Comparative phytochemical, anatomical, biological (antioxidant, larvicidal) and chemical profiling of leaf essential oils from *Aegle marmelos* (l.) Corrêa and *Citrus maxima* (burm.) Merr.

A Dissertation for

Course code and Course Title: BOO- Diss & Dissertation

Credits: 8

Submitted in partial fulfilment of Master's Degree

Botany

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DECLARATION BY STUDENT

I hereby declare that the data in this dissertation entitled "**Comparative phytochemical, anatomical, biological (antioxidant and larvicidal) and chemical profiling of leaf essential oils from *Aegle marmelos* (L.) Corrêa and *Citrus maxima* (Burm.) Merr.**" is based on the results of investigations carried out by me in the Botany Discipline at the School of Biological Sciences and Biotechnology, Goa University under the Supervision/Mentorship of **Dr. S. Krishnan** and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations/ experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation entitled "**Comparative phytochemical, anatomical, biological (antioxidant and larvicidal) and chemical profiling of leaf essential oils from *Aegle marmelos* (L.) Corrêa and *Citrus maxima* (Burm.) Merr.**" is a bonafide work carried out by **Ms. Flory Venie Nunes** under my supervision/mentorship in partial fulfilment of the requirements for the award of **degree of M.Sc. in the Discipline of Botany at the School of Biological Sciences and Biotechnology, Goa University**.

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ACKNOWLEDGEMENT

First, I am incredibly thankful to the almighty Lord, who has blessed and guided me. I thank all the people who have guided and helped me in the completion of my dissertation.

I express my sincere gratitude to my guide Dr. S. Krishnan, Professor, Botany discipline, Goa University, for his guidance, encouragement, and motivation rendered to me for the successful completion of this dissertation work.

I thank all the teachers Prof. B.F. Rodrigues, Prof. P. K. Sharma, Prof. Vijaya Kerkar, Dr. Rupali Bhandari, Dr. Siddhi Jalmi, and Dr. Aditi Naik for their advice, help, and support.

I would like to sincerely acknowledge the tremendous support and guidance provided by Dr. Ajeet Kumar Mohanty, Assistant Research Scientist & Officer-In-Charge of the ICMR-National Institute of Malaria Research Field Unit, Health Services Building, Campal, Panaji, Goa, for providing me with the laboratory facilities and guiding me with the mosquito larvicidal studies. I am thankful to each and every member of NIMR for their corporation and willingness to help whenever approached.

I thank Dr. Pranay Morajkar and Dr. Sandesh Budge, School of Chemical Sciences, Goa University, for the GC-MS analysis. I especially thank Research Scholar Mr. Leo Blaise D'Souza for his cooperation and valuable suggestions.

I am very grateful to Research Scholar Ms. Vaishali Gaonkar for being the source of motivation and her constant support in making this project successful.

I owe my sincere thanks to research scholars Mrs. Annie Nadar, Mrs. Divyarani Revankar, Ms. Arti Dabolkar and Ms. Ashwarya Gawade for their help and advice.

I would also like to thank the non-teaching staff of the department for their help and cooperation whenever needed. I also thank all my classmates and friends for their help in completing my work.

I am grateful to my parents for all the love, support, and encouragement during my dissertation work.

