dichloro-1-Propene	110.97	84.24	0	1	0	0	5	0	1.90
4-Methylcatechol	124.14	116.64	0	0	2	2	9	40.46	1.42
Coprostanol	388.68	429.34	1	5	1	1	28	20.23	7.80
Indole	117.15	113.02	0	0	1	1	9	15.79	2.16
Pyrocatechol	110.11	100.08	0	0	2	2	8	40.46	0.99

Table 4.29. Molinspiration results for selected *A. cymosa* compounds

Ligands	GPCR ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor
2,3-dichloro-1-Propene	-3.88	-3.85	-3.91	-3.85	-3.86	-3.83
4-Methylcatechol	-2.28	-1.66	-2.42	-2.24	-2.61	-1.80
Coprostanol	0.21	0.33	-0.34	0.66	0.22	0.51
Indole	-1.64	-1.02	-1.36	-2.04	-2.17	-1.29
Pyrocatechol	-3.04	-2.51	-3.10	-2.98	-3.24	-2.67

4.13.2. ADMET-SAR properties of *A. cymosa* compounds

The absorption (A) analysis predicts the Caco-2 cellular permeability of the study compounds. The results in Table 4.30 show that all the selected *A. cymosa* compounds possessed phytochemical compounds with highly greater potential to for binding to P-glycoprotein may facilitate the excretion of absorbed chemicals or phytocompounds from the cells through drug efflux mechanism. The results revealed that the phytochemicals were neither substrates nor inhibitors of P-glycoprotein (only CSL was a P-glycoprotein substrate). Coming to drug distribution (D) analysis, DCP, CSL and IDL were predicted to cross the blood-brain barrier (BBB). In the study of liver microsomal metabolism, the 2 key cytochrome P450s (CYP450s) involved in drug metabolism are CYP2D6 and CYP3A4; these two enzymes together perform the majority of the liver microsomal phase-I drug metabolism. The results showed that the *A. cymosa* compounds were non-inhibitors of CYP2D6; CSL alone was a substrate for CYP2D6 and CYP3A4. This data suggested that these phytochemical compounds would undergo metabolism in liver microsomes.The protein transporters, OATP1B1 and OATP1B3 are expressed on sinusoidal-membrane of hepatocytes and interaction of the phytocompounds with these transporters as either substrates or inhibitors would lead to drug resistance and clinical drug-drug reactions (Cheng *et al*., 2012).

Table 4.30. ADMET-SAR properties of the docked compounds of *A. cymosa*

Properties	2,3-dichloro-1-Propene	4-Methylcatechol	Coprostanol	Indole	Pyrocatechol
Ames mutagenesis	+	-	-	-	-
Acute Oral Toxicity (c)	II	II	III	III	II
Androgen receptor binding	-	-	+	-	-
Aromatase binding	-	-	+	-	-
Avian toxicity	-	-	-	-	-
Blood Brain Barrier	+	-	+	+	-
BRCP inhibitor	-	-	-	-	-
Biodegradation	-	-	-	+	+
BSEP inhibitor	-	-	-	-	-
Caco-2	+	+	+	+	+
Carcinogenicity (binary)	+	+	-	-	-
Carcinogenicity (trinary)	Non-required	Warning	Non-required	Non-required	Warning
crustacea aquatic toxicity	+	+	+	+	+

Properties	2,3-dichloro-1-Propene	4-Methylcatechol	Coprostanol	Indole	Pyrocatechol
CYP1A2 inhibition	-	-	-	+	-
CYP2C19 inhibition	-	-	-	+	-
CYP2C9 inhibition	-	-	-	-	-
CYP2C9 substrate	-	-	-	-	-
CYP2D6 inhibition	-	-	-	+	-
CYP2D6 substrate	-	-	+	-	-
CYP3A4 inhibition	-	-	-	-	-
CYP3A4 substrate	-	-	+	-	-
CYP inhibitory promiscuity	-	-	-	-	-
Eye corrosion	+	+	-	-	+
Eye irritation	+	+	-	+	+
Estrogen receptor binding	-	-	+	-	-
Fish aquatic toxicity	+	+	+	-	+
Glucocorticoid receptor binding	-	-	+	-	-
Honey bee toxicity	+	-	+	+	+
Hepatotoxicity	-	-	-	-	-
Human either-a-go-go inhibition	-	-	-	-	-
Human Intestinal Absorption	+	+	+	+	+
Human oral bioavailability	+	+	+	+	+
MATE1 inhibitor	-	-	-	-	-
Micronuclear	-	+	-	+	+
Acute Oral Toxicity	2.570429	1.918836	3.429071	2.016471	2.608541
OATP1B1 inhibitor	+	+	+	+	+
OATP1B3 inhibitor	+	+	+	+	+

Properties	2,3-dichloro-1-Propene	4-Methylcatechol	Coprostanol	Indole	Pyrocatechol
OATP2B1 inhibitor	-	-	-	-	-
OCT1 inhibitor	-	-	+	-	-
OCT2 inhibitor	-	-	-	-	-
P-glycoprotein inhibitor	-	-	-	-	-
P-glycoprotein substrate	-	-	+	-	-
PPAR gamma	-	-	-	-	-
Plasma protein binding	-0.10058	0.747179	1.17812	0.617306	0.592438
Subcellular localization	Mitochondria	Mitochondria	Mitochondria	Lysosomes	Mitochondria
Tetrahymena pyriformis	1.395906	0.480007	0.964528	0.586831	0.758359
Thyroid receptor binding	-	-	+	-	-
UGT catalysed	-	+	+	-	+
Water solubility	-2.25658	-0.37119	-4.54779	-2.64186	-0.02759

4.13.3. PPI network analysis of DEGs in breast cancer

Data obtained from the NCBI-GEO database (breast cancer) led to the identification of the differentially expressed genes (DEGs). The PPI interaction network shows the interactions between the various proteins (Figure 4.29). Among the top, 60 hits (in terms of greater expression) in breast cancer (GEO database; GSE 15852; GSE 2290 and GSE 1081180), MMP9, ESR (ER) and mTOR have significantly high importance in breast cancer. PIK3CA is within the 70 top DEGs too. Moreover, in literature, these proteins and their inhibitors have been pursued as cancer oncotargets. Based on the top 60 hits, this PPI network was constructed. Hence, clearly, genes like P1K3CA, MMP9, mTOR and ER are involved in the molecular pathophysiology of breast cancer. A shorter path between the proteins shown in the network indicates higher betweenness centrality values. These four proteins were selected for further *in silico* analyses (Castro *et al*., 2016).

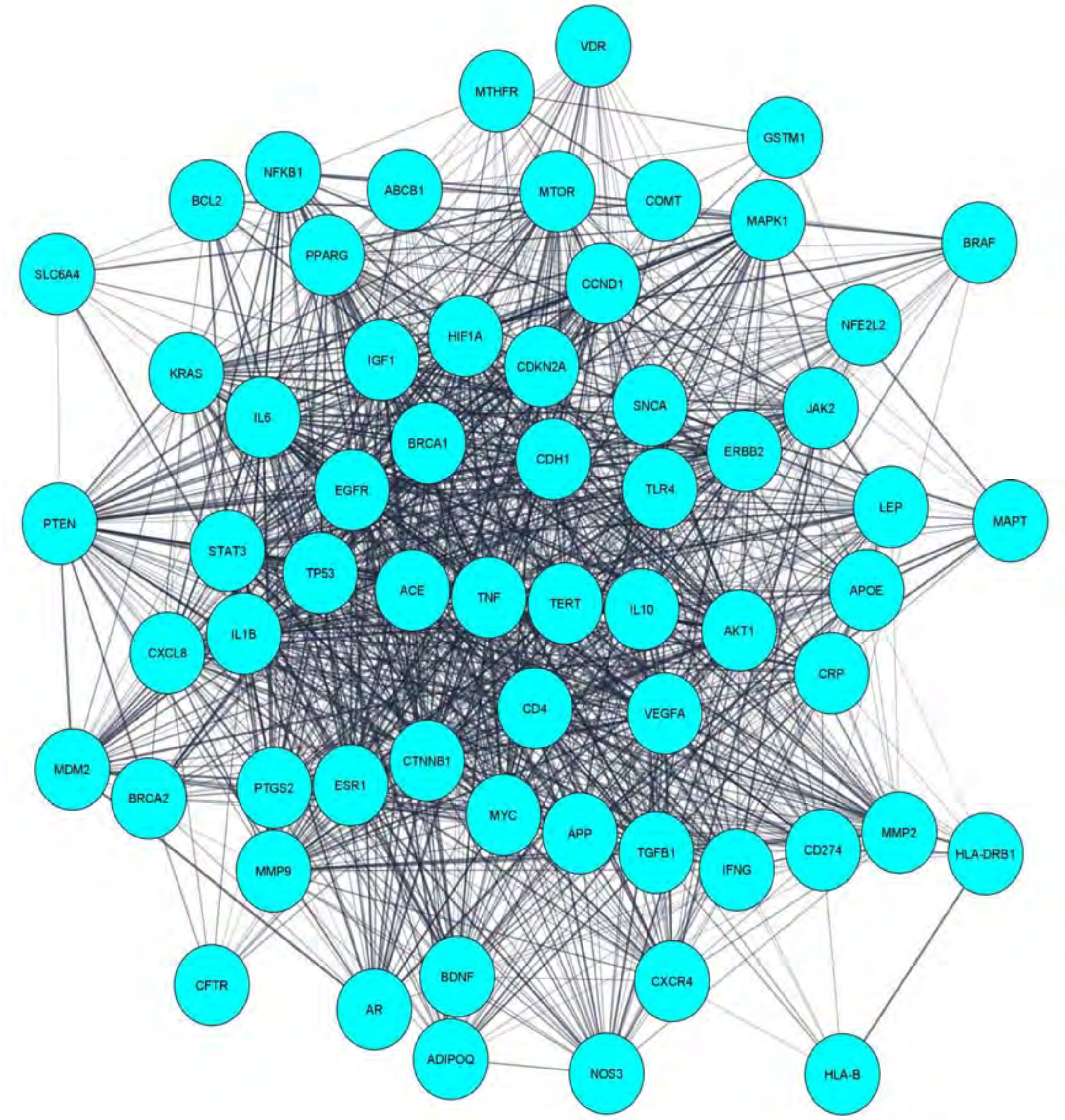

Figure 4.29. PPI network analysis of DEGs in breast cancer - Network analysis of key DEGs was performed using Cytoscape and some of the key genes of our study focus were found to be differentially expressed in the top 60-70 hits obtained from RNAseq data.

4.13.4. *In silico* affirmation of the bioactive potentials of *A. cymosa* phytochemical constituents

Computational studies for protein-ligand and protein-protein interactions have been affirmed for deciphering the bioactive potentials of phytochemical compounds. Phytochemical screening for potent bioactivities has been assessed through multidisciplinary *in vitro* and *in silico* studies for most diseases ranging from snake bites to COVID-19 (Dutta *et al*., 2021; Majeed *et al*., 2021; Rajendran *et al*., 2018). An extensive review of the phytochemical potentials as corroborated by in silico assessment and application perspectives has shown the prominent significance of computational affirmation (Sharanya *et al*., 2021).

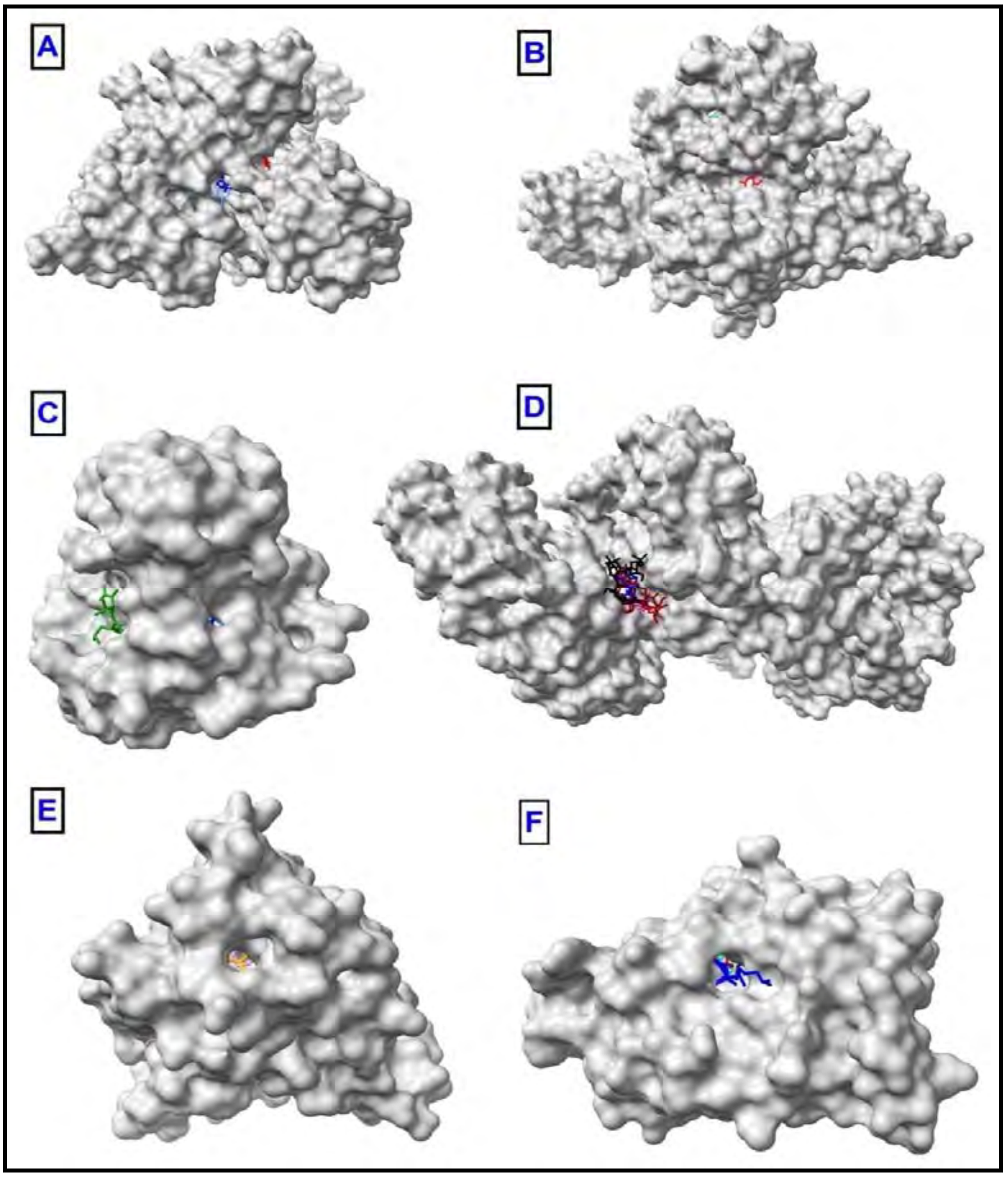

Figure 4.30. Ligand Interaction surface view of breast cancer protein targets [(PDB ID: A & B - 5NGB (PI3K); C - 5TH6 (MMP-9), D - 4JT6 (mTOR) and E & F - 3OS8 (ERβ)], plant compounds and drugs are coloured as follows: coprostanol - dark blue; 2,3-dichloro-1-propene - gold; estradiol - orange; indole - cyan; 4-methylcatechol - Red; marimastat - forest green; pyrocatechol - violet; ridaforolimus - black; rapamycin - dark red; toremifene - rosy brown and umbralisib - corn flower blue.

Table 4.31. Molecular docking of *A. cymosa* leaf extract identified through GC-MS: Best energy compounds. The respective control drugs (Italics) were compared with plant compounds

Targets	Ligands	Binding energy	Ligand efficiency	inhib_constant (μM)
5NGB (PI3K)	coprostanol	-11.38	-0.41	0.004
	indole	-5.95	-0.66	43.37
	4-methylcatechol	-5.17	-0.57	162
	pyrocatechol	-4.98	-0.62	224.22
	umbralisib	**-4.46**	**-0.11**	**534.66**
	2,3-dichloro-1-Propene	-3.84	-0.77	1530
5TH6 (MMP-9)	indole	-6.41	-0.71	19.88
	4-methylcatechol	-6.19	-0.69	29.15
	pyrocatechol	-5.62	-0.7	76.15
	2,3-dichloro-1-Propene	-4.24	-0.85	781.82
	marimastat	**-4.14**	**-0.18**	**920.32**
	coprostanol	-3.47	-0.12	2850
4JT6 (mTOR)	coprostanol	-8.66	-0.31	0.447
	rapamycin	**-8.31**	**-0.13**	**805.9**
	ridaforolimus	**-8.11**	**-0.12**	**1.14**
	indole	-5.41	-0.6	107.74
	4-methylcatechol	-5.37	-0.6	115.96
	pyrocatechol	-4.87	-0.61	268.83
	2,3-dichloro-1-Propene	-3.6	-0.72	2300
3OS8 (ERβ)	coprostanol	-10.67	-0.38	0.015
	estradiol	**-9.08**	**-0.45**	**0.219**
	toremifene	**-6.32**	**-0.22**	**23.32**
	indole	-5.37	-0.6	116.21
	4-methylcatechol	-5.01	-0.56	213.14
	pyrocatechol	-4.57	-0.57	447.26
	2,3-dichloro-1-Propene	-3.52	-0.7	2650

Molecular docking between protein-ligand interactions revealed the binding order in the series of, 5NGB (PI3K) > 5TH6 (MMP-9) > 4JT6ChainA (mTOR) > 3OS8ChainA (ERβ). The 44 phytochemical constituents were docked to the target proteins and the respective control drugs compared with plant compounds (Figure 4.30). Table 4.31 enlists the appropriate compounds and respective binding profile variations; DCP - 2,3-dichloro-1-Propene; MCL - 4-Methylcatechol; CSL - Coprostanol; IDL - Indole; PCL - Pyrocatechol; MMS - Marimastat; RFL - Ridaforolimus; RMN - Rapamycin; TMN - Toremifene; EDL - Estradiol; ULB - Umbralisib (Figure 4.31 - 4.34). Tables 4.32 - 4.35 depict the necessary types of interaction and the key interacting aminoacid residues. The bevy of weak intermolecular forces involved in the stabilization of the *A. cymosa* compounds at the active site of the breast cancer proteins (or alternatively, at other sites) reveals that these compounds sometimes have better binding energies w.r.t. the standard chemotherapeutic anticancer compounds. Network pharmacology assessment for the apt involvement of the 44 components in eliciting the interaction and target prediction with validation will advance our knowledge of phytomedicine. The present preliminary report aids in providing the scenario research of the plant *A. cymosa* and lays a solid platform for further explorations. RMSD, RMSF and hydrogen bonding patterns are structurally illustrated in Figure 4.35. The cohesive patterns and the trajectories depict that the selected receptor-drug combinations displayed stable interactions over a protracted period of 50 nanoseconds.

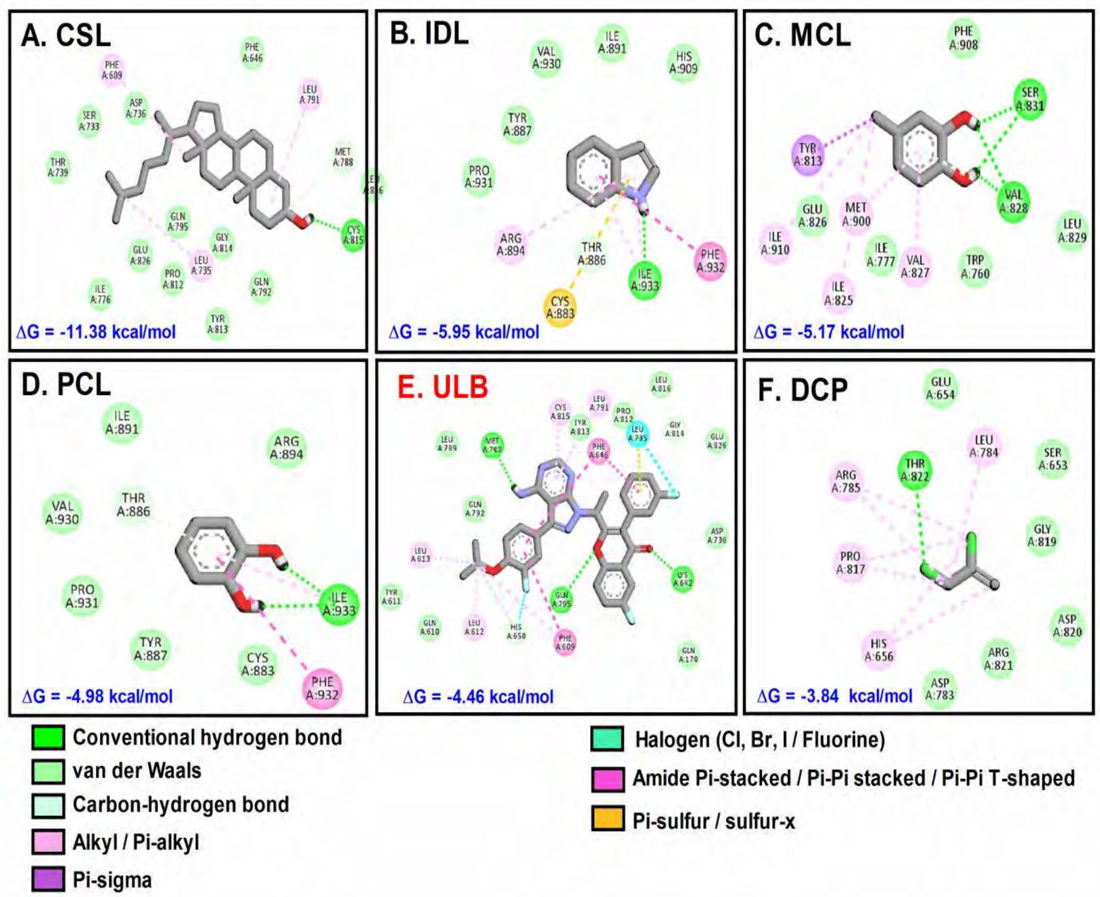

Figure 4.31. Receptor-ligand interactions between PI3K (5NGB) and phytocompounds obtained from *A. cymosa*. The binding of PCL, IDL, MCL and CSL was stabilized by hydrogen bonding of the ligands to PI3K. These compounds may serve as competitive inhibitors of PI3K by occupying the substrate (ATP) binding site. Other weaker interactions which may have stabilized the interactions between PI3K and the incoming ligands are demarcated by various colours, as given in the legend below the images. Interestingly, CSL exhibited a higher - ΔG than ULB, a control drug used for treating breast cancer.

Table 4.32. Types of interactions and interacting residues of PI3k receptor involved in docking of *A. cymosa* compounds and controls drugs

Types of interaction	5NGB (PI3K)					
	CSL	IDL	MCL	ULB	PCL	DCP
Conventional hydrogen bond	C815	I933	V828 and S831	M788, Q795 and K642	I933	T822
Van der waals	L816, Q792, Y813, G814, Q795, P812, E826, I776, Thr739, S733, D736 and F646	P931, Y887, V930, I891 and H909	F908, L829, W760 and I777	Y611, Q610, Q170, D736, E826, L816, P812, Y813, Q792 and L789	R894, I891, V930, P931, Y887 and C883	E654, S653, G819, D820, R821 and D783
Pi-sigma / Amide Pi-stacked	-	-	Y813	-	-	-
Pi-Pi Stacked / Pi-Pi T Shaped	-	F932	-	F609 and F646	F932	-
Pi-sulfur	-	C883	M788		-	-
Halogen (fluorine) / (Cl, Br, I)	-	-	-	L735	-	-
Alkyl/Pi-alkyl	F609, L791 and L735	R894	I910, M900, I825 and V827	C815, L791, L613 and L612	-	P817, R785, L784 and H656
Pi-Donor / Carbon hydrogen bond donor	M788	T886	E826	H650 and G814	T886	-

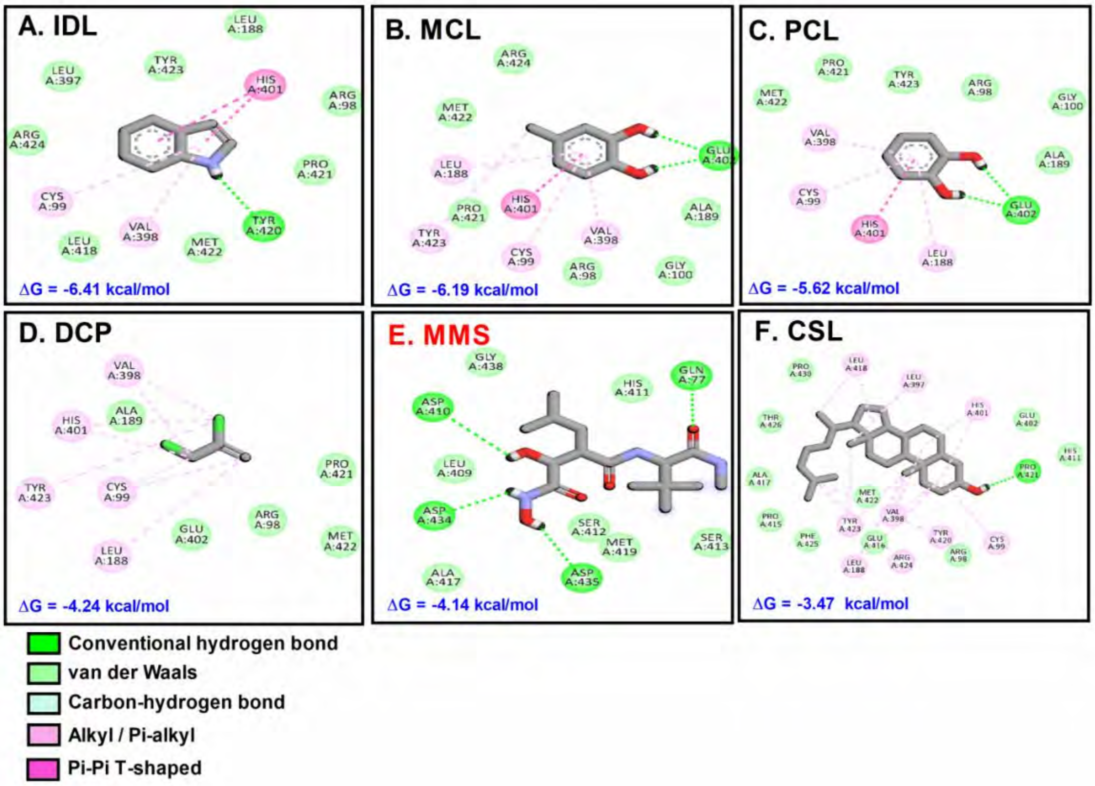

Figure 4.32. Molecular docking of *A. cymosa* compounds to MMP-9. IDL and MCL were found to have superior - ΔG values for binding to MMP-9 active site and the forces involved in the interaction between key active site amino acid residues and the docked ligands are displayed. IDL, MCL and PCL binding involved H-bonding and other weak forces like van der Waals, C-H bonds, pi-alkyl/alkyl and pi-pi interactions. MMS, the control inhibitor drug possessed a slightly lower - ΔG energy.

Table 4.33. Types of interactions and interacting residues of MMP-9 receptor involved in docking of *A. cymosa* compounds and controls drugs

Types of interaction	5TH6 (MMP-9)					
	IDL	MCL	PCL	DCP	MMS	CSL
Conventional hydrogen bond	Y420	E402	E402	-	D435, D434, D410 and Q77	P421
Van der waals	L418, M422, P421, R98, L188, Y423, L397 and R424	R424, M422, P421, R98, G100 and A189	M422, P421, Y423, R98, G100 and A189	A189, E402, R98, M422 and P421	H411, G438, L409, A417, S412, M419 and S413	P430, T426, A417, P415, F425, M422, E416, R98, H411 and E402
Pi-Pi Stacked/Pi-T Shaped	H401	H401	H401	-	-	-
Alkyl / Pi-alkyl	C99 and V398	L188, Y423, C99 and V398	V398, L188 and C99	V398, H401, Y423, C99 and L188	-	L418, L397, H401, C99 and Y420, R424, V398, L188, Y423

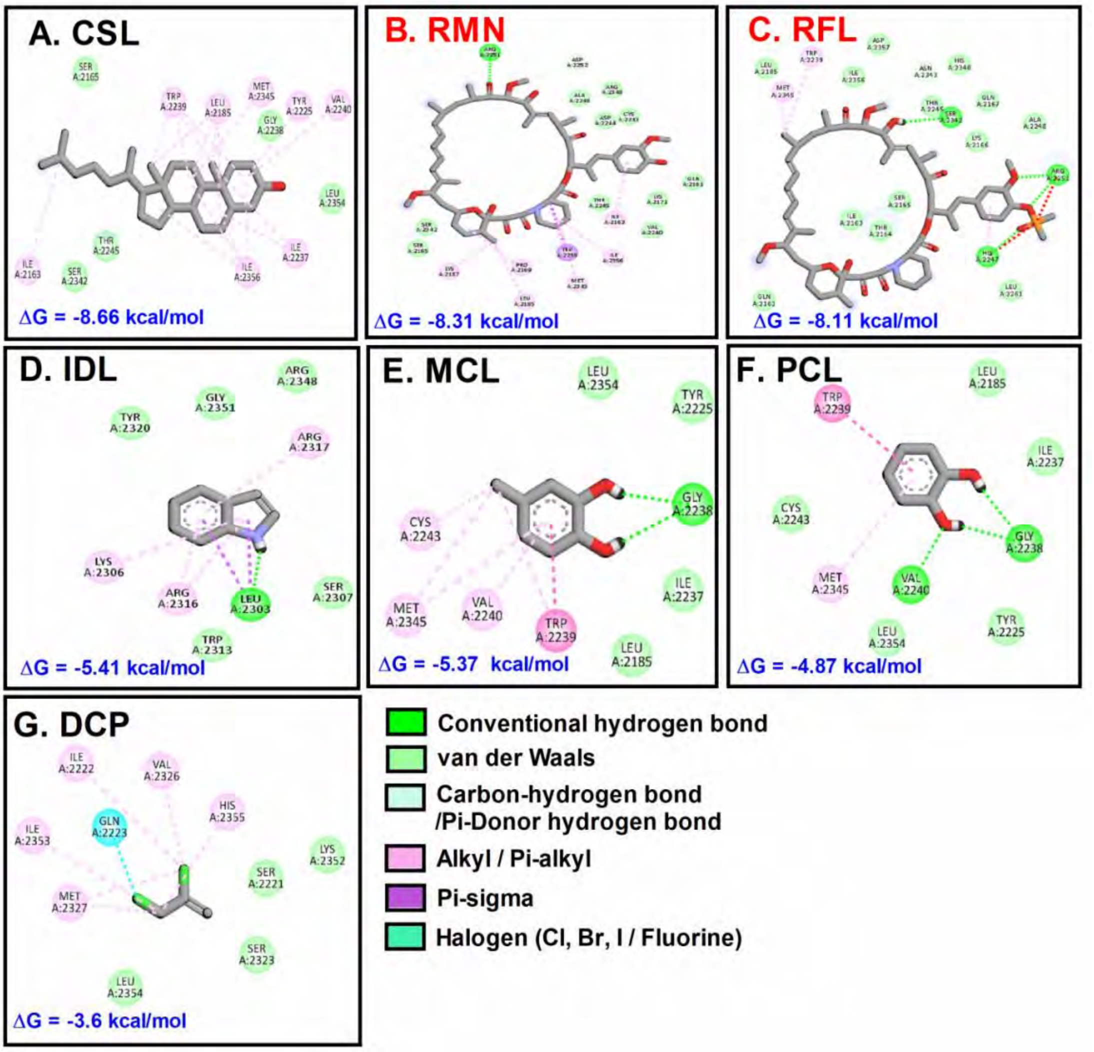

Figure 4.33. Docking interactions between mTOR and *A. cymosa* phytochemicals: Except CSL, all other phytochemicals from *A. cymosa* appeared to have weak binding to mTOR. The control ligands of mTOR, RMN and RFL, had much superior - ΔG values and correspondingly yielded low *K*i values. The numerous weak interactions and H-bond in CSL may be the reason for the very energetically favourable interaction between CSL and mTOR.

Table 4.34. Types of interactions and interacting residues of mTOR chain A receptor involved in docking of *A. cymosa* compounds and controls drugs

Types of interaction	4JT6 Chain A (mTOR)						
	CSL	**RMN**	**RFL**	**IDL**	**MCL**	**PCL**	**DCP**
Conventional hydrogen bond	-	Arg2251	S2342, R2251 and H2247	L2303	G2238	G2238 and V2240	-
Van der waals	S2165, G2238, L2354, T2245 and S2342	A2248, R2348, D2244, C2243, T2245, K2171, Q2161, V2240, S2342 and S2165	L2185, I2356, D2357, H2340, T2245, Q2165, K2166, A2248, S2165, I2163 and T2164	W2313, S2307, R2348, G2351 and Y2320	L2354, Y2225, I2237 and L2185	C2243, L2354, Y2225, I2237 and L2185	L2354, S2323, S2221 and K2352
Pi-sigma/ Amide Pi-Pi - stacked	-	W2239	-	-	W2239	W2239	-
Alkyl/Pi-alkyl / Halogen (Cl, Br, I)	I2163, I2356, I2237, V2240, Y2225, M2345, L2185 and W2239	K2187, P2169, L2185, M2345, I2356 and I2163	M2345 and W2239	-	C2243, M2345 and V2240	M2345	H2355, V2326, I2222, I2353, M2327 and Q2223
Carbon hydrogen bond donor / Pi-Donor hydrogen bond	-	D2252	Q2161, L2261 and N2343	-	-	-	-

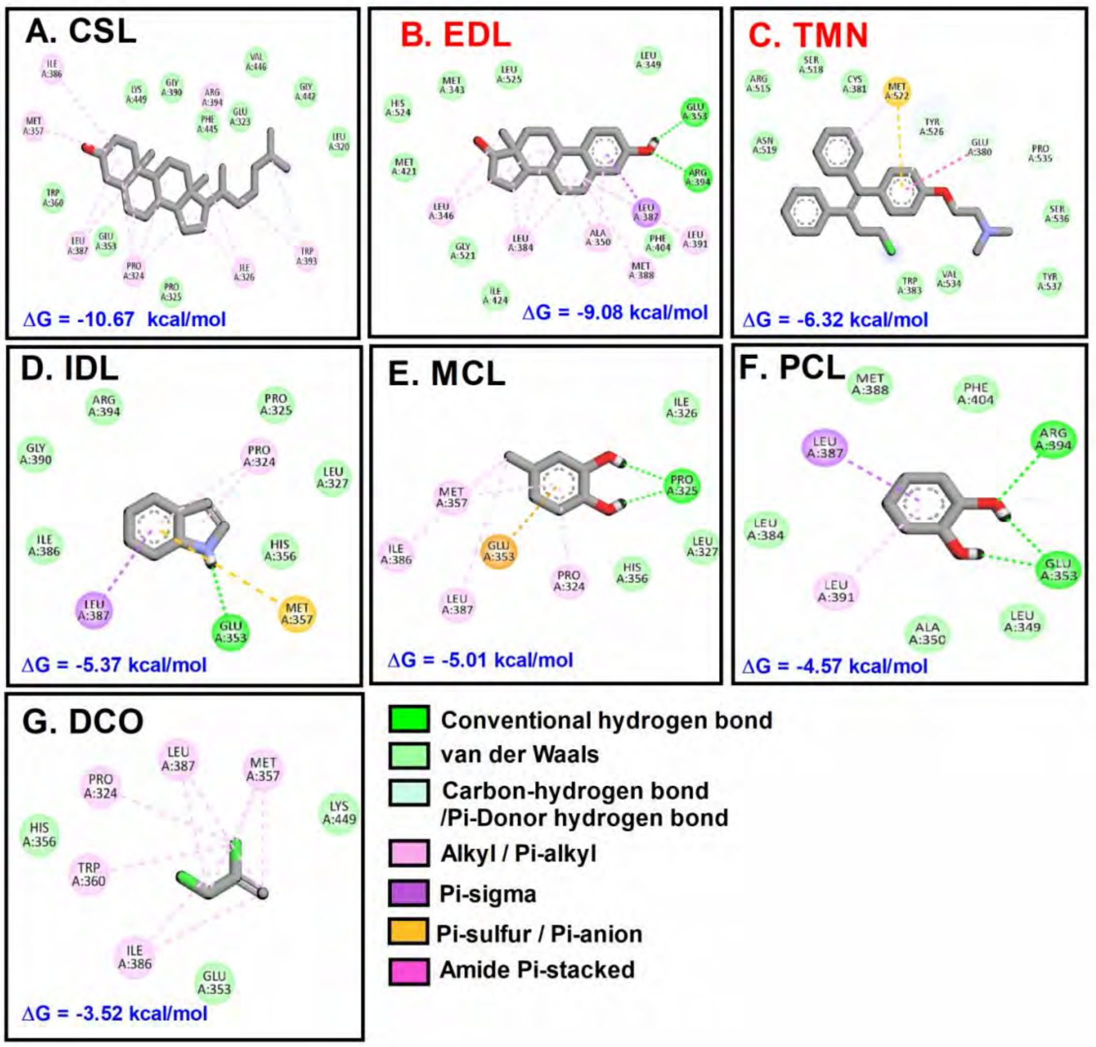

Figure 4.34. Docking interactions between ERβ and *A. cymosa* compounds: Binding of small molecules to ER may interfere with ER transactivation and aid in decreasing breast cancer growth and metastasis, especially in ER+ breast cancer cells. When compared to EDL, the natural ligand of ER, all other molecules except for CSL showed much poorer Gibbs free energy values. The intermolecular and weak forces which stabilized the receptor-ligand interactions are shown.

Table 4.35. Types of interactions and interacting residues of ERβ Chain A receptor involved in docking of *A. cymosa* compounds and controls drugs

Types of interaction	3OS8 Chain A (ERβ)						
	CSL	EDL	TMN	IDL	MCL	PCL	DCP
Conventional hydrogen bond	-	E353 and Arg394	-	E353	P325	R394 and E353	-
Van der waals	K449, G390, F445, E323, V446, G442, L320, P325, E353 and W360	L349, F404, I424, G521, M421, H524, M343 and L525	S536, Y537, V534, W383, N519, R515, S518 and C381	R394, P324, L327, H356, I386 and G390	I326, H356 and L327	M388, F404, L349, A350 and L384	H356, E353 and K449
Pi-sigma/ Amide Pi-stacked	-	L387	E380	L387	-	L387	
Pi-sulfir/Pi-Anion	-	-	M522	M357	E353	-	
Alkyl/Pi - alkyl	M357, I386, R394, I387, P324, I326 and W393	L391, M388, A350, L384 and L346	-	P324	M357, I386, L387 and P324	L391	P324, L387, M357, W360 and I386
Carbon hydrogen bond donor/Pi-Donor hydrogen bond	-	-	Y526, E380 and P535	-	-	-	-

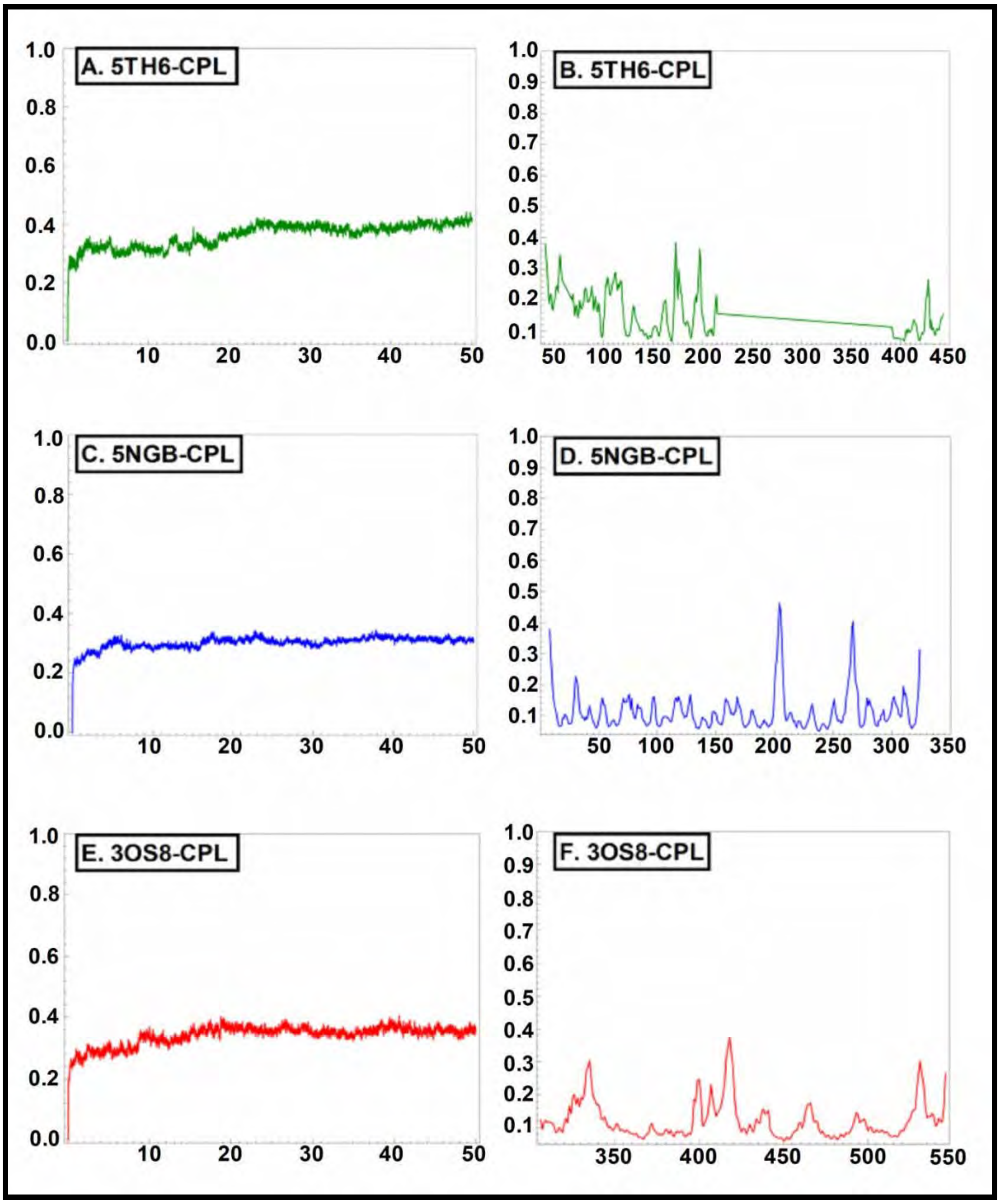

Figure 4.35. MD simulations of A & B - MMP-9; C & D - PI3k and E & F - ERβ with CSL, a common and promising ligand from *A. cymosa*. The MD simulations were performed for 50 ns and the RMSD (panels A, C, E) and RMSF (panels B, D and F) depict the real-time simulation and stability of receptor (PI3K/MMP-9/ER) - ligand (CSL) interactions. The RMSD plots show that the protein-ligand complexes were stable for almost the entire period of the simulation. The RMSF plots show that among residues of the entire protein analyzed, only a few notable residues had high fluctuations. Importantly, these fluctuations were not very high, and, understandably, a few amino acid side chains would experience a change in movement during the receptor-ligand interactions. Hence, these results indicate very high stability of the ligand interaction with the respective receptor protein docking sites over a period of 50 ns.

Future studies would involve bioavailability and assimilatory profiles that will promote the utility of these compounds as either leads or scaffolds for computer-aided drug design and confirm the traditional applications of *A. cymosa.* Similar study using medicagol, faradiol, and flavanthrin showed potent inhibition against SARS-CoV2 proteases with residues in the inhibitory zones containing H41, C45, M165, M49, Q189, T24, and T190 (Mahmud *et al*., 2021). The present study corresponds to differential binding with signalling cascade proteins in the present research encompassing PI3K / MMP-9 / mTOR / ERβ pathways affirming the roles for anti-inflammatory and antioxidant properties. Further, computational insights which help predict the structure-activity relationship, pharmacophore modelling and simulation modelling for virtual screening vs phytochemical catalogued compounds will provide conformational data for experimental validation. Hence, the present preliminary assessment summarizes the prolific benefits of the traditional medicinal plant's antimicrobial, antioxidant, anti-inflammatory properties and cytotoxicity profiles through *in vitro* and *in silico* methodologies.

4.14. Bioinformatics studies of *H. mystax*

Since *H. mystax* is a relatively poorly studied plant species thatare usually found in forests and hilly regions, it is not very accessible to the general population. There are very scanty reports of the bioactivity of this species in various traditional ayurvedic/Siddha literatures. Hence, the plant was studied in sufficient detail to justify its anti-inflammatory and anthelmintic activities reported in recent literature (Duarte Galhardo de Albuquerque *et al*., 2020). To the best of our knowledge, the exact mechanisms underlying the said activities are unknown, and hence we have employed some additional bioinformatics tools to understand it more. Even the phytochemical profile of this plant is as yet unknown. In this present study, we aimed to identify the phytochemical composition of the crude extract, as well as to analyze the intermolecular interactions between the identified compounds and six different breast cancer proteins such as Akt-1, MMP-9, HER2, mTOR, PI3K and ERβ. These proteins are prominently involved in breast cancer pathophysiology, and hence, these were chosen for docking studies. In the GC-MS studies, several compounds were identified (Table 4.27).

4.14.1. Molinspiration, PASSOnline server results and selection of receptors

The Molinspiration server results and biological activities predicted by PASSOnline server are given in Table 4.36 and Table 4.37, respectively. The data from Molinspiration server suggested that among the five compounds, judging from the positive values obtained, β-tocopherol had the highest bioactivity. This molecule was predicted to have nuclear receptor binding (score of +0.45), a protease inhibitor (+0.25), GPCR ligand (+0.21) and enzyme inhibitor (+0.20) activities (and a minor ion channel modulating activity, score = +0.09). In Molinspiration results, positive scores are correlated with higher biological activity. Carotol (CTL) also was predicted to possess nuclear receptor binding, ion channel binding and enzyme inhibitory activity. Neophytadiene was predicted to serve as an enzyme inhibitor. All the other compounds (loliolide, 2-methyl 4-vinyl phenol, 1,2-cyclooctanedione, desaspidinol and some other poorly explored compounds which were identified through GC-MS) were filtered out based on the results from Molinspiration and PASSOnline servers. The results for PASSOnline prediction showed that among these five compounds, four of them had anticancer (antineoplastic, antimetastatic, antileukemic) and anti-inflammatory properties and therefore, these compounds were considered to be involved in mediating the observed cytotoxic and anti-inflammatory activities that were observed *in vitro*. Also, the analysis of genes (gene weight scores) involved in breast cancer confirmed that these targets MMP9, HER2 (ErbB2), PI3K, mTOR, Akt1 and ERβ were among the top 100 odd genes among the 4979 genes (hits) from Gene (NCBI) search. Hence, we finalized these protein targets based on their rank in the NCBI gene search results for the keyword "breast cancer" and "*Homo sapiens*". We attempted to discover whether the compounds identified through GC-MS were capable of binding to these targets. Methaqualone showed no significant results.

Table 4.36. Molinspiration server predictions on *H. mystax* compounds

Natural compounds	GPCR ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor
BTP (β-tocopherol)	***0.21***	**0.09**	-0.22	***0.45***	***0.25***	***0.20***
MQL (Methaqualone)	-0.12	-0.18	-0.46	-0.60	-0.60	-0.32
DHA (Dihydroactinidiolide)	-0.58	-0.43	-1.02	-0.41	-0.64	-0.24
CTL (Carotol)	-0.09	***0.21***	-0.67	***0.42***	-0.21	***0.32***
NPD (Neophytadiene)	-0.12	-0.02	-0.35	***0.20***	-0.11	***0.14***

Table 4.37. PASSOnline server results for *H. mystax* compounds

1. DHA		
Pa	**Pi**	**Activity**
0,913	0,004	Antileukemic
0,888	0,005	Antineoplastic
0,622	0,004	Antimetastatic
0,621	0,030	Oxidoreductase inhibitor
0,558	0,006	BRAF expression inhibitor
0,549	0,033	Apoptosis agonist
0,539	0,023	MMP9 expression inhibitor
0,573	0,058	TP53 expression enhancer
0,478	0,015	Antineoplastic (lung cancer)

2. CTL		
Pa	**Pi**	**Activity**
0,748	0,004	Adenomatous polyposis treatment
0,708	0,015	Antiinflammatory
0,603	0,025	Prostaglandin-E2 9-reductase inhibitor
0,597	0,037	Oxidoreductase inhibitor
0,601	0,045	Antineoplastic
0,569	0,019	MMP9 expression inhibitor
0,556	0,007	Dementia treatment
0,518	0,005	Estrogen agonist
0,521	0,016	Antimetastatic

3. NPD		
Pa	**Pi**	**Activity**
0,860	0,014	Phobic disorders treatment
0,803	0,018	Mucomembranous protector
0,788	0,037	Ubiquinol-cytochrome-c reductase inhibitor
0,646	0,023	Antiarthritic
0,649	0,036	TP53 expression enhancer
0,545	0,033	Peroxidase inhibitor
0,516	0,007	BRAF expression inhibitor
0,548	0,056	Oxidoreductase inhibitor
0,482	0,017	Myc inhibitor

4. BTP		
Pa	**Pi**	**Activity**
0,950	0,002	Peroxidase inhibitor
0,936	0,002	Lipid peroxidase inhibitor
0,925	0,003	Antioxidant
0,913	0,005	TP53 expression enhancer
0,843	0,002	AR expression inhibitor
0,841	0,004	Nucleotide metabolism regulator
0,808	0,004	Reductant
0,814	0,013	Antiischemic, cerebral
0,800	0,009	Acute neurologic disorders treatment
0,771	0,003	Free radical scavenger
0,756	0,010	Antiinflammatory
0,771	0,043	Ubiquinol-cytochrome-c reductase inhibitor
0,698	0,015	Apoptosis agonist
0,682	0,010	Cholesterol antagonist
0,658	0,001	Cholesterol synthesis inhibitor
0,657	0,007	Cell adhesion molecule inhibitor
0,640	0,008	Proliferative diseases treatment
0,550	0,008	Myc inhibitor

4.14.2. POCASA and Molecular Docking Results

POCASA analysis was performed for all six chosen receptors. The number of cavities for the receptors was identified to be - Akt1 (12), MMP9 (7), HER2 (9), PI3K (24), mTOR (37) and ERβ (11 cavities); of these, the volume ($Å^3$) and volume depth (VD) were computed for the top 5 cavities for each protein (Table 4.38). Based on these results, the cavities which contained the active site alone were chosen for docking. To understand the mechanisms underlying the observed differences in cytotoxicity, all the 58 compounds (identified in GC-MS study) were evaluated for their action against key breast cancer protein targets using molecular docking. The Gibbs binding energies (ΔG), inhibition constant (K_i) and other key parameters (like logP) were assessed by comparing the data for the respective controls of these protein targets (commercially available drugs which are known to serve as active site ligands) to validate the efficacy of the plant compounds. The binding location of the compounds (both plant and respective control compounds) was explored; Figure 4.36 shows the active site of the six receptor targets and the binding location of the compounds. Surface views are also presented for the bound ligands, showing the active site cavities.

Table 4.38. POCASA analysis

PDB ID	Chain	Probe radius (Å)	Single Point Flag (SPF)	Protein Depth Flag (PDF)	Grid size (Å)	Volume (Å^3)	VD value	Average VD	Pocket
4JT6	A	2	16	18	1.0	2143	7607	3.55001	1244
						2153	7553	3.50844	1088
						649	1799	2.77247	1178
						176	454	2.58333	636
						120	417	3.48056	299
3OS8	A	2	16	18	1.0	233	2288	9.82117	260
						87	420	4.83525	132
						60	203	3.38889	394
						74	180	2.44144	107
						51	128	2.51634	83
5NGB	A	2	16	18	1.0	1622	7710	4.7536	815
						333	1440	4.32432	69
						184	480	2.61051	853
						137	446	3.26034	1288
						123	280	2.28184	653

PDB ID	Chain	Probe radius (Å)	Single Point Flag (SPF)	Protein Depth Flag (PDF)	Grid size (Å)	Volume (Å^3)	VD value	Average VD	Pocket
3PP0	A	2	16	18	1.0	318	1661	5.22537	125
						293	851	2.90557	34
						104	292	2.80769	411
						45	115	2.57037	468
						42	111	2.65873	341
3CQW	A	2	16	18	1.0	527	1950	3.70145	350
						110	277	2.52121	86
						85	202	2.38431	393
						68	166	2.44118	16
						61	155	2.54098	108
5TH6	A	2	16	18	1.0	157	696	4.43524	153
						107	836	7.81308	66
						73	172	2.36073	135
						42	99	2.36508	304
						40	98	2.45833	266

4.14.3. Molecular Docking Studies

To understand the mechanisms underlying the observed differences in cytotoxicity, all the 58 different compounds of *H. mystax* were evaluated for their action against key breast cancer protein targets using molecular docking. Among the six proteins studied herein, the compounds BTP, CTL, MQL, DHA and NPD were consistently among the top 3 phytochemicals in the docking results. The binding energy and K_i values of these compounds with the respective breast cancer protein receptors are compared with the results for the respective inhibitor ligands. For example, BTP (β-tocopherol) was found to dock with nanomolar or low μM inhibition constant (K_i) values to Akt1 (K_i = 30 nM), HER2 (K_i = 18 nM), mTOR (K_i = 0.3 μM), PI3K (K_i = 0.1 μM) and ERβ (K_i = 10 nM). This suggests that BTP could simultaneously bind to the various proteins. The ΔG value for BTP binding to Akt1 was more or less similar to that for control saracatinib, and this was better than the biding energy values of the respective control compounds to HER2 and ERβ. Both MQL and CTL had lower ΔG (and K_i) values than the control compounds that were docked to MMP9. In some cases, the plant compounds were found to have lower binding energies (w.r.t. the control drugs). Table 4.39 shows that the *H. mystax* phytocompounds (especially, BTP, CTL, NPD, DHA and MQL) could serve as active site ligands of key breast cancer proteins.

Table 4.39. Molecular docking of *H. mystax* compounds identified through GC-MS: Best energy compounds and control drugs

<table>
<tr><th colspan="4">Docking studies for key breast cancer proteins</th><th>Binding Analysis using ChimeraX</th></tr>
<tr><th rowspan="2">Protein targets</th><th rowspan="2">Ligands</th><th colspan="2">Active site docking</th><th rowspan="2">Binding location in exact active site?</th></tr>
<tr><th>Binding energy (kcal/mol)</th><th>Inhibition constant, K_i (μM)</th></tr>
<tr><td rowspan="5">A. 3CQW (Akt1)</td><td>β-tocopherol</td><td>-10.19</td><td>0.033</td><td>Yes</td></tr>
<tr><td>methaqualone</td><td>-6.72</td><td>11.95</td><td>near active site</td></tr>
<tr><td>dihydroactinidiolide</td><td>-6.26</td><td>25.96</td><td>Yes</td></tr>
<tr><td>Saracatinib</td><td>-10.43</td><td>0.022</td><td>Yes</td></tr>
<tr><td>Wortmannin</td><td>-7.88</td><td>1.67</td><td>near active site</td></tr>
<tr><td rowspan="5">B. 5TH6 (MMP9)</td><td>Caratol</td><td>-9.92</td><td>0.053</td><td>Yes</td></tr>
<tr><td>methaqualone</td><td>-9.84</td><td>0.061</td><td>Yes</td></tr>
<tr><td>neophytadiene</td><td>-8.17</td><td>1.02</td><td>No</td></tr>
<tr><td>Marimastat</td><td>-7.78</td><td>2.44</td><td>No</td></tr>
<tr><td>Batimastat</td><td>-7.02</td><td>7.06</td><td>No</td></tr>
<tr><td rowspan="5">C. 3PP0 (HER2)</td><td>β-tocopherol</td><td>-10.55</td><td>0.018</td><td>Yes</td></tr>
<tr><td>Caratol</td><td>-8.61</td><td>0.487</td><td>Yes</td></tr>
<tr><td>methaqualone</td><td>-8.35</td><td>0.751</td><td>Yes</td></tr>
<tr><td>Lapatinib</td><td>-8.71</td><td>0.409</td><td>Yes</td></tr>
<tr><td>Neratinib</td><td>-6.87</td><td>9.26</td><td>Yes</td></tr>
<tr><td rowspan="5">D. 4JT6 (mTOR)</td><td>β-tocopherol</td><td>-8.82</td><td>0.340</td><td>Yes</td></tr>
<tr><td>methaqualone</td><td>-7.87</td><td>1.72</td><td>Yes</td></tr>
<tr><td>Caratol</td><td>-7.27</td><td>4.66</td><td>Yes</td></tr>
<tr><td>Rapamycin</td><td>-9.05</td><td>0.234</td><td>near active site</td></tr>
<tr><td>ridaforolimus</td><td>-8.97</td><td>0.266</td><td>Yes</td></tr>
<tr><td rowspan="6">E. 5NGB (PI3K)</td><td>β-tocopherol</td><td>-9.37</td><td>0.135</td><td>near active site</td></tr>
<tr><td>Caratol</td><td>-8.55</td><td>0.540</td><td>near active site</td></tr>
<tr><td>methaqualone</td><td>-8.31</td><td>0.812</td><td>near active site</td></tr>
<tr><td>Dactolisib</td><td>-10.58</td><td>0.017</td><td>Yes</td></tr>
<tr><td>Taselisib</td><td>-10.04</td><td>0.043</td><td>Yes</td></tr>
<tr><td>Umbralisib</td><td>-9.24</td><td>0.168</td><td>near active site</td></tr>
<tr><td rowspan="5">F. ERβ (3OS8)</td><td>β-tocopherol</td><td>-10.91</td><td>0.010</td><td>Yes</td></tr>
<tr><td>methaqualone</td><td>-7.49</td><td>3.26</td><td>Yes</td></tr>
<tr><td>Caratol</td><td>-7.18</td><td>5.46</td><td>Yes</td></tr>
<tr><td>Toremefine</td><td>-9.69</td><td>0.078</td><td>Yes</td></tr>
<tr><td>Estradiol</td><td>-9.32</td><td>0.142</td><td>Yes</td></tr>
</table>

4.14.4. Protein-ligand interactions and binding site analysis

Visualization of the intermolecular interactions between *H. mystax* compounds (and control ligands for comparison) with the chosen receptor targets was carried out using Discovery Studio Viewer and the binding location of the molecules to the six selected receptors was visualized using ChimeraX (Goddard *et al*., 2018). The intermolecular interactions stabilised these molecules; binding to the active sites of the chosen receptors such as H-bonds, Van der Waals interactions, pi-pi, pi-alkyl, pi-sigma pi-lone pair, pi-sulfur, halogen bonding are shown in Figures 4.37-4.48 (Table 4.40 - 4.45). The involvement of different H-bonds and other weak forces in stabilizing the receptor-ligand interactions reveals that the interactions were thermodynamically feasible and stable. In Figure 4.36, the best configurations of docked outputs (PDB) of both *H. mystax* compounds (BTP, CTL, MQL, DHA and NPD) with the chosen protein receptors are shown.

Table 4.40. Molecular docking of *H. mystax* compounds identified through GC-MS: All the binding compounds

Docking studies for key breast cancer proteins				
Protein targets	**Ligands**	**Binding energy**	**Ligand efficiency**	**inhib_constant (μM)**
A. 3CQW (Akt1)	1,2-cyclooctanedione	-5.45	-0.55	101.07
	2-methoxy-4-vinylphenol	-5.33	-0.58	22.7
	1,2-Di-tert-butylbenzene	-5.67	-0.41	69.75
	4-Hydroxycyclohexanone	-5.68	-0.71	68.31
	Catechol	-4.39	-0.55	610.3
	Dasaspidinol	-5.54	-037	86.73
	Dihydroactinidiolide	-5.36	-0.41	117.25
	Loliolide	-6.16	-0.46	21.38
	Neophytadiene	-5.8	-0.29	56.05
	Phytol	-5.36	-0.26	117.02
	Tert-butylhydroquinone	-5.63	-0.47	74.96
	2,6-Dimethoxyphenol	-4.54	-0.41	472.79
B. 5TH6 chain A (MMP9)	1,2-cyclooctanedione	-6.57	-0.66	15.29
	2-methoxy-4-vinylphenol	-6.48	-0.59	17.94
	1,2-Di-tert-butylbenzene	-8.09	-0.58	1.18
	4-Hydroxycyclohexanone	-5.62	-0.7	75.89
	Catechol	-5.68	-0.71	68.58
	Dasaspidinol	-7.74	-0.52	2.13
	Dihydroactinidiolide	-7.42	0.57	3.64
	Loliolide	-7.92	-0.52	1.56
	Phytol	-6.34	-0.3	22.55
	Tert-butylhydroquinone	-7.28	-0.61	4.59
	2,6-Dimethoxyphenol	-5.81	-0.53	55.44
C. 3PP0 Chain A (HER2)	1,2-cyclooctanedione	-5.42	-0.54	106.38
	2-methoxy-4-vinylphenol	-5.26	-0.48	138.44
	1,2-Di-tert-butylbenzene	-6.88	-0.49	9.13
	4-Hydroxycyclohexanone	-5.02	-0.63	209.93
	Catechol	-4.52	-0.56	485.27
	Dasaspidinol	-6.27	-0.42	25.25
	Dihydroactinidiolide	-5.85	-0.45	52.27
	Loliolide	-6.74	-0.48	11.54
	Neophytadiene	-7.46	-0.37	3.43
	Phytol	-6.97	-0.33	7.74
	Tert-butylhydroquinone	-6.07	-0.51	35.49
	2,6-Dimethoxyphenol	-4.6	-0.42	421.42

Docking studies for key breast cancer proteins				
Protein targets	**Ligands**	**Binding energy**	**Ligand efficiency**	**inhib_constant (μM)**
D. 4JT6 chain A (mTOR)	1,2-cyclooctanedione	-6.08	-0.61	35.21
	2-methoxy-4-vinylphenol	-5.99	-0.54	40.4
	1,2-Di-tert-butylbenzene	-6.74	-0.48	11.41
	4-Hydroxycyclohexanone	-5.0	-0.63	215.34
	Catechol	-4.88	-0.61	262.89
	Dasaspidinol	-6.23	-0.42	27.15
	Dihydroactinidiolide	-6.27	-0.48	25.23
	Phytol	-5.84	-0.28	52.41
	Tert-butylhydroquinone	-6.75	-0.56	11.36
	2,6-Dimethoxyphenol	-5.27	-0.48	136.93
E. 5NGB (PI3K)	1,2-cyclooctanedione	-5.88	-0.59	49.26
	2-methoxy-4-vinylphenol	-5.79	-0.53	57.14
	1,2-Di-tert-butylbenzene	-7.53	-0.54	3.01
	Catechol	-4.99	-0.62	219.63
	Dasaspidinol	-6.33	0.42	23.11
	Dihydroactinidiolide	-6.16	-0.47	30.57
	Loliolide	-7.14	-0.51	5.83
	Neophytadiene	-8.13	-0.41	1.1
	Phytol	-6.85	-0.33	9.54
	Tert-butylhydroquinone	-5.87	-0.49	49.46
F. 3OS8 chain A (ER)	1,2-Di-tert-butylbenzene	-6.63	-0.47	13.92
	4-Hydroxycyclohexanone	-5.55	-0.69	86.12
	Dasaspidinol	-5.66	-0.38	71.4
	Dihydroactinidiolide	-5.8	-0.45	55.59
	Loliolide	-7.07	-0.53	23.37
	Neophytadiene	-6.84	-0.34	9.75
	Phytol	-7.5	-0.36	3.19
	Tert-butylhydroquinone	-6.02	-0.5	38.98
	2,6-Dimethoxyphenol	-4.96	-0.46	231.6

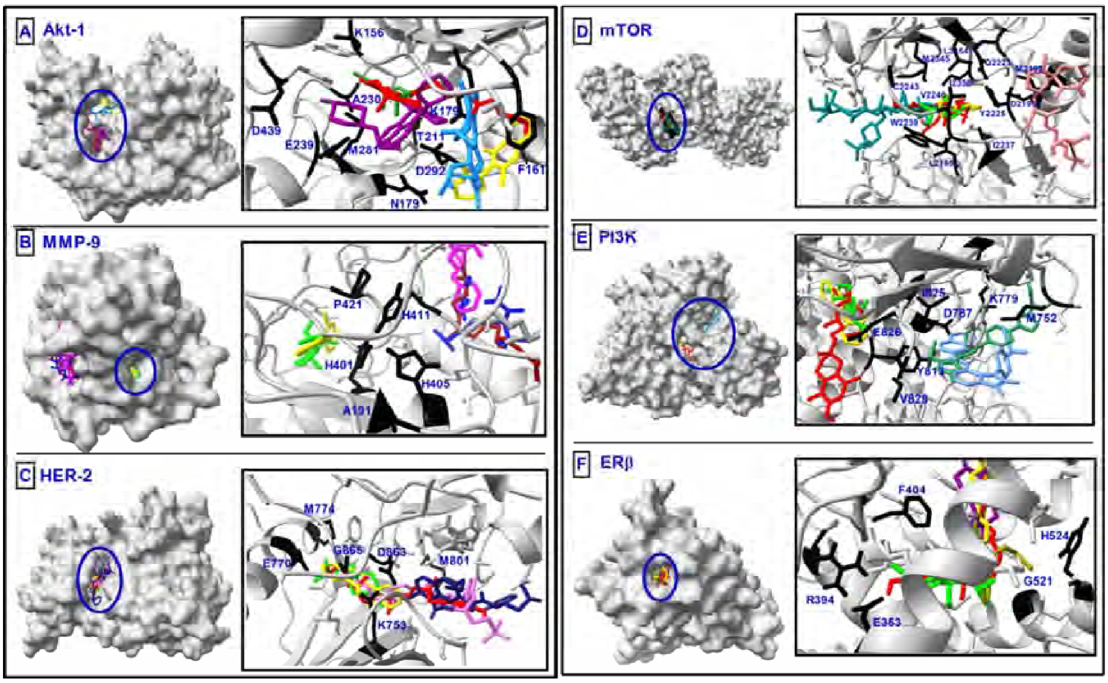

Figure 4.36. Ligandinteraction surface and active site view of breast cancer protein targets - The binding locations of the compounds were explored using ChimeraX. The surface view and active site views are presented for each receptor protein. In all panels, the plant compounds (represented as sticks) were coloured as follows: β-tocopherol (BTP) - bright red, Carotol (CTL) - lime green, methaqualone (MQL) - yellow, dihydroactinidiolide (DHA) - dark forest green and Neophytadiene (NPD) - dark red. For different control compounds, the colour representations are as follows: in Akt1 (panel A), saracatinib (STB) - dark magenta and wortmannin (WMN) - dodger blue; in MMP9 (panel B), batimastat (BMS) - magenta and marimastat (MMS) - blue; in HER2 (panel C), lapatinib (LTB) - orchid and neratinib (NTB) - midnight blue; in mTOR (panel D), ridafurolimus (RFL) - teal colour and rapamycin (RMN) - light coral; in PI3K (panel E), dactolisib (DLB) - cornflower blue, taselisib (TLB) - sea green and umbralisib (ULB) - cyan; and finally, in ERβ (panel F), estradiol (EDL) - purple and toremifene (TMN) - olive. The black sticks and labels indicate the active site amino acid residues.

A. Akt1: Akt1 is responsible for breast cancer progression *in vivo* via HER2 (ErbB2) (Ju *et al*., 2007). The active site of Akt1 is comprised of L156, F161, K179, T211, A230, E234, N279, M281, D292 and D439 (Guo *et al*., 2019). While BTP, DHA and the control drug STB were found to dock exactly inside the active site region (see Table 4.39, right side), the other compounds - WMN and MQL were found to bind close to the active site, in the substrate docking channel/cleft (Figure 4.36A). For efficient protein-protein interaction to occur, the Akt1 cavity must be completely free, and therefore, the binding of any organic molecule to the cleft can significantly affect substrate binding and phosphoryl group transfer at the kinase site. Hence, BTP, MQL and DHA, which docked favourably with Akt1, could prevent substrate (protein) binding and phosphorylation. Compared to the other two plant compounds (MQL and DHA), BTP yielded a much lower binding energy (<10 kcal/mol) for Akt1. BTP binding to Akt1 was stabilized by H-bonds with E228 and A230. A higher contribution of Van der Waals forces was observed, and alkyl/pi-alkyl interactions were also observed in BTP binding to Akt1 (Table 4.41, Figures 4.37 and 4.38).

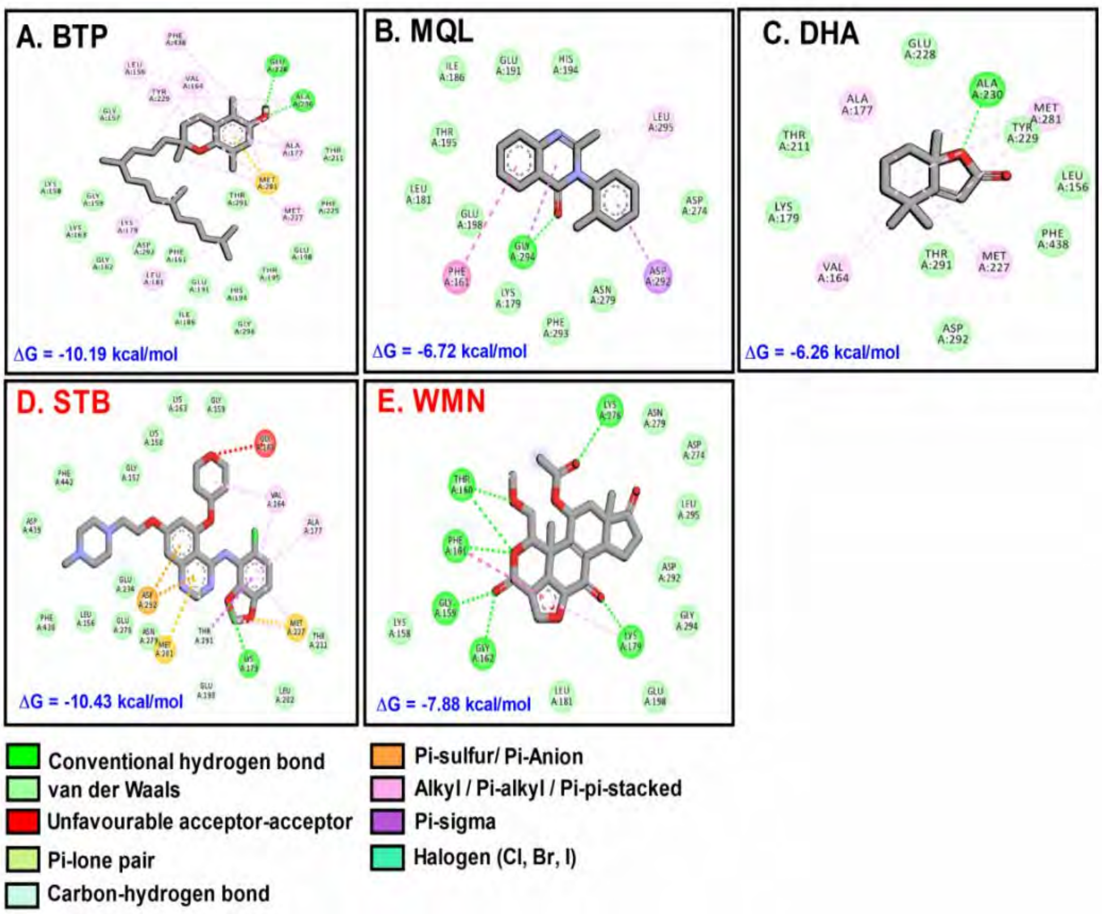

Figure 4.37. Interactions of Akt-1 with control drugs (STB and WMN) as well as *H. mystax* best energy compounds

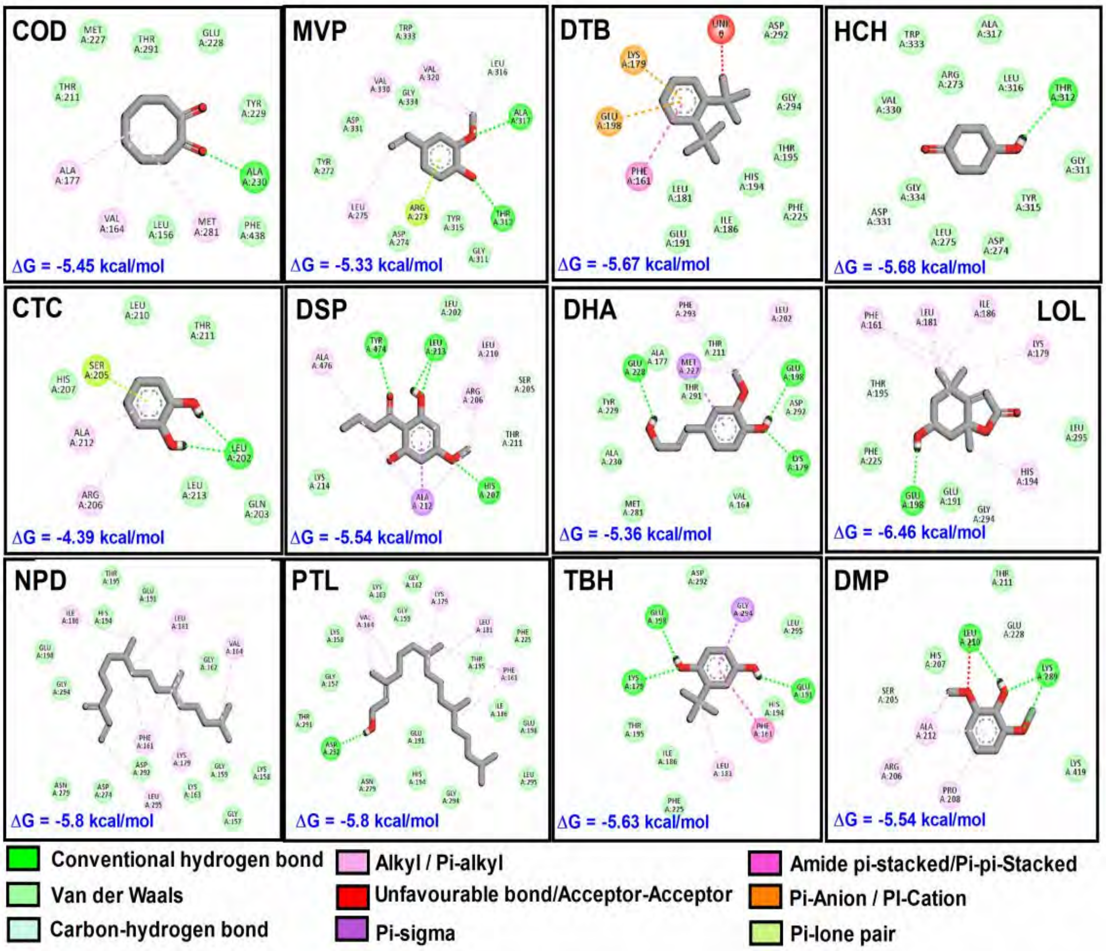

Figure 4.38. Interactions of Akt-1 with other binding compounds of *H. mystax*

Table 4.41. Types of interactions and interacting residues involved in docking of *H. mystax* compounds (and controls) with AKT-1 (3CQW)

3CQW (AKT-1)	Types of interactions and Interacting Residues					
	Conventional hydrogen bond	**Van der Waals**	**Unfavourable donor-donor / Pi-Sulfur / Pi-cation / Pi-anion / pi lone pair**	**Pi-Sigma / amide Pi-stacked / Pi-pi T-shaped**	**Carbon hydrogen bond donor**	**Alkyl/Pi – alkyl**
BTP	E228 and A230	G157, K158, G159, K163, G162, D292, F161, E191, I186, H194, G294, T195, E198, F225, T291 and T211	M281	-	-	F438, L156, Y229, V164, A177, M227, K179 and L181
MQL	G294	I186, E191, H194, D274, N279, F293, K179, E198, L181, T195	-	D292 and F161	-	L295
DHA	A230	Y229, L156, F438, T291, D292, K179, T211 and E228	-	-	-	A177, M281, M227 and V164
COD	A230	T211, M227, T291, E228, Y229, L156 and F438	-	-	-	A177, V164 and M281
MVP	A317 and T312	W333, G334, D272, Y272, D274, Y315 and G311	R273	-	L316	V320, V330 and L275
DTB	-	D292, G294, T195, H194, L181, E191, I186 and F225	K179 and E198	F161	-	-
HCH	T312	W333, A317, V330, R273, L316,G334, Y315, G311, L275 and D274	-	-	D331	-
CTC	L202	L210, T211, H207, L213 and Q203	S205	-	-	A212 and R206

3CQW (AKT-1)	Types of interactions and Interacting Residues					
	Conventional hydrogen bond	**Van der Waals**	**Unfavourable donor-donor / Pi-Sulfur / Pi-cation / Pi-anion / pi lone pair**	**Pi-Sigma / amide Pi-stacked / Pi-pi T-shaped**	**Carbon hydrogen bond donor**	**Alkyl/Pi – alkyl**
DSP	Y474, L213 and H207	L202 and Y214	-	A212	S205 and T211	A476, L210 and R206
LOL	E198	F195, E191 and L295,	-	-	T195 and G294	F161, L181, I186, K179 and H194
NPD	-	T195, E191, H194, E198, G294, G162, N279, D274, D292, K163, G159, K158 and G157	-	-	-	I186, L181, V164, F161, K179 and L295
PTL	D292	K163, G162, G159, G157, T291, F225, T195, I186, G198, L295, G191, N279, H194 and G294	-	-	-	V164, K179, L181 and F161
TBH	E198, K179 and G191	D292, L295, T195, I186, H194 and F225	-	G294 and F161	-	L181
DMP	L210 and K289	T211, H207, and K419	-	-	G228, S205 and K419	A212, R206 and P208
STB	Lys179	T211, L202, D279, E278, E234, L156, F438, D439, F442, G157, K158 and K163, G159	G162, D292, M281 and M227	H401	T291 and E198	V124 and A177
WMN	T160, F161, G159, G162, K179 and K276	N279, D274, L295, D292, G294, E198, L181 and K158	-	-	-	-

B. MMP9: Matrix metalloproteinase-9 is a breast cancer biomarker and a protease that cleaves the proteins present in the extracellular matrix (ECM), remodels the ECM, and paves the way for metastasis (Bergers *et al*., 2000). The active site of MMP9 is comprised of the amino acids A191, H401, H405, H411 and P421 (Rowsell *et al*., 2002). MMP9 and MMP-2 together represent the gelatinase subgroup of the MMP family; these MMPs facilitate invasion, metastasis and angiogenesis of cancer tumours (Huang, 2018). In MMP9, CTL and MQL were found to bind close to the active site (H401 and P421), while the other molecules were found to bind to the surface of the protein (Figure 4.36B). Only CTL and MQL appear to have docked into the active site channel. The ΔG (and K_i) values were in this order: CTL (-9.92 and K_i = 53 nM) > MQL (-9.84 and K_i = 61 nM), while MMS and BMS (unbeknownst to us) repeatedly bound at an alternative site. MQL binding to MMP9 was facilitated by H-bonding to H_{401}; other weak forces played a dominant role in the intermolecular interactions. CTL binding did not involve any H-bonds; receptor binding of CTL was mainly stabilized by weak forces (Table 4.42 and Figures 4.39 and 4.40).

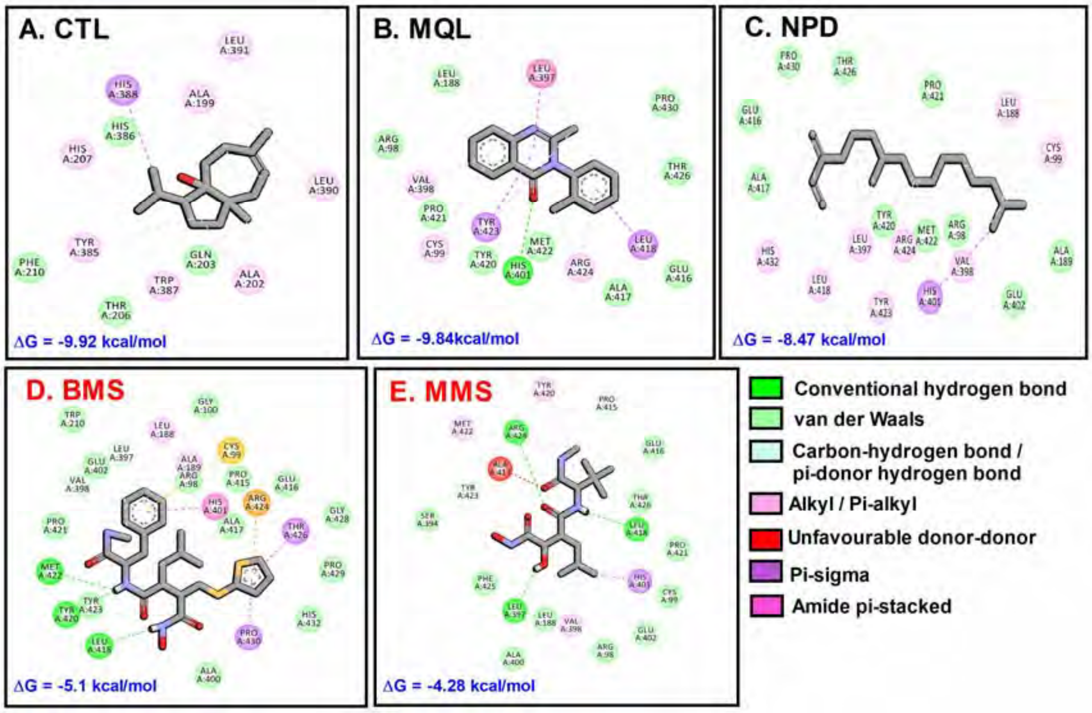

Figure 4.39. Interactions of MMP-9 with control drugs (MMS and BMS) as well as *H. mystax* best energy compounds

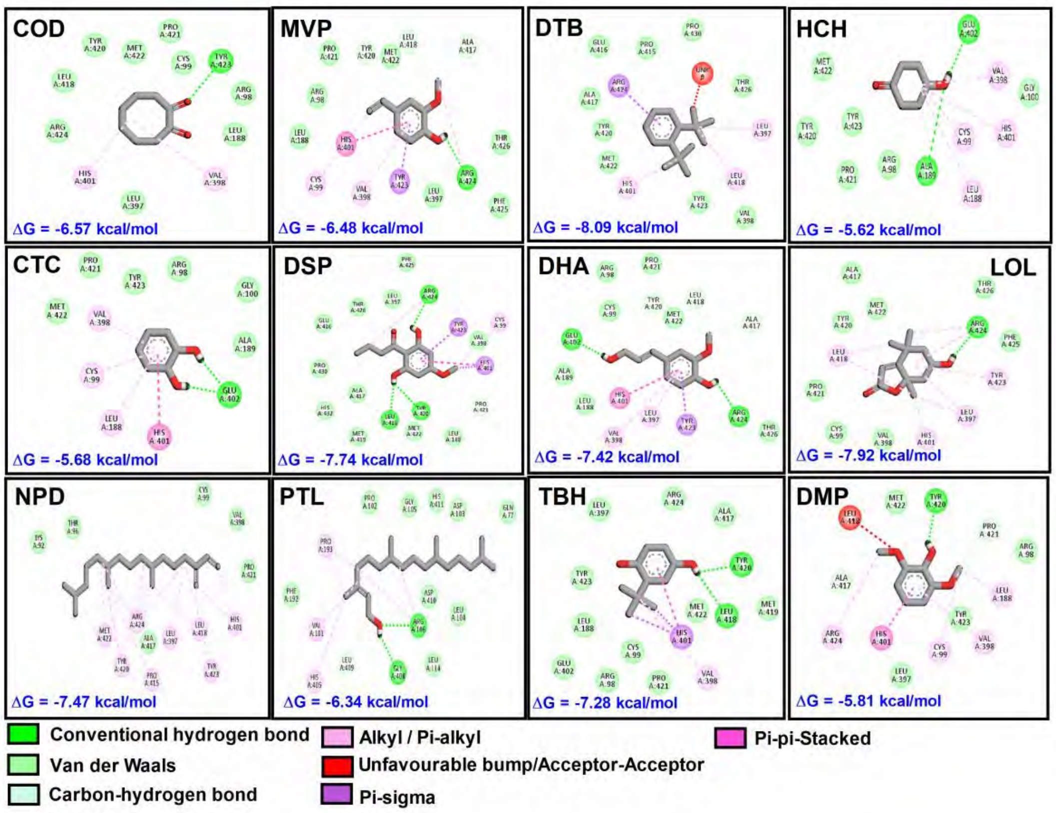

Figure 4.40. Interactions of MMP-9 with other binding compounds of *H. mystax*

Table 4.42. Interactions between MMP-9 and *H. mystax* phytocompounds in comparison with inhibitors of MMP-9

MMP9	Types of interactions and Interacting Residues					
	Conventional hydrogen bond	**Van der Waals**	**Unfavourable donor-donor / Pi-Sulfur / Pi-cation**	**Pi-Sigma / amide Pi-stacked**	**Carbon hydrogen bond donor**	**Alkyl / Pi-alkyl**
MQL	H401	L188, P430, T426, E416, A417, Y420, M422, P421 and R98	-	Y423, L418 and L397	-	V398, C99 and R424
NPD	-	P430, T426, P421, A189, E402, R98, M422, Y420, A417 and E416	-	H401	-	H432, L418, L397, R424, Y423, V398, L188 and C99
CTL	-	F210, T206, Q203 and H386	-	H388	-	L391, A199, L390, A202, W387, Y385 and H207
COD	Y423	P421, C99, M422, Y420, L418, R424, L397, L188 and R98	-	-	-	H401 and V398
MVP	R424	P421, M422, R98, L188, T426, L397 and F425	-	Y423 and H401	Y420, L418 and A417	C99 and V398
DTB	-	P430, P415, E416, A417, Y420, M422, Y423, V398 and T426	-	R424	-	L397, L418 and H401
HCH	E402 and A189	M422, Y423, Y420, R98, P421 and G100	-	-	-	V398, H401, C99 and L188
CTC	E402	M422, P421, Y423, R98, G100 and A189	-	H401	-	V398, C99 and L188
DSP	R424, L418 and Y420	F425, L397, T426, E416, P430, A417, V398, H432, M419, M422 and L188	-	H401 and Y423	P421	C99

MMP9	Types of interactions and Interacting Residues					
	Conventional hydrogen bond	**Van der Waals**	**Unfavourable donor-donor / Pi-Sulfur / Pi-cation**	**Pi-Sigma / amide Pi-stacked**	**Carbon hydrogen bond donor**	**Alkyl / Pi-alkyl**
DHA	E402 and R424	P421, R98, C99, M422, A189, L188 and T426	-	H401 and Y423	Y420, L418 and A417	L397 and V398
LOL	R424	A417, M422, Y420, P421, T426, F425, V398 and C99	-	-	-	L418, Y423, L397 and H401
PTL	R106 and G408	P102, G105, H411, D410, L104, Q77, D103, F192 and L114	-	-	L409	P193, V101 and H405
TBH	Y420 and L418	L397, R424, A417, Y423, L188, M422, M419, C99, E402, R98 and P421	-	H401	-	V398
DMP	Y420	M422, R98, Y423 and L397	L418	H401	P421 and A417	L188, V398, C99 and R424
MMS	R424, L418 and L397	S394, E416, T426, P421, C99, E402, R98, L188, A400 and F425	A417	H401	Y423 and P415	Y420, M422 and V398
BMS	M422, Y420 and L418	W210, G100, E402, Arg98, P415, E416, A417, G428, P429, H432, A400, Y423and P421	C99 and R424	H401, P430 and T426	V398 and L397	L188 and A189

C. HER2**:** In HER2, BTP, CTL and MQL were found to have occupied the exact active site, while the control compounds, NTB and LTB had bound at the mouth of the active site. HER2 (ErbB2), is the receptor for preferential binding of EGFR and is known to signal the effects of EDGF through its tyrosine kinase activity (Figure 4.36C). It is known that overexpression and aberrant signalling of HER2 is noted in several cancers; in breast cancer, the expression levels of HER2 increase by 15-30% (Mitri *et al*., 2012). The active site of HER2 is made up of K753, E770, M774, M801, D863 and G865 (Aertgeerts *et al*., 2011). Binding energies for CTL and MQL were similar to the value for lapatinib (LTB), but the ΔG value of BTP binding was much lower (-10.55, K_i = 18 nM). While the chromanol ring of BTP was not greatly involved in receptor binding (aside from one H-bond with L726), the isoprenoid chain was found to facilitate stronger binding of this ligand through multiple weak forces like Van der Waals and alkyl/pi-alkyl interactions (Table 4.43 and Figures 4.41 and 4.42).

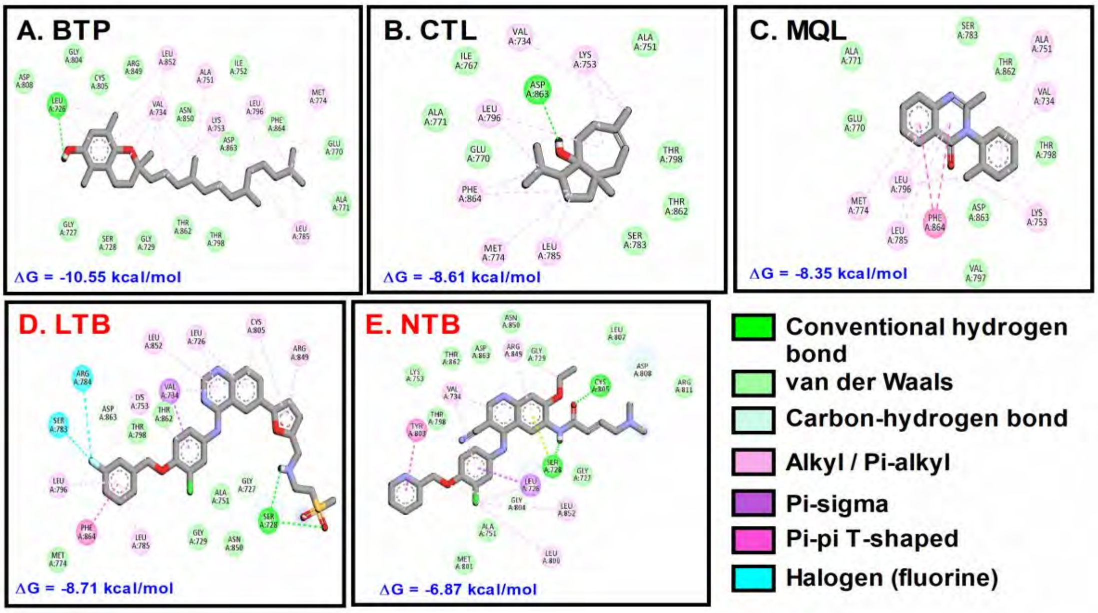

Figure 4.41. Interactions between various *H. mystax* best energy compounds and HER2 receptor compared to control HER2 drugs LTB and NTB. In the case of HER2, the control compounds were found to possess greater binding energy w.r.t the plant compounds. The colour codes for the interactions are shown.