FLORY VENIE NUNES

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ABBREVIATION

% - percent

µg - microgram

^{0}C - degree Celsius

A_0 - absorbance of the control

A_1 - absorbance of the extract

cm - centimeter

DPPH - α, α-Diphenyl-β- picrylhydrazyl

etc - Et cetera

eV - electron volt

g - grams

GC-MS - Gas Chromatography-Mass Spectroscopy

GPS - Global Positioning System

hrs - hours

IC_{50} - lethal concentration killing 50%

LC_{90} - lethal concentration killing 90%

LCL - Lower Confidence Limit

m - meter

mg - milligrams

mL - millilitres

mm - millimeter

NIST - National Institute of Standards and Technology

nm - nanometer

No. - number

ppm - parts per million

R - Replicate

ROS - Reactive Oxygen Species

SE - Standard Error

SPSS - Statistical Package for Social Sciences software

TS - transverse section

UCL - upper Confidence Limit

w/v - weight in volume

WHO - World Health Organization

X2 - Chi-square

CHAPTER 1: INTRODUCTION

INTRODUCTION

Plant extracts and secondary metabolites such as essential oils have been recognized as promising compounds in developing novel products, particularly in the agricultural, pharmaceutical, food, and perfumery industries (Gakuubi, 2016). The increasing therapeutic uses of plant essential oils in aromatherapy have urged scientists to investigate their biological potency (Mahomoodally *et al*., 2018). Plants with essential oils belong to broad genera scattered around 60 families (Raut and Karuppayil, 2014). Families such as Rutaceae, Alliaceae, Lamiaceae, Apiaceae, Poaceae, Asteraceae, and Myrtaceae are well known for their potential to produce essential oils of commercial and medicinal value (Hammer and Carson, 2011).

1.1. FAMILY: RUTACEAE

The Rutaceae family, also known as the "Rue family" or "Citrus family," comprises mainly perennial trees (*Aegle*, *Citrus*, *Feronia*), shrubs (*Murraya*, *Limonia*, *Zanthoxylum*) and herbs (*Ruta*, *Boenninghausenia*, *Dictamnus*) frequently with volatile aromatic oils contained in glands visible at the surface of leaves, young branches, inflorescences, flowers, fruit, or seeds. Members of this family have a high percentage of vitamin C and several alkaloids. It comprises about 160 genera and about 2,070 species, of which India contributes 71 species. It is distributed worldwide, especially in warm temperate, and tropical regions. The largest numbers are in Africa and Australia, often semi-arid woodlands (Roy *et al*., 2016).

Rutaceae exhibits stem of woody, erect, solid, cylindrical, branched, gland-dotted, and often with thorns or spines. Leaves are alternate (*Citrus*, *Murraya*) or opposite (*Evodia*), petiolate. Petiole may be winged (*Citrus aurantium*), simple or compound-pinnate (*Murraya*), palmate (*Aegle* and *Citrus*), smooth gland-dotted with essential oils, ex-stipulate, with entire margin or serrate, unicostate reticulate venation. In *Citrus*, the petiole is winged (Groppo *et al*.,

2008). The inflorescence is terminal or axillary cymes or panicles, sometimes racemose or solitary. Flowers are conspicuous for their colour, fragrance, and nectar; they are bisexual or unisexual (*Zanthoxylum*). This aromatic family consists of numerous economically important fruit trees and ornamental species. The essential genus in this family is *Citrus*, which includes *C. sinensis, C. limon, C. paradise, C. aurantifolia, C. latifolia*, and *C. reticulate. Citrus* is not only edible but also rich in vitamin C. Lemon oil is an effective mosquito repellent. *Barosma betulina* produces buchu from its leaves which is helpful in urinary diseases. Leaves of *Alurniya koenigii* are used in flavoring. *Luvunga scandens*, *Ruta, Ptelea, Calodendrum*, and *Murraya* are cultivated in gardens for their fragrant flowers (Hussain, 2021).

1.2. *Aegle marmelos* (L.) Corrêa

It belongs to the Rutaceae family, also known as bilva, bael, golden apple holy fruit, and stone apple. It is considered sacred by Hindus and offered in prayers of deities Lord Shiva and Parvati, thus, the tree is also known by the name Shivaduma (Neeraj and Johar, 2017) **(Plate 1).**