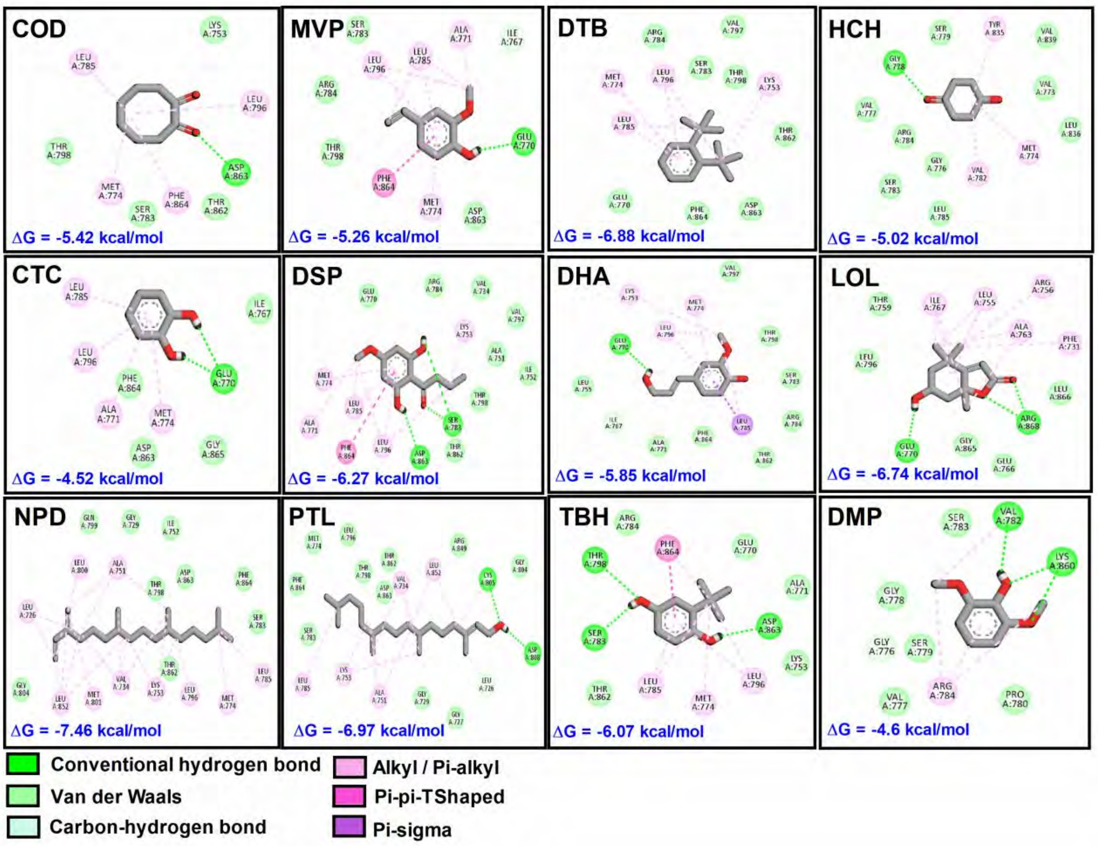

Figure 4.42. Interactions of HER-2 with other binding compounds of *H. mystax*

Table 4.43. Types of interactions and interacting residues involved in docking of *H. mystax* compounds (and controls) with HER2 (3PP0)

HER2 (3PP0)	Types of interaction and Interacting Residues						
	Conventional hydrogen bond	Van der waals	Unfavourable donor-donor / donor -acceptor	Pi-sigma / amide pi-stacked / pi-pi-stacked / pi-pi T-shaped	Carbon hydrogen bond donor	Alkyl/pi - alkyl	Halogen bond
BTP	L726	D808, G804, G805, R849, N850, D863, I752, F864, E770, A771, T798, T862, G729, S728 and G727	-	-	-	V734, L852, A751, K753, L796, M774 and L785	-
MQL	-	A771, S783, T862, T798, D863, V797 and E770	-	F864	Y281, S441 and H468	A751, V734, K753, L785, L796 and M774	-
CTL	D863	A751, T798, T862, S783, E770, A771 and I767	-	-	-	V734, K753, L796, F864, M774 and L785	-
COD	D863,	K753, T798, S783 and T862	-	-	-	L785, L796, M774 and F864	-
MVP	E770	S783, R784, T798 and D863	-	F864	I767	A771, L785, L796 and M774	-
DTB	-	V797, R784, S783, T798, T862, E770, F864 and D863	-	-	-	M774, L796, K753 and L785	-
HCH	G778	S779, V839, V777, R784, G776, V773, L836, S783 and L785	-	-	-	V782, M774 and Y835	-
CTC	E770	I767, F864, D863 and G865	-	-	-	L785, L796, A771 and M774	-

HER2 (3PP0)	Types of interaction and Interacting Residues						
	Conventional hydrogen bond	**Van der waals**	**Unfavourable donor-donor / donor -acceptor**	**Pi-sigma / amide pi-stacked / pi-pi-stacked / pi-pi T-shaped**	**Carbon hydrogen bond donor**	**Alkyl/pi - alkyl**	**Halogen bond**
DSP	L418, Y420 and R424	F425, L397, T426, E416, P430, A417, V398, H432, M419, M422 and L188	-	Y423 and H401	P421	C99	-
DHA	E770	V797, L755, T798, S783, R784, F864, T862 and A771	-	L785	I767	K753, M774 and L796	-
LOL	E770 and R868	T759, L796, L866, G865 and E766	-	-	-	I767, L755, R756, A763 and F731	-
NPD		Q799, G729, I752, T798, D863, F864, S783, T862 and G804	-	-	-	L800, A751, L726, L852, M801, V734, K753, L796, M774 and L785	-
PTL	C805 and D808	F864, M774, L796, T798, T862, D863, R849, G804, S783, G729 and G727	-	-	L726	V734, L852, K753, L785 and A751	-
TBH	S783, D863 and T798	R784, D770, A771, K753 and T862	-	F864	-	L785, M774 and L796	-
DMP	K860 and V782	S783, G778, S779, V777 and P780	-	-	G776	-	-
LTB	S728	T798, T862, A751, G729, N850 and M774	-	V734 and F864	D863 and G727	L785, L796, K753, L852, L726, C805 and R849	S783 and R784
NTB	C805 and S728	T798, K753, T862, D863, N850, G729, L807, R811, G727, A751 and M801	-	L726 and Y803	G804 and D808	R849, V734, L752 and L800	-

D. mTOR: The mammalian target of rapamycin is a PI3K-related Ser/Thr kinase which is responsible for cell growth in response to nutrients and growth factors through direct or indirect phosphorylation of ~800 proteins (Hua *et al*., 2019). The kinase site of mTOR possesses two lobes, the KD N lobe and the KD C lobe and the kinase domain of mTOR is located in chain A. Key residues of mTOR reported in literature for docking of substrates as well as inhibitors are located in the KD N lobe and the residue numbers are Y2225, M2349, M2345, C2243, L2354, Q2223, M2199, I2356, D2195, W2239, I2163, I2237, L2185, V2240 (Tanneeru and Guruprasad, 2012). The docking results show that all the molecules (both control RFL and the plant compounds) had bound at the same site, where the side chains of the aforementioned active site residues are present (Figure 4.36D). RMN alone had bound slightly away from this location. All of the ligands (control and plant compounds) were found to dock inside the active site (Table 4.44 and Figures 4.43 and 4.44).

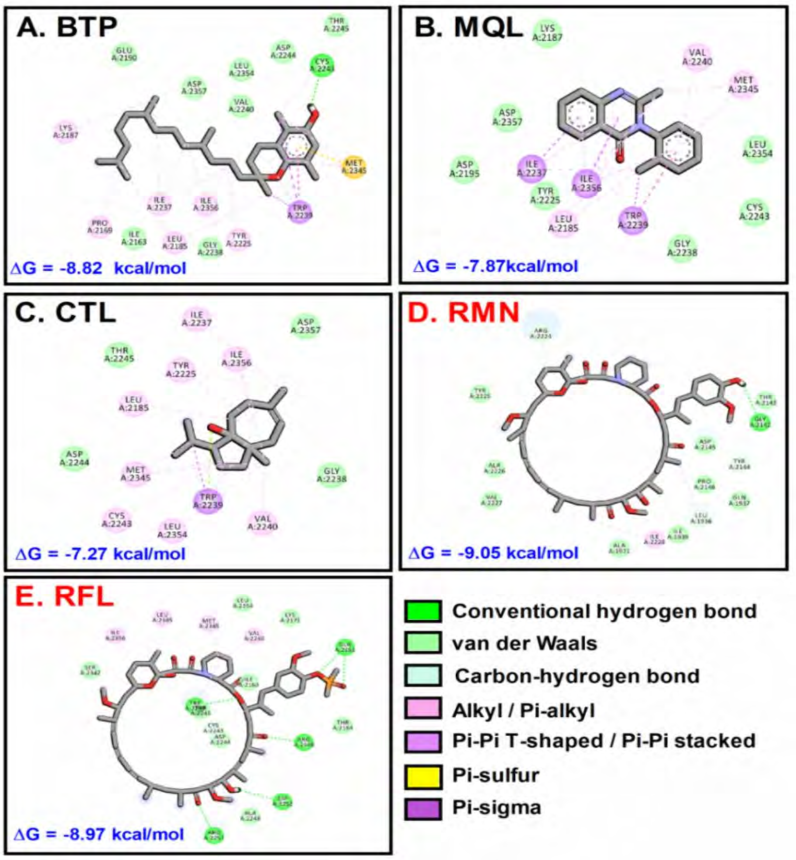

Figure 4.43. Interactions between *H. mystax* best energy compounds and control drugs with mTOR chain A

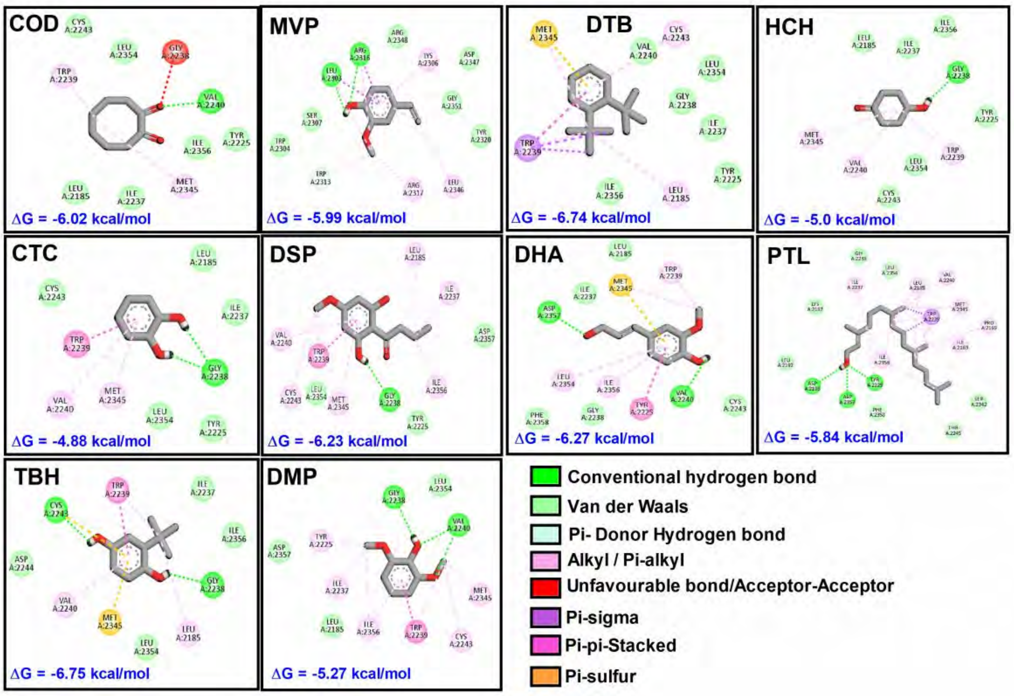

Figure 4.44. Interactions of mTOR with other binding compounds of *H. mystax*

Table 4.44. Types of interactions and interacting residues involved in docking of *H. mystax* compounds (and controls) with mTOR chain A

mTOR (4JT6)	A. Types of interactions and Interacting Residues					
	Conventional hydrogen bond	Van der waals	Unfavourable donor-donor / Pi-Sulfur / Pi-cation / Pi-anion / pi lone pair	Pi-Sigma/ amide Pi-stacked / Pi-pi T-shaped	Carbon hydrogen bond donor	Alkyl/Pi – alkyl
BTP	C2243	E2190, D2357, V2240, L2354, D2244, T2245, I2163 and G2238	M2345	W2239	-	K2187, P2169, I2237, L2185, I2356 and Y2225
MQL	-	K2187, D2357, D2195, Y2225, G2238, C2243 and L2354	-	I2356, I2237 and W2239	-	L2185, V2240 and M2345
CTL	-	D2357, G2238, D2244 and T2245	-	W2239	-	V2240, L2354, C2243, M2345, L2185, Y2225, I2356 and I2237
COD	V2240	C2243, L2354, I2356, Y2225, L2185 and I2237	G2238	-	-	W2239 and M2345
MVP	L2303 and R2316	R2348, D2347, G2351, S2307, W2304 and Y2320	-	K2306	W2313	R2317 and L2346
DTB	-	V2240, L2354, G2238, I2237, W2225 and I2356	M2345	W2239	-	C2243 and L2185
HCH	G2238	L2185, I2237, I2356, Y2225, L2354 and C2243	-	-	-	M2345, V2240 and W2239
CTC	G2238	C2243, L2185, I2237, L2354 and Y2225	-	W2239	-	V2240 and M2345

mTOR (4JT6)	A. Types of interactions and Interacting Residues					
	Conventional hydrogen bond	**Van der waals**	**Unfavourable donor-donor / Pi-Sulfur / Pi-cation / Pi-anion / pi lone pair**	**Pi-Sigma/ amide Pi-stacked / Pi-pi T-shaped**	**Carbon hydrogen bond donor**	**Alkyl/Pi – alkyl**
DSP	G2238	D2357, L2354 and Y2225	-	W2239	-	L2185, I2237, V2240, C2243, M2345 and I2356
DHA	D2357 and V2240	L2185	M2345	Y2225	I2237, F2358, G2238 and C2243	W2239, L2354 and I2356
PTL	D2195, D2357 and Y2225	G2238, L2354, K2187, L2192, F2358, S2342 and T2245	-	W2239	-	I2237, L2185, V2240, M2345, P2169, I2163 and I2356
TBH	C2243 and G2238	I2237, I2356, L2354 and D2244	M2345	W2239	-	V2240 and L2185
DMP	G2238 and V2240	L2354, D2357 and L2185	W2239	-	-	Y2225, I2237, I2356, M2345 and C2243
RMN	G2142	T2143, D2145, P2146, Q1937, I1939, A1971, V2227, A2226 and Y2225	-	-	R2224, Y2144 and L1936	I2228
RFL	Q2161, R2348, D2252, R2251 and W2183	S2342, L2354, K2171, I2163, T2245, D2244, T2164 and A2248	-	-	C2243	I2356, L2185, M2345 and V2240

E. PI3K: PI3K is an important player in several cancers and is a downstream effector of receptor tyrosine kinases such as insulin receptor and HER2, which transduce growth factor signalling (Mosesson and Yarden, 2004). PI3K catalyzes the production of phosphatidylinositol-3,4,5-triphosphate (PIP_3), which in turn activates Akt (protein kinase B) and other kinases. This protein has a catalytic domain (p110) and a regulatory domain (p85). The PI3K/Akt/mTOR pathway is commonly dysregulated in almost all human cancers, and hence, the proteins of this pathway are prime targets of anticancer therapeutic regimes (LoRusso, 2016). The PI3K-Akt-mTOR pathway is responsible for cellular longevity, cellular proliferation through nutrient uptake (as well as anabolism) and finally, cell survival through inhibition of apoptosis (Yu and Cui, 2016). Often, inhibitors of this pathway decrease cellular proliferation and induce cell death. The kinase domain of human p110 is located between residues ~696 to 1068 (Liu *et al*., 2014). The active site of PI3Kδ p110 domain is comprised of E826, I825, Y813, D787, K774, V828 and M752 (Berndt *et al*., 2010). In this work, the binding of the compounds MQL (ΔG = -8.31) and CTL (ΔG = -8.55) occurred close to Y_{813} and E_{826}, but the control compounds DLB (ΔG = -10.58), TLB (ΔG = -10.04) and ULB (-9.24) were found to bind exactly inside the active site pocket (Figure 4.36E). The interactions between PI3K and the docked compounds are shown in Figures 4.45, 4.46 and Table 4.45.

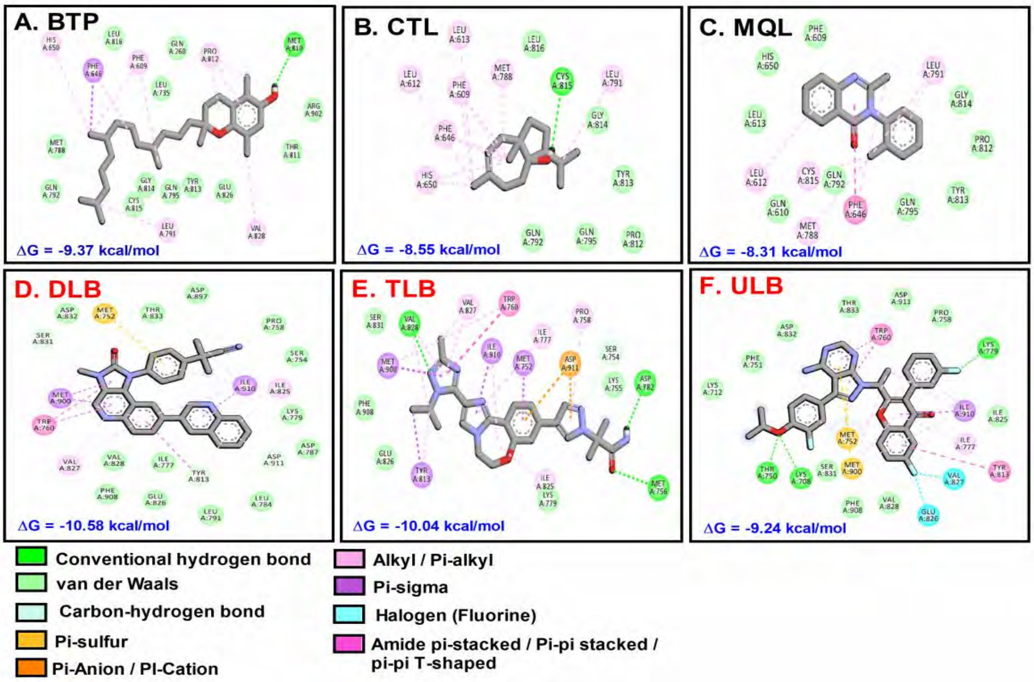

Figure 4.45. Interactions between PI3K (PDB ID: 5NGB) and *H. mystax* best energy compounds as well as standard PI3K inhibitors - The best energy compounds of the MeOH leaf extract are presented. The compounds such as CTL, MQL and BTP had the top ranked compounds in terms of ΔG. The relatively high inter-molecular interaction energies between PI3K and the control compounds ULB, DLB and TLB are seen when compared to top 3 plant compounds. A detailed account of the interacting residues and types of interactions are presented in Table 4.45.

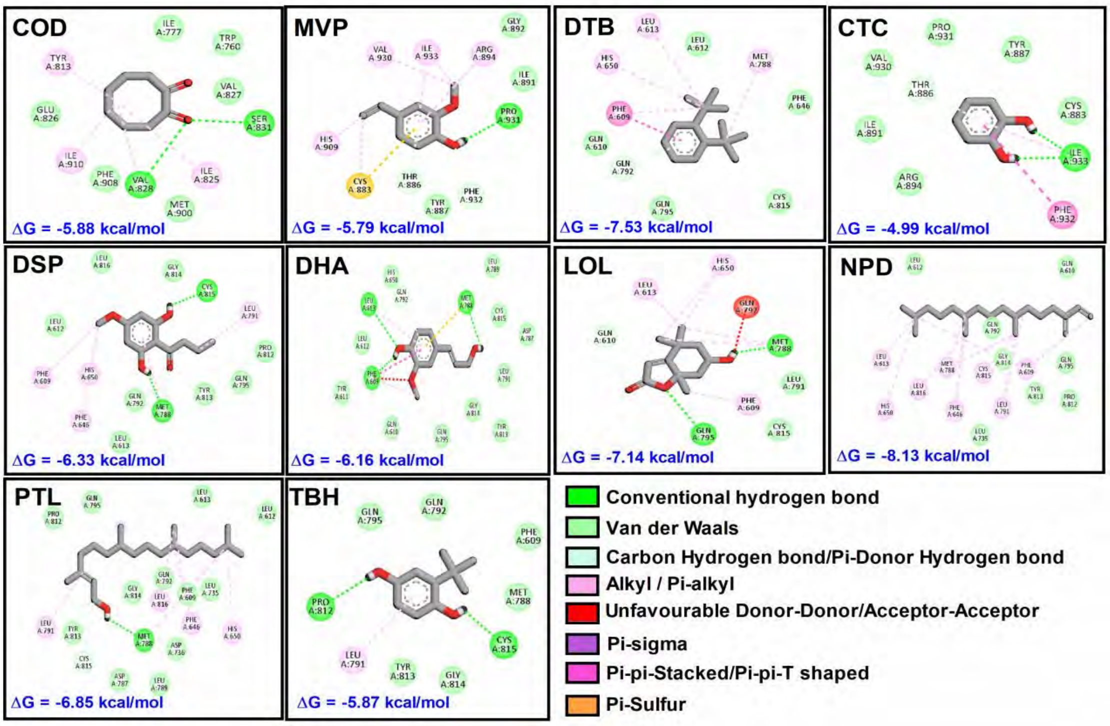

Figure 4.46. Interactions of PI3K with other binding compounds of *H. mystax*

Table 4.45. Differences in interactions between P13K and control inhibitors vs *H. mystax* compounds

PI3K (5NGB)	Types of interaction and Interacting Residues						
	Conventional hydrogen bond	**Van der waals**	**Pi-Sigma / amide Pi-staked / Pi-pi T-shaped**	**Unfavourable donor-donor / Pi-sulfur / Pi-cation / Pi-anion**	**Carbon hydrogen bond donor / pi-lone pair**	**Alkyl / Pi-alkyl**	**Halogen Bond**
BTP	M810	R902, T811, E826, Y813, Q795, G814, C815, Q792, M788, L816, L735 and Q260	F646	-	-	H650, F609, L791, P812 and V828	-
CTL	C815	L816, G814, Y813, P812, Q795 and Q792	-	-	-	L613, L791, L612, F609, M788, F646 and H650	-
MQL	-	F609, H650, L613, Q610, Q792, Q795, Y813, P812 and G814	F646	-	-	L612, C815, M788 and L791	-
COD	V828 and S831	I777, W760, V827, E826, F908 and M900	-	-	-	Y813, I910 and I825	-
MVP	P931	G892, I891 and Y887	-	C883	T886 and F932	V930, I933, R894 and H909	-
DTB	-	L612, Q610, F646, Q795 and C815	F609	-	Q792	L613, H650 and M788	-
CTC	I933	P913, V930, I891, R894, Y887 and C883	F932	-	-	-	-
DSN	C815 and M788	G814, L816 and L612	-	-	P812, Q795, Y813, Q792 and L613	L791, F609, H650 and F646	-
DHA	L613, M788 and F608	H650, L612, Y611, Q610, Q795, G814, Y813, L791, D787, C815 and L789	-	-	Q792	-	-

PI3K (5NGB)	Types of interaction and Interacting Residues						
	Conventional hydrogen bond	**Van der waals**	**Pi-Sigma / amide Pi-staked / Pi-pi T-shaped**	**Unfavourable donor-donor / Pi-sulfur / Pi-cation / Pi-anion**	**Carbon hydrogen bond donor / pi-lone pair**	**Alkyl / Pi-alkyl**	**Halogen Bond**
LOL	M788 and G795	L791 and C815	-	Q792	Q610	H650, L613 and P609	-
NPD	-	Q792, G814, Q795, Y813, P812 and L735	-	-	-	L613, H650, L816, F646, M788, C815, L791 and F609	-
PTL	M788	P812, Q795, L613, L612, Y813, G814, Q792, F609, L735, D736, L789 and D787	-	-	C815	L791, L816, F646 and H650	-
TBH	P812 and C814	Q795, Q792, F609, M788, Y813 and G814	-	-	-	L791	-
ULB	K779, K708 and T750	K712, F751, D832, T833, D911, P758, I825, V828, F908 and S831	I910, W760 and Y813	M752 and M900	-	I777	V827 and E826
DLB	-	D832, T833, D897, P758, S754, K779, D787, L784, L791, E826, F908, V828 and I777	M900, I910 and W760	M752	D911, Y813 and S831	V827 and I825	-
TLB	V828, M756 and D782	S831, F908, E826, K779 and K755	M900, Y813, I910, M754 and W760	D911	S754	V827, I777, P758 and I825	-

***F. ERβ*:** Estrogen receptors α and β are responsible for binding to estrogen and triggering the expression of estrogen-responsive genes, and both these proteins have 97% homology. 17-β-estradiol/EDL binding to the ligand-binding domain of ER causes its homo/heterodimerization (with ERα) and transactivation to the nucleus (Saadatian-Elahi *et al.*, 2004). ER signalling regulates protein kinase cascades, activates eNOS and phosphorylates other target proteins. When not directly bound to DNA, ER is known to activate PI3K and MAPK signalling cascades. Hence, ER ligands are used to inhibit ER signalling in ER-positive breast cancers. All the compounds (control and plant molecules) explored in this work were found to bind inside the active site of Erβ (Figure 4.36F). The ΔG of BTP binding to ERβ active site was ~ -11 kcal/mol (K_i = 10 nM), and this was significantly higher than that of MQL (-7.49) and CTL (-7.18) and the controls (EDL, ΔG = -9.32 and TMN with a value of -9.69) the docked compounds are shown in Figures 4.47, 4.48 and Table 4.46.

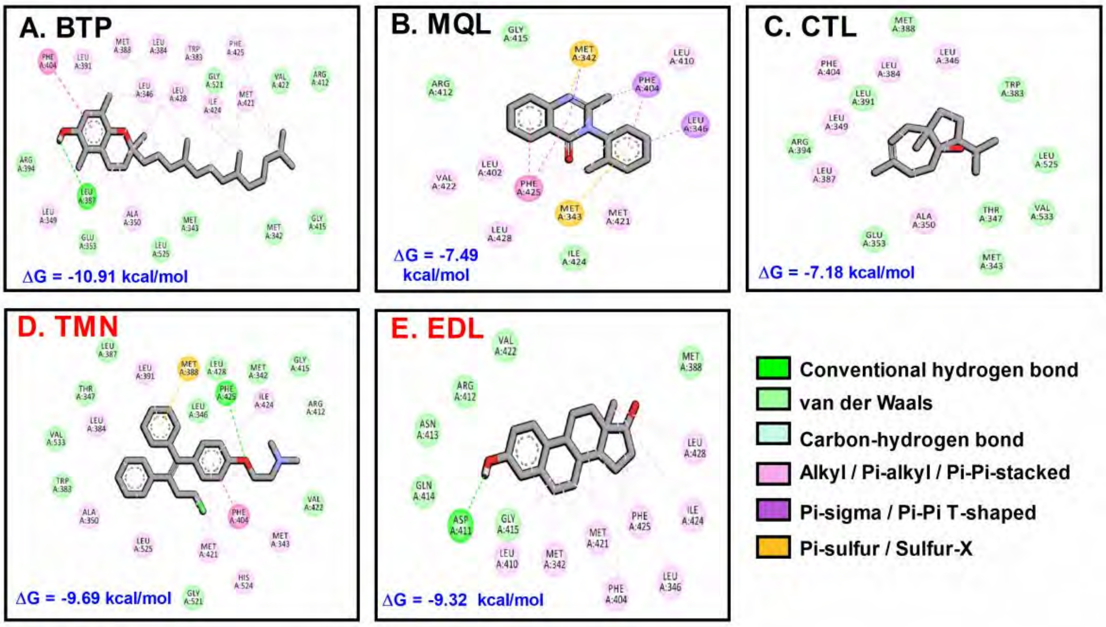

Figure 4.47. Molecular interactions between ERβ chain A and *H. mystax* best energy compounds

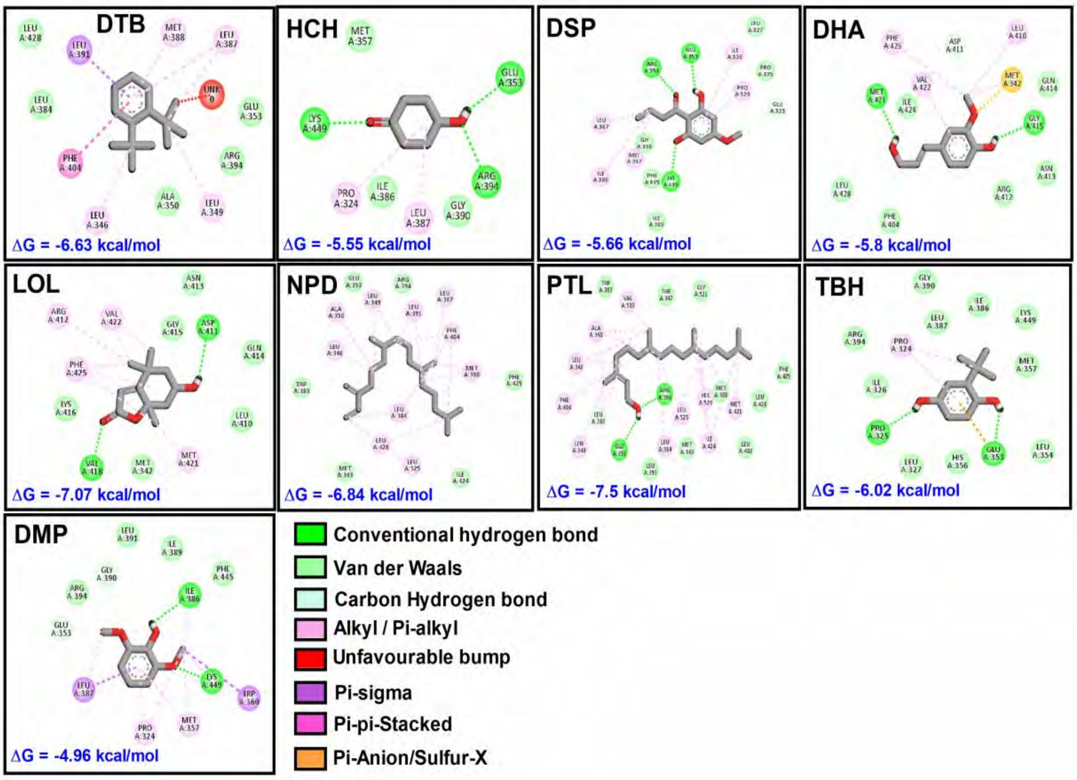

Figure 4.48. Interactions of ERβ with other binding compounds of *H. mystax*

Table 4.46. Types of interactions and interacting residues involved in docking of *H. mystax* compounds (and controls) with ERβ (3OS8) chainA

ERβ 3OS8 (Chain A)	Types of interactions and Interacting Residues					
	Conventional hydrogen bond	Van der waals	Pi-sigma / Pi-pi T-shaped	Sulfur-X / Pi-sulfur	Carbon hydrogen bond donor	Alkyl/Pi-Alkyl
BTP	L387	G521, V422, R412, G415, M342, M343, L525, E353 and R394	F404	-	-	L391, M388, L346, L384, L428, W383, I424, F425, M421, L349 and A450
MQL	-	I424, R412 and G415	F404, L346 and F425	M342 and M343	-	L410, V422, L402, L428 and M421
CTL	-	R394, L391, M388, W383, L525, Val533, T347, M343 and E353	-	-	-	A350, L387, L349, F404, L384 and L346
DTB		L428, L384, E353, R394 and A350	L391 and F404	-	-	M388, L387, L349 and L346
HCH	K449, E353 and R394	M357, I386 and G390	-	-	-	P324 and L387
DSP	E353, R394 and K449	L327, P325, G390, F445 and I389	-	-	-	I326, P324, L387, M357 and I386
DHA	E770	V797, T798, S783, L755, R784, F864, T862, A771	L785	-	I767	K753, L796 and M774
LOL	D411 and V418	N413, G415, Q414, K416, L410 and M342	-	-	-	R412, V422, F425 and M421

ERβ 3OS8 (Chain A)	Types of interactions and Interacting Residues					
	Conventional hydrogen bond	**Van der waals**	**Pi-sigma / Pi-pi T-shaped**	**Sulfur-X / Pi-sulfur**	**Carbon hydrogen bond donor**	**Alkyl/Pi-Alkyl**
NPD	-	E353, R394, W383, F425, M343 and I424	-	-	-	A350, L349, L391, L387, F404, M388, L346, M388, L384, L428 and L525
PTL	E353 and R394	W383, T347, G521, F425, M388, L428, M343, L402 and L391	-	-	L387	V533, A350, L346, F404, L525, H524, M421, I424 and L384
TBH	P325 and E353	G390, I386, L387, K449, R394, I326, M357, L327, H356 and L354	-	-	-	P324,
TMN	F425	V422, G521, Q383, V533, T347, L387, L346, L428, M342 and G415	F404	M388	F412	M343, H524, M421, L525, A350, L384, L391 and I424
EDL	D411	Q414, G415, N413, R412, V422 and M388	-	-	-	L410, M342, M421, F404, F425, L346, I424 and L428

4.14.5. Molecular SAR Properties

The cheminformatics parameters HBA (H-bond acceptors), HBD (H-bond donors), MR (molecular refractivity), TPSA (topology polar surface area), MlogP (lipophilicity or Octanol/Water partition coefficient), GIA (gastrointestinal absorption), BBB (blood brain barrier permeability) and Log K_p (skin permeability) of the selected plant compounds was calculated using SWISS-ADME tool and MedChem Designer 5.5. Drug-likeness was estimated by checking whether a given drug obeys Lipinski's rule of 5 (MW < 500, LogP < 5.0, RotB < 10, HBA < 10 & HBD < 5 (sum of NH and OH, and finally, PSA < 140.0). The data (Table 4.47) shows that all the five compounds obeyed Lipinski's RO5. BTP and NPD (with values > 5) had slightly higher than acceptable MlogP. The HBD and HBA numbers were very low and all five molecules were of molecular weight < 500 Da. Due to the higher MlogP values, BTP and NPD also had poorer GIA scores. MQL, DHA and CTL were predicted to cross the BBB and Log $K_p$scores were low for BTP and NPD, signifying their better ease of crossing the skin barrier when compared to MQL, DHA and CTL (with values in the range of -5 to -6 cm/s). These properties reveal that these *H. mystax* compounds (except the toxic compound MQL) could serve as good drug candidates/scaffolds.

Table 4.47. Molecular SAR properties of the selected plant compounds

TEST COMPOUNDS											
Plant Cpds.		Empirical formula	MW (g/mol)	HBA	HBD	MR	TPSA (Å²)	MlogP	GIA	BBB	Log K_p(cm/s)
H. mystax	BTP	$C_{28}H_{48}O_2$	416.68	2	1	134.31	29.46	5.94	Low	No	-1.51
	MQL	$C_{16}H_{14}N_2O$	250.30	2	0	77.27	34.89	2.99	High	Yes	-6.05
	DHA	$C_{11}H_{16}O_2$	180.24	2	0	51.35	26.30	2.37	High	Yes	-5.87
	CTL	$C_{15}H_{26}O$	222.37	1	1	70.46	20.23	3.67	High	Yes	-5.11
	NPD	$C_{20}H_{38}$	278.52	0	0	97.31	0	6.21	Low	No	-1.17

4.14.6. Molecular Dynamics Simulations (MDS) of *H. mystax*

MD simulations were performed to identify the stability of docked protein-ligand complexes. Using indices like RMSD and RMSF, the stability (as well as molecular fluctuations) of five breast cancer protein receptor-ligand docked complexes - MMP9 (5TH6)-MQL, mTOR (4JT6)-CTL, Akt1 (3CQW)-BTP, ERβ (3OS8)-CTL and HER2 (3PP0)-CTL was deciphered (Figure 4.49). RMSD indicates shift and dislocation of the atoms in the protein, and hence, it can be defined as a measure of the deviation from the overlap of two compared structures (Arnittali *et al.*, 2019). RMSF signifies the disturbances in sets of atoms of certain amino acid residues in the protein at each trajectory step throughout the course of the MD run. The RMSD and RMSF average values or cut-off points were fixed during the analysis of the runs. For RMSD, a cut-off value of 0.4-1.5 nm and for RMSF, a cut-off value of 0.4 - 0.8 nm were set to decide on the extent of displacement of atoms and docked molecules in the protein-ligand complexes. The docked complexes were run at 25000-50000 picoseconds (25-50 ns) to gauge the complexes' stability (and time duration thereof). In all RMSD profiles of the five different receptor-ligand complexes, the RMSD values were in the range of 0.2-04 nm (except for the combination of mTOR-CTL, where the deviation was in the range of 0.2-0.5 nm). Also, in all cases, the RMSF values were <0.4 nm. RMSF plots were prepared only to cover the amino acid regions associated with the respective protein active sites (where ligand binding is expected). In this region, RMSF is expected to be greater, owing to more significant fluctuations in the amino acid residues when compared to areas of the protein that are stable throughout the MDS run. Based on these results, the different receptor-ligand complexes studied herein affirmed the stability of the different receptor-ligand complexes studied herein. The results indicated good structural stability of binding between the breast cancer target proteins and phytocompounds of *H. mystax*.

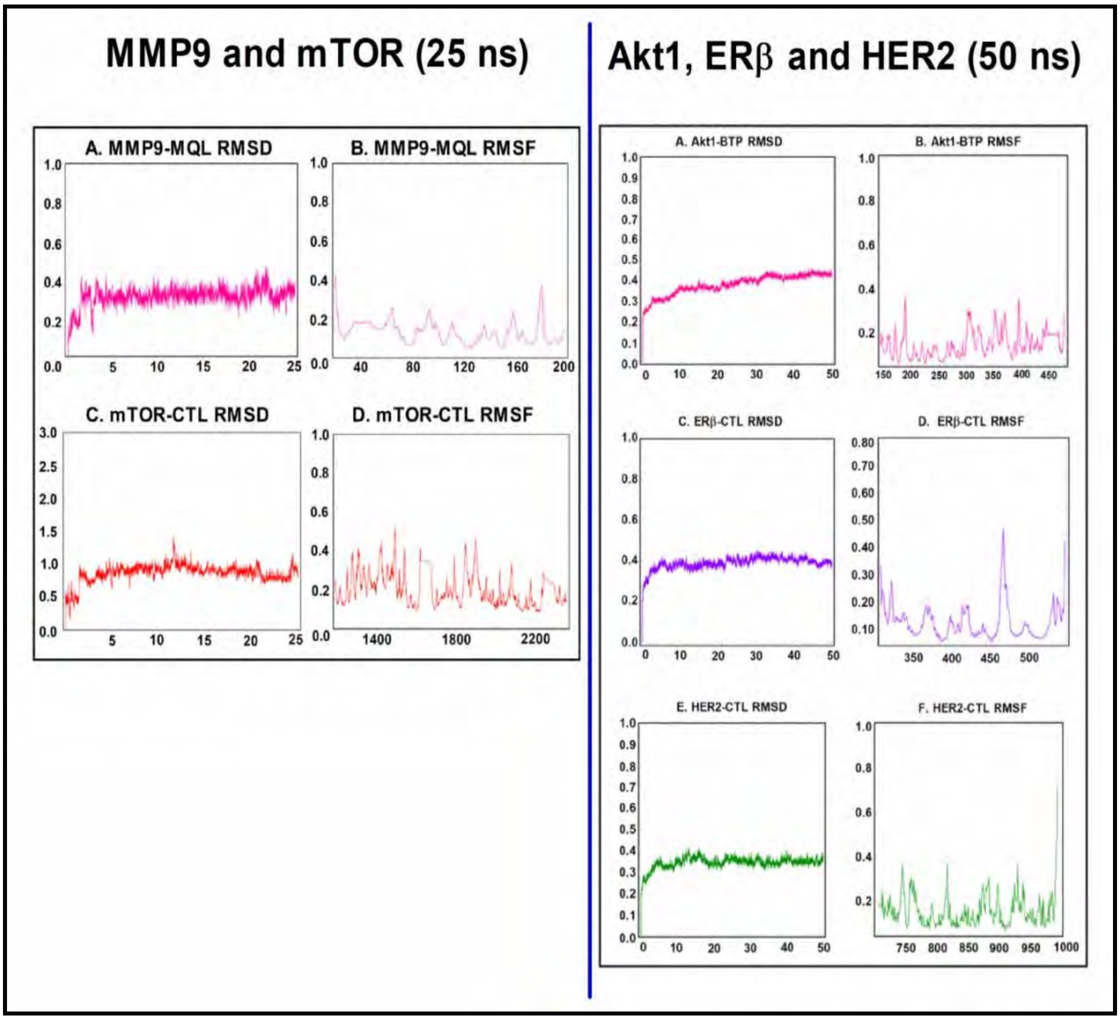

Figure 4.49. Molecular Dynamics simulations studies on *H. mystax* compounds: MD simulations were performed for 25 ns and the stability of the docked complexes - RMSD and RMSF profiles for a 25-50 ns MD simulation run of the best energy (-ΔG) docked complexes of protein and ligand are shown.

4.14.7. DFT Studies

The highest occupied molecular orbital - lowest unoccupied molecular orbital (HOMO–LUMO) energy gaps were calculated (Gece and Bilgiç, 2009) by density functional theory (DFT) for the studied compounds are shown in Figure 4.50. In all the compounds, the HOMO orbitals overlap on their corresponding active binding site, which defines the region on the molecule(s) capable of facilitating interaction with the pertinent receptors. The contour maps which were obtained for the compounds show the active binding sites. From the contour map analysis, it was noted that the compound DHA shows the presence of electron charge density overlapping on the oxygen atom of actinidiolide ring. BTP shows the presence of electron charge density clouded with chromene ring. NPD shows the presence of charge density clouded on the diene group. CTL shows the presence of electron charge density clouded on the 7-membered ring with -OH group of carotol. It is noted that the compounds NPD and CTL have no aromatic rings; they possess only aliphatic rings and chains. Among the five compounds extracted from *H. mystax*, MQL possesses less HOMO-LUMO energy gap of 4.99 eV compared to other molecules due to the presence of pyrimidinone ring, which is clouded with more electron charge density on the aromatic ring and hence, might facilitate better interactions with target receptor proteins.

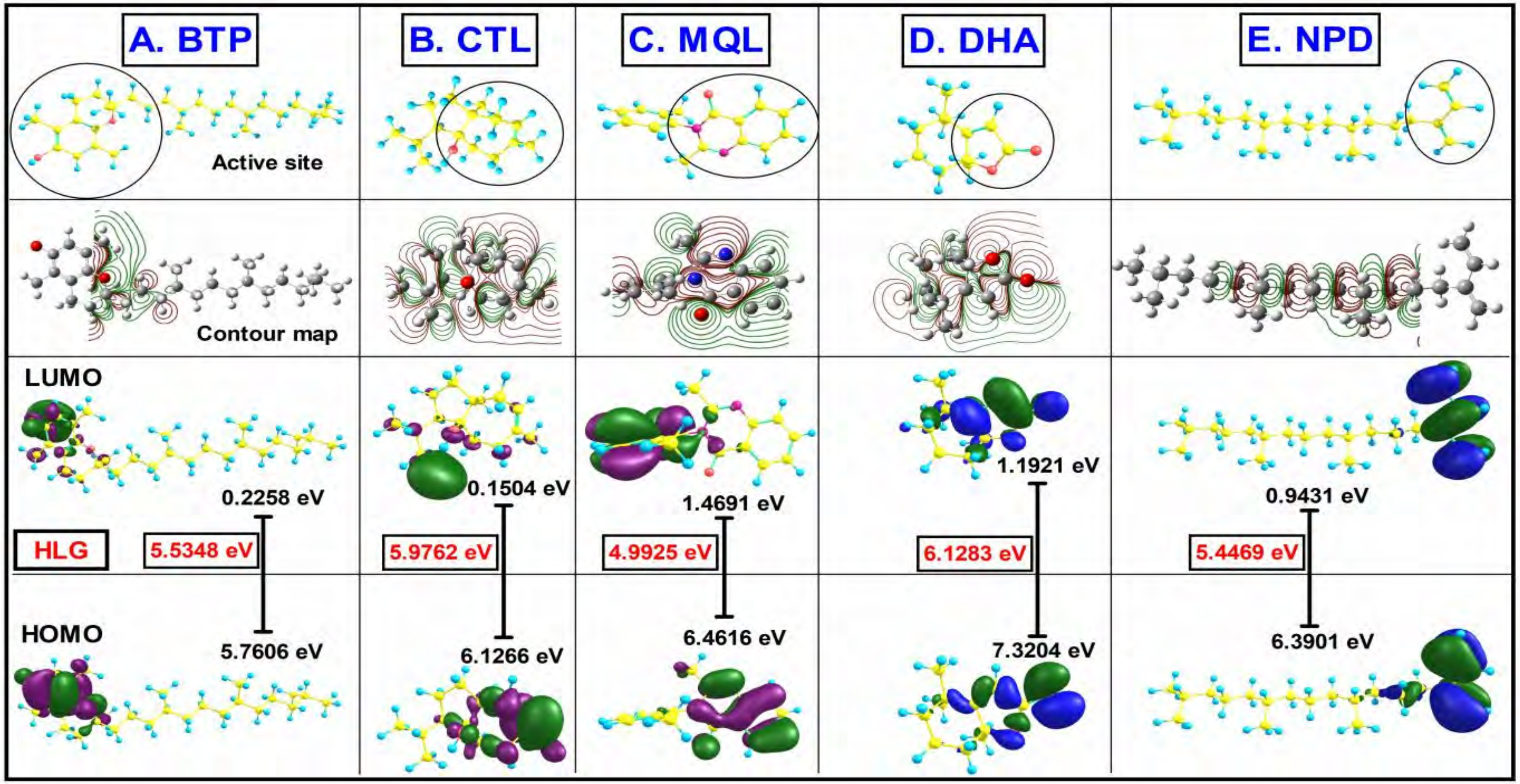

Figure 4.50. DFT analysis of the five *H. mystax* compounds -The five compounds are presented column-wise and the aspects like active site, contour map, LUMO and HOMO for these compounds are presented row-wise. The HOMO-LUMO gap (HLG) is shown in red letters within boxes.