1.2.1. Scientific Classification (Nigam and Nambiar, 2015).

Kingdom : Plantae

Clade : Angiosperms

Order : Sapindales

Family : Rutaceae

Genus : *Aegle* Corrêa

Species : *A. marmelos*

Binomial name: *Aegle marmelos* (L.) Corrêa

1.2.2. Origin and Distribution

Bael is native to India and found throughout Southeast Asia. In India, this fruit is grown in Indo-Gangetic plains and Sub-Himalayan tracts up to 500 m, in North-East India and dry and deciduous forests of central and southern India (Neeraj and Johar, 2017). It is a subtropical plant that grows up to 1,200m from sea level and grows well in dry forests in hilly and plain areas. It is a widely distributed plant found in Ceylon, China, Nepal, Sri Lanka, Myanmar, Pakistan, Bangladesh, Nepal, Vietnam, Cambodia, Thailand, Indonesia, Malaysia, Tibet, Sri Lanka, Philippines, and Fiji (Seema, 2011).

1.2.3. Botanical Description

Bael is the only member of the monotypic genus *Aegle* (Nigam and Nambiar, 2015). *A. marmelos* is a deciduous, slow-growing, medium-sized tree, up to 12-15 m tall, with a short trunk, thick bark, flaking and spreading, and sometimes the lower ones drooping (Sharma and Dubey, 2013). Leaves are composed of 3 to 5 leaflets. The petiole is absent in lateral leaflets, whereas the terminal leaflet has a long petiole 1 to 1.5 inches. The leaflets are ovate to lanceolate, with shallowly toothed margins and reticulate pinnate venation (Nigam and Nambiar, 2015). New foliage is pinkish-maroon and glossy in nature. Mature leaves emit a peculiar fragrance when bruised (Neeraj and Johar, 2017). Flowers are sweetly scented, white in colour, bisexual, actinomorphic, bracteates, hypogynous stalk which holds the flower is 8 mm long. The calyx is five-lobed and gamosepalous. Petals five in number, leathery, imbricate. Stamens polyandrous, with numerous basified and dehiscence longitudinally. Gynoecium has capitate stigma hosting terminal style (Karki, 2022). The fruit is spherical to oval in shape. Shell is woody and hard, showing the presence of minute oil glands that are aromatic. The fruit is green when unripe and yellowish when ripe. Pulp is pale orange in appearance, aromatic, and sweet, resinous, more or less astringent. 10 to 15 seeds are embedded in the fruit pulp and seeds

are flattened-oblong, bearing woolly hairs and each is enclosed in a sac of adhesive, transparent mucilage that solidifies on drying (Sharma, 2013).

1.2.4. Importance and Uses

1.2.4.1. Traditional uses

A. marmelos is considered a sacred tree by the Hindus as its leaves are offered to Lord Shiva during worship. All parts of the tree are used as ethno-medicine to treat several human ailments (Gupta *et al*., 2013). The roots and bark are used to treat dog bites, gastric disorders and fever by preparing a decoction. The unripe dried fruit is astringent and used to cure diarrhea, dysentery and pulp mixed with milk and sugar cures urogenital disorders. An easy treatment for dyspepsia is ripe fruit. The fruit powder of bael produces anti-cancer and anti-proliferative activity (Bhar *et al*., 2018). The bark is used for diarrhea and bone fracture therapy. The leaf portion of the tree is used to treat inflammation, asthma, diabetes, constipation and recurrent diarrhea. The gum around the seed is used to improve the adhesive strength of water paint (Raghuveer, 2018).

1.2.4.2. Nutritional uses

Fruits of the *A. marmelos* are rich in nutritional value. The fruit pulp is a rich source of glucose, protein, vitamin C, vitamin B1 and B2 riboflavin, carotene and contains a good amount of calcium, fibers, minerals and good fats (Behera *et al*., 2014).

1.2.4.3. Products

Wood is used for construction of cart. Stem gum is used for book binding and as an adhesive. Fruit squash, ready-to-serve (RTS) drinks, bael powder, jam, slab, candy and toffee are prepared from fruits (Gurjar *et al*., 2015).