4.14.8. ADMET-SAR results

Table 4.48. Salient results from ADMET-SAR server

Properties \ Ligands	BTP	MQL	DHA	CTL	NPD
Ames mutagenesis	-	-	+	-	-
Acute Oral Toxicity (c)	III	II	II	III	III
Avian toxicity	-	-	-	-	-
Blood Brain Barrier	+	-	+	+	+
Caco-2	+	+	+	+	+
Carcinogenicity (binary)	-	-	-	-	+
Carcinogenicity (trinary)	NR	NR	NR	NR	NR
CYP1A2 inhibition	+	+	-	-	+
CYP2C19 inhibition	+	+	-	-	-
CYP2C9 inhibition	+	-	+	-	-
CYP2C9 substrate	-	-	-	+	-
CYP2D6 inhibition	-	-	-	-	-
CYP2D6 substrate	-	+	-	+	-
CYP3A4 inhibition	-	-	-	-	-
CYP3A4 substrate	-	-	+	-	-
CYP inhibitory promiscuity	+	+	-	-	-
Estrogen receptor binding	+	-	+	-	-
Hepatotoxicity	+	-	+	-	-
Human Intestinal Absorption	+	+	+	+	+
Human oral bioavailability	+	+	+	+	-
Acute Oral Toxicity	2.383	1.739	2.379	1.913	2.885
OATP1B1 inhibitor	+	+	+	+	+
OATP1B3 inhibitor	+	+	+	+	+
OATP2B1 inhibitor	-	-	-	-	-
P-glycoprotein inhibitor	-	-	-	-	-
P-glycoprotein substrate	-	-	-	-	-
PPAR gamma	-	-	-	-	-
Plasma protein binding	1.034	0.676	0.854	0.851	0.903
Subcellular localization	M	M	M	M	M

BTP - β tocopherol; CTL - carotol; MQL - methaqualone; DHA - dihydroactinidiolide; NPD - neophytadiene.

Using the online tool ADMET-SAR 2.0, (Malik *et al*., 2017) the molecular toxicology profiles of the study compounds (and control inhibitor ligands of various proteins) was computed (Table 4.48). Among the five compounds from *H. mystax*, DHA was flagged as Ames mutagenesis positive, while the other study compounds were predicted to be Ames mutagenesis negative. Except for MQL, all the other compounds were predicted to cross the BBB. NPD was predicted to cause binary carcinogenicity, while the other compounds were not deemed to cause either binary or trinary carcinogenicity. All the five compounds were predicted to have good Caco2 permeability, and hence, the bioavailability of these compounds (if administered orally) would be high. ADMET-SAR server also predicted the CYP450 inhibitors and substrates; while BTP was predicted to inhibit CYP2C19 and 2C9, it was not particularly deemed to be metabolized by any CYP450. MQL was deemed to inhibit CYP1A2 and CYP2C19, and it was deemed to be a CYP2D6 substrate. CTL was envisaged to act as a CYP2C9 substrate and as an inhibitor of CYP2D6. All five compounds were predicted to inhibit OATP1B1 and OATP1B3, and none of them was OATP2B1 inhibitors. A downside of these compounds is their inhibition of OATP1B1 and OATP1B3, which indicates potential drug-drug interactions that could be envisaged. Usually, negatively charged compounds are known to bind to OATP (organic anion transporter proteins), which are expressed in high amounts in hepatocytes. None of the five molecules was envisaged to be P-glycoprotein substrates or inhibitors. Plasma protein binding of these molecules is expected to be higher owing to their higher MlogP values. All the five compounds were foreseen to localize in the mitochondria.

5. SUMMARY

5. SUMMARY

- Siriya Kalvarayan hill was our study area, and it possessed a tremendous amount of flora. The plant survey study led to the identification of 100 different species belonging to 46 families and 90 genera.
- Many (61 rare plants) species were identified through this study (for which very little published material was found). These identified plants were not studied hitherto, and their phytochemical constituents are unidentified/unknown.
- Information was gathered from 105 residents of 8 villages. Following are no. of residents interviewed from those eight villages: Sirukkalur - 16, Edapattu - 18, Athikkuzhi - 3, Vazhakuli - 2, Vanjikkuli - 30, Moolakadu - 6, Aanaimaduvu - 5 and Puliyankottai - 25. Among those who were interrogated, 86 were male, and 19 were female.
- The ethnopharmacological survey revealed that among the 100 plants surveyed, the local/ethnic uses of 52 plants were identified through the population. Unique diseases/ailments of about 32 were identified as health problems for which the residents used some of the 100 surveyed species as medicine. For those selected species, quantitative indices such as ICF, FL and UV were analyzed.
- Upon surveying the National Medicinal Plants Board database, it was observed that around ten species identified and recorded in this research work were not mentioned in the flora of different Indian states (all states given on the website) namely Tamil Nadu, Kerala, Karnataka, Andhra Pradesh (& Telangana), West Bengal, Sikkim, Odisha, Chattisgarh, Rajasthan and Maharashtra.
- We chose six unexplored plants (*Aganosma cymosa* (Roxb.) G. Don, *Dalbergia lanceolaria* L.f., *Hugonia mystax* L., *Loeseneriella obtusifolia* (Roxb.) A.C. Sm,

Plecospermum spinosum Trecul. and *Walsura trifoliata* (A. Juss) Harms) for *in vitro* and *in silico* studies from the plant survey list, which were found to be relatively less, explored (and even unexplored) from Siriya Kalvarayan hills.

- Five different antioxidant assays were performed in MeOH leaf extract, which are DPPH radical scavenging activity, ABTS assay, Ferric reducing power, Nitric oxide and Phospho-molybdenum.
- DPPH scavenging activity - The order of DPPH scavenging ability is in the order - *W. trifoliata* > *A. cymosa* > *D. lanceolaria* > *H. mystax* > *L. obtusifolia* > *P. spinosum.*
- ABTS radical scavenging activity -The order of ABTS radical cation scavenging ability at lower concentrations of the extracts is in the range of *D. lanceolaria / L. obtusifolia / A. cymosa* (these 3 had almost comparable activities) > *W. trifoliata* > *H. mystax / P. spinosum* (these had similar activities).
- Reducing power -When comparing reducing power, amongst all the species, the plant species closest (~80%) to ascorbic acid control (across all concentrations compared), was *A. cymosa*. The order of reducing power is in the order - *A. cymosa* > *L. obtusifolia* > *D. lanceolaria* > *W. trifoliata* > *H. mystax* > *P. spinosum*.
- $NO^{\bullet}$ radical scavenging activity - Based on the comparative profiles obtained, to summarize, the following is the order of their activity, *A. cymosa* > *L. obtusifolia* > *D. lanceolaria* > *P. spinosum* > *H. mystax* > *W. trifoliata*.
- Antimicrobial assays were done through well diffusion method, with six different bacterial strains (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Shigella flexneri*, *Salmonella typhimurium* and *Escherichia coli)* and four different fungal strains (such as *Aspergillus fumigatus*, *Candida glabrata*, *Candida tropicalis* and *Echinodontium tinctorium*).

- The standard antibiotic drugs were used for the positive control against bacterial and fungal strains. For bacteria - Nitrofurantoin, Vancomycin, Gentamicin, Bacitracin, Amoxyclav and Methicillin were used, and for fungal - Penicillin-G, Methicillin, Chloramphenicol, Amikacin and Ampicillin were employed.
- *A. cymosa* extract showed modest activity against *E. coli* and *P. aeruginosa* and *E. tinctorium. D. lanceolaria* extract was found to inhibit *P. aeruginosa*, *S. aureus*, *S. typhimurium* and *E. coli* and *E. tinctorium*. *H. mystax* extract was found to inhibit *P. aeruginosa*, *S. aureus*, *S. typhimurium*, *E. coli*, *K. pneumoniae, S. flexneri*, *E. tinctorium.* The extract of *L. obtusifolia* was found to inhibit *P. aeruginosa*, *S. aureus, S. typhimurium, E. coli* and *E. tinctorium.* In *P. spinosum* leaf extract the highest zone of inhibition was obtained against *P. aeruginosa and E. tinctorium. W. trifoliata* extract inhibited all of the six bacterial strains, viz., *P. aeruginosa*, *S. aureus*, *S. typhimurium*, *E. coli*, *K. pneumonia, S. flexneri* and *E. tinctorium*.
- Using MTT assay, the cytotoxic effect of the crude extracts of the six study plants were evaluated by supplying the extract at various concentrations to MCF-7 cells and VERO cells and IC_{50} values was obtained.
- *A. cymosa* extract was > 10 times more effective against MCF-7 cells (w.r.t. control Vero cells); this fold difference in activity was lower (>8.13 times) when *P. spinosum* extract was studied and also much higher when *H. mystax* was tested in the two cell lines.
- The leaf extract of all the six species were studied for the anti-inflammatory effect through HRBCs membrane stabilization (inhibition of hemolysis) method in comparison to aspirin (positive control). The inhibition (%) was obtained in a dose dependent manner. The plant species *D. lanceolaria* and *A. cymosa* recorded better anti-inflammatory activity.

- Preliminary qualitative screening was performed for all the plants with six different solvents (water, methanol, ethanol, acetone, chloroform and hexane) and ten different tests (triterpenoids, sugars, catechins, flavonoid, saponins, tannins, anthraquinones, amino acid, sterols and carbohydrates).
- It was evident that the methanolic extract possessed the greatest variety of phytochemical classes (triterpenoids, sugars, saponins, tannins, sterols and carbohydrates) followed by ethanolic extracts (catechins), aqueous and chloroform extracts (anthraquinones) and chloroform (carbohydrates).
- Quantitative estimation of total chlorophyll, total carotenoids, total sugar, total protein, total lipids, total amino acids, total flavonoids, total phenol and total tannin were done for all the six plant extracts.
- Through GC-MS analysis 192 different types of volatile compounds were identified collectively among the six selected plants. Among the identified compounds, it was observed that 21 kinds of compounds are commonly found in other plants.
- Bioinformatics studies were carried out based on the GC-MS results for two selected plants (*A. cymosa* and *H. mystax*). Studies included Molinspiration, ADMET-SAR, Network analysis, Molecular docking and Molecular dynamics.
- Molinspiration tool results of *A. cymosa* revealed that among the five ligands studied (DCP, MCL, CSL, IDL, PCL), Indole and Coprostanol were the best and potent inhibitors since they possessed lower negative or positive values for the different receptor types. In *H. mystax* among the five compounds from *H. mystax*, DHA was flagged as Ames mutagenesis positive, while the other study compounds were predicted to be Ames mutagenesis negative.

- Network analysis Data obtained from the NCBI-GEO database (breast cancer) led to the identification of the differentially expressed genes (DEGs) in *A. cymosa*. The PPI interaction network recorded interactions between the various proteins. Among the top 60 hits (in terms of greater expression) in breast cancer (GEO database; GSE15852; GSE2290 and GSE1081180), MMP9, ESR (ER) and mTOR have significantly high importance in breast cancer. *H. mystax* didn't show significant results.
- Molecular docking between protein-ligand interactions revealed the binding order in the series of 3CQW (AKT-1) > 5TH6 (MMP-9) > 3PP0 (HER-2) > 4JT6 ChainA (mTOR) > 5NGB (PI3K) > 3OS8 ChainA (ERβ). The *H. mystax* phytochemical constituents were docked to the target proteins along with the respective control drugs in comparison with plant compounds. For *A. cymosa* docking was done for the following receptors 5NGB (PI3K) > 5TH6 (MMP-9) > 4JT6ChainA (mTOR) > 3OS8ChainA (ERβ).
- The Molecular Dynamic simulations studies were performed 50 ns for *A. cymosa* compound with PI3K/MMP-9/ERβ; in *H. mystax* compounds 25 ns for MMP-9/mTOR and 50 ns for Akt-1/ERβ/HER-2.
- A density functional theory (DFT) study was carried out for *H. mystax* compounds such as beta-tocopherol, carotol, methaqualone, dihydroactinidiolide and neophytadiene. Among the five compounds extracted from *H. mystax*, MQL possesses less HOMO-LUMO energy gap of 4.99 eV compared to other molecules due to the presence of pyrimidinone ring, which is clouded with more electron charge density on the aromatic ring and hence, might facilitate better interactions with target receptor proteins.
- ADMET-SAR properties of toxicology studies have been done for both *H. mystax* and *A. cymosa* plants compounds, and the drugs can be considered for tests in live models in future.

6. CONCLUSION

6. CONCLUSION

The present work aimed at identifying 1. The distribution of flora in the Siriya Kalvarayan hills in Kallakurichi District, Tamil Nadu, India and 2. The phytochemical and biological activities of six selected medicinal plants, *Aganosma cymosa* (Roxb.) G. Don, *Dalbergia lanceolaria* L.f., *Hugonia mystax* L., *Loeseneriella obtusifolia* (Roxb.) A. C. Sm , *Plecospermum spinosum* Trecul. and *Walsura trifoliata* (A. Juss.) Harms. The crude extracts of these compounds were obtained in a pilot scale using a range of solvents of differing polarities. Among all the solvents, methanol was found to contain the widest variety of phytochemical classes (in all six plants). The methanolic extracts of the six plants were obtained and used for further analyses. The qualitative estimation of i) flavonoids, ii) triterpenoids, iii) saponins, iv) catechins, v) anthraquinones, vii) sugar, viii) sterols and ix) tannins was performed. The six plants had unique qualitative phytochemical profiles. In quantitative analysis, a) total flavonoid, b) total tannins, c) total phenols, d) total free amino acids, e) total sugar, f) total lipid, g) total carotenoids, and h) total chlorophyll content was estimated. The data revealed the variations in the composition of primary and secondary metabolites in the methanolic leaf extracts of the chosen plant species. To identify the exact phytochemicals present in the extracts and their relative abundance, the methanolic leaf extracts of the six plants were subjected to GC-MS analysis and the phytochemicals identified through GC-MS studies were checked for their potential biological activities using Molinspiration and ADMET-SAR server. The *in vitro* studies pertained to the assessment of the methanolic leaf extracts of the selected six plants for their a) antioxidant assay using ABTS and DPPH radical scavenging assays, phosphomolybdenum assay, ferric reducing power assay and nitric oxide radical scavenging assay; b) anti-inflammatory assay using HRBC stabilization method; c) cytotoxicity assessment using MTT assay and d) antimicrobial effect of the crude extracts of these plants. To find the molecules which could be responsible for the *in vitro* biological activities (beta-tocopherol, carotol, methaqualone, dihydroactinidiolide and neophytadiene.), each compound in the GC-MS results for each of the plant extracts (for six plants) was searched for bioactivity reports in published literature. Preliminary docking screening with AUTODOCK Vina led to identifying potent biomolecules against key receptors involved in breast cancer pathogenesis and pathophysiology: PI3K, Akt, MMP9, mTOR, ERβ and HER2. Molecular dynamics simulations with selected best docked receptor-drug combinations revealed the mechanism of binding for the best-docked poses. Future studies would involve deeper study into bioavailability and assimilatory profiles that will promote the utility of these compounds as either leads or scaffolds for computer-aided drug design and confirm the traditional applications of the promising bioactive compounds which were investigated through *in vitro* studies (obtained from two plants - *H. mystax* and *A. cymosa*).

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PUBLICATIONS

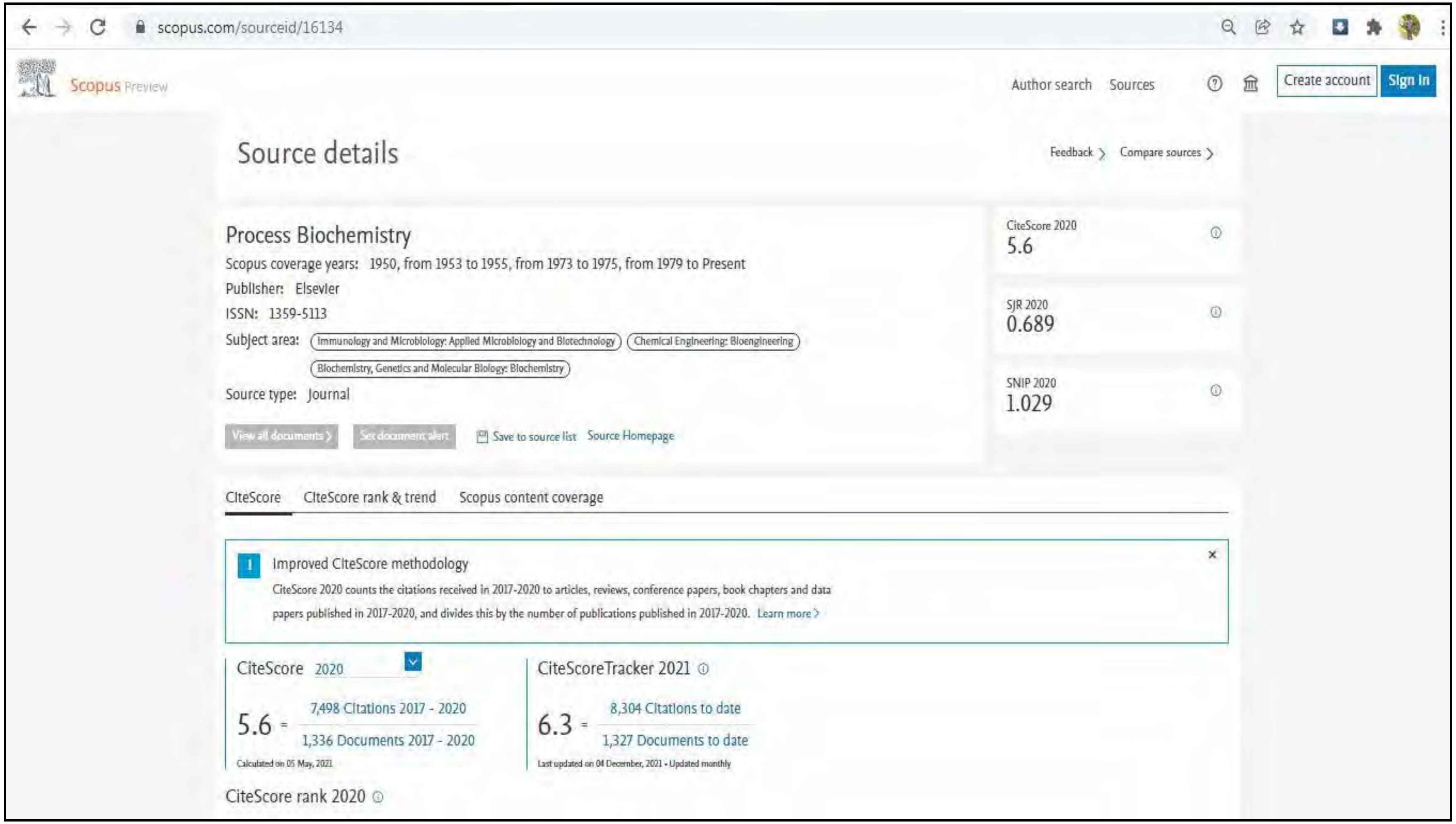
LIST OF PUBLICATIONS

1. **Pushparaj Annadurai**, Vinothkanna Annadurai, Ma Yongkun, Arivalagan Pugazhendhi, and **Kandavel Dhandayuthapani**. “Phytochemical composition, antioxidant and antimicrobial activities of Plecospermum spinosum Trecul.” **Process Biochemistry** 100 (2021): 107-116. **DOI: 10.1016/ j.procbio. 2020.09.031. (IF: 3.757) [UGC-CARE List-Group-II (Scopus/Web of Science) ISSN: 1359-5113].**

2. **Annadurai Pushparaj**, Gideon Daniel Andrew, Annadurai Vinothkanna, Ganesan Mohan, **Dhandayuthapani Kandavel**. “Indigenous uses of ethnomedicinal plants among Malayali tribals in Siriya Kalvarayan Hills, Eastern Ghats, India.” International Journal of Botany Studies 6 (5) (2021): 1243-1260. **Print ISSN: 2455-541X [UGC-CARE List-Group-II (Web of Science)**].

3. Pushparaj Annadurai, Daniel Andrew Gideon, Vijay Nirusimhan, Sivaramakrishnan Ramachandran, Arivalagan Pugazhendhi and Kandavel Dhandayuthapani. “Deciphering the pharmacological potential of *Aganosma cymosa* (Roxb.) G. Don. Using *in vitro* and computational methods”. Process Biochemistry 114 (2022): 119-133. DOI: 10.1016/j.procbio.2022.01.024. (Impact Factor 3.7).

4. Pushparaj Annadurai, Daniel Andrew Gideon, Vijay Nirusimhan, Bella Antony Paulraj, Sangavi Jeyachandran, Antony Sebastin Raj, Langeswaran Kulanthaivel and Kandavel Dhandayuthapani. “Identification of Hugonia mystax L. phytochemical and their molecular targets responsible for cytotoxic effects against MCF-7 breast cancer cells. ~ Under communication in Journal of Biomolecular Structure and Dynamics (Tyalor & Francis).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ISSN: 1359-5113

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Process Biochemistry 100 (2021) 107–116

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Phytochemical composition, antioxidant and antimicrobial activities of *Plecospermum spinosum* Trecul.

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ARTICLE INFO

Keywords:
Plecospermum spinosum
Phytochemicals
Antioxidant
Anti-inflammatory
Bioactive compounds
GC–MS

ABSTRACT

Plecospermum spinosum Trecul. leaves collected from Eastern Ghats, India were assessed for antioxidant potential using the methanolic extract *in vitro* for free radical scavenging and phosphomolybdenum assays. The (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) ($ABTS^{\cdot+}$) assay exhibited potent free radical scavenging capacity at 100 μg/mL (86.48 ± 0.8 %). Antimicrobial activity of the methanolic leaf extract greatly inhibited the growth of *Pseudomonas aeruginosa* (9 ± 0.7 mm) and *Echinodontium tinctorium* (12 ± 0.9 mm) when compared to the other tested pathogens. The hemolysis (%) was calculated for anti-inflammatory activity and the results showed dose dependent effects. The lowest inhibition (53.08 ± 1.5 %) was obtained for 800 μg/mL extract when compared with control aspirin (90.41 ± 1.3 %) at the same concentration. Qualitative phytochemical tests confirmed the presence of triterpenoids, flavonoids, saponins, tannins, sterols, sugars and free aminoacids. Totally, 35 different phytochemical compounds were found using gas chromatography-mass spectrometry (GC–MS). Furthermore, the phenolics (70.34 ± 2.1 mg/g), flavonoids (164.28 ± 1.1 mg/g) and other metabolites were estimated quantitatively. These results exhibited considerable antioxidant, and antimicrobial activities due to the presence of bioactive phytochemicals. *P. spinosum* may be employed for the separation of novel bioactive metabolites with potential pharmaceutical activities.

1. Introduction

For thousands of years, plants have been used around the world as an important source in traditional medicinal systems to treat a wide variety of diseases. Almost 70–80 % of natural medicines originate from plant sources of which, 122 bioactive potentials were obtained from 94 plant species [1,2]. In developing countries, plants are important sources of medicines to develop novel/newer drugs for treating various diseases [1,3]. Numerous efforts have been employed to find out novel antimicrobial drugs from different types of natural plant sources. Similarly, several medicinal plants and their formulations have exhibited antimicrobial activities [3]. In recent times, secondary bioactive metabolites have been used as direct/main components for drug synthesis in pharma industries. Nearly, 25 % of the prescribed organic drugs are derived directly/indirectly from plant sources in industrialized countries. In current years, transmission of antibiotic resistant organisms has increased by alarming rates [1,4]. The unprecedented rise in antibiotic resistant pathogenic microorganisms (superbugs) in the last few decades has increased the demand for novel/alternative antimicrobial agents. Plant secondary metabolites with potent antimicrobial activities are attractive alternatives to synthetic biochemical compounds. Several ethnomedical herbs/plants possess various pharmacological and therapeutic activities [5].

Plecospermum spinosum Trecul. is a large rambling shrub, which climbs using its stout, long and straight thorns. It belongs to the Moraceae family and its vernacular (Tamil) name is ‘Korratimul’. This plant is used in silk textile industries for dyeing [6]. A study has reported that the leaf extract was efficacious against neuropathic pain in streptozotocin (STZ) induced diabetic rats [7].

Reactive oxygen species (ROS) are known to be responsible for several chronic diseases such as coronary atherosclerosis, diabetes, cancer, gastritis, neurodegenerative diseases, cataract, Alzheimer’s

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https://doi.org/10.1016/j.procbio.2020.09.031
Received 29 April 2020; Received in revised form 24 September 2020; Accepted 25 September 2020
Available online 28 September 2020
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Fig. 1. Photographical view of *Plecospermum spinosum* Trecul. leaves and their extracts.

disease, paralysis and aging [8]. Polyphenols are known to be key antioxidants, which neutralize the free radicals and therefore, are used in antioxidant formulations [8,9]. Natural antioxidant compounds derived from crude plant extracts and their derivatives are more efficient in suppressing/inhibiting the cellular damages caused by the free radicals generated by the ROS system [10]. Plant-derived polyphenolics serve as reducing agents, singlet and triplet oxygen scavengers and hydrogen donors [8]. Antioxidants derived from natural sources are known to be safer when compared to synthetic antioxidant molecules [11,12]. Plants are known to produce diverse secondary metabolites to guard themselves against numerous pathogens [2,13]. Previous reports suggested that the plant extracts showed better antimicrobial activities due to the presence of bioactive metabolites [1,2]. It is proposed that active principle(s) from plant sources responsible for antimicrobial activity possess little or no side effects when compared to the synthetic pharmaceutical agents [14]. Since chronic inflammation leads to weight gain and disease [15], a number of plant extracts and their bioactive compounds which possess anti-inflammatory activities have been documented [16,17].

Phytochemicals are classified into two major kinds, based on their relative abundance (mg/100 g of dry weight) and the roles they play in energy metabolism and biochemical processes. Plant metabolites are classified into macro and microconstituents; while carbohydrates, lipids and proteins are present in superabundant quantities (grams), the minor constituents are not so abundant [18]. Secondary metabolites, vitamins, and minerals are the chief minor constituents and are present at microgram to milligram levels per 100 g of dried plant material. Plant secondary metabolites are maintained at minimal amounts, and are known to be distributed throughout the plant in a spatiotemporal manner [19]. Carotenoids, isoflavones, flavonoids, phenolic compounds and sterols are classic examples of active principles with wide ranging active groups and cellular targets [20]. Different groups of phytochemicals have various activities such as free radical scavenging/antioxidant properties, antimicrobial effects and immunomodulatory effects.

The present study was aimed at identifying the bioactive potentials of methanolic extract of *P. spinosum* and to study its antioxidant, antimicrobial and anti-inflammatory properties. Further, this article is the first ever report on the antioxidant, antimicrobial activities of *P. spinosum* extract. Till now, even the GC–MS profiling of *P. spinosum* has not been carried out. Hence, the focus of our study was to identify the phytochemical classes (and the metabolites) present in the methanolic leaf extract and assess the extract for its antioxidant, anti-inflammatory and antimicrobial properties.

2. Materials and methods

2.1. Plant sample collection

Plecospermum spinosum Trecul. leaves were collected from Siriya Kalvarayan hills (Kallakurichi district) situated in the Eastern Ghats (Tamilnadu, India). *P. spinosum* (Fig. 1) is potentially a new source of natural medicines. A specimen of the plant was authenticated by the Rapinat Herbarium at St. Joseph's College, Tiruchirappalli, Tamil Nadu, India. A copy of the herbarium sheet is attached in the supplementary section (Fig. S1).

2.2. Chemicals and reagents used

General chemicals, reagents and solvents were purchased from Himedia and SRL, India. Unless otherwise stated, all the chemicals were certified as analytical grade. 2,2′-diphenyl 1-picryl hydrazyl (DPPH), [2, 2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] ($ABTS^{\cdot+}$) were procured from Sigma Aldrich, India.

2.3. Solvent extraction

P. spinosum leaves were collected from the Siriya Kalvarayan hills. After shade drying, the leaves were ground to powder using a blender-mixer. Furthermore, the powder was weighed and packed into a thimble made of α-cellulose cotton fibre (Whatmann, supplied by GE healthcare Ltd.). The plant powder was then placed in the soxhlet apparatus and refluxed with methanol (63 °C) over a heating mantle for about 8 h until solvent concentrates were obtained. The resulting extract was poured into glass petridishes and then kept for evaporation of the excess solvent (room temperature).

2.4. In vitro antioxidant assay

2.4.1. DPPH radical scavenging assay

DPPH free radical scavenging ability of the leaf extract of *P. spinosum* was investigated [21]. Initially, 1 mL radical solution of DPPH (0.2 mM) was added to 1 mL of the leaf extract of *P. spinosum* at various concentrations (10−100 μg/mL). L-ascorbic acid was used as positive control. The disappearance of DPPH radical was monitored at 517 nm using a UV–vis spectrophotometer (Jasco V-650, Tokyo, Japan) after 20 min incubation at 27 °C in the dark. Radical scavenging ability (%) was calculated as follows:

DPPH radical scavenging % = (Ac-As/Ac) × 100; Where, Ac- control; As -sample. (1)

2.4.2. ABTS$^{\bullet+}$ radical scavenging assay

The antioxidant activity of the methanolic leaf extract of *P. spinosum* was performed by the decolorization of ABTS$^{\bullet+}$ radical cation [9]. It was diluted with double distilled water to 7 mM concentration. By reaction with potassium persulfate (2.45 mM) in a 1:1 ratio, ABTS$^{\bullet+}$ radical cation (ABTS$^{\bullet+}$) was generated and this reaction was permitted in room temperature (dark) for 12−16 h. After incubation, the solution (ABTS$^{\bullet+}$) was dissolved in double distilled water to adjust the optical density (OD) value to 0.7 (734 nm). Further, 1 mL of the freshly prepared solution (ABTS$^{\bullet+}$) was mixed with 1 mL of the leaf extract at different concentrtions (10−100 μg/mL). The OD value was measured at 734 nm using a UV–vis spectrophotometer (Jasco V-650, Tokyo, Japan). Ascorbic acid was used as the standard.

2.4.3. Reducing power assay

Ferric reducing power was evaluated as per the methodology described earlier [21]. Different concentrations of the plant extract (10−100 μg/mL) were separately diluted with 2.5 mL of phosphate buffer saline (PBS) (0.2 M, pH 6.6), 2.5 mL of potassium ferricyanide (1%) and incubated at 50 °C in a water bath for 20 min. After addition of 2.5 mL of TCA (10 %), the tubes were centrifuged at 10,000 rpm in a centrifuge for 10 min. Then, 2.5 mL of the collected supernatant was mixed with 2.5 mL of double distilled water and subsequently, 0.5 mL of ferric chloride (0.1 %) solution was added. The OD value of the reaction mixture was obtained at 700 nm. L-ascorbic acid was used as the standard antioxidant control and reducing power (%) was calculated.

Reducing power assay (%) = (Test sample – Control sample)/ Test sample × 100 (2)

2.4.4. Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity of the methanolic leaf extract of *P. spinosum* was assessed using the method of Sharma et al. [21]. Initially, 1 mL of different concentrations (10−100 μg/mL) of the methanolic leaf extract was added to 3 mL of sodium nitroprusside (10 mM) and incubated at room temperature for 150 min. Further, 3 mL of Griess reagent [1% sulfanilamide, 2% H_3PO_4 and 0.1 % N-(1-Naphthyl) ethylenediamine dihydrochloride] was added to all the samples. Then, the OD value of the chromophore was measured against the blank at 546 nm. L-ascorbic acid served as the postive control. Nitric oxide scavenging (%) activity was calculated and the efficacy of the plant extract was compared with the results obtained for L-ascorbic acid standard.

2.4.5. Phosphomolybdenum assay

The antioxidant capacity of the leaf extract was determined by phosphomolybdenum method [21]. Initially, 1 mL of sample was added with an equal volume of reagent solution containing sulphuric acid (0.6 M), sodium phosphate (28 mM) and ammonium molybdate (4 mM). Then the mixture was incubated at 95 °C (in water bath) for 90 min. Further, it was allowed to cool at room temperature and the OD value was obtained against the blank at 765 nm using UV–vis spectrophotometer. These findings were stated as ascorbic acid equivalents (AAE)/g extract.

2.5. Microorganisms involved in antimicrobial activity

The antimicrobial activity of the five chosen concentrations of the methanolic leaf extract of *P. spinosum* (6, 7, 8, 9, and 10 mg/mL) were tested against different fungal and bacterial strains using agar well diffusion method. Standard antibiotic discs such as nitrofurantoin, vancomycin, bacitracin, amoxyclav, gentamicin, penicillin-G, amikacin, chloramphenicol, ampicillin, and methicillin were used as positive controls (in μg quantities; refer to Tables 1 and 2 for details).

The bacterial and fungal pathogens were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. Different bacterial (*Pseudomonas aeruginosa* MTCC 1034, *Staphylococcus aureus* MTCC 9542, *Klebsiella pneumoniae* MTCC 8911, *Shigella flexneri* MTCC 9543, *Salmonella typhimurium* MTCC 3224 and *Escherichia coli* MTCC 584) and fungal pathogens (*Candida tropicalis* MTCC 2795, *Candida glabrata* MTCC 3983, *Echinodontium tinctorium* MTCC 1038 and *Aspergillus fumigatus* MTCC 2483) were used in this study. The bacterial and fungal pathogens were subcultured on nutrient broth and Sabouraud dextrose broth (Himedia), respectively.

2.5.1. Well diffusion method

The antimicrobial activity was evaluated with modified agar well diffusion method [1]. The bacterial and fungal cultures were swabbed onto the agar surface of Mueller Hinton agar and Sabouraud dextrose agar plates, respectively. Then the wells were filled with

Table 1
Antibacterial activity of methanolic leaf extract of *P. spinosum* against bacterial pathogens.

Methanolic extract (mg/mL)	Radius of zone of clearance (mm)					
	Pseudomonas aeruginosa	*Staphylococcus Aureus*	*Klebsiella pneumoniae*	*Shigella Flexneri*	*Salmonella typhimurium*	*Escherichia coli*
6.0	6 ± 2.3	3 ± 2.3	NE	NE	6 ± 0.7	2 ± 1.9
7.0	7 ± 1.0	4 ± 1.1	NE	NE	6 ± 0.4	3 ± 1.1
8.0	8 ± 0.5	4 ± 1.5	NE	NE	7 ± 0.9	4 ± 0.5
9.0	8 ± 0.7	5 ± 2.4	NE	NE	8 ± 1.6	5 ± 1.7
10.0	9 ± 0.7	6 ± 0.7	NE	NE	8 ± 0.5	7 ± 0.6
Antibiotics tested (positive control)						
Nitrofurantoin (10 μg)	8 ± 1.2	14 ± 1.3	8 ± 1.4	9 ± 1.9	2 ± 1.0	12 ± 0.9
Vancomycin (10 μg)	9 ± 1.3	11 ± 1.4	NE	NE	10 ± 1.5	9 ± 2.0
Gentamicin (10 μg)	16 ± 1.4	17 ± 1.6	12 ± 1.5	11 ± 0.9	17 ± 1.6	5 ± 1.2
Bacitracin (10 μg)	7 ± 1.3	13 ± 1.6	NE	NE	5 ± 1.0	7 ± 1.0
Amoxyclav (30 μg)	18 ± 1.4	22 ± 1.7	17 ± 1.4	2 ± 0.5	22 ± 1.7	13 ± 2.1
Methicillin (5 μg)	NE	NE	NE	NE	NE	NE

Values expressed as mean ± standard deviation. NE – No Effect detected.

Table 2
Antifungal activity of methanolicleaf extract of *P. spinosum* against fungal pathogens.

Methanolic extract (mg/mL)	Radius of zone of clearance (mm)			
	Echinodontium tinctorium	*Candida tropicalis*	*Candida glabrata*	*Aspergillus fumigatus*
2.0	8 ± 0.2	NE	NE	NE
4.0	8 ± 0.3	NE	NE	NE
6.0	9 ± 0.3	NE	NE	NE
8.0	10 ± 0.3	NE	NE	NE
10.0	12 ± 0.9	NE	NE	NE
Antibiotics tested (positive control)				
Penicillin-g (10 μg)	NE	NE	NE	NE
Methicillin (5 μg)	NE	NE	NE	NE
Chloramphenicol (30 μg)	7 ± 0.1	11 ± 0.4	11 ± 0.1	9 ± 0.2
Amikacin (30 μg)	6 ± 0.2	8 ± 0.3	8 ± 0.3	7 ± 0.1
Ampicillin (10 μg)	NE	NE	NE	NE

Values expressed as mean ± standard deviation. NE – No Effect detected.

P. spinosum extract at different concentrations mentioned above. Sterile antibiotic discs (positive controls) were employed to check for the formation of zones of inhibition. Solvent (Methanol) served as negative control. The plates were incubated at 37 °C for 24 h for bacteria and 28 °C for 48 h for fungi and the zones of inhibition were recorded accordingly.

2.6. Anti-inflammatory activity of P. spinosum leaf extract

The anti-inflammatory activity of the *P. spinosum* leaf extract was done using the method of Human Red Blood Cell (HRBCs) membrane stabilization. Fresh blood samples were taken from healthy volunteers and an equal ratio of disinfected Alsever solution [containing dextrose (2 %), sodium citrate (0.8 %), citric acid (0.9 %) and sodium chloride (0.72 %)], which could act as an anticoagulant was added. Further, the mixture was spun (10,000 rpm) for 15 min at 37 °C. The supernatant was discarded and the red blood cells were washed in normal saline buffer. Until the supernatants turned clear, the process of washing and centrifugation were performed repeatedly. This red blood cell suspension was taken for the evaluation of the anti-inflammatory property. The plant leaf extracts (at various concentrations) were mixed with phosphate buffer (2 mL), hypo saline (4 mL) and RBC suspension (0.9 mL). The mixtures were incubated at room temperature (37 °C) for 30 min. The supernatant was discarded and the hemoglobin content was evaluatedusing a UV–vis spectrophotometer at 620 nm. The hemolysis (%) was calculated using the formula;

$$\text{Hemolysis (\%)} = \text{Test sample/Control sample x 100} \tag{3}$$

2.7. Qualitative analysis

The method of Brindha *et al.* [22] was followed for analyzing the phytochemical constitution of *P. spinosum.* The reagents required for this qualitative assay were prepared freshly. Different concentrations of plant extracts (test solutions) were also prepared freshly. Care was taken to reconfirm all the assays in order to rule out false positives.

2.8. Quantitative biochemical and phytochemical evaluation

2.8.1. Quantitative biochemical analysis

Estimation of chlorophyll a, b, total chlorophyll and carotenoid content was performed using 100 mg of dried leaf powder. Total sugar content was evaluated by Dubois method. Estimation of total lipid was performed using gravimetric method, total protein content was determined by lowry's method and total free amino acids were estimated using a standard method.

2.8.2. Total phenol content

Estimation of total phenolics was done using Folin-Ciocalteau method. Briefly, 1 mL of methanolic leaf extract of *P. spinosum* (100 μg/mL) was mixed with 0.5 mL of Folin-Ciocalteau reagent (1 N) and incubated at 37 °C for 5 min. 2.5 mL of sodium carbonate solution (5%) was added. Further, the mixture was incubated at room temperature (in dark) for 90 min. Gallic acid (GA) served as the standard. The absorbance was measured against blank at 765 nm using a UV–vis spectrophotometer (Jasco V-650, Tokyo, Japan). The total phenolic content in the extract was calculated and expressed as milligrams of gallic acid equivalent per gram of leaf extract [23,24].

2.8.3. Total tannin content

Methanolic leaf extract was evaluated for the presence of total tannins by the modified method [24]. 500 μL of the methanolic leaf extract (1 mg/mL) was diluted with an equal volume of distilled water and polyvinyl polypyrrolidone (100 mg) was added. The mixture was incubated for 4 h at 4 °C. Afterwards, the mixture was spun (at 3000 rpm for 10 min at 4 °C) and the supernatant was used as non-tannin phenolics. About 1 mL of non-tannin phenolics solution thus obtained was mixed with 0.5 mL of Folin-Ciocalteau reagent (1 N), vortexed and incubated at room temperature for 5 min. Then, 2.5 mL of sodium carbonate solution (5%) was added and the mixture was incubated at room temperature (in dark) for 90 min. Tannic acid was used as the standard. The absorbance was read against blank at 765 nm using a UV–vis spectrophotometer (Jasco V-650, Tokyo, Japan). Expression of results were as mg tannic acid equivalents/g sample.

$$\text{Tannins (g)} = \text{Total phenolics (g)} - \text{Non- tannin phenolics (g)} \tag{4}$$

2.8.4. Flavonoid content

The total flavonoid content of the methanolic leaf extract was performed based on the aluminium chloride ($AlCl_3$) method [23,24]. Initially, 1 mL of leaf extract (1 mg/mL) was mixed with 150 μL of sodium nitrite (5%) and incubated at 37 °C for 5 min. Further, 150 μL of $AlCl_3$ (10 %) solution was added, voxtexed and allowed to stand for 6 min at room temperature. Then, 2 mL of sodium hydroxide (4%) was added and made up to 5 mL using double distilled water. The reaction mixture was then incubated for 15 min at room temperature. The presence of flavonoid was indicated by pink colour and the absorbance was measured against blank at 510 nm by a UV–vis spectrophotometer (Jasco V-650, Tokyo, Japan). Rutin was used as the flavonoid standard. Results were expressed as rutin equivalents (RE).

2.9. GC–MS profiling of the methanolic leaf extract of P. spinosum

The phytochemical evaluation of the methanolic leaf extract of *P. spinosum* was carried out using a Gas Chromatography Mass Spectrophotometer (GC–MS) (Perkin Elmer Clarus 500, Connecticut, USA) equipped with flame ionization detector, capillary column (30 m length × 0.25 mm ID coated with 5 % phenyl 95 % dimethylpolysiloxane) with a film thickness of 0.25 μm. Helium gas (mobile phase) was the carrier gas and its flow rate was fixed at 1 mL/min. The temperature of injection port was maintained at 280 °C and the volume of sample injection was 1 μL. The stationary phase (capillary column) temperature was set in the range of 60–300 °C (raising rate of 10 min). Mass spectra were programmed as scan type: full scan mode and scan range: 40–450 Daltons. The peaks of the compounds were matched with the standard peaks available in NIST (The National Institute of Standards and Technology) library.

3. Results and discussion

3.1. Antioxidant activity of the methanolic leaf extract of P. spinosum

In the recent decades, more consideration has been given to plant derived natural antioxidants and their health benefits to consumers. Besides, these natural antioxidants do not usually cause toxicity to human patients. Antioxidant based medicines are used to prevent as well as treat several complex diseases [8,12]. Plants possess a repertoire of natural antioxidants, which also contains medicinal properties [10,12, 24]. Polyphenols possess arguably the highest free radical scavenging activity among the phytochemicals. Antioxidant activity is dependent on the redox potential of these compounds; since they are reductants, they react with singlet oxygen, superoxide, nitric oxide as well as hydroxyl radicals and are also known to chelate metal ions, which accelerate in Fenton and Haber-Weiss reactions [8,23,25]. Commonly, medicinal plants possess appreciable amounts of bioactive phytochemicals such as flavonoids, phenolics, tannins, stilbenes, coumarins, lignans and lignins. These potential compounds possess several biological as well as antioxidant activities [9,12,20]. DPPH is a stable free radical exhibiting violet colour in solution, which after mixing with antioxidants, changes to a stable yellow colour, indicating antioxidant activity [8]. While *P. spinosum* extract (100 μg/mL) yielded 70 % free radical scavenging activity, it was comparatively lower than that of L-ascorbic acid (100 μg/mL) standard as shown in Fig. 2a. In the ABTS$^{\bullet+}$ decolourization assay, the antioxidant activity of *P. spinosum* extract (100 μg/mL) was found to be 86.48 ± 0.8 %, which was less when compared with the activity of ascorbic acid (which showed marginally greater inhibition as shown in Fig. 2b). Reducing power is a good indicator of antioxidant activity. It is evaluated by the transformation of Fe^{3+} to Fe^{2+} through reduction facilitated by antioxidant molecules [8]. Nitric oxide radical was generated by the addition of sodium nitroprusside. The scavenging

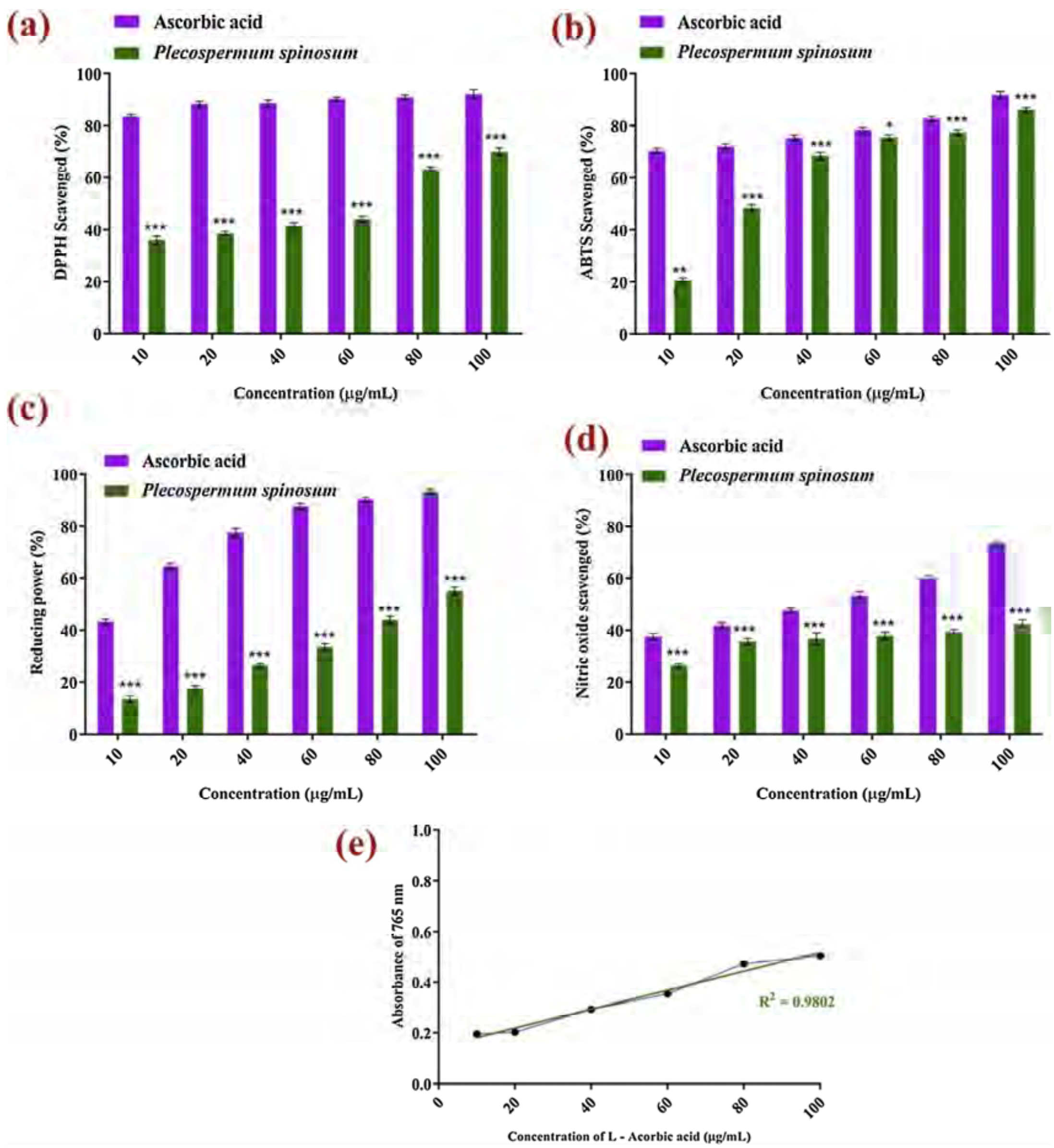

Fig. 2. Antioxidant activities of methanolic leaf extract of *Plecospermum spinosum* Trecul. The values indicate mean ± standard deviation (n = 3). The mean difference is significant at the levels of *p < 0.05, **P < 0.01 and ***P < 0.001 Vs standard. ns – non significant; (a) DPPH (b) ABTS$^{\bullet+}$ (c) Reducing power (d) Nitric oxide and (e) Phosphomolybdenum.