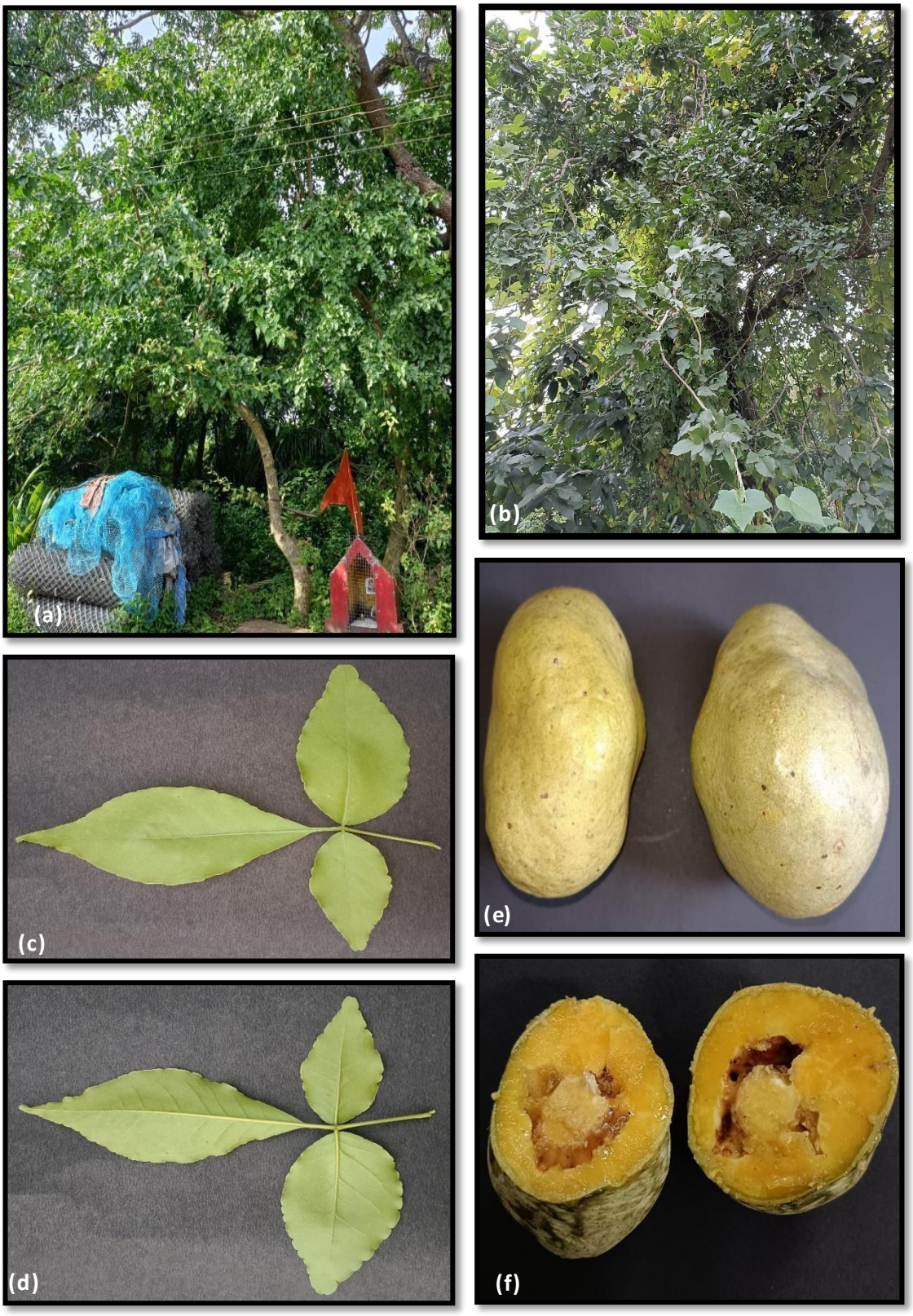


Plate 1: ***Aegle marmelos*** **(a)** Habitat, **(b)** Habit, **(c)** Leaves (adaxial side), **(d)** Leaves (abaxial side), **(e)** Fruits, **(f)** Cut open fruit.

1.3. *Citrus maxima* (Burm.) Merr.

Citrus maxima, means "the biggest citrus". It is a perennial tree commonly known as Pomelo and Shaddock. In Goa, it is locally known as “Toring”. It is commonly consumed and used during festivals throughout Southeast Asia (Kharjul, 2012) **(Plate 2)**.

1.3.1. Scientific Classification

Kingdom : Plantae

Clade : Angiosperms

Order : Sapindales

Family : Rutaceae

Genus : *Citrus*

Species : *C. maxima*

Binomial name: *Citrus maxima* (Burm.) Merr.

1.3.2. Origin and Distribution

C. maxima originated from Southeast Asia. It is widely distributed in India, China, Japan, Indonesia, Malaya, Indonesia, USA, Philippine and Thailand (Ajeet, 2017).

1.3.3. Botanical Description

C. maxima is a medium-sized perennial tree with somewhat crooked trunk. Spines are present on the branchlets and trunk. Leaves with small winged petioles, simple, alternate, ovate-oblong, leathery, entire margin, dotted oil glands, glossy above and dull-green below, 4 to 8 inches long, with acute apex, and characteristic odour (Kharjul, 2012). Flowers are bisexual, sweet smell, borne singly or in clusters of 2 to 10 in the leaf axils in terminal racemes, with 5 petals, dotted with yellow-green glands. Stamens are 16-24 in number, prominent, in bundles of 4 to 5 anthers with orange colouration. Ovary deeply inserted in the disk, stigma broadly

capitate. The fruit is large, pale yellow; rind is thick with prominent oil glands, pericarp spongy, pulp varying in colour from crimson to pale pink or yellow. Seeds are irregular in shape (Vijaylakshmi and Radha, 2015).

1.3.4. Importance and Uses

1.3.4.1. Traditional uses

C. maxima is used as folk medicine in many countries as an antidiabetic, anti-inflammatory, antiplatelet, antimicrobial and hepatoprotective. Fruit juice is nutritive and it is consumed for cholesterol reduction and weight loss. The fruit rind is used as an antiasthmatic, brain tonic, cardiotonic, and useful during vomiting and headaches. Leaves are used to treat stomach pain due to indigestion. Oils from the leaves have antifungal properties. Hot leaf decoction is useful to treat ulcers and swelling (Ajeet, 2017).

1.3.4.2. Nutritional uses

Many species of *Citrus* are cultivated for their fruits which is eaten fresh or processed into juice. The juice contains high amount of citric acid giving its characteristics sharp taste and flavour; they are also a good source of vitamin C and flavonoids. The popularity of *Citrus* juice is certainly due to its pleasant and refreshing flavour (Ajeet, 2017).

1.4. ANATOMY

A plant is a complex structure comprising several parts that constitute the whole plant. Anatomical studies helps in understanding the importance of plant parts to aromatherapists making aware of the part of the plant from which essential oil is derived as there is often a connection between the location of oils in the plant and its therapeutic action (Raut and Karuppayil, 2014). The Rutaceae family is characterized by the presence of secretory cavities, particularly in the leaves and reproductive structures (Groppo *et al*., 2008). Pellucid glands are

Plate 2: ***Citrus maxima*** **(a)** Habitat, **(b)** Habit along with fruits, **(c)** Leaf (adaxial side), **(d)** Leaf (abaxial side), **(e)** Fruit, **(f)** Cut open fruit.

found on the leaves and are responsible for the aromatic smell of Rutaceae family members (Muntoreanu *et al*., 2011).

1.5. PHYTOCHEMISTRY

Phytochemicals are naturally occurring substances found in various plant parts to protect themselves from herbivores but recent research demonstrates that many phytochemicals can protect humans against diseases (Mujeeb *et al*., 2014). The medicinal value lies in the bioactive phytochemical constituents that produce definite physiological action (Akinmoladun *et al*., 2007). Different phytoconstituents present in medicinal plants are flavonoids, carotenoids, alkaloids, anthocyanidins, phenolics and tannins, carboxylic acids, terpenes, amino acids, and inorganic acids (Showmiya and Ananthi, 2018).