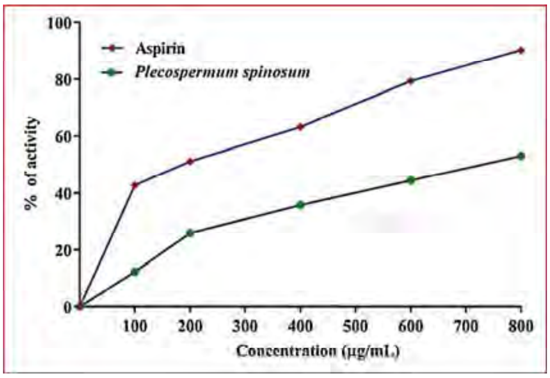

Fig. 3. Anti-inflammatory activity of *Plecospermum spinosum* Trecul. methanolic leaf extract.

activity of *P. spinosum* leaf extract (100 µg/mL) exhibited 51.16 ± 1.5 %, was less compared with the ascorbic acid standard (Fig. 2d). Reducing power of *P. spinosum* leaf extract was investigated and the data are shows in Fig. 2c. The leaf extract exhibited 32.45 ± 1.2 % at the maximum concentration, which was low when compared with the standard ascorbic acid. In the phosphomolybdenum assay, molybdenum VI was reduced into molybdenum V in the presence of antioxidant molecules of the leaf extract [26]. A linear profile was observed for the standard concentrations of ascorbic acid (10−100 µg/mL) with $R^2 = 0.9802$ (and a straight line equation of $y = 0.003\ x - 1.44$) and the significantly higher antioxidant activity obtained (reduction of molybdenum VI to V) was 93.33 ± 1.7 mg AAE/g. Ascorbic acid was the standard and the results are shown in Fig. 2e.

3.2. Antimicrobial activity of the methanolic leaf extract of P. spinosum

Natural plant sources have been utilized for the treatment of several human as well as animal diseases. Plants contain various types of chemical compounds which possess numerous pharmacological applications. Mortality rate is increased by the incidence of infectious diseases; about 70 % of hospital deaths are mostly due to diverse infectious diseases caused by bacteria, fungi and viruses [27,28]. Natural drugs have been to possess efficacy against antibiotic resistant microorganisms [13]. Botanists, ethnopharmacologists, and natural chemists are using medicinal plants for isolating a plethora of phytochemicals with potent activity against several clinically relevant pathogens, some of which are multidrug resistant and extensively drug resistant [13,29]. The potential antimicrobial activity of plants and herbs were already documented against different pathogens [1,2]. *P. spinosum* leaf extract was tested for inhibitory action against six bacterial strains and four fungal strains and some commercially available antibiotic discs were used as positive controls respectively. The highest zone of inhibition (9 ± 0.7 mm) was obtained against *P. aeruginosa* at the concentration of 10 mg/mL, which was equal to the zone obtained with 10 µg of vancomycin (9 ± 1.3 mm). The other pertinent details of zones of inhibition against various strains (*P. aeruginosa*, *K. pneumoniae*, *S. flexneri*, *S. typhimurium* and *E. coli*) are provided in Table 1. Against the fungal strain of *E. tinctorium*, the extract (2 mg/mL) exhibited 8 ± 0.2 mm zone of inhibition (Table 2). However, the zones obtained were several orders of magnitude lesser than those which were yielded by the standard antibiotics. It is important to note that the purified antimicrobial phytochemicals would generally possess substantial antimicrobial activity when compared with the crude extracts.

3.3. Anti-inflammatory activity of the methanolic leaf extract of P. spinosum

Inflammation is characterized by phagocyte activation, leading to discharge of chemical mediators such as prostaglandins, TNF-α and interleukins [15]. Leaky gut syndrome is known to be a leading cause of inflammation. Astonishingly, inflammation itself is the underlying cause of several diseases such as cardiovascular disease, cancer and arthritis. Escalation of oxidative stress mediated tissue damage leads to vascular changes [30]. Plant formulations and hydroalcoholic extracts have the potential to improve digestive health and increase memory power. Antioxidants with anti-inflammatory activity were also reported [16,31]. In the present study, the leaf extract of *P. spinosum* was studied for the anti-inflammatory effect through HRBCs membrane stabilization (inhibition of hemolysis) in comparison to aspirin (positive control) (Fig. 3). The inhibition (%) was obtained in a dose dependent manner. At high concentrations of the plant extact (800 µg/mL), 53.08 ± 1.5 % inhibition was obtained. However, aspirin control exhibited very high (90.41 ± 1.3 %) activity at the same concentration (800 µg/mL). This shows that aspirin is a far better anti-hemolytic (and anti-inflammatory) agent than the plant extract. However, if active principles responsible for this activity are purified and used in the assay, better efficacies can be obtained.

3.4. Qualitative and quantitative phytochemical analysis

It was evident that the methanolic extract possessed the greatest variety of phytochemical classes (triterpenoids, sugars, saponins, tannins, sterols and carbohydrates) followed by ethanolic extracts (catechins), aqueous and chloroform extracts (anthroquinones) and chloroform (carbohydrates) (Table 3). The other solvents were effective at extracting other phytochemical types. This was based on the relative polarities of the solvents and the affinities of the respective solvents for different plant compounds. Plant secondary metabolites are often unique and possess distinctive biological activities. Phenolic compounds and tannins possess antimicrobial properties [29]. There is a close relationship between the phenolic content and the antioxidant activity of plants and phenolics alone have diverse biological activities [8,16].

Table 3
Qualitative phytochemical screening of *P. spinosum* leaf extract.

Name of the test	Different solvents used for extraction of *P. spinosum* leaf extract					
	Aqueous	Methanol	Ethanol	Acetone	Chloroform	Hexane
Triterpenoids	–	+	+	–	–	+
Sugars	–	+	+	+	+	+
Catechins	–	–	+	–	–	–
Flavonoids	+	+	+	–	–	–
Saponins	+	+	–	–	+	+
Tannins	+	+	+	+	–	–
Anthroquinones	+	–	–	–	+	–
Amino acids	–	+	–	–	–	–
Sterols	+	+	–	–	+	+
Carbohydrates	–	–	–	–	+	–

(+) present, (-) absent.

Table 4
Quantitative analysis of *P. spinosum* leaf extract.

Name of the test	Amount of compounds present (mg/g)
Chlorophyll a	6.64 ± 0.1
Chlorophyll b	11.55 ± 0.3
Total chlorophyll	12.22 ± 0.5
Total carotenoids	1.34 ± 0.4
Total sugar	360 ± 1.7
Total protein	0.8 ± 0.1
Total lipids	160 ± 1.0
Total free amino acids	0.2 ± 0.2
Total phenol	70.34 ± 2.1
Total tannin	43.34 ± 1.2
Total flavonoids	164.28 ± 1.1

Catechins are a group of flavonoids exhibiting several therapeutic activities against Parkinson's disease, Alzheimer's disease, type II diabetes and prion diseases these molecules also possess anti-inflammatory, antiallergic, antiplatelet, antiviral and antioxidant activities etc [32, 33]. Crozier et al. reported that the plant derived saponins showed antimicrobial and anti-inflammatory activities [34]. Previous studies demonstrated that the tannins exhibited antimicrobial, antimutagenic, anticarcinogenic, antidiarrhoeal and antiseptic properties [35,36]. Anthraquinone glycosides were found to be minimally distributed among the plant species. Phytosterols are useful in lowering blood cholesterol levels and are relatively under used. Among the phytochemicals, phenols are a major group of antioxidants due to their therapeutic potential and ability to inhibit free radical generation. The presence of a multitude of secondary metabolites shows the promising nature of this medicinal plant. Chlorophyll a (6.64 ± 0.1 mg/g), chlorophyll b (11.55 ± 0.3 mg/g), total chlorophyll (12.22 ± 0.4 mg/g) and total carotenoids (1.34 ± 0.4 mg/g) were estimated (Table 4). The plant hadrich pigments contributing to its photosynthetic potential. Carbohydrate content in the plant extract was 360 ± 1.7 mg/g of total sugar. The total protein was 0.8 ± 0.1 mg/g protein as bovine serum albumin (BSA) equivalent. Total free amino acids could not be detected and perhaps, might have been present in minute quantities. Phenolic content was found to be 70.34 ± 2.1 mg TAE/g extract. Tannin content was 43.34 ± 1.2 mg TAE/g extract. Total flavonoid content was 164.28 ± 1.1 mg/g. In this study, the *P. spinosum* leaf extracts were found to contain a wide range of potential phytochemicals, which would possess multiple therapeutic efficacies and may be relatively than the synthetic drugs. Futher studies are required to prove the therapeutic potential of purified active principles.

3.5. GC–MS profiling of methanolic leaf extract of P. sinosum

The major bioactive phytochemicals present in the methanolic leaf extract of *P. spinosum* were identified using GC–MS (Fig. 4). There were 35 different phytochemical compounds obtained from the leaf extract and their retention time, names, % of peak area, molecular weight, molecular formula and therapeutic uses are represented (Table 5). Another study has suggested that 4-hydroxy benzyl alcohol (naturally occurring phenolic compound) was a potential inhibitor of lipid peroxidation and protein oxidation, indicating it could be considered as a

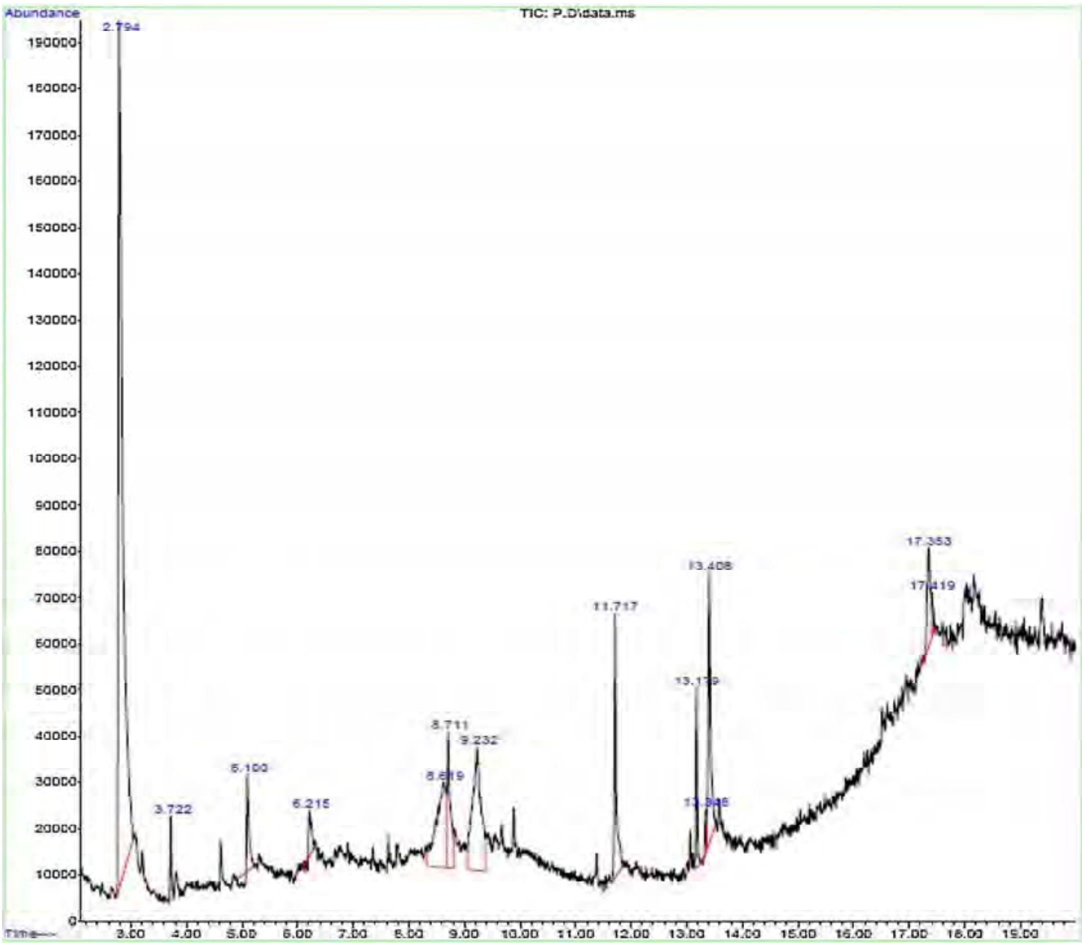

Fig. 4. GC–MS chromatogram of methanolic leaf extract of *Plecospermum spinosum* Trecul.

Table 5
Phytochemical analysis of methanolic leaf extract of *P. spinosum* using GC–MS.

Retention Time	Peak Area %	Compound Name	Molecular wt. g/mol.	Molecular formula	Therapeutic uses
2.796	45.45	1-Pentanol	88.15	$C_5H_{12}O$	Antioxidant
2.796	45.45	L-Serine, N-methyl-	119.12	$C_4H_9NO_3$	Diabetes
2.796	45.45	Diethylhydroxylamine	89.138	$C_4H_{11}NO$	No significant report
3.723	1.31	2-Butanethiol, 2-Methyl-	104.211	$C_5H_{12}S$	No significant report
3.723	1.31	4-Cyanobenzoic acid, 2-Phenylethyl ester	251.285	$C_{16}H_{13}NO_2$	No significant report
3.723	1.31	2-Phenylethyl pivalate	206.285	$C_{13}H_{18}O_2$	No significant report
5.103	1.74	4-Hydroxybenzyl alcohol	124.139	$C_7H_8O_2$	Antioxidant, anti-angiogenic and antidepressant
5.103	1.74	Isonicotinic acid	123.11	$C_6H_5NO_2$	Treatment of tuberculosis
5.103	1.74	2-Pyridinecarboxaldehyde, N-oxide	123.111	$C_6H_5NO_2$	antimicrobial and antineoplastic
6.219	1.36	N,N-Dimethyl-m-phenylenediamine	136.198	$C_8H_{12}N_2$	No significant report
6.219	1.36	Benzaldehyde, 2-Hydroxy-4-methyl-	136.15	$C_8H_8O_2$	Anti-inflammatory
6.219	1.36	1H-Inden-1-one, 2,3,4,5,6,7-Hexahydro	136.194	$C_9H_{12}O$	Inhibition of AChE and BuChE
8.621	9.90	Decanoic acid, 3-Methyl-	186.295	$C_{11}H_{22}O_2$	Antibacterial and anti-inflammatory
8.621	9.90	2-Butenoic acid, 4-Hydroxy-, Ethyl ester	116.116	$C_5H_8O_3$	No significant report
8.621	9.90	Dimethylcyanophosphine	87.062	C_3H_6NP	No significant report
8.715	5.23	.Beta.-D-Mannofuranoside, Methyl-	194.183	$C_7H_{14}O_6$	No significant report
9.235	12.56	2-Butenoic acid, 4-Hydroxy-, Methyl ester	116.116	$C_5H_8O_3$	No significant report
9.235	12.56	Silane, Ethyltrimetyl-	102.252	$C_5H_{14}Si$	No significant report
11.712	6.17	Palmitic acid	256.43	$C_{16}H_{32}O_2$	Antitumor and anti-HIV infection
11.712	6.17	Tridecanoic acid	214.349	$C_{13}H_{26}O_2$	No significant report
13.178	3.03	Phytol	296.539	$C_{20}H_{40}O$	Antiproliferative, anxiolytic and antidepressant
13.178	3.03	Butanoic acid, 3-Methyl-, 3,7-Dimetyl-6-Octenyl ester	–	–	–
13.178	3.03	11,13-Dimethyl-12-tetradecen-1-ol acetate	282.468	$C_{18}H_{34}O_2$	No significant report
13.348	0.68	7-Pentadecyne	242.831	$C_{15}H_{27}Cl$	No significant report
13.348	0.68	Z,Z-3,13-Octadecedien-1-ol	266.469	$C_{18}H_{34}O$	No significant report
13.348	0.68	E-11,13-Tetradecadienal	208.345	$C_{14}H_{24}O$	No significant report
13.405	8.01	Linolenyl alcohol	264.453	$C_{18}H_{32}O$	Anti-proliferative and antibacterial
13.405	8.01	Methyl (Z)-5,11,14,17-Eicosatetraenoate	318.501	$C_{21}H_{34}O_2$	No significant report
13.405	8.01	Cis,Cis,Cis-7,10,13-Hexadecatrienal	234.383	$C_{16}H_{26}O$	No significant report
17.357	3.84	Silane, Trimethyl[5-methyl-2-(1-methylethyl) phenoxy]-	222.403	$C_{13}H_{22}OSi$	No significant report
17.357	3.84	Propanamide, N-(4-methoxyphenyl)-2,2-Dimethyl	207.273	$C_{12}H_{17}NO_2$	No significant report
17.357	3.84	Arsenous acid, Tris(trimethylsily) ester	–	–	–
17.423	0.73	2-Ethylacridine	207.276	$C_{15}H_{13}N$	No significant report
17.423	0.73	2-Methyl-7-phenylindole	207.276	$C_{15}H_{13}N$	No significant report
17.423	0.73	Brallobarbital	287.113	$C_{10}H_{11}BrN_2O_3$	Sedative

better free radical scavenger [37]. Further, HBA was the potent anti-angiogenic agent for the treatment of different angiogenesis-related diseases including arthritis, diabetic retinopathy, cancer and endometriosis [38]. The compound HBA ameliorates cerebral ischaemic injury by the suppression of apoptotic pathways and upregulation of protein disulphide isomerase without causing any toxicity [39]. HBA showed antidepressant activity in rat by reducing monoamine metabolism and modulated cytoskeleton protein expression in the pathway of Slit-Robo [40]. A previous study has also demonstrated that isonicotinic acid was active against the pathogenic *Mycobacterium* sp. [41]. The Compound 2-pyridinecarboxaldehyde conjugated with metals such as cobalt, manganese, nickel, copper and other chemical derivatives and thereby, exhibited antimicrobial and antineoplastic activities [42–44]. 5-Bromo-2-hydroxy-4-methyl-benzaldehyde suppressed lipopolysaccharide stimulated inflammatory mediators by the inactivation of NF-κB, ERK and p38 in RAW 264.7 macrophages [45]. The compound 2-(4-alkoxybenzylidine)-2,3-dihydro-5,6-dimethoxy-1H-inden-1-one derivatives exhibited significant inhibition of human butyrylcholinesterase and acetylcholinesterase enzymes when compared with the standard donepezil [46]. Research findings demonstrated that palmitic acid was a potential therapeutic agent because it specifically targeted multiple myeloma cells [47]. Further, this compound suppressed HIV-1 infection by binding to CD4 receptor and efficiently blocked gp120-to-CD4 attachment and by this mechanism, is known to prevent transmission of HIV infection through sexual intercourse. Phytol (diterpene) and its derivatives exhibited a wide range of therapeutic applications in pharma and biotechnological industries. Phytol exerts phytopharmacological activity on the central nervous system, and causes anxiolytic and antidepressant effects [48]. Moreover, linolenyl alcohol showed considerable antibacterial effect and was particular effective against dental caries and periodontal disease [49]. Conjugation of two compounds with γ-linolenyl alcohol exhibited potential antiproliferative effect against colon, breast and prostate cancer in *in vitro* as reported [50].

4. Conclusion

It is concluded that *P. spinosum* exhibited a great potential to inhibit oxidative stress mediated damage, reduce high valent metal ions, decrease inflammation and inhibit microbial growth. The plant possesses novel compounds, which are yet to be isolated and characterized. Hence, further investigations are required to isolate novel phytochemicals which possess potent biological activities in clinical/animal trails. The ongoing *in silico* studies will lead to the identification of prospective biochemical targets of these phytochemicals (for most of which, there are little or no reports).

In this present study, the quantification of total phenolic content, total tannin and flavonol components present in methanolic solvent leaf extract of *P. spinosum* was performed. The phytochemicals present in this plant have been shown to scavenge free radicals through their antioxidant actions. Since ROS have a key role in inflammation, the phytocompounds with antioxidant activities were able to prevent inflammation, which are often seen in cancer. The extract showed promising ability to prevent hemolysis of HRBC membrane in a manner comparable to that of the commercial drug, aspirin. To the best of our knowledge, the present study is the first report on *P. spinosum* and its biological activities. From the GC–MS profile, it is inferred that the methanolic leaf extract of *P. spinosum* possessed higher concentrations of secondary metabolites such as phenol and flavonoids. 4-hydroxy benzyl alcohol (naturally occurring phenolic compound) is known to be a prospective inhibitor of lipid peroxidation and protein oxidation, signifying that it could be considered to be a good free radical scavenger. Hence *P. spinosum* possesses good antioxidant and anti-inflammatory activities.

CRediT authorship contribution statement

Pushparaj Annadurai: Conceptualization, Methodology, Writing - original draft. **Vinothkanna Annadurai:** Conceptualization, Methodology, Writing - original draft. **Ma Yongkun:** Writing - review & editing. **Arivalagan Pugazhendhi:** Supervision, Project administration. **Kandavel Dhandayuthapani:** Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.procbio.2020.09.031.

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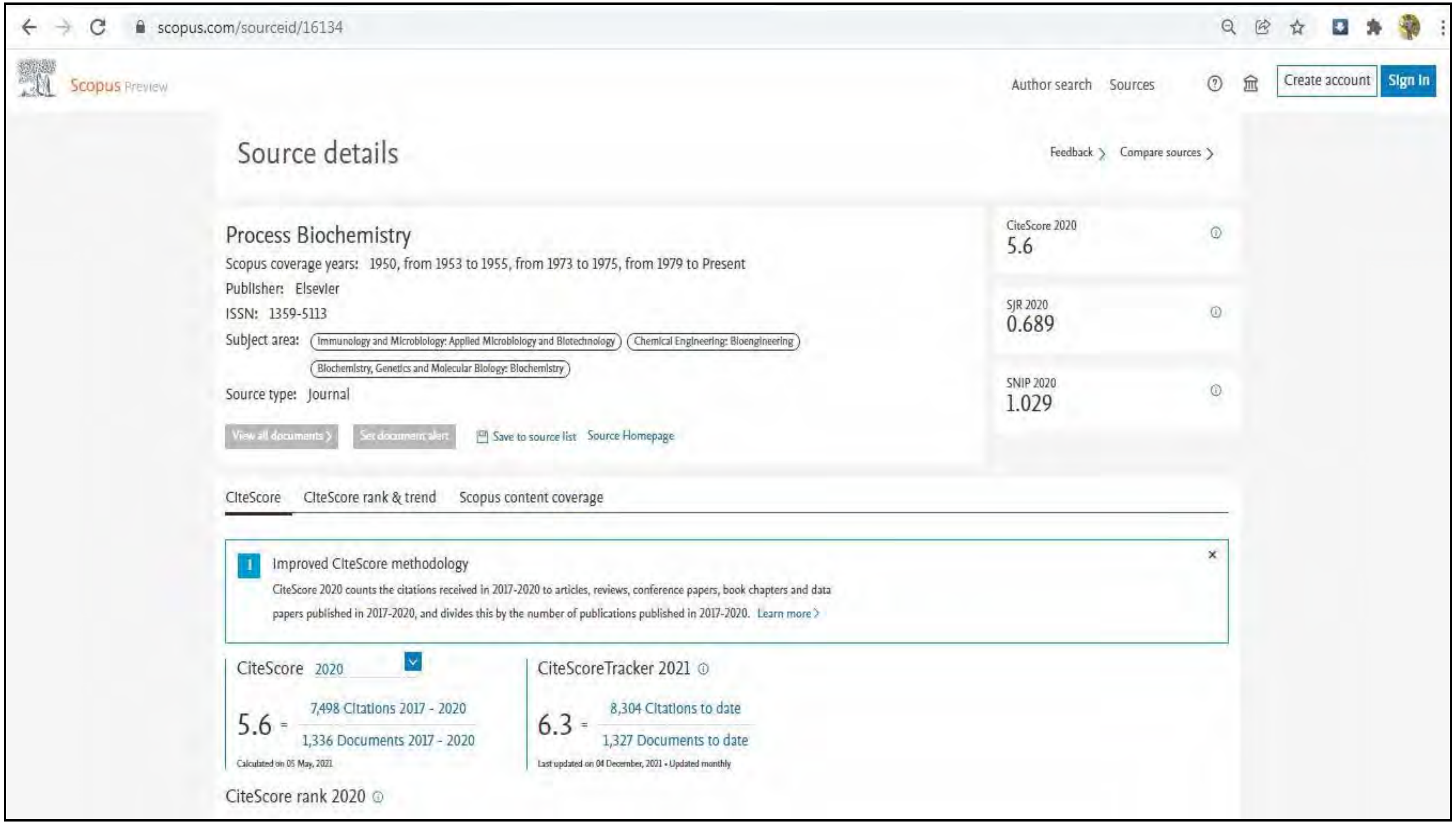

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Process Biochemistry 114 (2022) 119–133

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Deciphering the pharmacological potentials of *Aganosma cymosa* (Roxb.) G. Don using *in vitro* and computational methods

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A R T I C L E I N F O

Keywords:
Aganosma cymosa
GC-MS
Antioxidant
Cytotoxicity
Anti-inflammatory Molecular docking

A B S T R A C T

The methanolic leaf extract of *A. cymosa* yielded significant (60-75%) free radical scavenging activity in ABTS [2,2`-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] and DPPH (2,2`-diphenyl 1-picryl hydrazyl) assays, in the concentration range of 10-100 μg/mL. The extract showed antioxidant efficacy of 30-65% in reducing power assay and 55% inhibition in nitric oxide scavenging assay against ascorbic acid positive control. The plant extract exhibited modest inhibition against *Escherichia coli*, *Pseudomonas aeruginosa* and the fungal strain, *Echinodontium tinctorium*. The IC_{50} value of the extract was 100 μg/mL against MCF-7 breast cancer cells in MTT assay. The extract yielded 22-65% inhibition at 100-800 μg/mL concentration range in RBC membrane stabilization assay. Qualitative phytochemical screening revealed the presence of several classes of primary and secondary metabolites. Quantitative analysis revealed a high content of phenolic and flavonoid molecules. GC-MS profile of the plant extract revealed the presence of 44 bioactive compounds. Network analysis of key breast cancer proteins from differentially expressed genes aided in selecting the key proteins involved in breast cancer pathophysiology. ADMET-SAR analysis was done to assess the potential bioactivity of selected compounds. From the molecular docking results, coprostanol, 4-methylcatechol, pyrocatechol and indole were found to act as ligands of breast cancer target proteins. Furthermore, in molecular dynamics simulations (50 ns), the receptor-ligand complexes were stable for 50 nanoseconds. The results indicate that coprostanol is the principal component for binding to the selected breast cancer receptor proteins. Together, the results suggest that the phytochemicals explored herein exert the beneficial biological activities of *A. cymosa*.

1. Introduction

Medicinal plants have been used for treating majority of diseases and specific formulations or concoctions have been successfully used worldwide for managing adverse complications of a host of ailments since the beginning of time. Plants contain a repertoire of chemical

Abbreviations: AAE, ascorbic acid equivalents; AUC, area under the curve; ANOVA, ANalysis Of VAriance; ABTS, 2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid; CSL, Coprostanol; DCP, 2,3-dichloro-1-Propene; DPPH, (2,2`-diphenyl 1-picryl hydrazyl); EDL, Estradiol; ERβ, Estrogen receptor β; GC-MS, gas chromatography and mass spectroscopy; IDL, Indole; IC_{50}, inhibitory concentration; MCF, 7 – Michigan cancer foundation-7; MCL, 4-Methylcatechol; MeOH, methanol MTCC – microbial type culture collection; MMP, 9 – Matrix metallopeptidase 9; mTOR, Mammalian target of rapamycin; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]; MMS, Marimastat; NE, no effect detected; NCCS, national centre for cell science; NO, nitric oxide; NVT, normalization visualization tool; NPT, number of particles pressure and temperature; OD, optical density; PCL, Pyrocatechol; PI3K, Phosphoinositide 3-kinase; RET, rare endangered threatened; RFL, Ridaforolimus; RMSD, Root means square deviation; RMSF, Root means square fluctuation; RMN, Rapamycin; ROS, reactive oxygen species; RNS, reactive nitrogen species; TMN, Toremifene; ULB, Umbralisib; WRT, with respect to.

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https://doi.org/10.1016/j.procbio.2022.01.024
Received 5 December 2021; Received in revised form 20 January 2022; Accepted 27 January 2022
Available online 29 January 2022
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constituents that encompass six major classes of macronutrients/primary metabolites - carbohydrates, lipids, alkaloids, phenolics, terpenoids and other nitrogen containing compounds; each plant species possesses unique secondary metabolite profiles [1]. India possesses ubiquitous biodiversity hotspots which contain an enormous variety of medicinal plants and the estimated number of flora is ~ 47,000 to 50,000 species as per the Botanical Survey of India [2]. The Western Ghats of India have been studied extensively for biodiversity and several plants from these parts were explored for their prospective medicinal values [3]. However, the Eastern Ghats have not been so widely explored [4]. With the view to dissect the bioactive potentials of plants of the Eastern Ghats, the Siriya Kalrayan hills were surveyed for their floral diversity. One of the rarely explored plants identified in this region, *Aganosma cymosa* (Roxb.) G. Don, is a climber which belongs to Apocynaceae and is known to possess anthelmintic, emetic and anti-bronchitis properties as per traditional medicinal knowledge of tribals indigenous to the region. This rare endangered and threatened/RET climber is relatively rare in the biodiversity-rich Western Ghats [5] but is rather abundant in the Siriya Kalvarayan hills.

Cancer is one of the biggest killers among non-infectious diseases in humans; in terms of mortality, it comes only second to cardiovascular diseases. The six key hallmarks of cancer include - sustained proliferative signalling, evasion of growth suppressors, resistance to apoptosis, enablement of high replicative immortality, angiogenesis and activation of invasion/dissemination to sites other than the site of origin (metastasis) [6]. In 2020 alone, nearly 2.3 million new cases of breast cancer were confirmed worldwide and 685,000 deaths had been recorded [7]. Breast cancer is the leading cancer type in women [8]. Moreover, inflammation is an important process that is involved in pathogenesis and pathophysiology of breast cancer [9], and hence, cancer-related inflammation ought to be addressed, apart from inhibitor ligand-based blockage of breast cancer receptors.

Cancer stem cells have the ability to self-renew, differentiate and also initiate/sustain tumour growth and the presence of these stem cells is known to be responsible for cancer drug resistance, cancer recurrence, metastasis and replenishment of cancer cells [10,11]. When compared to standard chemotherapeutic drug regimen, phytochemicals may treat breast cancer by lowering its recurrence and metastatic spread; phytochemicals are known to possess lower side-effects and exhibit wide safety profiles [10–12]. Therefore, natural compounds and phytochemicals have been found to be promising alternatives to conventional cancer chemotherapeutic medications [13,14].

Aganosma cymosa (Roxb.) G. Don belongs to the climber family and the whole plant has been used for treating worm infestations and bronchitis in traditional Indian medicine. While *A. cymosa* was reported to possess anti-inflammatory [15] and hepatoprotective [16] activities, no other reports on the plant's medicinal properties are available. The present study is the first report cataloguing *A. cymosa's* phytochemical constitution using GC-MS. Earlier, this plant was shown to possess over 10.3% fats and therefore, was proposed as a raw material for hydrocarbon production [17]. Nanoparticles containing *A. cymosa* extract possessed mosquito larvicidal activity [18]. This might be the first ever comprehensive report on the phytochemical profile and biological activity spectrum of *A. cymosa.* A Google Scholar search of the plant name yielded no reports on the medicinal properties of this species. Hence, to investigate the primary and secondary metabolite composition of the plant and to identify bioactive principles, this work was carried out. The methanolic crude extract of *A. cymosa* leaf was explored for its antimicrobial, anti-inflammatory, cytotoxic and antioxidant properties. Initially, preliminary phytochemical screening was carried out to qualitatively identify the diverse phytochemical classes present in *A. cymosa.* Using quantitative biochemical analyses, the concentrations of various phytochemicals present in the extract were determined. GC-MS profiling was done to identify the repertoire of phytochemicals present in the extract. Further, the phytochemical components were rigorously assessed using *in silico* approaches to compare their binding affinities for key receptor proteins which were identified through network analysis of differentially expressed genes during breast cancer, using hits obtained from the NCBI Gene database. From comparing the protein-ligand docking scores for *A. cymosa* phytochemicals with well-known drugs which are used in breast cancer pharmacotherapy, the potential usefulness of these compounds as natural breast cancer therapeutic agents was assessed. Molecular dynamics (MD) simulations were performed for selected receptor-drug combinations to check the stability of the drug-receptor complexes.

2. Material and methods

2.1. Plant collection

About 1 kg of leaves of *A. cymosa* was obtained from the Siriya Kalvarayan hills, which belong to the Eastern Ghats and are located in Kallakurichi District in the southern Indian state of Tamil Nadu. Since *A. cymosa* is an RET climber [19], it could be a potentially novel source of natural medicines. This relatively rare plant was found to grow abundantly in the study area. The herbarium specimen was authenticated by a competent taxonomist at the Rapinat Herbarium located in St. Joseph's College campus, Tiruchirappalli, India (Supplementary Fig. S1 is a copy of the herbarium specimen of the entire plant).

2.2. Chemicals and reagents

Unless otherwise stated explicitly, all the chemicals and reagents used for this study were procured from reputed vendors (SRL, India and HiMedia, Mumbai, India) and were of certified, analytical grade. All the reagents/chemicals were prepared freshly and used for the assays.

2.3. Qualitative analysis

Phytochemical screening was carried out [20] to identify the major classes of phytochemicals present in *A. cymosa.* The reagents required for this qualitative assay were prepared freshly. All the assays were repeated in order to rule out experimental errors and hence, data were acquired in either duplicate or triplicate. A wide range of extracts was obtained with solvents of various polarities such as water (aqueous extract), methanol, ethanol, acetone, chloroform and hexane. After testing the various extracts, methanol was chosen for soxhlet extraction since it showed the presence of a broad range of compounds. Plant metabolites belongs to diverse classes such as-triterpenoids, sugars, catechins, flavonoids, tannins, saponins and free aminoacids. Hence, further quantitative and biological activity assays were performed and GC-MS profiling was done to search for any potential bioactive molecules which could be responsible for specific biological activities (like antioxidant, anti-inflammatory and cytotoxic action).

2.4. Solvent extraction

The leaves were shade dried for over three days and the leaf samples were weighed, and powdered using a mixer/blender jar. 45 g of the powder was filled into a cellulose cotton fibre thimble (Whatmann, GE healthcare Ltd.). The extraction was carried out after pouring 200 mL methanol into the distillation flask, which was placed over a heating mantle at 63 °C for 12 h. The solvent concentrate obtained was dried by pouring into a glass petridish at room temperature and then, weighed and collected. The extract was dissolved again in methanol and this sample was used for the assays in this work.

2.5. Quantitative phytochemical evaluations

Biochemical assay of chlorophyll a and b, carotenoids and total chlorophyll content was carried out by taking 100 mg of the leaf powder. A popular method was used for assaying total sugar content [21]. Total lipid content was assessed using gravimetric method [22] and total protein content was estimated using Lowry's method [23]. Using a standard method [24], total free aminoacid content was quantified. Total phenol content was determined and the mg gallic acid equivalents/g of sample was calculated from the results [25]. The total tannin content was estimated by subtracting the value of non-tannin phenolics from that of the total phenolic content assayed earlier using the Folin-Ciocalteau method and the results were expressed as mg tannic acid equivalents/gram of the sample. Using the aluminium chloride method, assay of flavonoid content was performed. Rutin was employed as the flavonoid standard (100 μg/mL) and the results were expressed as rutin equivalents [26].

2.6. Antioxidant assays

DPPH radical scavenging assay was employed to assess the efficacy of the compound [27]. To each test tube, 1 mL of the *A. cymosa* MeOH leaf extract was added in the concentration range of 10-100 μg/mL to 1.0 mL of 0.2 mM DPPH radical solution. Corresponding blank solutions were prepared and the positive control was L-ascorbic acid, in the concentration range of 10-100 μg/mL. $ABTS^{\bullet+}$ radical scavenging assay was performed as per a popular method. 1 mL of plant sample (10-100 μg/mL) was added to 1 mL of freshly diluted $ABTS^{\bullet+}$ solution. Using ascorbic acid as standard (10-100 μg/mL), the antioxidant efficacy of the plant extract was compared [28].

Ferric ion reducing power assay was performed [29]. Ascorbic acid was used as the antioxidant positive control. To complement the antioxidant activity results obtained using the aforementioned methods, phosphomolybdenum assay was performed additionally as per a standard protocol [26] and the results were represented as ascorbic acid equivalents (AAE)/g extract. Also, the nitric oxide radical scavenging activity of the plant extract was determined [26].

2.7. Antimicrobial activity

The bacterial strains utilized for the determination of antimicrobial activity were -*Escherichia coli* MTCC584, *Klebsiella pneumoniae* MTCC8911, *Pseudomonas aeruginosa* MTCC1034, *Staphylococcus aureus* MTCC9542, *Salmonella typhimurium* MTCC3224 and *Shigella flexneri* MTCC9543. The fungal strains employed were - *Candida glabrata* MTCC3983, *Candida tropicalis* MTCC2795, *Echinodontium tinctorium* MTCC1038 and *Aspergillus fumigatus* MTCC2483. Nutrient broth was used to subculture the bacterial species at 37 °C for 18-24 h (overnight) to revive the organisms. Well diffusion method was followed [30] and antimicrobial activity of *A. cymosa* extract at 6,7,8,9 and 10 mg/mL was assessed. Amikacin, ampicillin, amoxyclav, bacitracin, chloramphenicol, gentamicin, methicillin, nitrofurantoin, penicillin-G and vancomycin discs were used as positive controls.

2.8. *In vitro* cytotoxicity of *A. cymosa*

MTT assay was performed to determine the *in vitro* cytotoxic efficacy of the *A. cymosa* extract [31]. The breast cancer cell line, MCF-7, was procured from the National Center for Cell Science (NCCS), Pune, India. 1×10^5/well cells were seeded onto 24-well plates. After attainment of cell confluence, the plant extract (in the range of 7.8-1000 μg/mL) was added to various wells containing equal cell numbers (for controls and treatments) incubated for 24 h. After incubation, the excess sample was removed from the wells by washing with sterile PBS (pH 7.4) or DMEM without serum, after which, MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] was added to each of the wells and the culture was incubated for another 4 h. After this, 1 mL of cell culture grade DMSO was added to each of the wells and the absorbance was measured using a UV-Vis spectrophotometer (570 nm). DMSO was used as the blank and the concentration of the plant extract required for half maximal inhibition (50% inhibition/IC_{50}) was determined. Cell viability (%) was calculated as follows:

$$\% \text{ Cell viability} = \text{Control cell - treated cells / control cells} \times 100$$

2.9. Anti-inflammatory effect of *A. cymosa* leaf extract

The anti-inflammatory efficacy of the *A. cymosa* methanolic leaf extract was assessed as per the method followed earlier [32,33]. The plant extract as well as the standard aspirin (in the concentration range of 10-100 μg/mL) were mixed with 2 mL of phosphate buffer, 4 mL of hypo-saline and finally, 0.9 mL of RBC suspension. After mixing, and incubation for 30 min at 37 °C, the supernatant was decanted and the concentration of hemoglobin was estimated at 620 nm. Percentage (%) of haemolysis was calculated using the formula:

$$\% \text{ Haemolysis} = \text{T/C x } 100$$

T-Test sample; C-Control sample

2.10. GC-MS profiling of *A. cymosa*

Gas Chromatography-Mass Spectrometry (GC-MS) was carried out using a Perkin Elmer Clarus 500 (Connecticut, USA) instrument equipped with a flame ionization detector with capillary column, as per the method followed by us earlier [32].

3. Computational studies

3.1. Bioinformatics prospecting of biological activities of *A. cymosa* phytochemicals

3.1.1. Molinspiration

Chemdraw Ultra 8.0. was used to draw all the chemical structures of compounds for *in silico* explorations. The online tool of Molinspiration Property Calculator and ACD/I-LAB service was used for predicting the drug-likeness of the compounds which were identified through GC-MS; parameters of structure-activity like Lipinski's rule of five were evaluated. Also, the values yielded in the results enabled prediction of the bioavailability and potential biological activities of the selected compounds. The analysis yielded insights into the medicinal properties and plausible generic target protein classes – eg., kinase inhibition, nuclear receptor binding, etc. and this aided in zeroing-in on plausible protein targets (https://www.molinspiration.com/).

3.1.2. ADMET-SAR

The PK properties, such as Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET), of the *A. cymosa* compounds (results from the GC-MS report) were predicted using the admetSAR v2.0 server (http://lmmd.ecust.edu.cn/admetsar2/). This tool is a convenient platform for biochemical and drug discovery research investigations. Various chemicophysical characteristics of the phytocompounds of *A. cymosa* were predicted.

3.1.3. Network Analysis

The mode of interactions between differentially expressed genes (DEGs) was probed using Search Tool for the Retrieval of Interacting Genes (STRING, http://string.embl.de/) database and the results were viewed with the help of Cytoscape. The cut-off value was set at a degree >25. Network analysis was performed with the aid of network analysis and the small-world networks were obtained by calculating network properties like node degree distribution, shortest path distribution, average aggregation coefficient and proximity to the center. A plugin for Cytoscape, Molecular complex detection (MCODE), was employed for PPI network module analysis, and the scores of MCODE > 3 and number of nodes > 5 were determined as cut-off criteria with the default

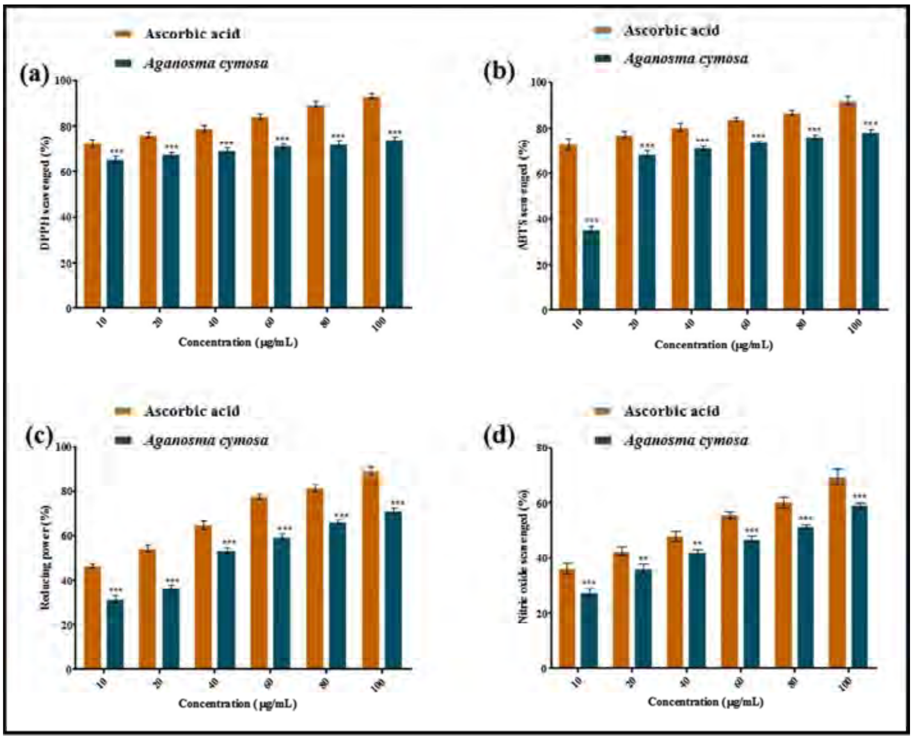

Fig. 1. Antioxidant activity of methanolic leaf extract of *Aganosma cymosa* (Roxb.) G. Don. Values indicate mean ± standard deviation (n = 3). The mean difference is significant at the levels of *p < 0.05, **P < 0.01 and ***P < 0.001 Vs. standard. ns – non significant; (a) DPPH (b) $ABTS^{\bullet+}$ (c) Reducing power (d) Nitric oxide.