A. marmelos contain a number of phytochemical constituents which reveals its uses for various therapeutical purposes and have a great potential to develop Ayurvedic, modern medicines and athletic supplements by pharmaceutical industries (Rahman and Parvin, 2014). It contains a good amount of flavonoids and polyphenols. Polyphenols found in bael depend greatly on the maturity stage of the bael. Its leaves contain a great number of phytochemicals which include rutin, marmeline, marmesinin, aegelin, γ-sitosterol, β-sitosterol, halfordiol, lupeol, glycosides, catechin, flavanols, flavones, lignin, tannins, and isoflavones. Lignan glucoside, 7, 8- dimethoxy-1- hydroxyl2-methyl anthraquinone and 6-hydroxy-1-methoxy-3-methyl anthraquinone compounds have been isolated from the bark (Sarkar *et al.*, 2020).

The bark and root of *C. maxima* contain β-sitosterol, acridone alkaloid. Essential oil from the leaves and unripe fruits contain limonin, nerolol, nerolyl acetate and geraniol. Like other citrus plant C. maxima fruit is rich in Vitamin C. Fruits also contains polyphenolic compound like hesperidin, naringin, caffeic acid, P-Coumaric acid, Ferulic acid and vanillic

acid (Showmiya and Ananthi, 2018). Ethanol extract of leaves showed the active phytochemical classes as alkaloids, amino acids, carbohydrates, carotenoids, coumarins, flavonoids, monoterpenes, sesquiterpenes and steroids (Balamurugan *et al*., 2014).

1.6. ANTIOXIDANT ACTIVITY

Compounds capable of either delaying or inhibiting the oxidation processes that occur under the influence of atmospheric oxygen or reactive oxygen species are antioxidants (Pisoschi and Negulescu, 2012). Free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are known to cause aging-related damage to proteins, enzymes, lipids, and nucleic acids which cause injury to cells or tissues (Mosquera *et al*., 2011).

Catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GPX), beta-carotene, vitamin A, vitamin C, and vitamin E have a broad variety of enzymatic and non-enzymatic antioxidant defenses. Both the behaviours and the intracellular levels of these metabolites are interrelated, shielding themselves from oxygen toxicity (Pisoschi and Negulescu, 2012).

The stable free radical method of DPPH is a simple, fast, and sensitive way of investigating the antioxidant activity of a particular compound or plant extract (Sharmila and Vasundra, 2011). The free-radical method of DPPH creates a violet solution in alcohol and, due to the presence of an antioxidant molecule, changes to a colourless solution. Because of the delocalization of the lone pair of electrons over the molecule as a whole, molecule - α, α-Diphenyl-β- picrylhydrazyl (DPPH) is defined as a stable free radical to avoid dimerization, as would be the case for most other free radicals. The delocalization results in deep violet colour, distinguished by an absorption band centered at about 517 nm in the methanol solution. When

a DPPH solution is combined with a product that may donate a hydrogen atom, due to the still presence of pecryl residue, this gives decreased shape with the transition of the violet colour to the pale colour at the end (Rajan *et al*., 2011).

1.7. ESSENTIAL OILS

Essential oils, or volatile oils as they are often known, have been widely employed in fragrances, flavourings, and medications since the dawn of civilization. It is a mixture of a volatile, aromatic, hydrophobic compound from a specific part of a plant such as leaves, flowers, seeds, rind, or bark, also commonly found in bryophytes, such as liverworts. They are biologically regarded as metabolites of secondary importance to plants. The aqueous solubility of individual components of an essential oil varies. While essential oils are slightly soluble in water, it is commonly predicted that components with more polar functional groups would be more soluble in water compared to other components (Poonkodi *et al*., 2019). Essential oils can play an evident environmental role, such as in fire tolerance, seed dispersal, attraction of pollinators, drought tolerance, or plant-to-plant biosemiotics (Sadgrove and Jones, 2015). They fight infection, containing hormone-like compounds, initiate cellular regeneration, and work as chemical defences against microbial, fungal, and viral diseases (Rao and Pandey, 2007).