Table 1
Antibacterial activity of methanolic leaf extract of *A. cymosa* by well diffusion method.

Antibiotic (µg/disc)	Zones are represented as radius (mm)						
	Concentration of the plant extract (mg/mL)	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Klebsiella pneumoniae*	*Shigella flexneri*	*Salmonella typhimurium*	*Escherichia coli*
-	6	4 ± 0.2	NE	NE	NE	NE	3 ± 0.2
-	7	4 ± 0.2	NE	NE	NE	NE	4 ± 0.2
-	8	5 ± 0.5	NE	NE	NE	NE	4 ± 0.5
-	9	7 ± 0.6	NE	NE	NE	NE	5 ± 0.7
-	10	8 ± 0.5	NE	NE	NE	NE	6 ± 0.8
Nitrofurantoin (10)	-	8 ± 1.7	13 ± 1.3	9 ± 1.1	8 ± 1.2	3 ± 0.8	11 ± 0.7
Vancomycin (10)	-	10 ± 1.3	11 ± 1.4	NE	NE	9 ± 1.5	NE
Gentamicin (10)	-	15 ± 1.3	15 ± 1.2	13 ± 1.8	9 ± 0.8	16 ± 1.8	6 ± 1.0
Bacitracin (10)	-	7 ± 1.4	11 ± 1.1	NE	NE	6 ± 1.4	9 ± 1.2
Amoxyclav (30)	-	17 ± 1.9	20 ± 1.0	15 ± 1.6	3 ± 0.7	21 ± 1.3	14 ± 2.0
Methicillin (5)	-	NE	NE	NE	NE	NE	NE

NE- no effect detected

Table 2
Antifungal activity of methanolic leaf extract of *A. cymosa* by well diffusion method.

Antibiotics (µg/disc)	Zones are represented as radius (mm)				
	Concentration of the plant extract (mg/mL)	*Echinodontium tinctorium*	*Candida tropicalis*	*Candida glabrata*	*Aspergillus fumigatus*
-	2	6 ± 1.4	NE	NE	NE
-	4	12 ± 0.1	NE	NE	NE
-	6	11 ± 0.1	NE	NE	NE
-	8	12 ± 0.2	NE	NE	NE
-	10	14 ± 0.4	NE	NE	NE
Penicillin-G (10)	-	N.E.	NE	NE	NE
Methicillin (5)	-	NE	NE	NE	NE
Chloramphenicol (30)	-	9 ± 0.7	10 ± 0.9	10 ± 0.8	9 ± 1.2
Amikacin (30)	-	5 ± 0.4	7 ± 0.5	9 ± 1.2	8 ± 0.7
Ampicillin (10)	-	NE	NE	NE	NE

NE- no effect detected

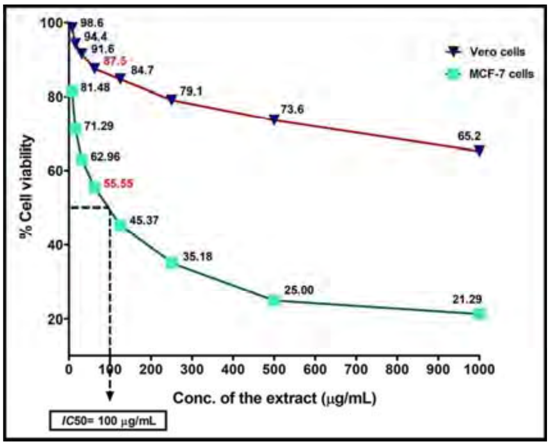

Fig. 2. Cytotoxic effect of *Aganosma cymosa* (Roxb.) G. Don methanolic leaf extract on Vero cell line and MCF-7 cell line after exposure of 24 h.

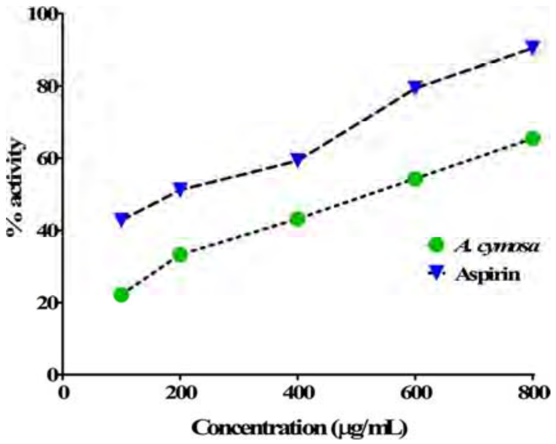

Fig. 3. Anti-inflammatory activity of methanolic leaf extract of *Aganosma cymosa* (Roxb.) G. Don.

parameters. Clue Go is also a Cytoscape software for gene ontology and pathway enrichment analysis. Different criteria are applied for annotation analysis of studied modules (protein complexes). These include Kappa statistic ≥ 0.4, enrichment (Right-sided hypergeometric test), and Bonferroni step down method for probability value correction.

3.2. Ligands and protein preparation

The 44 phytochemical constituents identified from the GC-MS analysis were retrieved in canonical SMILES format from the Pubchem database (http://pubchem.ncbi.nlm.nih.gov/). The two dimensional structures were converted to the appropriate three dimensional PDB format using OPENBABEL 3.1.1 toolbox [34]. Employing the clean geometry tool in Arguslab 4.0.1, optimized structures for the ligands were generated *in vacuo* based on the Root-mean-square deviation (RMSD) and initial minimization was added with hydrogens and Gasteiger charges utilizing CHARMM force field by unusual bond fixation and three dimensional structures were derived [35]. Target protein structures were retrieved from the PDB database and protein preparation was performed using Autodock Vina by adding polar hydrogen atoms, hetatm, water addition and final conversion from PDB format to PDBQT format was performed [36].

3.3. Active site validation, analysis of clefts and binding pockets

Due to their roles key roles in breast cancer, PI3K (PDB ID: 5NGB), MMP-9 (5TH6), mTOR (4JT6) and ERβ (3OS8) were chosen as target receptors to decipher the plausible underlying mechanisms of *A. cymosa* compounds' bioactivity. Active sites of the protein targets were validated using Autodock Vina based on RMSD values and geometry optimization [37]. Analysis of pockets, clefts and the necessary solvent accessibility profiles with a water probe of 1.4 Å radius were performed, and electrostatic potential at 80.0 dielectric constant was calculated for 20 iterative cycles using the CASTp server [38]. From published literature reports, the key active site residues of the receptors were identified and docking was performed by preparing grids to cover the active site areas. Pertinent details regarding the key active site residues identified from literature reports are given in Table S1.

Table 4
Quantitative analysis of *A. cymosa* methanolic leaf extract.

Test	Amount of compounds present (mg/g)
Chlorophyll *a*	8.70 ± 0.4
Chlorophyll *b*	16.41 ± 1.1
Total chlorophyll	17.15 ± 0.5
Total carotenoids	1.94 ± 0.1
Total sugar	43 ± 0.6
Total protein	2.1 ± 1.1
Total lipids	9 ± 0.6
Total free amino acid	2.4 ± 0.4
Total phenol	97.93 ± 0.3
Total tannin	30.93 ± 0.4
Total flavonoids	40.84 ± 1.8

Table 3
Preliminary phytochemical screening of *A. cymosa* leaf extract.

Test	Extraction using various solvents					
	Aqueous	Methanol	Ethanol	Acetone	Chloroform	Hexane
Triterpenoids	-	+	+	-	-	-
Sugars	+	+	+	+	+	+
Catechins	+	+	+	+	-	-
Flavonoids	+	+	+	-	-	-
Saponins	+	+	+	-	+	+
Tannins	+	+	+	+	-	-
Anthraquinones	-	-	-	-	-	-
Aminoacids	-	+	+	-	-	-
Sterols	-	-	+	+	+	+
Carbohydrates	-	-	-	-	+	-

(+) – Present; (-) – absent

Table 5
GC-MS analysis of methanolic leaf extract of *A. cymosa*.

Peak No.	Retention time	Area (%)	Height (%)	Compound name	Molecular Weight g/mol	Molecular Formula	Uses
1	5.05	0.11	0.64	Cyclomethicone	296.61	$C_8H_{24}O_4Si_3$	Removing cuticular hydrocarbons from human head lice
2	5.15	0.16	0.24	1,3-Difluoroacetone	94.061	$C_3H_4F_2O$	Pesticidal activity
3	6.832	0.35	0.77	Isoamyl Formate	116.16	$C_6H_{12}O_2$	No significant report
4	8.299	1.26	1.37	Pyrocatechol	110.112	$C_6H_6O_2$	Antitumor potency, to prevent late growth of sugar beet and increase sucrose content
5	8.545	0.1	0.28	2,3-dichloro-1-Propene	110.965	$C_3H_4Cl_2$	No significant report
6	8.617	0.43	0.97	4-Vinylphenol	120.151	C_8H_8O	Anti-angiogenic activity (Yue, Grace Gar-Lee., et al 2015)
7	8.823	0.11	0.22	3-Ethyl-4-methyl-1H-pyrrole-2,5-dione	139.15	$C_7H_9NO_2$	No significant report
8	9.004	0.09	0.2	Phenylacetic acid	136.15	$C_8H_8O_2$	Plant growth regulating properties, anti-fungal activity
9	9.645	0.06	0.11	4-Methylcatechol	124.139	$C_7H_8O_2$	Stimulator of endogeneousnerve growth factor synthesis, neurotrophic and antioxidant activity
10	9.755	0.06	0.23	Indole	117.151	C_8H_7N	Antimicrobial agent and anticancer activity
11	9.979	0.07	0.34	2-Methoxy-4-vinylphenol	150.177	$C_9H_{10}O_2$	Antitumor activity, anticancer activity
12	10.451	0.07	0.28	2,6-Dimethoxyphenol	154.165	$C_8H_{10}O_3$	Used for food preparation and bio fuel
13	10.549	0.04	0.11	4-(1H-Pyrrol-3-yl)butanoic acid	167.208	C9H13NO2	No significant report
14	11.495	0.11	0.33	Tyrosol	138.166	C8H10O2	Anticancer activity, antioxidant activity and also used for bacterial growth medium
15	11.76	0.42	0.43	Tris(Hydroxymethyl)Nitromethane	151.118	C4H9NO5	No significant report
16	11.959	0.09	0.19	[Butoxy bis(trimethylsilyloxy)silyl] tris (trimethylsilyl) silicate	591.229	$C_{19}H_{54}O_7Si_7$	No significant report
17	12.419	0.14	0.22	.beta.-D-Glucopyranose, 1,6-anhydro-	164.132	$C_9H_9FO_4$	No significant report
18	12.762	0.05	0.13	2-Oxovaleric acid, TBDMS derivative	230.379	$C_{11}H_{22}O_3Si$	No significant report
19	12.927	0.07	0.34	Dihydroactinidiolide	180.247	$C_{11}H_{16}O_2$	Used for fragrance
20	13.115	0.06	0.19	Capric Acid	172.268	$C_{10}H_{20}O_2$	Larvicidal and anticonvulsant effect
21	13.525	2.55	4.5	Mome Inositol	-	-	No significant report
22	13.927	2.71	2.8	1,3,4,5-Tetrahydroxycyclohexane-1-carboxylic acid	192.167	$C_7H_{12}O_6$	No significant report
23	14.31	0.05	0.11	Citral diethyl acetal	226.36	$C_{14}H_{26}O_2$	Antitumor activity
24	14.37	0.04	0.17	Diethylene glycol diacetate	190.195	$C_8H_{14}O_5$	No significant report
25	15.403	12.55	7.67	Undecanoic acid	186.295	$C_{11}H_{22}O_2$	Antifungal activity
26	15.7	1.3	3.32	Ethyl-1-thio-.beta.-d-glucopyranoside	224.271	$C_8H_{16}O_5S$	No significant report
27	16.718	0.14	0.41	Phytol	296.539	$C_{20}H_{40}O$	Anticancer activity, cytotoxicity
28	17.164	0.19	0.84	Methyl Palmitate	270.457	$C_{17}H_{34}O_2$	Fuel production, ant-inflammatory activity and antifibrotic effect
29	17.511	1.85	7.35	Palmitic Acid	256.43	$C_{16}H_{32}O_2$	Anticancer activity
30	17.901	0.12	0.3	4-Decenal, (4z)-	154.253	$C_{10}H_{18}O$	Using for making fragrance
31	17.96	0.09	0.17	2,3,4-Trimethyl-3-pentanol	130.231	$C_8H_{18}O$	No significant report
32	18.161	0.05	0.15	Cyclohexyl Laurate	282.468	$C_{18}H_{34}O_2$	No significant report
33	18.861	0.15	0.75	Methyl hexadeca-9,12-dienoate	266.425	$C_{17}H_{30}O_2$	No significant report
34	19.203	1.25	3.43	9-Tetradecenal, (9Z)-	210.361	$C_{14}H_{26}O$	No significant report
35	19.41	0.25	0.9	Stearic Acid	284.484	$C_{18}H_{36}O_2$	Therapeutic efficacy in hepatic cancer and cosmetic products
36	21.161	0.15	0.35	1,3-O-Benzylidene glyceryl-2-myristate	374.565	$C_{24}H_{38}O_3$	No significant report
37	22.38	0.26	0.91	Glyceryl 2-palmitate	330.509	$C_{19}H_{38}O_4$	No significant report
38	23.46	0.05	0.08	Nonadecanoic acid, dimethyl (isopropyl)silyl ester	398.747	$C_{24}H_{50}O_2Si$	No significant report
39	23.664	0.16	0.27	22,23-Dibromostigmasterol acetate	614.547	$C_{31}H_{50}Br_2O_2$	Antibacterial activity
40	23.846	0.32	0.36	Glyceryl Trielaidate	885.453	$C_{57}H_{104}O_6$	No significant report
41	24.06	0.15	0.21	Glyceryl Monostearate	358.563	$C_{21}H_{42}O_4$	Anticancer activity
42	24.41	3.42	4.98	Cholest-5-en-3-ol (3beta)-	386.664	$C_{27}H_{46}O$	No significant report
43	24.624	0.35	0.5	Coprostanol	388.68	$C_{27}H_{48}O$	Used as biomarker
44	24.82	0.04	0.07	5-Chlorovaleric acid, tridec-2-ynyl ester	314.894	$C_{18}H_{31}ClO_2$	No significant report

Table 6
SAR properties of *A. cymosa* compounds.

Ligands	Molecular Weight	Volume	nViolations	nrotb	nON (HBA)	nOHNH (HBD)	nAtoms	TPSA	miLogP
2,3-dichloro-1-propene	110.97	84.24	0	1	0	0	5	0	1.90
4-methylcatechol	124.14	116.64	0	0	2	2	9	40.46	1.42
Coprostanol	388.68	429.34	1	5	1	1	28	20.23	7.80
Indole	117.15	113.02	0	0	1	1	9	15.79	2.16
Pyrocatechol	110.11	100.08	0	0	2	2	8	40.46	0.99

3.4. Molecular docking and Dynamics simulation studies

Molecular interactions between the 3D structures of (PDB ID: 5NGB 5TH6, 4JT6 and 3OS8) and the bound phytochemical compounds were assessed using MGL tools (AutoDock 4.2) [39]. For converting both receptor and the selected ligands (and pertinent drug positive controls/inhibitor controls for each protein target) into PDBQT format from PDB files, the standard AutoDock protocol was followed. After adjusting AutoGrid dimensions of XYZ at $60 \times 60 \times 60$ Å^3, the grid spacing was set at 0.375 Å. Both polar hydrogen atoms and Gasteiger charges were

Table 7
Molinspiration results for selected *A. cymosa* compounds.

Ligands	GPCR ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor
2,3-dichloro-1-propene	−3.88	−3.85	−3.91	−3.85	−3.86	−3.83
4-methylcatechol	−2.28	−1.66	−2.42	−2.24	−2.61	−1.80
Coprostanol	0.21	0.33	−0.34	0.66	0.22	0.51
Indole	−1.64	−1.02	−1.36	−2.04	−2.17	−1.29
Pyrocatechol	−3.04	−2.51	−3.10	−2.98	−3.24	−2.67

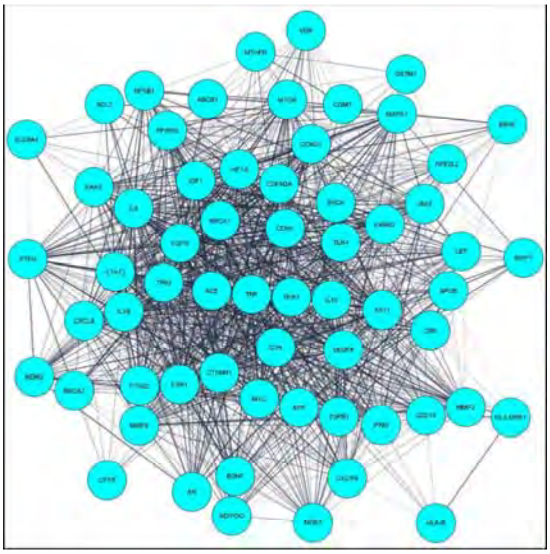

Fig. 4. PPI network analysis of DEGs in breast cancer – Network analysis of key DEGs was performed using Cytoscape and some of the key genes of our study focus were found to be differentially expressed in the top 60-70 hits obtained from RNAseq data (reported in NCBI-GEO databases).

added to the target macromolecules. Prior to commencement of docking runs, the active torsions and degrees of torsional freedom were assessed for the small molecules. The Lamarckian principle was selected and 10 docking runs were set. Gibbs free energy, ΔG in kcal/mol, was utilized as a pivotal parameter in assessment of binding-based inhibitory potentials of phytochemical compounds. Molecular visualization was done using PyMoL and ChimeraX. The best poses depicting potential interactions were undertaken using the 2D poses of the best hits for the necessary binding of ligands with the necessary protein targets in Accelrys Discovery Studio Visualizer 2.5 [40]. Binding sites and necessary aminoacid interactions which depicted the protein-ligand affinity were verified using Chimera 1.14 [41].

A modified protocol was followed by combining two molecular dynamics simulation methods [42,43]. GROMACS 5.1.5 software was utilized based on the optimized and default parameters for topology assessment and simulation [44]. Ligand topology files were prepared using PRODRG online tool (http://davapc1.bioch.dundee.ac.uk/cgi-bin/prodrg) and further using the force field parameters in GROMACS, namely GROMACS96 43a1 and the proteins were solvated employing Simple Point Charge water model with the coordinates of X, Y & Z: 144 × 144 × 144 Å^3, respectively. The final optimization was performed by addition of sodium ions for neutralization of charges. Periodic Boundary Condition (PBC) plays a vital role in avoiding the boundary effects which might lead to detachment among the protein, ligand and water molecule; also, it helps to maintain the connectivity among the protein, ligand, and water. PBC was calibrated by the mathematical principle of Velocity Verlet algorithm and Leap-frog algorithm. Protein Energy Minimization (EM) was performed by the steepest descent minimization algorithm with the maximum force <10.0 kJ/mol (1000.0) and was converged by maximum 50000 number of steps to minimize the protein. Also, this cut-off scheme was fixed to find the neighbouring set of atoms / molecules and to calculate the non-bond interactions like electrostatic and vander waal's force with the help of Verlet algorithm and Particle Mesh Ewald (Lennard Jones and Coulomb potential). Protein Restrain Topology equilibration was carried out by using the NVT and NPT phases with the help of Parrinello-Rahman method; NVT –Conjugated gradient algorithm (Leap-frog integrator) helps to minimize the complex upto 100 ps; Berendsen thermostat and V-rescale weak coupling method helps to maintain the temperature coupling upto 300 K; LINCS helps to analyze the hydrogen bond interactions between the protein and ligand; Maxwell distribution assigns the velocity to minimize the complex and NPT - Isotropic Berendsen thermostat pressure coupling helps to minimize the complex upto 25 ns (25000 ps) steps; time constant and pressure, tau_p & ref_p was maintained upto 2.0 ps & 1.0 atm, respectively.

4. Results and discussion

4.1. Antioxidant assays

Oxidative stress is a significant player in health and disease and often is known to drive the pathogenesis of several disorders and diseases [45]. The generation of free radicals (and oxidants) such as superoxide ($O_2^{\cdot-}$), hydroxyl radical (OH.), hydroperoxyl radical (·OOH), hydrogen peroxide (H_2O_2) and singlet oxygen has been demonstrate to be much higher during oxidative stress [46]. *A. cymosa* possesses prominent antioxidant and anti-inflammatory properties. The methanolic extract of the plant had shown satisfactory scavenging/inhibitory activity against ROS and RNS, as depicted in Fig. 1. The plant extract showed 60-70% inhibition (in the concentration range of 10-100 μg/mL) against DPPH radical when compared to the ascorbate control (70-90% inhibition in the concentration range of 10-100 μg/mL). Against ABTS metastable radical cation, *A. cymosa* extract showed considerable effect, yielding 35-70% inhibition in the 10-100 μg/mL concentration range. The ABTS radical cation is one-electron oxidized and donation of an electron by the reductants (antioxidants) present in the plant extract essentially quenched the free radical (against 70-90% proffered by the ascorbate control) and ascorbic acid exerted similar dose-dependent effects in all cases, barring the first data point (10 μg/mL). In the ferric reducing power assay (which measures the ability of the chemical compounds/extracts to convert Fe^{3+} ion to Fe^{2+}), the plant extract possessed roughly 30-65% activity when compared to the ascorbate control (~40-85%), while both the control and test samples inhibited in the range of 10-100 μg/mL. Nitric oxide scavenging efficacy of the plant extract was close to that of the ascorbate control with over 25->55% activity . The phosphomolybdenum assay result showed the antioxidant activity to be 63.33% with respect to ascorbic acid control (standard plot slope). Using two-way ANOVA, the results were found to be significant when the data points were compared between each ascorbate control point (same concentration of ascorbate as the test); Bonferroni post-tests revealed that the P-value for all the four assays (between the control and test points in all the cases) were significant (*** at $P < 0.001$); however, in isolated instances, the level of significance was ** (at $P < 0.01$).

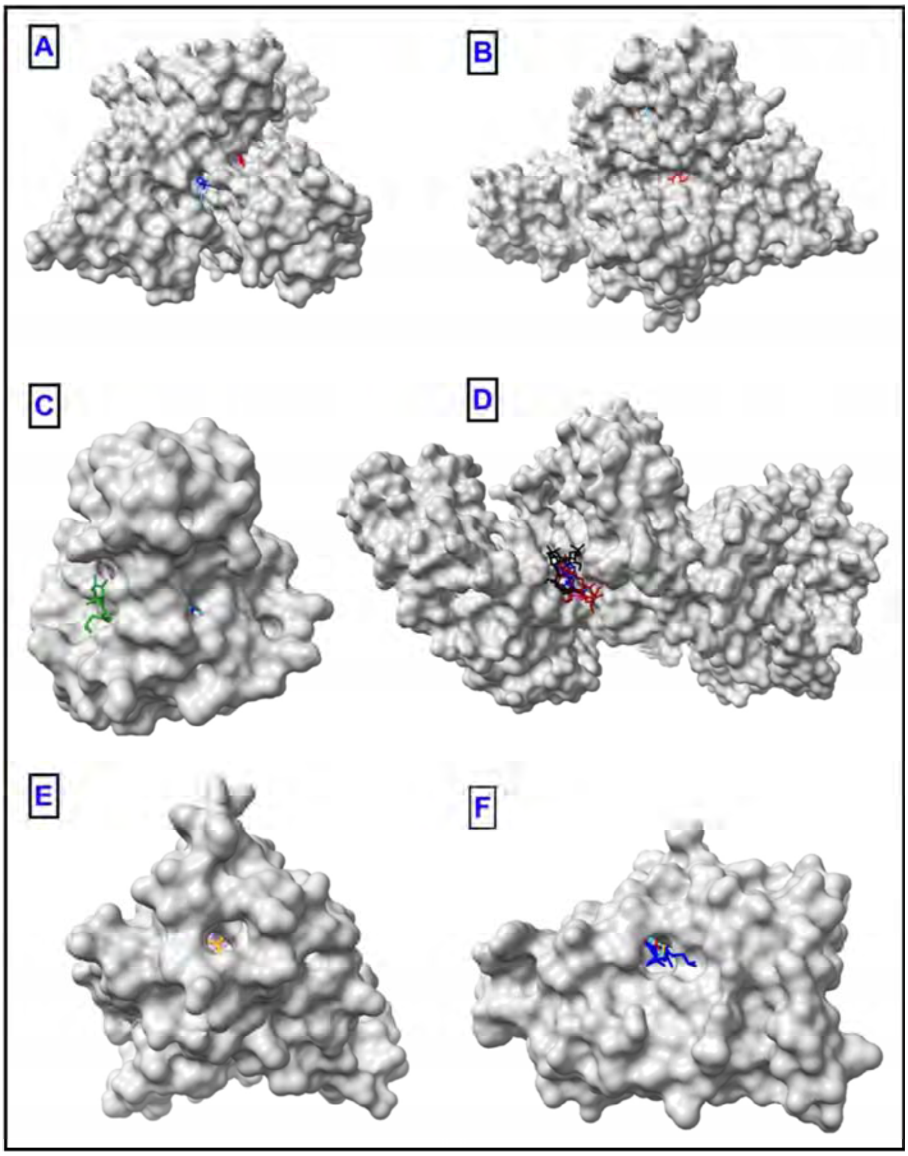

Fig. 5. Ligand Interaction surface view of breast cancer protein targets [(PDB ID: A & B - 5NGB (PI3K); C - 5TH6 (MMP-9), D - 4JT6 (mTOR) and E & F - 3OS8 (ERβ)], plant compounds and drugs are coloured as follows: CSL (coprostanol) - dark blue; DCP (2,3-dichloro-1-propene) - gold; EDL (estradiol) - orange; IDL (indole) - cyan; MCL (4-methylcatechol) - Red; MMS (marimastat) - forest green; PCL (pyrocatechol) - violet; RFL (ridaforolimus) - black; RMN (rapamycin) - dark red; TMN (toremifene) - rosy brown and ULB (umbralisib) - corn flower blue.

4.2. Antimicrobial assays

Plant extracts contain several compounds, which have potent antibacterial and antifungal activities. Plants contain secondary metabolites, which aid in the defence against potential pathogens [47]. Several classes of metabolites/phytochemicals have been reported to elicit antimicrobial activity- such as, essential oils, terpenoids, alkaloids, phenolics - flavonoids and polyphenols [48]. These substances may either act directly by binding to the biochemical targets inside the microbial cells, or may indirectly inhibit their growth (static activity), or even cause microbial cell death (microbicidal activity) [49]. These effects may be confirmed through docking (*in silico*) studies, which are underway to identify specific ligand-receptor interactions; however, *in vitro* (and *in vivo*) studies ought to be performed to back up *in silico* predictions. Amongst the bacterial strains studied (Table 1), the plant extract was efficacious against *Escherichia coli* (6 mm) and *Pseudomonas aeruginosa* (8 mm), but it did not show any effect (even at high concentrations of 10 mg/mL) against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Shigella flexneri* and *Salmonella typhimurium* at any of the concentrations studied (in the range of 6-10 mg/mL). The standard antibiotics were efficacious against the bacterial species at much lower (μg) amounts; for example, gentamicin had profound inhibitory action against almost all the bacterial species used in this work. As for the antifungal activity of the plant extract (Table 2), it was effective (14 mm) against *Echinodontium tinctorium*, a plant pathogen; however, the extract did not inhibit any other fungi cultured in this study which are clinically important human pathogens (*Candida tropicalis*, *Candida glabrata* and *Aspergillus fumigatus*) which are commonly encountered in clinical settings. Antimicrobial efficacy also largely depends on the rate of drug uptake and physicochemical properties of the compounds (eg., LogP and pKa). Hence, as purified compounds have not been used for antimicrobial assays in this study, it was impossible to check the rate of drug uptake. Since the plant extract was prepared with methanol, the effect of methanol on microbial growth was checked using a solvent control (methanol alone) and no detectable zone of inhibition was observed in the control wells.

ROS generation from biological redox systems has been repeatedly found to assist in bactericidal killing by bactericidal antibiotics and

Table 8
Molecular docking of *A. cymosa* leaf extract identified through GC-MS: Best energy compounds. The respective control drugs (shaded in red) were compared with plant compound.

Targets	Ligands	Binding energy	Ligand efficiency	inhib_constant (μM)
5NGB (PI3K)	Coprostanol	−11.38	−0.41	0.004
	Indole	−5.95	−0.66	43.37
	4-methylcatechol	−5.17	−0.57	162
	Pyrocatechol	−4.98	−0.62	224.22
	Umbralisib	*-4.46*	*-0.11*	*534.66*
	2,3-dichloro-1-propene	−3.84	−0.77	1530
5TH6 Chain A (MMP-9)	Indole	−6.41	−0.71	19.88
	4-methylcatechol	−6.19	−0.69	29.15
	Pyrocatechol	−5.62	−0.7	76.15
	2,3-dichloro-1-propene	−4.24	−0.85	781.82
	Marimastat	*-4.14*	*-0.18*	*920.32*
	Coprostanol	−3.47	−0.12	2850
4JT6 Chain A (mTOR)	Coprostanol	−8.66	−0.31	0.447
	Rapamycin	*-8.31*	*-0.13*	*805.9*
	Ridaforolimus	*-8.11*	*-0.12*	*1.14*
	Indole	−5.41	−0.6	107.74
	4-methylcatechol	−5.37	−0.6	115.96
	Pyrocatechol	−4.87	−0.61	268.83
	2,3-dichloro-1-propene	−3.6	−0.72	2300
3OS8 Chain A (ERβ)	Coprostanol	−10.67	−0.38	0.015
	Estradiol	*-9.08*	*-0.45*	*0.219*
	Toremifene	*-6.32*	*-0.22*	*23.32*
	Indole	−5.37	−0.6	116.21
	4-methylcatechol	−5.01	−0.56	213.14
	Pyrocatechol	−4.57	−0.57	447.26
	2,3-dichloro-1-propene	−3.52	−0.7	2650

therefore, ROS generation is a secondary mechanism which augments primary target binding of antibiotics [50]. Although most phytochemicals are known to possess antioxidant functions, some plant compounds may increase ROS production by interacting with the cellular components and thereby, exert antimicrobial effects by acting as pro-oxidants [51]. Bioactive principles in the plants bind and inhibit enzymes/proteins responsible for pathophysiological manifestations. Ample reports of gene expression changes (and epigenetic changes) when plant extracts/isolated active principles are added to cell cultures have been published. Reactive nitrogen species (RNS) such as nitric oxide (NO·), or peroxynitrite (OONO), the product of NO reaction with $O_2^{\cdot-}$, have been detected during nitrosative stress. These radicals are known to cause macromolecular damage and also initiate carcinogenesis, diabetes and other metabolic disorders [52]. Since oxidative stress arises due to excess free radical production, antioxidants have been proposed to act as agents which neutralize ROS and RNS [53].

4.3. Cytotoxicity of A. cymosa extract

Cancer remains a devastating disease which has increased in an unprecedented manner, leading to high mortality rates worldwide [54]. While the treatment of cancer depends on the type and stage of cancer, radiation and chemotherapeutic regimen (and chemoradiotherapy, a combination of both) are the usual treatment modalities for both early and later stages of several cancers [55]. Although monoclonal antibodies and other novel therapeutic regimes (alternative medicine) have been explored for cancer chemotherapy, radiation therapy still serves as an important avenue for treating several kinds of cancers [56]. Plant based compounds such as vinca alkaloids, etoposide and paclitaxel, have been used for cancer chemotherapy and the major classes of plant compounds which are known to possess anticancer activity are flavonoids, polyphenols and brassinosteroids [57]. Diverse plant compounds possessing anticancer activities (as well as their specific mechanisms of action) have been reported in literature [58]. In this study, the cytotoxic potential of the plant extract was evaluated by performing MTT assay (Fig. 2) and the IC_{50} value of the extract was found to be 100 μg/mL. Cancer cells are usually immortal (MCF-7 is an immortalized breast cancer cell line) and hence, cell death is dysregulated in cancer cells. A low IC_{50}value indicates the pro-apoptotic/cytotoxic effects of the plant extracts . The cytotoxic potential of the plants was confirmed by the formation of insoluble purple formozan by the metabolically active cells. Cell death led to lower product (formozan dye) formation, as evident from the fact that a low plant extract concentration (62.5 μg/mL) yielded 55.5% cell viability (~45% cell death). At a concentration of 1000 μg/mL, cell viability was only 21.29%. Nonetheless the extract caused

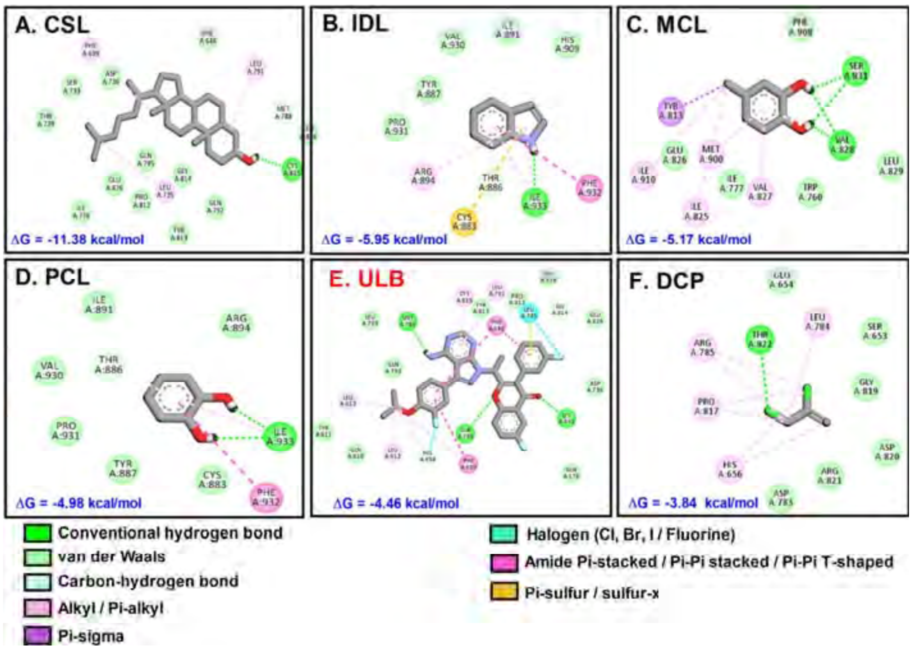

Fig. 6. Receptor-ligand interactions between PI3K (5NGB) and phytocompounds obtained from *A. cymosa*. Binding of pyrocatechol (PCL), indole (IDL), 4-methylcatechol (MCL) and coprostanol (CSL) was stabilized by hydrogen bonding of the ligands to PI3K. These compounds may serve as competitive inhibitors of PI3K by occupying the substrate (ATP) binding site. Other weaker interactions which may have stabilized the interactions between PI3K and the incoming ligands are demarcated by various colours, as given in the legend below the images. Interestingly, coprostanol exhibited a higher - ΔG than umbralisib, a control drug used for treating breast cancer.

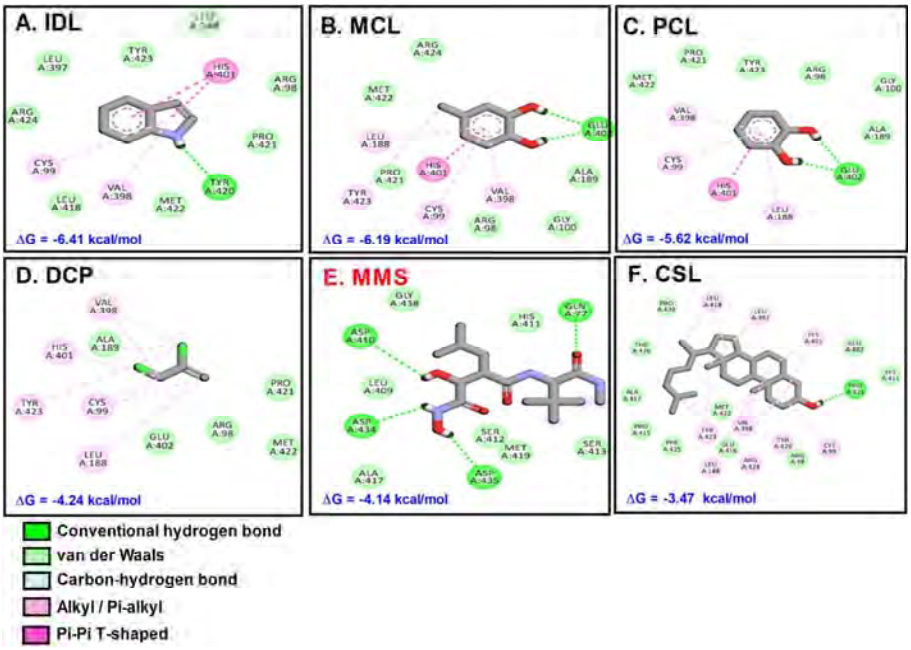

Fig. 7. Molecular docking of *A. cymosa* compounds to MMP-9. Indole (IDL) and 4-methylcatechol (MCL) were found to have superior - ΔG values for binding to MMP-9 active site and the forces involved in interaction between key active site amino acid residues and the docked ligands are displayed. Indole, 4-methylcatechol and pyrocatechol (PCL) binding involved H-bonding as well as other weak forces like van der Waals, C-H bonds, pi-alkyl/alkyl and pi-pi interactions. Marimastat (MMS), the control inhibitor drug possessed a slightly lower - ΔG energy.

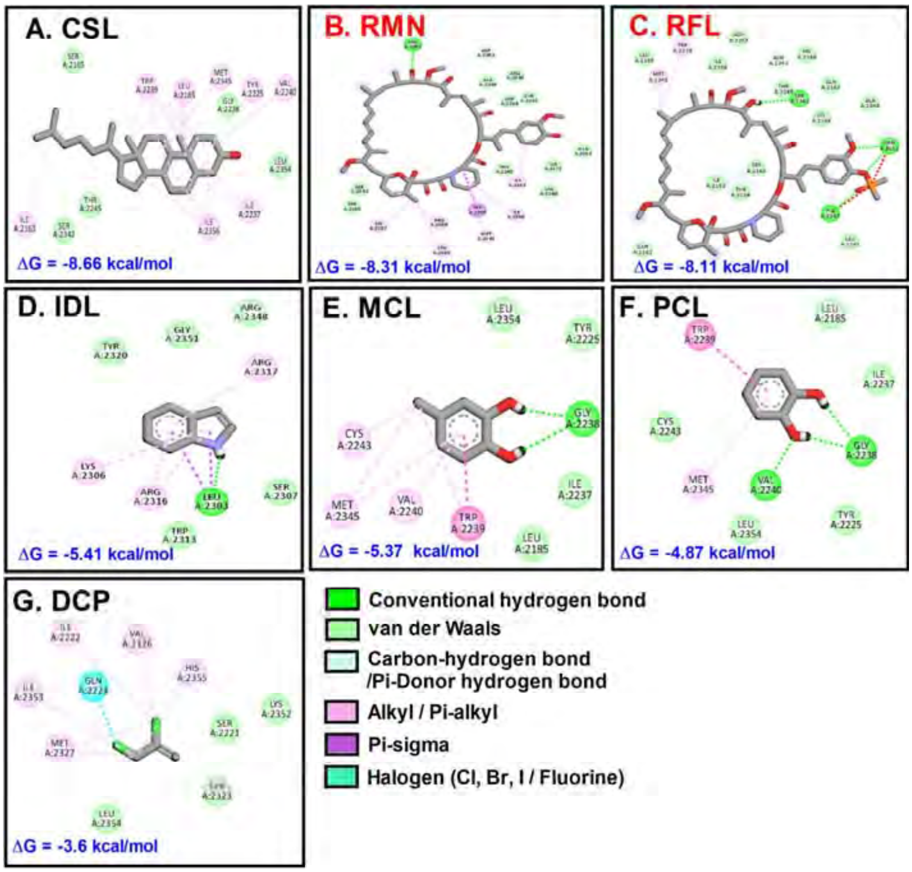

Fig. 8. Docking interactions between mTOR and *A. cymosa* phytochemicals: Except for coprostanol (CSL), all other phytochemicals from *A. cymosa* appeared to have weak binding to mTOR. The control ligands of mTOR, rapamycin (RMN) and ridaforolimus (RFL), had much superior - ΔG values and correspondingly, yielded low Ki values. The numerous weak interactions as well as H-bond in coprostanol may be the reason for the very energetically favourable interaction between coprostanol and mTOR.

12.5% cytotoxicity (cell viability of 87.5%) against Vero cells (Fig. S3 B) at the same concentration (62.5 μg/mL). Thus, the cytotoxicity in MCF7 cells was 40% higher than in Vero cells. The toxicity obtained for Vero cells could be further minimized by identifying and purifiying the active principle responsible for this cytotoxic effect. These results proved that the *A. cymosa* methanolic leaf extract was more cytotoxic to the MCF-7

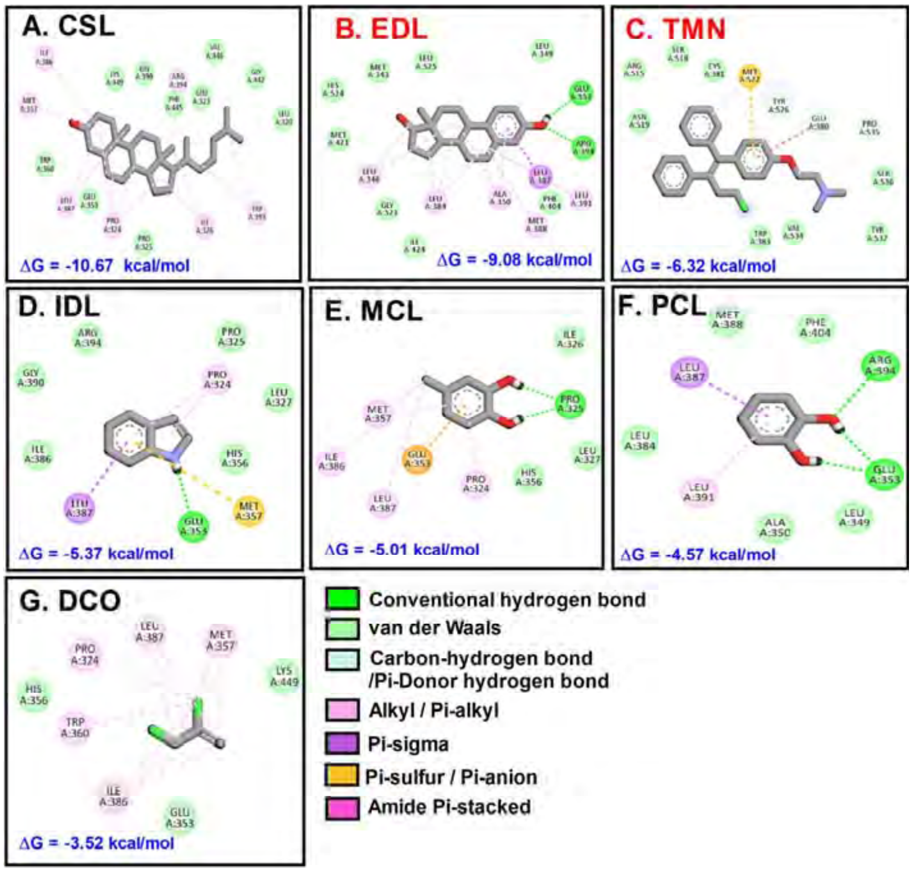

Fig. 9. Docking interactions between ERβ and *A. cymosa* compounds: Binding of small molecules to ER may interfere with ER transactivation and aid in decreasing breast cancer growth and metastasis, especially in ER + breast cancer cells. When compared to estradiol (EDL), the natural ligand of ER, all other molecules except for coprostanol (CSL) showed much poorer Gibbs free energy values. The intermolecular and weak forces which stabilized the receptor-ligand interactions are shown.

cells when compared to the control (Vero cells).

For a very effective cytotoxic activity, much higher concentrations are required [59]. However, the present results correspond to the crude extract, and hence, further research explorations are needed to evaluate the biological activities of the identified bioactive principles. GC-MS analysis aids in detecting volatile compounds present in plant extracts [60]. Further in-depth analysis of the effects of purified phytochemicals and cross-comparisons of their effects vis-a-vis the activities of known anticancer compounds will help in unravelling their mechanistic mode (s) of action [61].

4.4. Anti-inflammatory activity

The ability of the plant extract to prevent RBC lysis is attributed to its potent anti-inflammatory activity (Fig. 3). When compared against aspirin control (which yielded 43-90% inhibition), the plant extract exhibited considerable anti-inflammatory activity in the range of 22-65% (both aspirin and plant extract sample were employed in the range of 100-800 μg/mL). Inflammation corresponds to the infiltration of circulating WBCs at the site of infection, leading to oedema and erythema [62]. Most of the anti-inflammatory plant compounds like phenolics and phytochemicals have the ability to lower inflammation by exerting antioxidant action [63]. These compounds are important for the alleviation of rheumatic diseases (and other chronic inflammatory diseases).

4.5. Phytochemical analysis of A. cymosa methanolic leaf extract

Phytochemical qualitative analysis (screening) showed the presence of various phytochemicals based on the extraction solvent. In the methanolic extract, triterpenoids, sugars, flavonoids, catechins, saponins, tannins and amino acids were present (Table 3). While the methanolic and ethanolic extracts revealed the presence of the most diverse classes of phytochemicals, the other solvents (especially, the more nonpolar ones) were able to extract only fewer classes of phytochemicals. Quantitative analyses were performed to identify the concentrations of chlorophylls, carotenoids, sugars, protein, lipids, free amino acids, phenolics and tannins. Substantial amounts of phenolics (97 mg/g of leaf powder) and flavonoids (40 mg/g) were found and the other phytochemical classes - sugars (43 mg/g), tannins (30 mg/g) and lipids (9 mg/g), were not very abundant (Table 4). GC-MS analysis of the methanolic extract was performed since it contained diverse classes of phytochemicals. The GC-MS spectrum (Fig. S4) revealed the presence of over 44 compounds. The peaks were integrated and the area under the curve (AUC) was calculated (in %) to find the overall abundance of the phytochemicals (see Table 5). After peak identification using a compound library, each of the compounds was searched for earlier reports of bioactivity using Google Scholar as well as PubChem. The uses of the compounds (identified from literature survey) are mentioned in Table 5. For compounds with no significant literature reports, the description has been given as “no significant report”. These may be novel compounds and bioinformatics explorations may pave the way to identify their potential biochemical targets. These compounds can be isolated and further *in vitro* assays will lead to the discovery of their mechanisms of action as well as their specific macromolecular targets. Big data analytics for specific protein interactions will be more advantageous in deciphering the pivotal roles of the plants and molecular dissection of the same can lead to formulation of applications to take plant-based breakthroughs from the bench to the bedside.

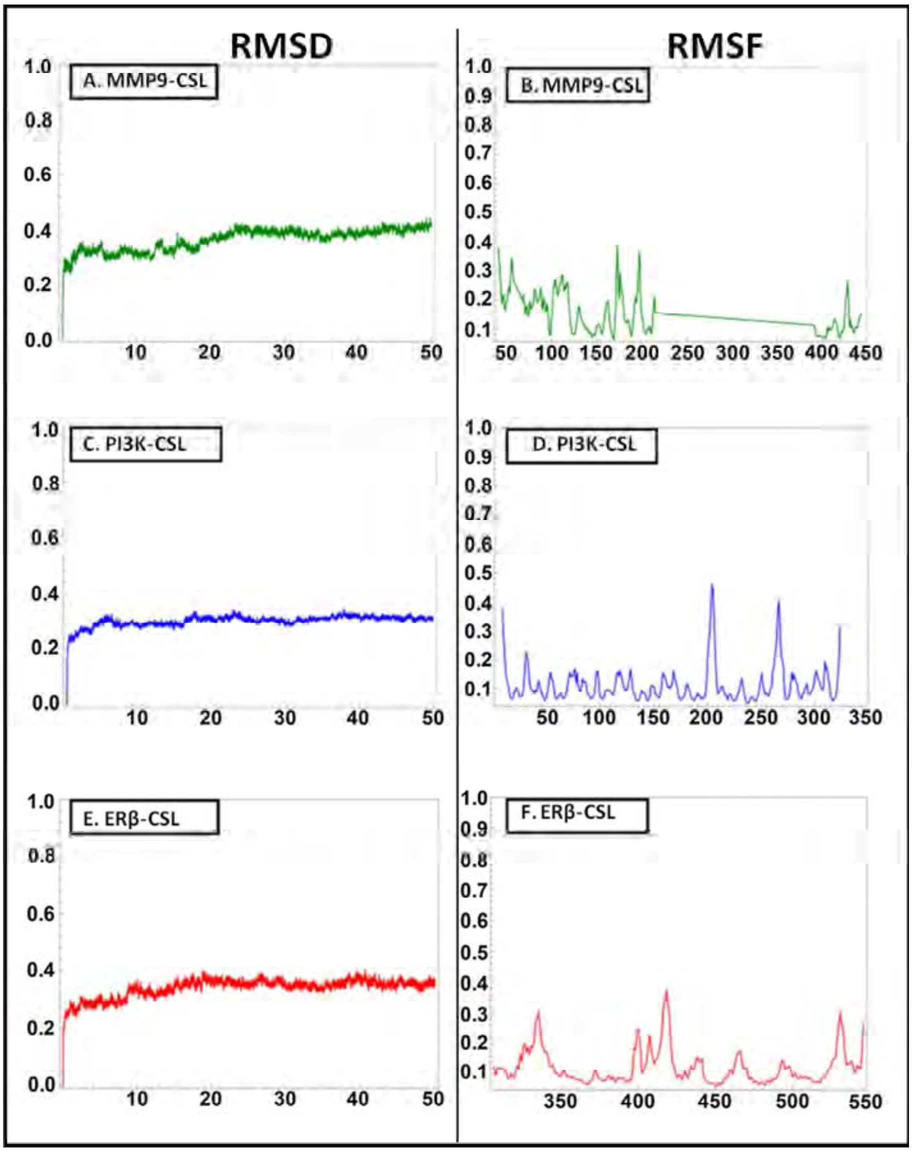

Fig. 10. MD simulations of A & B - MMP-9; C & D - mTOR and E & F - ER with coprostanol, a common and promising ligand from *A. cymosa*. The MD simulations were performed for 50 ns and the RMSD (panels A, C, E) and RMSF (panels B, D and F) depict the real-time simulation and stability of receptor (PI3K/mTOR/ER)-ligand (coprostanol) interactions. The RMSD plots show that the protein-ligand complexes were stable for almost the entire period of the simulation and the RMSF plots show that among residues of the entire protein analyzed, only a few notable residues had high fluctuations. Importantly, these fluctuations were not very high and it is understandable that a few amino acid side chains would experience a change in movement during the receptor-ligand interactions. Hence, these results indicate a very high stability of the ligand interaction with the respective receptor protein docking sites over a period of 50 ns.

4.6. Bioinformatics prospecting of biological activities of *A. cymosa* phytochemicals

4.6.1. Molinspiration study

The biochemical/biological activities of therapeutic drugs mainly depend on structure of the compounds and their physicochemical properties. Molinspiration is a convenient online tool that is widely used for prediction of drug-likeness and bioactivity of molecules such as phytochemical secondary metabolites and synthetic organics. This tool measures the $_{mi}$logP value (Octanol-water partition coefficient logP) and TPSA (Topological polar surface area) values of the compounds using Bayesian statistics (see Table 6). Among the major drugs known to man and in clinical use, the key classes of drugs target these protein receptor classes: G protein-coupled receptors (GPCR), ion channels (eg. Na^+/K^+, Cl- etc.), kinases, nuclear hormone receptors (eg. vitamin D receptor, steroid and xenobiotic receptor, aryl hydrocarbon receptor etc.), proteases, and other critical enzymes. Moreover, prediction of *in silico* activity of the compounds identified through GC-MS yields valuable information for commencing experimental studies, as the type of target which the drug/test molecule could exhibit the greatest efficacy/activity can be predicted. In this context, among the five phytochemicals that were chosen for *in silico* studies, indole and coprostanol were the most potent inhibitors, since they possessed lower negative or positive values for the different receptor types (see Table 7).

4.6.2. ADMET-SAR

The absorption (A) analysis predicts the Caco-2 cellular permeability of the study compounds. The results in Table S6 show that all the selected *A. cymosa* compounds possessed phytochemical compounds with potential for binding to plasma proteins. Usually, P-glycoprotein binders facilitate the excretion of chemotherapeutic chemicals or phytocompounds from the cells through drug efflux mechanism and increase drug resistance. The results revealed that the phytochemicals were neither substrates nor inhibitors of P-glycoprotein (only coprostanol was a P-glycoprotein substrate). Coming to drug distrubution (D) analysis, coprostanol and indole were predicted to cross the blood-brain barrier (BBB). In the analysis of liver microsomal metabolism, the 2 key cytochrome P450s (CYP450s) which are involved in drug metabolism are CYP2D6 and CYP3A4; these two enzymes together perform the majority of the liver microsomal phase-I drug metabolism. The results showed that *the A. cymosa* compounds were non-inhibitors of CYP2D6; CSL alone was substrate for CYP2D6 and CYP3A4. This data suggested that these phytochemical compounds would undergo metabolism in liver microsomes. The protein transporters, OATP1B1 and OATP1B3 are expressed on sinusoidal-membrane of hepatocytes and interaction of the phytocompounds with these transporters as either substrates or inhibitors would lead to drug resistance and clinical drug-drug reactions.

4.6.3. Network analysis

Data obtained from NCBI-GEO database (breast cancer) led to identification of the differentially expressed genes (DEGs). The PPI interaction network shows the interactions between the various proteins (Fig. 4). Among the top 60 hits (in terms of greater expression) in breast cancer (GEO database; GSE 15852; GSE2290 and GSE1081180), MMP9, ESR (ER) and mTOR have significantly greater importance. PIK3CA is within the 70 top DEGs too. Moreover, in literature, these proteins and their inhibitors have been pursued as oncotargets. Based on the top 60 hits, this PPI network was constructed. Hence, clearly, genes like P1K3CA, MMP9, mTOR and ER are involved in the molecular pathophysiology of breast cancer. A shorter path between the proteins shown in the network indicates higher between-ness centrality values. These four proteins were selected for further *in silico* analyses (Fig. 5).

4.7. In silico affirmation of the bioactive potentials of A. cymosa phytochemical constituents

Computational studies for protein-ligand and protein-protein interactions have been pursued for deciphering bioactive potentials of phytochemical compounds. After phytochemical screening, bioactivity assessments through multidisciplinary *in vitro* and *in silico* studies have yielded prospective drugs for several human ailments, ranging from snake bites to COVID-19 [64–66]. An extensive review illustrating the phytochemical potentials as corroborated by *in silico* assessment and application perspectives have shown the prominent significance of computational studies [67].

Molecular docking between protein-ligand interactions revealed the binding order to be in the order of, 5NGB (PI3K)> 5TH6 (MMP-9)> 4JT6ChainA (mTOR)> 3OS8ChainA (ERβ). The 44 phytochemical constituents were docked to the target proteins along with the respective control drugs in comparison with plant compounds. Table 8 enlists the appropriate compounds and respective binding profile variations; DCP-2,3-dichloro-1-Propene; MCL- 4-Methylcatechol; CSL - Coprostanol; IDL - Indole; PCL - Pyrocatechol; MMS - Marimastat; RFL - Ridaforolimus; RMN - Rapamycin; TMN - Toremifene; EDL - Estradiol; ULB -Umbralisib (Figs. 6–9). Tables S2-S5 depict the necessary types of interaction and the key interacting amino acid residues. The bevy of weak intermolecular forces involved in stabilization of the *A. cymosa* compounds at the active site of the breast cancer proteins (or alternatively, at other sites) reveals that at instances, these compounds docked better with the cancer target proteins when compared to the standard chemotherapeutic anticancer compounds. Network pharmacology assessment for the apt involvement of the 44 components in eliciting the interaction and target prediction with validation will advance our knowledge of phytomedicine. The present preliminary report aids in providing a solid platform for further explorations using *A. cymosa* in phytomedicinal studies. The MD simulations show that the root-mean square deviation (RMSD) values of the selected receptor (PI3K, MMP9 and ER)-ligand (coprostanol) combinations were stable throughout the study. The very minimal RMSD changes after attainment of a stable complex (as witnessed in the formation of a plateau after the initiation of the MD runs) show the stable interaction of coprostanol in the active site of the receptor targets. Root-mean square fluctuations (RMSF) values were also negligible in the studied amino acid regions encompassing the active site and these data show that the interaction of the compound with the target proteins did not cause massive perturbations in the RMSF values. RMSD, RMSF and hydrogen bonding patterns are structurally illustrated in Fig. 10. The cohesive patterns and the trajectories clearly depict that the selected receptor-drug combinations displayed stable interactions over a protracted period of 50 nanoseconds.

4.8. Discussion on phytochemicals explored in the work and their biological activities

Coprostanol is a metabolite of cholesterol and is a phytosterol molecule. It is often used as an indicator of fecal metabolism of cholesterol in human waste/sewage and is prominently used to gauge the activity of gut microbiomes. 4-methylcatechol is a phenolic compound (polyphenol) [68]. Pyrocatechol is a benzenediol (a diphenol) and is also a phenolic compound [69]. Indole is a product of tryptophan metabolism; it is a natural compound in plants and has plant-protective roles as it attracts insect pollinators and repels herbivores [70]. 2, 3-dichloro-1-propene is an impurity that was present in the plant. It is not a phytochemical, but is an organochlorine compound which may have gone into the soil due to pesticide/insecticide use. Moreover, this compound is very toxic in humans because it undergoes P450-mediated reaction to form 1,2-dichloroacetone, a potent carcinogenic compound. It is not clear how this impurity found its way into the soil of the Siriya Kalvarayan hills.

Phenolic compounds have long been known to inhibit oxidative stress, which is widely recognized as a prominent hallmark of several pathological conditions like cancer [71]. For example, 4-methylcatechol was found to exert cytotoxicity *in vitro* in murine tumour cells through induction of apoptotic cell death through “extracellular pro-oxidant action” [72]. A very comprehensive and interesting work showed that 4-methylcatechol selectively exhibited cytotoxicity against malignant melanoma cells, but spared normal melanocytes by inhibiting Akt-mediated cancer cell survival [73]. Moreover, 4-methycatechol also caused accumulation of melanoma cells in G_2/M phase (cell cycle arrest) and induced apoptosis through the intrinsic mitochondrial pathway by enhancing ROS production via its prooxidant action [73]. Pyrocatechol is a product that is formed from chlorogenic acid when coffee beans are roasted and this component of coffee was found to be a potent anti-inflammatory compound, as evidenced by its ability to inhibit NFκB in RAW264.7 cells [74]. Pyrocatechol also possesses considerable antioxidant activity [75]. This compound also activates Nrf2, which suppresses oxidative stress [76]. However, no direct evidence is shown for the ability of pyrocatechol to inhibit cancer. Hence, we plan to study the effect of pyrocatechol in normal and cancer cell lines. Coprostanol is a phytoestrogen and many studies indicate that phytoestrogens regulate cholesterol levels and also serve as anticancer agents [77]. Phytoestrogens inhibit human breast cancer cells [78]. Hence, the observed cytotoxic, antioxidant and anti-inflammatory actions of *A. cymosa* in this work can be presumed (based on the earlier findings from literature) to be mediated by biomolecules such as coprostanol, pyrocatechol and 4-methylcatechol (and possibly, synergistic effects of fatty acids and other phytochemicals) present in the extract.

5. Conclusion

Based on the findings, it could be concluded that the methanolic leaf extract of *A. cymosa* exhibited a high antioxidant activity against DPPH as well as ABTS free radicals and also had a significantly high potential to reduce Fe^{3+} ions. Moreover, the plant extract demonstrated modest anti-inflammatory activity. The phytochemical constitution of the plant was analyzed using preliminary screening protocols and the plant extract possessed phenolics, sugars, catechins, tannins, flavonoids and saponins. Using the quantitative analytical methods, the plant extract was found to contain significant amounts of phenolics and flavonoids; these compounds are attributed for the antioxidant, antimicrobial, anti-inflammatory as well as cytotoxic activities of the plant extract. *In silico* assessment based on molecular docking and molecular dynamics simulation experiments reveal that coprostanol, 4-methylcatechol, pyrocatechol and indole serve as PI3K/ MMP-9/ mTOR/ ERβ inhibitors. These compounds could modulate breast cancer signalling pathways. Out of the 44 phytochemical constituents identified herein, coprostanol showed better binding affinity patterns, compared to the standard control drugs.

As mentioned earlier, this account might be the first ever comprehensive report on the phytochemical profile and biological activity spectrum of *A. cymosa*. Future studies would involve bioavailability and assimilatory studies that will promote the utility of these compounds as either leads or scaffolds for computer-aided drug design and confirm the traditional applications of *A. cymosa*. A similar study showed that medicagol, faradiol, and flavanthrin showed potent inhibition against SARS-CoV 2 proteases by interacting with His41, Cys45, Met165, Met49, Gln189, Thr24, and Thr190 [79]. The present study corresponds to differential binding with signalling cascade proteins in the present study encompassing PI3K/ MMP-9/ mTOR/ ERβ pathways, thereby affirming the anti-inflammatory and antioxidant properties of *A. cymosa* phytochemicals. Further, computational validations of the structure activity relationship, pharmacophore modelling and simulation modelling for virtual screening vs. phytochemical catalogued compounds will provide conformational data for experimental validation. Hence, the present preliminary assessment summarizes the prolific benefits of the traditional medicinal plant's antimicrobial, antioxidant, anti-inflammatory properties and cytotoxicity profiles through a combination of *in vitro* and *in silico* methodologies.

6. Author contributions

Pushparaj Annadurai - Conceptualization, methodology, writing- original draft
Daniel A. Gideon – Data curation, writing- original draft
Vijay Nirusimhan - Investigation, writing- original draft
Ramachandran Sivaramakrishnan - Writing - review & editing
Kandavel Dhandayuthapani - Supervision, Writing - review & editing
Arivalagan **Pugazhendhi -** Supervision, Writing - review & editing

Funding

No funding was obtained for this study.

Declaration of Competing Interest

The authors report no conflicts of interest in this work

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.procbio.2022.01.024.

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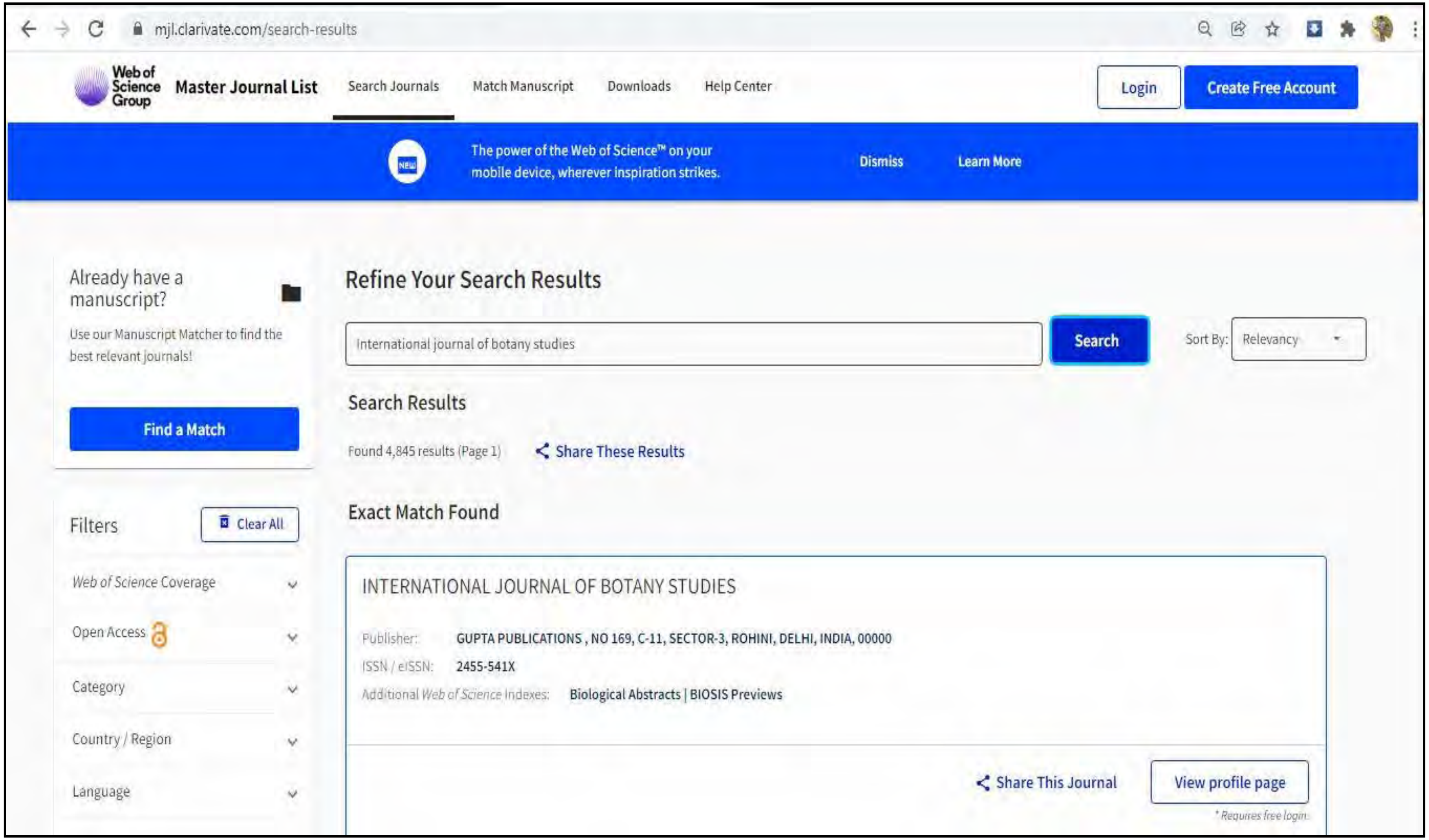
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International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 16-09-2021, Accepted: 30-09-2021, Published: 16-10-2021
Volume 6, Issue 5, 2021, Page No. 1243-1260

Indigenous uses of ethnomedicinal plants among Malayali tribals in siriya Kalvarayan Hills, Eastern Ghats, India

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Abstract

The Eastern Ghats of India are rich in biodiversity and contain several flora and fauna. We surveyed the Siriya Kalvarayan hills from Puliyankottai to Serapattu. The study area is ~20 km lengthwise and 10 km breadth wise and is located~55 kilometers away from Kallakurichi in Villupuram district. We identified nearly 90 genera containing close to 100 species, which belong to over 46 families. The Periya (large) and Siriya (small) Kalvarayan hills form an integral part of the Eastern Ghats of India with an elevation of 2500-4000 feet above sea level. These hills contain several angiosperms, gymnosperms, shrubs as well as trees, which have long been considered to have tremendous potential for use in ethnomedicine, as per traditional ethnobotanical literature. We document the ethnobotanical uses and phytochemical constitution of some novel, and as yet, little explored plant species (totally 61/100 surveyed) and discuss the potential uses of these plants in treating a range of health issues. We surveyed the local population (mostly Malayalis) through a questionnaire-based interview to assess their knowledge of traditional medicine. Also, we checked the dependence of residents of 8 villages upon different forms of traditional medicine (Ayurveda, Siddha and Naturopathy). When plant photographs were shown to the respondents, they identified them and shared the local names by which those plants are called. Also, we identified the extent of inclination/dependence of the respondents towards allopathic medicine for treating a range of ailments. The specific plant parts and the species used by the tribals were identified through a survey to assess the ethnobotanical awareness of the Malayali people. Quantitative indices such as Information Consent Factor (ICF), Fidelity Level (FL) as well as Use Value (UV) were also measured. Our study led us to identify 10 species which are not listed in the National Medicinal Plants Board website. Also, we came across a few plant species which are either endangered or threatened, as per the IUCN red list. We wish to study the secondary metabolites of all the plants for which, little or no published literature exists.

Keywords: siriya Kalvarayan hills, ethnobotany, tribal medicine, rare plants and malayalis

Introduction

Ethnic medicine has been in use since time immemorial, and has historically been utilized by village shamans for treating a wide array of human diseases, ranging from the common cold to serious illnesses such as cancer [1]. Custodians of these ancient secrets are adept at the art of identification and utilization of either the whole plant or specific parts thereof for various medicinal purposes [2]. Extracts (which would contain particular active principles) are usually prepared in a specified manner and these crude medicinal formulations are administered in the form of churna (powder), water-based concoctions and raw concentrates produced by squeezing plant parts using mortar and pestle. Traditionally, this knowledge (of the plant parts used and the specific methodologies of preparation and mode of administration) has been passed on from one generation to the next [3]. Shrubs, herbs and trees and their constituent active principles (from different plant parts thereof) have been extracted using a range of methods that are common in ayurvedic, siddha and unani traditions of Indian ethnomedicine. The government of India has integrated the alternative medicine fronts and created the ministry of AYUSH (Ayurveda, Unani, Siddha and Homeopathy) [4]. The market value for traditional medicine was estimated to be 6 billion dollars at the end of the year 2018 [5].

The basis of traditional ayurvedic medicine is to balance the body humors to treat vatha, pitta and kapha, which are three distinct prakriti types [6]. Siddha is another form of traditional medicine which is practised in Tamil Nadu. It involves certain aphorisms which were advocated by 18 Siddha Rishis of ancient Tamil culture and comprises of an array of techniques such as mind concentration, mastery over the senses, meditation, exercise, controlled breathing (yoga) and other medicinal formulations which together combined, bring about the restoration of psychosomatic harmony [7]. Naturopathy also is practiced by some members of the community in the study area. This form of medicine integrates traditional medicine with scientific advancements/research by which, the practitioner recognizes the innate healing capacity, identifies the patient's underlying cause(s) of disease and corrects the identified maladies using a range of treatment modalities such as herbal medicine, minor surgery, hydropathy, exercise, counseling and dietary-lifestyle changes [8].

Allopathic medicinal formulations typically rely on the use of extracts obtained from medicinal plants, which are purified from natural sources or synthesized *in vitro*. These chemicals specifically target receptors/biomolecules (usually enzymes/proteins) in the human body. An estimated 50% of compounds used in allopathic medicine are derived from plant sources [9].

The Siriya Kalvarayan hills of the Eastern Ghats are home to several (as many as 61) rare plants (identified in this survey alone and not an exhaustive number), for which, very little or no published material was found (Table 1). This paper would act as a catalyst in the search for potential compounds of therapeutic value and also to accelerate the quest to find lead compounds for treating specific illnesses, thus accentuating the value of India's rich biodiversity. Also, advancements in knowledge pertaining to the mechanisms of isolated compounds' action would validate our ancestors' knowledge and reveal exactly which compounds act as the active principles responsible for alleviation of specific conditions.

Materials and methods

Survey region

The present florestic studies were carried out in the Siriya Kalvarayan hills, Eastern Ghats, Kallakurichi district (just months ago, the study area belonged to Villupuram district). The location of the study area is shown in Fig. 1. The survey was conducted between January 2017 and November 2019. We used a questionnaire to conduct a survey among Malayali (meaning in Tamil - mountain dwellers) tribals and other residents (total number of respondents = 105) of 8 villages in the study area – specifically, Sirukkalur, Edapattu, Vazhakuli, Athikkuzhi, Vanjikkuli, Moolakkadu, Aanaimaduvu, and Puliyankottai (in order of high-low altitude).

Study area

The Siriya Kalvarayan hills start with a road beginning at Moolakkadu, which winds up to Serappattu, which is roughly 800 m above sea level. To reach this point, the road consists of five large hair-pin bends winding through the terrain (a map of the study area is given in Fig. 1). While this survey focuses on specific plants that have been identified in the region, it is not exhaustive. Whenever possible and when different species of flora were located, photographs were captured using a high-resolution phone camera (13 MP, Xiaomi Mi4i). The images were collated using Microsoft Office PowerPoint and refined using GIMP (ver. 2.10.6). The graphs were plotted using GraphPad Prism (v.5.02).

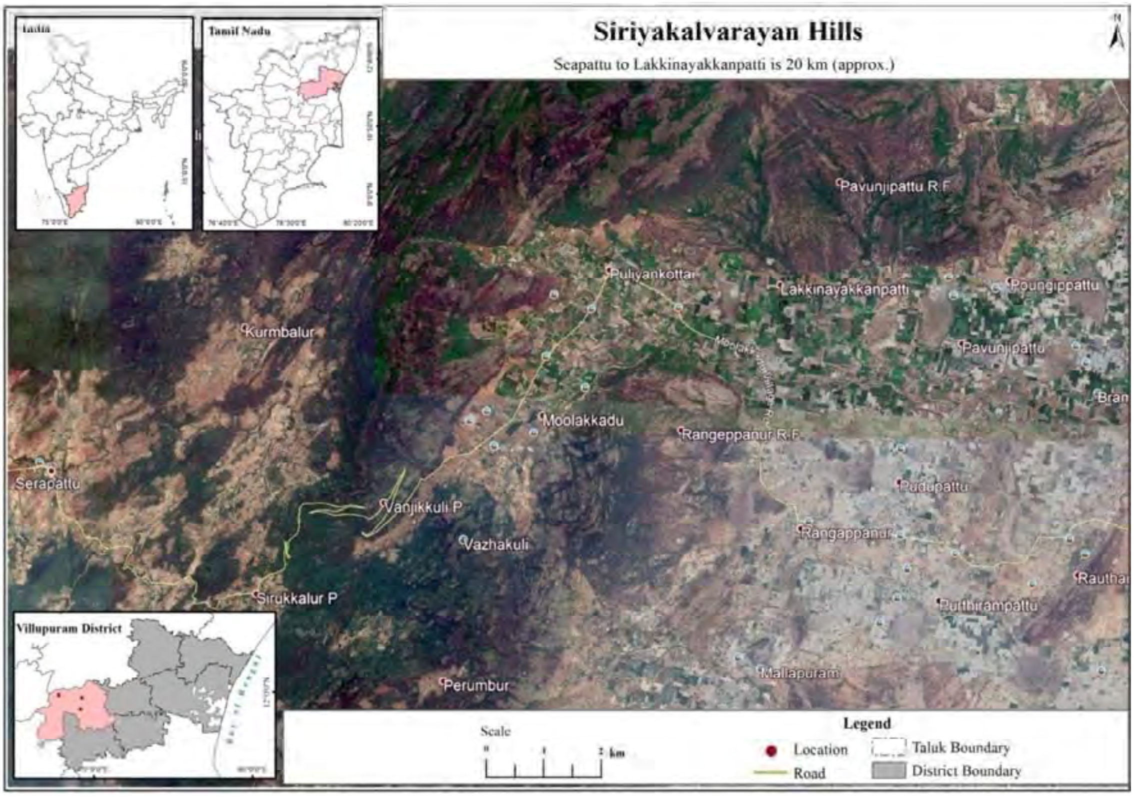

Fig 1: A close-up map of the study area – The study region from Serappattu to Puliyankottai is shown in this map, covering a distance of ~20 km lengthwise and a breadth of 10 km. The study area is indicated on the map by the dotted yellow oval marking.

Photography and botanical identification

The plants were photographed using a digital camera and the taxonomic identification of these species was carried out at the Rapinat Herbarium, St. Joseph's College, Tiruchirappalli. The photographs of the surveyed species have been arranged in alphabetical order and presented in Fig. 4-8 and the total number of plants surveyed is 100 species (Table 1). The binomial names of these plants were verified by cross-checking the entire list with the names given on the plant list website (http://www.theplantlist.org). Information pertaining to the plant species which were identified by most of the people (and which they used regularly for specific ailments), the local names of the plants as well as the plant parts used by the Malayali tribals are given in Table 2. After this, the species endemic to the region and the IUCN classification of the plants was also scrutinized to identify any critically endangered or threatened species. This information is provided in Table 4.

Ethnobotanical survey

A total of 105 subjects from eight villages (as mentioned before) which lie within the study area shown in the map (marked by a yellow dotted line, Fig. 1) Sirukkalur, Edapattu, Vazhakuli, Athikkuzhi, Vanjikkuli, Moolakkadu, Aanaimaduvu, and Puliyankottai. A questionnaire comprising of 7 critical questions was used to interview the residents (informants). Using a non-probabilistic method of sampling [10], the residents of these villages who were willing to talk were interviewed. We explained the aim of the study to the residents and showed them all the photographs of the plants that had been surveyed. While some of the traditional uses of nearly 40% of the plants are known (from citations/references to those particular plants in ancient medicinal literature such as the Charaka Samhita) as well as the National Medicinal Plants Board (Government of India) website, information pertaining to the medicinal uses of the remaining 60% of the plants is either obscure or completely absent both in ancient texts and in published scientific works (when searching for publications/reports using Google Scholar). Hence, locals were asked for the medicinal uses of each of the plants, and in particular, about those plants whose medicinal properties were hitherto unknown to us. A few medicine men (shamans) of these villages who expressed willingness to share their knowledge with us were also interviewed. Photographs of the plants (Fig. 4-8) were shown to them and they were interviewed regarding the plants (and the parts thereof), their traditional medicinal uses and if applicable, ritualistic/other uses. Based on the survey findings, it is realized that the residents had an appreciable (if not thorough) extent of knowledge regarding most of the plants and their medicinal uses. Based on the information that was gained, the species' names and their traditional uses were presented in Table 2. The distribution of the flora (herbs, shrubs, climbers, lianas, succulents and trees) are presented in table S1 and in Fig. 9. The demographics of the local population in 8 villages of the study area (age, sex and the dependence level of these residents on natural medicine) are given in Fig. 2. Also, it is indicated which of the plants have been used in ancient Indian as well as Tamil Nadu folk medicine (Supporting information table S1). Particular attention is also given to the specific uses of these plants by the local population. Demographics such as age, sex and their dependence on allopathic medicine is presented in Fig. 2. The percentage of subjects (total n=105) who use traditional medicinal formulations (ayurveda & siddha) and other streams of medicine such as allopathy and naturopathy to cater to their healthcare needs is given in Fig. 3. The informants' responses were recorded through a questionnaire-based inquiry approach and the responses were tabulated and counted. Using the numbers obtained through the survey, the following were calculated:

Table 1: Families, genera and species identified in the study The names of various families, genera, species and the plant types (based on morphology) are presented in the table below

S. No.	Name of the family	No. of genus/genera	No. of species	Herb	Shrub	Climber	Lianas	Succulents	Tree	Common plants	Rare plants	Total no. of plants
1	Acanthaceae	3	4	4	-	-	-	-	-	4	-	4
2	Apocynaceae	5	5	-	1	3	-	1	-	1`	4	5
3	Aponogetonaceae	1	1	1	-	-	-	-	-	-	1	1
4	Amaryllidaceae	1	1	1	-	-	-	-	-	-	1	1
5	Anacardiaceae	2	3	-	-	-	-	-	3	1	2	3
6	Arecaceae	1	1	-	-	-	-	-	1	1	-	1
7	Asclepiadaceae	1	1	-	-	1	-	-	-	-	1	1
8	Asparagaceae	1	1	-	-	-	-	1	-	1	-	1
9	Asteraceae	2	2	2	-	-	-	-	-	1	1	2
10	Boraginaceae	1	1	-	-	-	-	-	1	-	1	1
11	Burseraceae	1	1	-	-	-	-	-	1	-	1	1
12	Caesalpiniaceae	2	3	2	-	-	-	-	1	2	1	3
13	Capparaceae	1	1	-	-	-	-	-	1	-	1	1
14	Celastraceae	2	2	-	-	2	-	-	-	-	2	2
15	Colchicaceae	1	1	1	-	-	-	-	-	1	-	1
16	Comaceae	1	1	-	-	-	-	-	1	-	1	1
17	Combretaceae	2	5	-	-	-	-	-	5	1	4	5
18	Convolvulaceae	1	1	1	-	-	-	-	-	1	-	1
19	Dioscoreaceae	1	1	1	-	-	-	-	-	-	1	1
20	Euphorbiaceae	4	5	3	1	-	-	-	1	2	3	5
21	Fabaceae	6	6	-	-	1	1	-	5	4	3	7
22	Hernandiaceae	1	1	-	-	-	-	-	1	-	1	1
23	Lamiaceae	4	4	2	1	-	-	-	1	2	2	4
24	Linaceae	1	1	-	-	-	1	-	-	-	1	1
25	Malvaceae	4	4	1	1	-	-	-	2	1	3	4
26	Meliaceae	4	4	-	1			-	3	2	2	4
27	Melastomataceae	1	1	-	1	-	-	-	-	-	1	1
28	Moraceae	1	1	-	-	-	1	-	-	-	1	1
29	Olacaceae	1	1	-	1	-	-	-	-	-	1	1
30	Oleaceae	2	3	-	1	2	-	-	-	-	3	3
31	Opiliaceae	1	1	-	-	1	-	-	-	-	1	1
32	Phyllanthaceae	2	2	-	1	-	-	-	1	-	2	2
33	Poaceae	4	4	4	-	-	-	-	-	3	1	4
34	Proteaceae	1	1	-	-	-	-	-	1	-	1	1
35	Pteridaceae	1	1	1	-	-	-	-	-	-	1	1
36	Rhamnaceae	2	3	-	-	1	-	-	2	1	2	3

37	Rubiaceae	7	7	1	2	-	-	-	4	4	3	7
38	Rutaceae	2	2	-	1	-	-	-	1	1	1	2
39	Salicaceae	1	1	-	-	-	-	-	1	-	1	1
40	Salvadoraceae	1	1	-	1	-	-	-	-	-	1	1
41	Sapindaceae	4	4	1	1	1	-	-	1	2	2	4
42	Symplocaceae	1	1	-	-	-	-	-	1	-	1	1
43	Tiliaceae	1	1	-	-	-	-	-	1	1	-	1
44	Verbinaceae	1	1	-	1	-	-	-	-	1	-	1
45	Violaceae	1	1	1	-	-	-	-	-	1	-	1
46	Vitaceae	1	1	1	-	-	-	-	-	-	1	1
No.	46	90	99	28	15	12	3	2	40	39	61	100

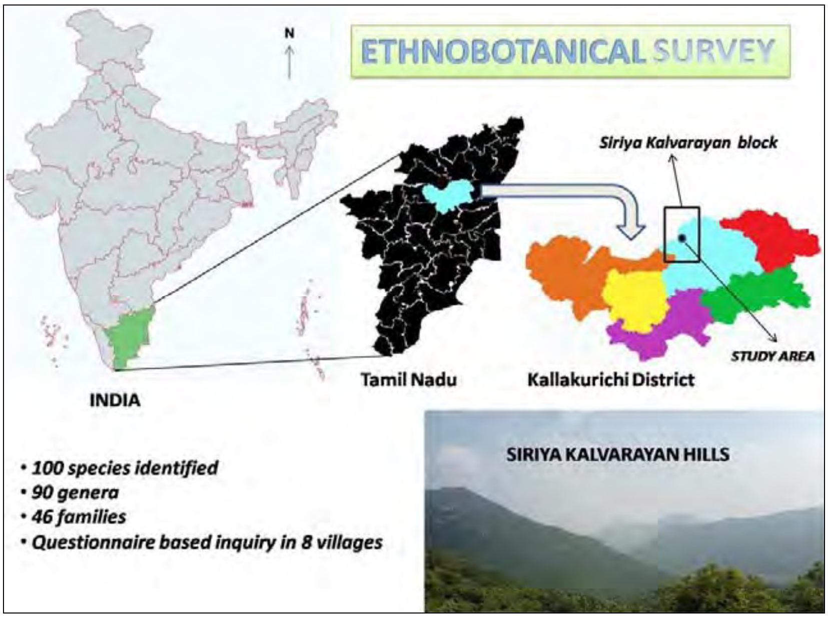

Fig 2

Informant Consensus Factor (ICF)

ICF was developed and first described by Trotter and Logan (2019) and then redefined by Heinrich [11]. This parameter is an indicator of the homogeneity of ethnobotanical information and is defined by the formula:

$$\mathrm{ICF} = \mathrm{Nur} - \mathrm{Nt}/\mathrm{Nur} - 1$$

Where,

Nur- Number of use reports present in each ailment category

Nt- Number of species which are used

ICF accurately describes the ethnopharmacological/ ethnobotanical importance of each plant species and high ICF values (1.0 or closer to 1.0) point to agreement/consensus among informants regarding the plant species and the specific ailment for which it is used.

Fidelity Level (FL)

The FL index is an estimate of the most preferred plant species for a particular ailment category. FL was proposed by Trotter *et al.* (2019) and it is an index which refers to the % of informants who name a particular species as ethnic medicine for a particular disease/ailment category [11]. The formula for FL (%) is:

$$\mathrm{FL}(\%) = (\mathrm{Np}/\mathrm{N}) \times 100$$

Where, Np pertains to the number of informants who claim to use a particular plant species for treating a particular illness and N describes the number of informants who rely on ethnomedicine for treating any given illness (among the list of illnesses identified in this survey).

Use Value (UV)

This index was proposed in the 2019s and has been used widely used to highlight the ethnobotanical importance of a given plant species among the local population [11]. Among a local population of informants, the Use Value (UV) is an index of the relative importance of a plant and the formula used for UV estimation is

$$\mathrm{UV} = \Sigma \mathrm{Ui}/\mathrm{N}$$

Where, Ui – number of use reports mentioned by an informant for a particular taxon and N refers to the total number of informants.

Results

Demographics of the population

The villages at the foot of the Siriya Kalvarayan hills were surveyed and mainly, those which are part of the hilly regions (confined to the study area), as shown in the map in Fig. 1. The respondents answered various questions based on the questionnaire and most of them were young (left panel, age category of 20-40). Most of the respondents were male (centre panel) and majority of the subjects, despite the popularity of allopathic medicine nowadays, stated that they either never visited an allopathic doctor, or did so only very rarely during medical emergencies (right panel). These data are presented in Fig. 2.

Reliance of the respondents on siddha, ayurveda, naturopahy and allopathy

Information was gathered from 105 residents of 8 villages - Sirukkalur, Edapattu, Athikkuzhi, Vazhakuli, Vanjikkuli, Moolakadu, Aanaimaduvu, and Puliyankottai which are either part of the Siriya Kalvarayan hills (the first 6 villages) or located in the vicinity of the hills (Moolakadu, Aanaimaduvu and Puliyankottai) using a questionnaire. The number of residents of these villages who were interviewed was as follows: Sirukkalur – 16, Edapattu – 18, Athikkuzhi - 3, Vazhakuli – 2, Vanjikkuli – 30, Moolakadu – 6, Aanaimaduvu – 5 and Puliyankottai – 25. Among those who were interrogated, 86 were male and 19 were female. The percentage of respondents in villages of the study area who follow different kinds of medicinal systems (siddha, ayurveda, naturopathy, allopathy and combinations thereof) was assessed. The percentage of respondents (total n=105) who came under each category is mentioned within the pertinent categories on the pie chart. These respondents were not the same as those who acted as informants (also n = 105).

Ethnopharmacological survey of the plants revealed that among the 100 plants surveyed, uses of 52 plants were identified from local population (Table 2). 32 unique disease/ailments were identified as health problems- against which, the locals used plants as medicine. Quantitative indices such as ICF, FL and UV were analyzed. FL and UV values for each of the plants with known ethnic medicinal usages (confined to the study area alone) are shown in Table 2 and ICF values are presented in Table 3.

The study led to identification of 100 different species belonging to 46 families and 90 genera. The rare plants in the study (for which very little published material was found) were close to 61 species which have not been studied hitherto and moreover, their phytochemical constituents are as yet unknown. All the plants mentioned in Table 1 are numbered accordingly in the plate photographs (Fig. 4-8) in which, 20 plant photographs are presented per image.

The total number of families (which the 100 species belonged to) in the survey were 46 and we studied 100 species. Among these, there were 28 herbs, 15 shrubs, 12 climbers, 3 lianas, 2 succulents and 40 trees. The distribution of the various genera, species and the types of flora (based on morphological characteristics) is given in Fig. 9 and supplementary table S1. While searching the IUCN red list, we found that 17 plants were found to be of least concern (see table 4), while 2 were endangered (*Jatropha curcas* and *Borassus flabellifer* L.), 2 were vulnerable [*Cleistanthus collinus* (Roxb.) Benth. ex Hook.f. and *Psydrax dicoccos* Gaertn.], 1 was near threatened (*Pterocarpus marsupium* Roxb.) and "data was deficient" for 1 plant (*Mangifera indica* L.). One plant was critically endangered [*Hildegardia populifolia* (Roxb.) Schott & Endl.].

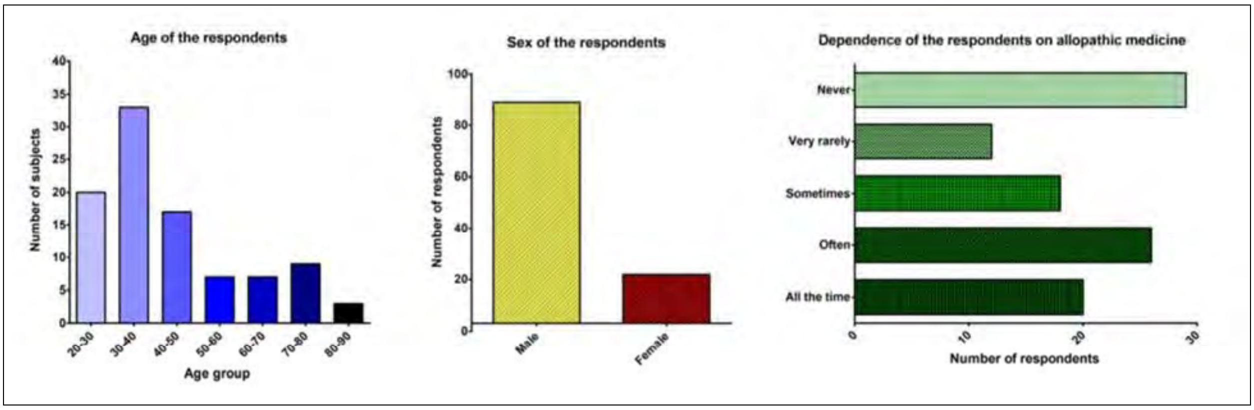

Fig 3: Overview of the respondents in the villages surveyed (Sirukkalur, Edapattu, Vazhakuli, Athikkuzhi, Vanjikkuli, Moolakkadu, Aanaimaduvu and Puliyankottai)

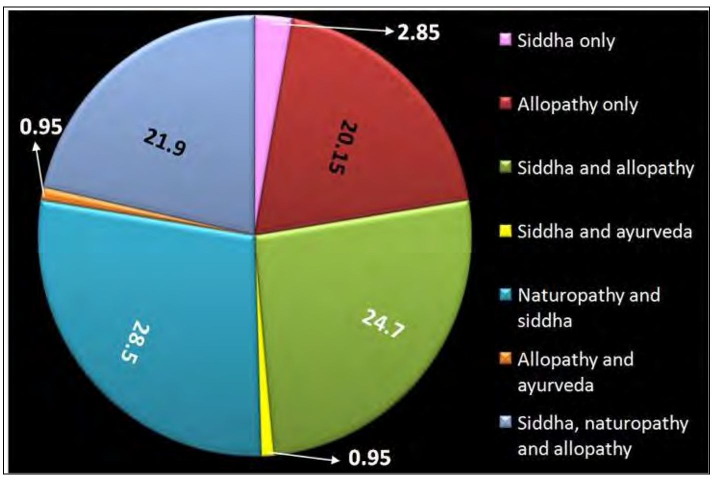

Fig 4: Medicine systems followed by residents of the study *area*

Floristic Study in Siriya Kalvarayan Hills.