In earlier days extraction was carried out using alcohol and a fermentation process leading to alteration in the therapeutic properties of essential oils. The use of CO_2 and Super Critical CO_2, turbo distillation, steam distillation, hydro distillation, and microwave-assisted hydro-distillation are examples of techniques for extracting essential oils (Rao and Pandey, 2007). Different methodologies lead to variations in the composition and yield of the resulting essential oil, both qualitatively and quantitatively (Poonkodi *et al*., 2019).

Chemical characterization of essential oils uses gas chromatography coupled with mass spectrometry (GC-MS). Based on their molecular mass and volatility, this approach identifies the structure of a mixture of organic compounds (Sadgrove and Jones, 2015). The first instrumental element is a gas chromatograph that separates the components of a mixture using a temperature-controlled capillary column and distinguishes the constituents based on the boiling points (volatility) and molecular weights. The high volatility rate component passes from the column earlier, and components with high boiling points and high molecular weights pass later. The mass spectrometer is the second instrumental component. In this segment, each pulse is broken down and offers the pattern of mass fragmentation (mass spectra). Then the mass spectra for structure validation are compared to the available database (Rao and Pandey, 2007).

1.8. CHEMISTRY OF ESSENTIAL OILS

Essential oils are concentrated volatile aromatic compounds produced by plants, easily evaporated essences that give plants their wonderful scents containing over 300 different compounds (Rao and Pandey, 2007). The compounds found in essential oils are from various chemical classes, mostly terpenes. It also contains phenylpropanoids and other compounds in lesser amounts in essential oil (Moghaddam and Mehdizadeh, 2017). Essential oils are hydrocarbons and their derivatives; they may also contain nitrogen or sulphur. They are low molecular weight compounds with limited solubility in water (Başer and Demirci, 2007). They may also exist in form of alcohols, acids, esters aldehydes, ketones and amines (Husnu *et al.*, 2007).

Essential can be classified into two groups (Rao and Pandey, 2007).

Volatile fraction	Non-volatile residue
Essential oil constituting 90–95% of the oil in weight, containing the monoterpene and sesquiterpene hydrocarbons, including their oxygenated derivatives along with aliphatic aldehydes, alcohols and esters.	Comprises 1–10% of the oil, containing hydrocarbons, fatty acids, sterols, carotenoids, waxes, and flavonoids.

1.8.1. Hydrocarbon

Essential Oils consist of chemical compounds that have hydrogen and carbon as their building blocks. The basic hydrocarbon found in plants is isoprene (Rao and Pandey, 2007).

1.8.2. Terpenes

They generally have names ending in "ene." For examples: Limonene, Pinene, Piperene, Camphene, etc. Terpenes are anti-inflammatory, antiseptic, antiviral, and bactericidal. Further, categorized into monoterpenes, sesquiterpenes and diterpenes (Rao and Pandey, 2007).

1.9. MOSQUITO LARVICIDAL ACTIVITY

According to WHO, the vectors responsible for vector-borne diseases such as *Anopheles, Culex* and *Aedes* species are considered the biggest threats to public health (WHO, 2017). Finding suitable ways and tools to control the vector population are essential components and strategies to control the spread of vector-borne diseases. Mosquito-borne diseases such as malaria, dengue, filariasis, chikungunya, etc are major public health problems in Southeast Asian countries because of their tropical or subtropical climate, poor drainage systems,

especially during rainy seasons and the presence of many fish ponds, irrigation ditches and rice fields which provide abundant mosquito breeding places (Das *et al*., 2015). WHO has declared the mosquito as "public enemy number one" because mosquitoes are responsible for the transmission of various disease-causing pathogens (WHO, 1998). Synthetic insecticides have an adverse effect on the ecosystem (Mahanta *et al*., 2017).