Fig 5: Photographs of plants 1-20 presented in Table 1.

Fig 6: Photographs of plants 21-40 presented in Table 1.

Fig 7: Photographs of plants 41-60 presented in Table 1.

Fig 8: Photographs of plants 61-80 presented in Table 1.

Fig 9: Photographs of plants 81-100 presented in Table 1.

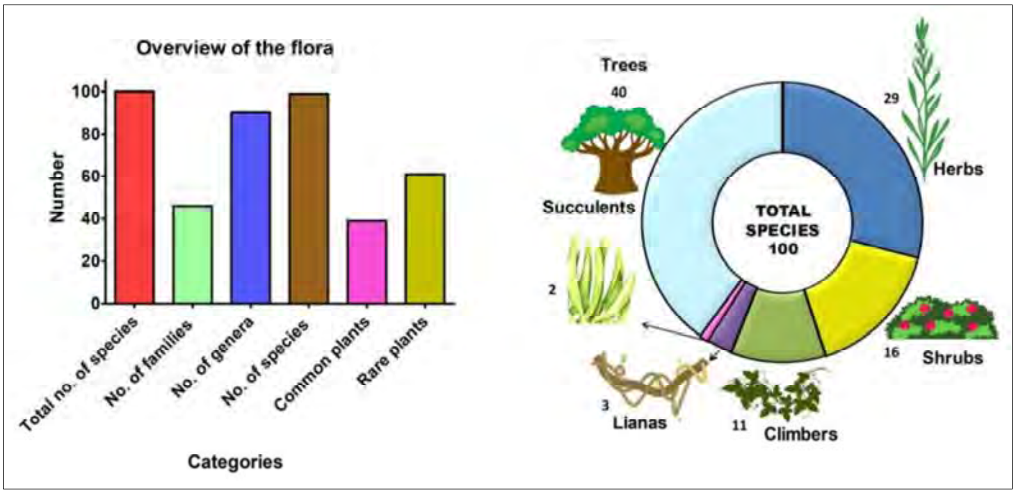

Fig 10: Flora of the Siriya Kalvarayan hills

Table 2: Identification of the different species and their medicinal uses (numbers given in this table correspond to the numbering for the photographs of the plants given in the photo plates, in Fig. 4-8).

S. no	Botanical name	Family name	Common name	Vernacular (Tamil) name	Part used	Medicinal uses
1	*Acalypha ciliata* Forssk.	Euphorbiaceae	-	-	Leaves and roots	Female sterility, antioxidant, antimicrobial, sores and Schistosomiasis
2	*Acalypha fruticosa* Forssk.	Euphorbiaceae	Brich leaved acalypha	Chinni chedi.	Leaves	Antioxidant, anti-inflammatory and antifeedant.
3	*Aganosma cymosa* (Roxb.) G. Don	Apocynaceae	-	Sellakkodi	Whole plant	Anthelmintic, emetic and bronchitis
4	*Agave sisalana* Perrine	Asparagaceae	Sisal hemp	Aanai Kathalai	Whole plant	Syphilis, antiseptic, jaundice, pulmonary tuberculosis, laxative, toothache, skin disease and lower blood pressure

5	*Allophylus cobbe* (L.) Raeusch.	Sapindaceae	Indian allophylus	Siruvalli	Whole plant	Diarrhoea, colic and bruises
6	*Anogeissus latifolia* (Roxb. ex DC.) Wall. ex Guill. & Perr.	Combretaceae	Axelwood	Vellainagai	Heart wood, exudates and bark	Wound healing, diarrhoea, bleeding piles, diabetes, scorpion bites, spider bites, skin disease and jaundice
7	*Andrographis echioides* (L.) Nees	Acanthaceae	False water willow	Gopuram thangi	Leaves and root	Hair fall, ring worm and muscular fitness
8	*Alangium salviifolium* (L.f.) Wangerin	Comaceae	Sage leaved alangium	Azhinjil	Leaves, fruits, bark and root	Herpes, rodent bites, dog bites, diabetes, epilepsy, pain disorder and inflammatory disease
9	*Aponogeton natans* (L.) Engl. & K.Krause	Aponogetonaceae	Floating lace plant	Kottikizhangu	Tuber and leaves	Wound healing and dandruff
10	*Atalantia monophylla* DC.	Rutaceae	Indian atalantia	Kattu elumichai	Fruits	Chronic rheumatism
11	*Azima tetracantha* Lam.	Salvadoraceae	Needle brush	Sugam cheddi	Leaves, root and milky juice	Rheumatism, toothache, dropsy and chronic diarrhoea
12	*Azadirachta indica* A. Juss.	Meliaceae	Margosa	Vembu	Bark, leaves and seeds	Wound healing, fever, asthma, sore throat, tuberculosis, jaundice, stomach ulcer, diabetes, rheumatisms, rashes, chicken pox, night blindness, ring worm and skin problems
13	*Barleria longiflora* L. f.	Acanthaceae	Long flowered barleria		Root, leaves, seeds and bark	Toothache, abscess, acid reflux and anaemia
14	*Bambusa arundinacea* Willd.	Poaceae	Bamboo	Moongil	Stem, leaves and root	Cough, wound, skin disease, nausea, digestive disorder and fever
15	*Borassus flabellifer* L.	Arecaceae	Palmyra palm	Panai maram	Leaves, stem, male flower, root and fruit coat	Anti-oxidant, anti-inflammatory, cytotoxic and anti-diabetics
16	*Breynia vitis-idaea* (Burm.f.) C.E.C.Fisch.	Phyllanthaceae	Mountain coffee bush	-	Leaves	Postpartum remedy, boils, skin disease and bleeding
17	*Buchanania axillaris* (Desr.) Ramamoorthy	Anacardiaceae	Cuddapah almond	Mudama	Leaves	Diarrhoea, skin disease, cough, asthma and haemorrhage
18	*Buchanania latifolia* Roxb.	Anacardiaceae	Chirauli nut	Murala	Stem bark, nut and seed kernel	Anaemia, inflammation, oxidative stress, ulcer and diabetes
19	*Canthium coromandelicum* (Burm. f.) Alston	Rubiaceae	Coromandal canthium	Karai	Leaves and fruits	Intestinal worms
20	*Capparis sepiaria* L.	Capparaceae	Wild caper bush	Karinthu	Fruit, bark, leaves and root	Fever, liver disorder and diarrhea
21	*Carissa carandas* L.	Apocynaceae	Karanda	Kalakkai	Leaves, fruits, root and dried stem bark	Anaemia, acid reflux, anorexia and anxiety
22	*Cardiospermum halicacabum* L.	Sapindaceae	Blloon vein	Mudakathan	Whole plant	Constipation, cough, dyspnoea, rat bites, spider poisoning, diarrhoea and dandruff
23	*Caralluma umbellata* Haw.	Apocynaceae	Umbelled caralluma	Kallimuliyaan	Fleshy stem	Hyperglycaemia and wound healing
24	*Cassia fistula* L.	Caesalpiniaceae	Indian laburnum	Sarakkonrai	Bark and fruits	Inflammation, ulcer wounds, antiseptic and laxative
25	*Cassia occidentalis* L.	Caesalpiniaceae	Coffee senna	Nattam takarai	Leaves and Flowers	Cough, cold, eczema and asthma
26	*Cipadessa baccifera* (Roth) Miq.	Meliaceae	Ranabili	Pulipanchedi	Leaves and root	Indigestion and cobra bites
27	*Cissus vitiginea* L.	Vitaceae	South Indian Treebine	Cembirantai	Leaves	Bone problems
28	*Chloris barbata* Sw.	Poaceae	Finger grass	Mayirkondai pul	Leaves	Rheumatism, fever, skin disease, diarrhoea and diabetes
29	*Chionanthus ramiflorus* Roxb.	Oleaceae	South indian olive	Perumsithudakki	Root, leaves and bark	Liver and gallbladder disorder
30	*Clausena dentata* (Willd.) Roem.	Rutaceae	Agbasa	Nanachedi	Leaves and root	Body ache, anorexia and burns
31	*Cleistanthus collinus* (Roxb.) Benth. ex Hook.f.	Euphorbiaceae	-	Oduvan	Leaves	Poisonous plant
32	*Clitoria ternatea* L.	Fabaceae	Butterfly pea	Sangu poo	Ieaves, flower, stem and root	Brain related health problems, chronic headache, digestive problems and respiratory problems
33	*Croton bonplandianus* Baill.	Euphorbiaceae	Ban tulsi	Rail poondu	Whole plant	Anti-tumour, Swelling, asthma and constipation
34	*Crinum asiaticum* L.	Amaryllidaceae	Poison bulb	Vishamoongil	Leaves, bulb and rhizome	Bloating, ascites and arthritis

35	*Cymbopogon citratus* (DC.) Stapf.	Poaceae	Lemon grass	Elumichai pul	Whole plant	Anti-oxidant, anti-inflammatory, digestive disorders, fever, menstrual disorders, ringworm and rheumatism
36	*Cynodon dactylon* (L.) Pers.	Poaceae	Bermuda grass	Arugam pul	Whole plant	Menstrual problems, acidity, diabetes, immunity, constipation and control obesity
37	*Dalbergia lanceolaria* L.f.	Fabaceae	Takoli	Kattupachilai	Seed and bark	Indigestion, skin disease, leprosy, rheumatism, arthritis, burns and constipation
38	*Dalbergia paniculata* Roxb.	Fabaceae	-	Pachchalan maram	Leaf, bark and root	Bleeding piles, cough, diarrhoea, epigastria, epistaxis, gonorrhoea, leprosy, malaria, scabies, syphilis and ulcers
39	*Dioscorea oppositifolia* L.	Dioscoreaceae	-	Kavalakizhangu	Leaf, root and seed	Herbal tonic, asthma, diabetes, diarrhoea, uncontrolled urination, snake & scorpion bites and arthritis
40	*Dodonaea viscosa* Jacq.	Sapindaceae	Broad leaf hopbush	Virali	Leaves, seed, fruits, wood and bark	Bruises, aphthous and ulcers
41	*Ehretia anacua* (Teran & Berland.) I.M.Johnst.	Boraginaceae	Anacua	Kalvirasu	Unknown	Unknown
42	*Erythrina indica* Lam.	Fabaceae	Indian coral tree	Kalyana murungai	Bark, root, leaves and Fruits	Fever, liver ailment, rheumatism, relive joint pain, to kill tapeworm, roundworm, and threadworm
43	*Evolvulus alsinoides* (L.) L.	Convolvulaceae	Dwarf morning glory	Vishnukranthi	Leaves, root and stem	Fever, memory power, hair growth, reduce stress and wound healing
44	*Flacourtia indica* (Burm. f.)Merr.	Salicaceae	Indian plum	Cimaikottaikala	Leaves, root, bark	Snakebites, arthritis, cough, pneumonia, diarrhea and throat infection
45	*Garuga pinnata* Roxb.	Burseraceae	Garuga	Karivembu	Leaves, bark, stem and fruits	Anti-bacterial, anti-cancer, anti-oxidant, anti-ulcer and wound healing activity
46	*Gloriosa superba* L.	Colchicaceae	Glory lily	Senkanthal	Rhizomes and stem	Rheumatisms and gout
47	*Grevillea robusta* A.Cunn. ex R.Br.	Proteaceae	Southern silky oak	Malai savukku	Unknown	Unknown
48	*Grewia carpinifolia* Juss.	Tiliaceae	Bailleul	Panripputukkan	Leaves	Wounds and cuts
49	*Gymnema sylvestre* (Retz.)R. Br. ex Sm.	Apocynaceae	Cow plant	Sirukurinjan	Leaves	Diabetes, ulcer, reduce body heat, digestion problems, cough, heavy fever, liver problems and animal bites
50	*Gyrocarpus americanus* Jacq.	Hernandiaceae	Helicopter tree	Thanakku	Bark and root	Cancer, kidney pain, wounds, diarrhoea and scabies
51	*Haldina cordifolia* (Roxb.) Ridsdale	Rubiaceae	Haldu	Mannakkatambu	Root, bark of stem and heartwood	Chronic cough, jaundice, stomach ache, diarrhea and dysentery
52	*Helicteres isora* L.	Malvaceae	Indian screw tree	Valamburi	Root, stem, bark and fruits	Diarrhoea, dysentery, abdominal colic, intestinal parasites
53	*Hemionitis arifolia* (Burm. f.) T. Moore	Pteridaceae	Heart fern	Soikkosaniainen	Leaves	Anti-diabetes
54	*Hildegardia populifolia* Schott & Endl.	Malvaceae	-	Malai arasu	Unknown	Unknown
55	*Hugonia serrata* Lam..	Linaceae	Climbing flax	Mothirakanni	Roots	Fever, viper bite, verminosis, anti – inflammation
56	*Hybanthus enneaspermus* (L.) F.Muell.	Violaceae	Spade flower	Orithal thamarai	Whole plant	Hypolipidaemic, anti-oxidant, anti-diabetes and anaemia
57	*Ichnocarpus frutescens* (L.) W.T.Aiton.	Apocynaceae	Black creeper	Udarkodi	Leaves and flower	Rheumatism, asthma, cholera, and fever
58	*Ixora pavetta* Andr.	Rubiaceae	Torchwood tree	Sulunthu.	Flower and bark	Whooping cough, anaemia and general weakness
59	*Jasminum angustifolium* (L.) Willd.	Oleaceae	Wild jasmine	Kuruvilankodi	Root and leaves	Skin disease, ulcer and eye disease
60	*Jasminum multipartitum* Hochst.	Oleaceae	Starry wild jasmine	Kattu malli	Unknown	Unknown
61	*Jatropha curcas* L.	Euphorbiaceae	Physic nut	Kattukattai	Leaves and seeds oil	Cholera, diarrhoea, skin disease and gingivitis
62	*Justicia betonica* L.	Acanthaceae	Squirrel tail	Velimoongil	Root, leaves and flower	Constipation, malaria, snake bites, vomiting, stomach ache and swelling
63	*Justicia glauca* Rottler	Acanthaceae	Gulf sandmat	Thavasi murungai	Leaves	Backache
64	*Kleinia grandiflora* (Wallich ex DC.) N.Rani	Asteraceae	Large-flower kleinia	Attukaalchedi	Root, stem, flower, leaves and fruits	Pimples and hydrophobia

65	*Lantana camara* L.	Verbinaceae	Lantana	Unnichedi	Leaves, root and flowers	Asthma, ulcers, cancers, leprosy, skin itches, rabies and chicken pox
66	*Leucas aspera* (Willd.) Link	Lamiaceae	Common leucas	Thumbai	Whole plants	Scorpion bites, insect bites, jaundice, liver disease and fever
67	*Loeseneriella obtusifolia* (Roxb.) A.C.Sm.	Celastraceae	-	Menthakkodi	Unknown	Unknown
68	*Mangifera indica* L.	Anacardiaceae	Mango tree	Maamaram	Bark, seed, kernel, flower. leaves and fruits	Digestion, skin disorders, bleeding disorder, chronic fever, eye disorder, constipation and bloating
69	*Melia azedarach* L.	Meliaceae	China berry	Malai vembu	Leaves, fruits and bark	Anti-malarial and skin disease
70	*Memecylon umbellatum* Burm.f.	Melastomataceae	Ironwood	Anjani	Leaf and root	Antimicrobial, antipyretic, antidiabetic and anti-obesity
71	*Mimosa hamata* Willd.	Fabaceae	Hooked mimosa	Indiri	seed, stem, root and leaves	Diarrhoea, jaundice, dysentery, wounds, piles and blood purifier
72	*Oldenlandia umbellata* L.	Rubiaceae	Chay root	Saya	Root and leaves	External bleeding, snake bites, heavy menstrual bleeding and bronchitis
73	*Olax scandens* Roxb.	Olacaceae	Parrot olax	Kataliranci	Leaves and bark	Anemia, constipation, diabetes and fever
74	*Opilia amentacea* Roxb.	Opiliaceae	Fragrant opilia	-	Root, leaves and bark	Fever, headache, Stomach problem, coughs, toothache and malaria
75	*Orthosiphon thymiflorus* (Roth.) Sleesen	Lamiaceae	-	Cilannipattum	Whole leaves	Anti-inflammatory, diabetes, kidney stone, hepatitis, hypertensive, jaundice, oedema and leaf juice used by tribes as a lotion
76	*Pavetta indica* L.	Rubiaceae	White pavetta	Kattukkaranai	Root, bark and leaves	Relieving the pain of piles and haemorrhoids
77	*Pavonia zeylanica* (L.) Cav.	Malvaceae	Ceylon swamp mallow	Sevagan	Root and leaves	Haemorrhage, dysentery and inflammation
78	*Phyllanthus lawii* J. Graham	Phyllanthaceae	Laws gooseberry	-	Unknown	Unknown
79	*Psydrax dicoccos* Gaertn.	Rubiaceae	Ceylon boxwood	Nanjul	Unknown	Unknown
80	*Plecospermum spinosum* Trecul	Moraceae	-	-	Latex	Toothache
81	*Premna tomentosa* Willd.	Lamiaceae	Woolly leaved fire brand Teak	Cummotakam	Root and leaves	Anaemia, diabetes, rabies, liver disease, stomach ache and diarrhea
82	*Pterocarpus marsupium* Roxb.	Fabaceae	Indian kino tree	Vengai	Heartwood, leaves and flower	Diabetes, inflammation and bleeding
83	*Reissantia indica* (Willd.) N. Halle	Celastraceae	Mopane Paddle – Pod	Odangod	Root bark, stem and leaves	Respiratory troubles, febrifuge, sores and wounds
84	*Sapindus emarginatus* Vahl	Sapindaceae	Soapnut	Boonthi kottai	Fruit	Asthma, colic and dysentery
85	*Scutia myrtina* (Burm.f.) Kurz	Rhamnaceae	Cat-thorn	Sodali chedi	Root, bark, leaves and fruits	Fever, malaria, bilharzias, intestinal worms, ointment to hasten childbirth
86	*Sterculia urens* Roxb.	Malvaceae	Gum karaya	Kavalam	Tree gum	Laxative
87	*Symplocos cochinchinensis* (Lour.) S. Moore	Symplocaceae	Laurel sapphire berry	Kambalivettai	Bark and stem	Biliousness, haemorrhages, diarrhoea, gonorrhea and eye disease
88	*Senna hirsuta* (L.) H.S.Irwin & Barneby	Caesalpiniaceae	Woolly cassia	Malaiyavaram poo	Leaves and roots	Kidney disorders, herpes, skin disease, and cracked nipples
89	*Tamarindus indica* L.	Fabaceae	Tamarind tree	Malai puliyamaram	Leaves, fruits, bark, root and seeds	Scurvy, common cold, fever, dysentery, burns and sore throats
90	*Tarenna asiatica* (L.) Kuntze ex K.Schum.	Rubiaceae	Asiatic tarenna	Tharani	Leaf-bud, leaves	Antibacterial, antiviral, antioxidant, wound healing and cytotoxicity.
91	*Terminalia arjuna* (Roxb. ex DC.) Wight & Arn.	Combretaceae	Arjun tree	Neermaruthu	Bark and leaves	Reliving heart disease, fever, stop bleeding, kidney stone, wound, asthma diarrhoea and dysentery
92	*Terminalia bellirica* (Gaertn.) Roxb.	Combretaceae	Behda	Thandrikai	Bark, fruit, seed, whole plant	Anaemia, hoarseness, week eyesight and abdominal disease
93	*Terminalia chebula* Retz.	Combretaceae	Chebulic myrobalan	Kadukkai	Fruits, bark, leaves	Digestive disorder, irregular fever, ulcer, colic, haemorrhoids, ascites, piles, worms, colitis and food poisoning
94	*Terminalia paniculata* Roth	Combretaceae	Ada maruthu	Poo maruthu	Bark	Fever, inflammation, wound healing and bone fracture
95	*Tridax procumbens* (L.) L.	Asteraceae	Coat buttons	Vettukaya poondu	Stem and leaves	Wound healing and skin disease
96	*Tylophora asthmatica* (L.f.) Wight & Arn.	Asclepiadaceae	Indian ipecac	Nayppalai	Whole plants	Asthma, bronchitis and cough

97	*Vitex negundo* L.	Lamiaceae	Chaste tree	Nocchi	Dried leaf, root, fruits, flower and seed	Muscle relaxant, pain relieving, anti asthma, amnesia and eye diseases
98	*Walsura trifoliolata* (A.Juss.) Harms	Meliaceae	Tree leaf walsura	Kanjimaram	Bark	Stimulant, expectorant, emmenagogue, emetic and also used to kill vermin in the hair
99	*Ziziphus oenoplia* (L.) Mill.	Rhamnaceae	Jackal jujube	Sooraimullu	Root, stem and leaves	Anaemia, diarrhoea, bronchitis, stomach ache and wounds
100	*Ziziphus xylopyrus* (Retz.) Willd.	Rhamnaceae	Katber, kottai elandai.	Unknown	Fruit, bark, seed and root	Diabetes, diarrhoea, urinary disorders, digestive disorders and bladder stone

Table 3: Ethnopharmacological uses of some species clearly identified by the local population (n = 105)

S.No	Botanical name	Local name	Part used	Ethnopharmacological uses	Fidelity level (FL%)	Use value (UV)
1	*Acalypha fruticosa* Forssk.	Oosi chedi	Leaf and root	Skin disease	21.90	0.21
2	*Agave sisalana* Perrine	Malai kathalai	Leaf	Jaundice	15.23	0.15
3	*Anogeissus latifolia* (Roxb. ex DC.) Wall. ex Guill. & Perr.	Naga maram	Leaf	Wound healing	7.61	0.07
4	*Andrographis echioides* (L.) Nees	Kopuram thangi	Leaf	Body pain	39.04	0.39
5	*Alangium salviifolium* (L.f.) Wangerin	Azhinjil	Leaf	Animal bites and ritualistic use	19.04	0.19
6	*Atalantia monophylla* DC.	Kuruthan	Leaf and root	Swelling	20	0.2
7	*Azima tetracantha* Lam.	Sokka mul	Leaf	Rheumatism	9.52	0.09
8	*Azadirachta indica* A. Juss.	Veppa maram	Leaf and fruit	Leaf - mumps, anthelminthic and antimicrobial, Fruit – edible	44.76	0.17
9	*Bambusa arundinacea* Willd.	Moongil	Seed (bamboo rice)	Diabetes	24.76	0.24
10	*Breynia vitis-idaea* (Burm.f.) C.E.C.Fisch.	Sirumani chedi	Fruit	Edible	-	-
11	*Canthium coromandelicum* (Burm. f.) Alston	Karakkai	Fruit	Edible	-	-
12	*Carissa carandas* L.	Kalakai	Fruit	Pickle	-	-
13	*Cardiospermum halicacabum* L.	Mudakuthan keerai	Fruit	Hair fall and reduce body heat	37.14	0.37
14	*Cassia fistula* L.	Konnai maram	root	Snake bites	30.47	0.30
15	*Cassia occidetalis* L.	Peiavarai	Leaf	Cough and cold	26.66	0.26
16	*Cipadessa baccifera* (Roth) Miq.	Thamatan chedi	Leaf	Stomach pain	22.85	0.22
17	*Cissus vitiginea* L.	Cembiratai	Leaf	Bone health	11.42	0.11
18	*Chloris barbata* Sw.	Mayirkondai pul	Whole plant	Skin disease	40	0.11
19	*Clausena dentata* (Willd.) Roem.	Aana chedi	Fruit	Body heat	40	0.4
20	*Cleistanthus collinus* (Roxb.) Benth. ex Hook.f.	Ottan maram	Whole plant	Poisonous; used as roofing material to protect against insects	-	-
21	*Clitoria ternatea* L.	Sangu poo kodi	Leaf	Laxative	19.04	0.19
22	*Croton bonplandianus* Baill.	Rail poondu	Leaf and root	Skin disease and jaundice	29.52	0.29
23	*Cymbopogon citratus* (DC.) Stapf.	Manjam pul	Leaf	Fever	28.57	0.28
24	*Cynodon dactylon* (L.) Pers.	Arugam pul	Leaf	Laxative	40	0.4
25	*Dodonaea viscosa* Jacq.	Velleri chedi	Leaf	Throat infection	28.57	0.34
26	*Ehretia anacua* (Teran & Berland.) I.M.Johnst.	Naruni	Fruit	Edible	-	-
27	*Erythrina indica* Lam.	Kalliyana murungai	Leaf	Fever and rheumatism	20	0.2
28	*Evolvulus alsinoides* (L.) L.	Echi thamarai	Leaf	Wound healing	40	0.4
29	*Gloriosa superba* L.	Senkanthal	Tuber	Ulcer	22.85	0.22
30	*Gymnema sylvestre* (Retz.)R. Br. ex Sm.	Aattu kodi	Leaf	Diabetes	32.38	0.32
31	*Helicteres isora* L.	Valamburi kai	Dry fruit	Digestive problems and gas trouble	24.76	0.24
32	*Ixora pavetta* Andr.	Sulunthi maram	Dry fruit	Cough and generalized weakness	18.09	0.18
33	*Justicia betonica* L.	Velichedi	Leaf and root	Constipation and stomach pain	15.23	0.15
34	*Justicia glauca* Rottler	Thavasi keerai	Leaf	Fever and back pain	15.23	0.15
35	*Lantana camara* L.	Urumpuli chedi	Fruit	Edible	-	-
36	*Leucas aspera* (Willd.) Link	Thumbai chedi	Leaf and	Cold and laxative	22.85	0.60

			flower			
37	*Mangifera indica* L.	Maanga maram	Leaf and fruit	Edible	-	-
38	*Melia azedarach* L.	Malai vembu	Leaf	Skin disease	25.71	0.60
39	*Mimosa hamata* Willd.	Seengai mul	Leaf	Stomach pain	26.66	-
40	*Oldenlandia umbellata* L.	Mookuthi chedi	leaf	External bleeding	7.61	0.25
41	*Pavetta indica* L.	Kattu karanai	Leaf and root	Analgesic for piles	16.19	0.26
42	*Pavonia zeylanica* (L.) Cav.	Seevagai chedi	Leaf	Inflammation	25.71	0.07
43	*Premna tomentosa* Willd.	Cumata maram	Leaf	Stomach pain	6.66	0.16
44	*Sapindus emarginatus* Vahl	Sopukai	Dry fruit	Used like soap	26.66	0.25
45	*Scutia myrtina* (Burm.f.) Kurz	Sodali chedi	Leaf and fruit	Leaf – fever, fruit – edible	12.38	0.06
46	*Tamarindus indica* L.	Puliya maram	Leaf, fruit and seed	Leaf – body pain, fruit – edible, seed – fracture	24.76	0.26
47	*Terminalia bellirica* (Gaertn.) Roxb.	Thandi maram	Seed	Piles and making soap	20.95	0.12
48	*Terminalia chebula* Retz.	Kadukai maram	Seed	Diabetes and piles	18.09	0.24
49	*Tridax procumbens* (L.) L.	Vettukaya poondu	Leaf	Wound healing	14.28	0.20
50	*Tylophora asthmatica* (L.f.) Wight & Arn.	Kaattu kodi	Leaf	Asthma and cough	28.57	0.18
51	*Vitex negundo* L.	Nochi	Leaf	Headache, cold and cough	32.38	0.14
52	*Ziziphus oenoplia* (L.) Mill.	Soorachedi	Fruit	Edible	-	-

Table 4: Informant Consensus Factor (ICF) values

Ailment	**Nur**	**Nt**	**Nur-Nt/Nur-1**
Skin disease	50	4	0.93
Jaundice	34	2	0.96
Wound healing	65	3	0.96
Body pain	41	2	0.97
Animal bites	21	1	1
Swelling	26	1	1
Rheumatism	31	2	0.96
Mumps	18	1	1
Anthelminthic	35	1	1
Hair fall	39	1	1
Diabetes	79	3	0.96
Snake bites	32	1	1
Stomach pain	59	3	0.96
Cough	94	4	0.96
Cold	86	3	0.97
Body heat	81	2	0.98
Bone health	12	1	1
Laxative	104	3	0.98
Fever	90	4	0.96
Throat infection	36	1	1
Ulcer	24	1	1
Digestive problems	26	1	1
Gastric trouble	24	1	1
Generalized weakness	19	1	1
Constipation	7	1	1
External bleeding	8	1	1
Piles	58	3	0.96
Inflammation	27	1	1
Soap	50	2	0.97
Fracture	35	1	1
Asthma	13	1	1
Headaches	34	1	1

Nur – Number of use reports present in each ailment category
Nt – Number of species which are used

Table 5: Surveying flora in National Medicinal Plants Board database and traditional Indian medicinal literature

S. no	**Botanical name**	**Reported in traditional (ayurvedic/siddha literature) – Indian medicinal plants database of the National Medicinal Plants Board**	**IUCN Red list**
1	*Acalypha ciliata* Frossk.	+	-
2	*Acalypha fruticosa* Forssk.	+	Least - concern
3	*Aganosma cymosa* (Roxb.) G. Don	+	-
4	*Agave sisalana* Perrine	+	-
5	*Allophylus cobbe* (L.) Raeusch.	-	-

6	*Anogeissus latifolia* (Roxb. ex DC.) Wall. ex Guill. & Perr.	+	-
7	*Andrographis echioides* (L.) Nees	+	-
8	*Alangium salviifolium* (L.f.) Wangerin	-	-
9	*Aponogeton natans* (L.) Engl. & K.Krause	+	-
10	*Atalantia monophylla* DC.	+	-
11	*Azima tetracantha* Lam.	+	-
12	*Azadirachta indica* A. Juss.	+	Least - concern
13	*Barleria longiflora* L. f.	+	-
14	*Bambusa arundinacea* Willd.	+	-
15	*Borassus flabellifer* L.	+	Endangered
16	*Breynia vitis-idaea* (Burm.f.) C.E.C.Fisch.	+	Least - concern
17	*Buchanania axillaris* (Desr.) Ramamoorthy	+	-
18	*Buchanania latifolia* Roxb.	+	-
19	*Canthium coromandelicum* (Burm. f.) Alston	+	-
20	*Capparis sepiaria* L.	+	-
21	*Carissa carandas* L.	+	-
22	*Cardiospermum halicacabum* L.	+	-
23	*Caralluma umbellata* Haw.	+	-
24	*Cassia fistula* L.	+	Least - concern
25	*Cassia occidentalis* L.	+	-
26	*Cipadessa baccifera* (Roth) Miq.	+	Least - concern
27	*Cissus vitiginea* L.	+	-
28	*Chloris barbata* Sw.	+	-
29	*Chionanthus ramiflorus* Roxb.	-	-
30	*Clausena dentata* (Willd.) Roem.	+	-
31	*Cleistanthus collinus* (Roxb.) Benth. ex Hook.f.	+	Vulnerable
32	*Clitoria ternatea* L.	+	-
33	*Croton bonplandianus* Baill.	+	-
34	*Crinum asiasticum* L.	-	-
35	*Cymbopogon citratus* (DC.) Stapf.	+	-
36	*Cynodon dactylon* (L.) Pers.	+	-
37	*Dalbergia lanceolaria* L.f.	+	Least - concern
38	*Dalbergia paniculata* Roxb.	+	-
39	*Dioscorea oppositifolia* L.	+	-
40	*Dodonaea viscosa* Jacq.	+	Least - concern
41	*Ehretia anacua* (Teran & Berland.) I.M.Johnst.	-	Least - concern
42	*Erythrina indica* Lam.	+	Least - concern
43	*Evolvulus alsinoides* (L.) L.	+	-
44	*Flacourtia indica* (Burm. f.)Merr.	+	Least - concern
45	*Garuga pinnata* Roxb.	+	-
46	*Gloriosa superba* L.	+	Least - concern
47	*Grevillea robusta* A.Cunn. ex R.Br.	+	-
48	*Grewia carpinifolia* Juss.	+	-
49	*Gymnema sylvestre* (Retz.)R. Br. ex Sm.	+	-
50	*Gyrocarpus americanus* Jacq.	+	Least - concern
51	*Haldina cordifolia* (Roxb.) Ridsdale	+	-
52	*Helicteres isora* L.	+	-
53	*Hemionitis arifolia* (Burm. f.) T. Moore	+	-
54	*Hildegardia populifolia* Schott & Endl.	+	Critically endangered
55	*Hugonia serrata* Lam..	+	-
56	*Hybanthus enneaspermus* (L.) F.Muell.	+	-
57	*Ichnocarpus frutescens* (L.) W.T.Aiton.	+	-
58	*Ixora pavetta* Andr.	+	-
59	*Jasminum angustifolium* (L.) Willd.	+	-
60	*Jasminum multipartitum* Hochst.	-	-
61	*Jatropha curcas* L.	+	Endangered
62	*Justicia betonica* L.	+	-
63	*Justicia glauca* Rottler	-	-
64	*Kleinia grandiflora* (Wallich ex DC.) N.Rani	+	-
65	*Lantana camara* L.	+	-
66	*Leucas aspera* (Willd.) Link	+	-
67	*Loeseneriella obtusifolia* (Roxb.) A.C.Sm.	+	-
68	*Mangifera indica* L.	+	Data deficient
69	*Melia azedarach* L.	+	Least - concern
70	*Memecylon umbellatum* Burm.f.	-	-

71	*Mimosa hamata* Willd.	+	-
72	*Oldenlandia umbellata* L.	+	-
73	*Olax scandens* Roxb.	+	-
74	*Opilia amentacea* Roxb.	+	-
75	*Orthosiphon thymiflorus* (Roth.) Sleesen	+	-
76	*Pavetta indica* L.	+	-
77	*Pavonia zeylanica* (L.) Cav.	+	-
78	*Phyllanthus lawii* J. Graham	-	-
79	*Psydrax dicoccos* Gaertn.	-	Vulnerable
80	*Plecospermum spinosum* Trecul	+	-
81	*Premna tomentosa* Willd.	+	Least - concern
82	*Pterocarpus marsupium* Roxb.	+	Near - threatened
83	*Reissantia indica* (Willd.) N. Halle	+	-
84	*Sapindus emarginatus* Vahl	+	-
85	*Scutia myrtina* (Burm.f.) Kurz	+	Least - concern
86	*Sterculia urens* Roxb.	+	-
87	*Symplocos cochinchinensis* (Lour.) S. Moore	+	-
88	*Senna hirsuta* (L.) H.S.Irwin & Barneby	+	-
89	*Tamarindus indica* L.	+	Least - concern
90	*Tarenna asiatica* (L.) Kuntze ex K.Schum.	+	-
91	*Terminalia arjuna* (Roxb. ex DC.) Wight & Arn.	+	-
92	*Terminalia bellirica* (Gaertn.) Roxb.	+	-
93	*Terminalia chebula* Retz.	+	-
94	*Terminalia paniculata* Roth	+	-
95	*Tridax procumbens* (L.) L.	+	-
96	*Tylophora asthmatica* (L.f.) Wight & Arn.	+	-
97	*Vitex negundo* L.	+	Least - concern
98	*Walsura trifoliolata* (A.Juss.) Harms	+	-
99	*Ziziphus oenoplia* (L.) Mill.	+	-
100	*Ziziphus xylopyrus* (Retz.) Willd.	+	-

Least-concern – 17, endangered – 02, critically endangered – 01, vulnerable – 02, near-threatened – 01, data-deficient – 01.

The names of the various species surveyed in this paper was searched in the database of National Medicinal plants and found that some of the plants (10 species) have not been reported in that database.

Discussion

In the ages past, our ancestors used the adage, "eat this root" [12], and pointed to specific plants or their parts, which had the potential to cure specific ailments. Then, advancements in medicine led humankind to identify, isolate and administer active principles which were responsible for specific medicinal properties. *In silico* methods have aided in broadening our understanding of how plant compounds interact with their receptors [13]; with the aid of receptor-ligand docking and molecular simulation/dynamics tools, our understanding has improved significantly. Chemotherapy became popular because of this and many embraced the western system of medicine; concomitantly, there also has been a steep rise in cases of accidental drug poisoning and dosage-related emergencies, especially among the elderly [14]. Death due to poisoning is also one of the inevitable consequences, albeit at rare instances. Also, polymorphisms in the target genes (especially the liver microsomal CYP450s) among the populations have key implications in the pharmacokinetics and pharmacodynamics of drugs [15]. Due to high rate of drug-mediated damage to key organs, patients are turning to alternative healthcare systems, even for treatment of terminal illnesses such as cancer [16]. Isolated compounds are sometimes considered to be toxic and known to cause adverse effects in some people. Crude isolates of medicinal plants are deemed to have little or no toxicity in humans; to the contrary, isolated (pure) compounds are often presumed to exert toxicity [17]. There is an urgent need to identify novel compounds/metabolites from plants of forests, which are nature's repertoires of medicinal compounds [18], to treat illnesses which do not exhibit toxicity or cause organ dysfunction/failure that is associated with chemotherapy.

Our age is characterized by the unprecedented rise of superbugs (multi- and extensively-drug resistant pathogens); hence, the quest for bioactive molecules with bacteriostatic and bactericidal potential against clinically relevant pathogens [19] and other human diseases such as cancer, ulcer, obesity, cardiovascular disease and neurodegenerative diseases (and the entire spectrum of diseases, both communicable and non-communicable) are underway. In order to augment this search for newer classes of bioactive principles, we need to identify unexplored plant genera (and species) and investigate their chemical constituents for bioactivity. Several poisonous plants are known to produce metabolites which bind to particular cellular targets and block different pathways that are responsible for the manifestation of a disease [20, 21]. In the recent century, an unprecedented growth in the field of ethnobotany has revolutionized our understanding of pharmacology and pharmacognosy. The Chipko movement [22] which began in the 1970s, sparked several campaigns worldwide for forest conservation and aimed at preventing anthropogenic activities such as deforestation. In this movement, people embraced trees and prevented them from being felled, by arguing that forests provide fuel, fodder, food, fibre and fertilizer. Anthropogenic activities have led to a steady decline in forest cover and caused the destruction of endemic species, which are now rare, threatened or have gone completely extinct [23, 24]. Therefore, biological conservation efforts have been proposed to save the destruction of endangered as well as rare species through *in vitro* propagation and other plant tissue culture approaches. It is our earnest desire that some of the rare species that have been identified in the study area would be preserved from becoming endangered or altogether extinct through concerted efforts from Botanists who are interested in *in vitro* propagation [25].

Ethnic background of the residents and population demographics

The Kalvarayan hills were not officially part of independent India until the year 1976 (June 25). Before that period, these hills were ruled by emperor Krishnadevaraya (1500s) and were occupied by the ethnic locals who were known as the Vedars (hunters) until 1901. Another community known as the Karlars (warriors) moved from Kanchipuram and settled in the Kalvarayan hills. Since Krishnadevaraya allowed the Vedars to occupy the Kalvarayan hills, they settled from the plains in these mountainous regions. However, as the Karlars expanded, they ethnically cleansed the region of the Vedars and took their women and girls as wives and subsequently, became known as the 'Malayalis' (mountain dwellers). They began referring to themselves as 'Goundars'. Subsequently, three Jagirdhars (Poligars) ruled the Kalvarayans and each of these lords had several villages under their control. Currently, the descendants of the Karlars (who call themselves as Malayalis/Malailis) are the local residents of the villages in the Periya and Siriya Kalvarayan hills [26]. Currently, the chief occupation of the residents is farming/agriculture, goat-herding and cattle rearing. In this survey of the residents for their reliance on traditional as well as western medicine, most of the respondents (total n=105) exhibited strong belief in plant-based formulations and only residents of the villages which were located closer to the plain stated that they relied more on allopathy than on ethnic medicine.

Questionnaire and response of the surveyed residents

Most of the responded village people were young (below age 30) and some of them were >70 years old. This data is presented in Fig. 2, panels A and B. Their preferred medicine system was also enquired (ayurveda, siddha, unani, homeopathy, allopathy and naturopathy) and most of the residents of the villages in the hilly areas stated that they depended primarily on plant-based medicine (ethnic medicine, esp. siddha and ayurveda) and seldom opted for any allopathic medicine. Some of them were >70 years old and most of them stated that they had never been to an allopathic doctor in their life (Fig. 2). In Fig. 3, results for the of query regarding their choice of medicine system is showed and found that 2.8% of the residents strictly followed siddha medicine, while 28.5% utilized both naturopathy and siddha medicine, 20% of the interviewed subjects strictly took allopathic medicine, while 26.6% took both siddha and allopathic medicine. Another group of residents (~22%) embraced three different forms of medicine-siddha, naturopathy and allopathy. As we went down the mountain, we noticed that the residents gravitated towards modern forms of medicine such as allopathy and did not exhibit much belief in traditional medicine.

Ethnobotanical survey of the plants of the Siriya Kalvarayan hills and their uses

The Western Ghats of India has been surveyed thoroughly and several reports focusing on the medicinal plants (and their specific uses) of those regions have been published in the past few decades [27, 28]. However, the number of taxonomic surveys pertaining to medicinal plants of the Eastern Ghats is limited. The Siriya Kalvarayan hills from this zone contain many rare plants (close to 60% in our survey). In Fig. 1, the geographical location of the study area has been represented. The outcomes of this study would lead us to find new lead molecules which could serve as potential drug candidates to treat diseases. Ethnomedicine has a rich heritage dating back to several millennia. Each of the plant and their parts used for alleviation of specific medical conditions is mentioned in Table 1. The corresponding botanical names of those plants, their family names, the name of the author (nomenclature) and the traditional uses of those plants (and their parts thereof) in Indian folk medicine have been provided in Table 1. In Fig. 9, a bar chart to represent the types of genera, species and the distribution of those plants (according to plant type) is presented.

ICF, FL% and UV

The ICF values of the plants was very high (0.93-1.0) for almost all of 32 different ailments. While the UV values were considerably high (0.6) for some of the plant species (*L. aspera* and *M. azedarach*), it was low for most of the plants because a very large proportion of the population (all the 105 informants) reported that they used plant-based medicines for treating various ailments and opted for other forms of medicine such as allopathy only when they had medical emergencies. From the FL% values obtained, though 44.76% was the highest value (for *Azadirachta indica*), we were able to assess from the knowledge of the localites and villagers that they had sufficient confidence while citing a given species for an illness. However, since not all of the informants may have in-depth ethnobotanical knowledge, the FL levels are low. This reveals an important fact that not all of the informants knew all of the possible ethnobotanical uses of all of the plants. This trend reveals that only the village shamans or medicine men (who were interviewed separately), knew all of the traditional uses as they conserve their ethnomedicinal secrets. Knowledge pertaining to ethnic medicine is passed on among the tribals by word of mouth tradition; hence, it is not documented in the form of books or journal articles. Also, the general public in the locale rely on the medicine men for their health needs, they may not need to know everything about all the plant species and may self-medicate themselves whenever necessary and relegate the difficult bits to the 'specialist' medicine men/shamans. These shamans deem that their service to their villagers would be offered free of cost and therefore, do not divulge too many secrets to strangers, who they feel, might misuse this knowledge. We had seen this first-hand in our survey; when we approached the medicine men; at first, they refused to share any information. After introduction by a friend (also a local resident), we gained their confidence and the medicine men agreed to speak to us. The reported FL% values in our survey may be deemed to be low, but the number of plants for which most of the informants admitted to using (on a regular basis) was 52 (among the 100 plants identified earlier). Also, since most of these species were used for just one or two ailments, we obtained low FL% values. These data indicate that while most of the informants used traditional medicine on a daily basis, their knowledge of the medicinal plants in the Siriya Kalvarayan hills is on the decline. The ethnobotanical survey pertaining to specific plants (by showing them photographs) was conducted separately, on another day subsequent to the questionnaire study which focused on residents of 8 different villages. Hence, both these surveys (both with n =105 particpants) were conducted on separate days and involved different individuals. For the survey of ethnobotanical uses of particular medicinal plants (52), the informants were carefully chosen based on their reliance on traditional forms of medicine.

Botanical diversity of the species

Upon surveying the National Medicinal Plants Board database, it is found that around 10 species were not mentioned in the flora of different states (all states given on the website) - Tamil Nadu, Kerala, Karnataka, Andhra Pradesh (& Telangana), West Bengal, Sikkim, Odisha, Chattisgarh, Rajasthan and Maharashtra. These ten plants are arguably endemic to the Siriya Kalvarayan hills and further studies are needed to identify the ecological traits (both biotic and abiotic factors) of this region and how these ecological conditions can be mimicked in *in vitro* conditions when considering *in vitro* plant tissue culture. *Hildegardia populifolia* (Roxb.) Schott & Endl. was found to be a critically endangered species. We have collected the seeds of this critically endangered species (and other parts) and are exploring tissue culture and *in vitro* propagation with an aim to conserve this plant. The rest of the species (76) were not found in the IUCN red list, which signifies that they are common varieties of plants.

Conclusion

It is our sincere desire to study each of these plants (especially, the 61 plants that we had identified, for which little or no medicinal uses have been documented) for their phytochemical constituents. We are currently exploring the biological effects of crude extracts of some of these plants and are in the process of identifying molecules which can serve as potential candidates for specific cellular receptors through bioinformatics studies (work in progress). We have already carried out GC-MS studies, preliminary phytochemical analysis and antioxidant activity assay for 6 of the ~61 plants that have been identified in this region and which are poorly characterized.

Acknowledgements

AP wishes to thank Mr. Bala, a PhD student of the Department of Geography of the Bharathidasan University Campus, Tiruchirappalli, for his help in drawing the map of the study area.

Conflict of Interests

The authors declare that they have no conflicts of interest to disclose.

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