Plant-origin products, especially essential oils, have received considerable attention because they are rich sources of bioactive compounds that are effective against the developmental stages of mosquitoes and are naturally biodegradable into non-toxic products (Mahanta *et al*., 2017).

Larviciding is an effective method to reduce mosquito density before they emerge as adults (Osanloo *et al*., 2017). The control of mosquitoes at their larval stage is effective due to their low mobility (Ishtiaq *et al*., 2019).

Anopheles stephensi is responsible for severe malaria outbreaks in urban areas, particularly where there is active construction activity. This is one of the thermophilic species which has a longer flight range and thus maintains a high degree of contact with the human population (Kalra, 1991). It is the main urban mosquito vector of *Plasmodium falciparum* and *Plasmodium vivax* which is a main malaria agent in India (Govindarajan, 2010).

Culex quinquefasciatus the southern house mosquito is considered an "urban bridge vector" which bridges different hosts to humans because of its confrontation with different vertebrates. It is found in tropical and subtropical regions of the world and has been a vector of *Wuchereria bancrafti*, avian malaria and arboviruses including St. Louis encephalitis virus, Western equine encephalitis virus, West Nile virus, etc. Lymphatic filariasis which is caused

by *Wuchereria bancrafti and transmitted by C. quinquefasciatus* can affect the lymphatic system of human beings (Mahanta *et al*., 2017).

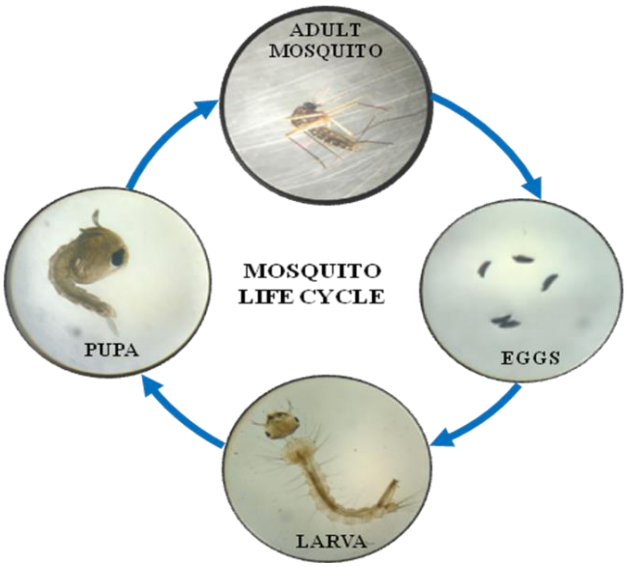


Figure 1: Mosquito life cycle

CHAPTER 2:
REVIEW OF LITERATURE

REVIEW OF LITERATURE

More than 250 types of essential oils are traded in the world market. They are known for volatile nature, and are present in low concentrations in plants specialized structures such as oil cells, glandular trichomes, or resin ducts. The purity of essential oil is determined by its chemical constituents.

The relevant literature on the present study has been briefly reviewed to understand the different aspects of the study carried out.

2.1. ANATOMY

The anatomical studies of vegetative organs of plant species can provide additional data regarding the morphological characteristics, and could thereby help resolve taxonomic issues. *Luvunga crassifolia* is an underutilized plant in the *Citrus* family. Studies showed that *L. crassifolia* leaves are amphistomatic. Pellucid dots were observed on both adaxial and abaxial leaf surfaces. Leaf cross-sections consisted of schizogenous secretory cavities, whereas stem and petiole cross-sections show abundant schizolysigenous secretory cavities. Xylem, phloem, and pericyclic fibers were also found in the leaf, stem, and petiole sections. Calcium oxalates were present in the leaf and stem sections, while non-glandular trichomes were detected on the stem and petiole sections (Shunmugama *et al*., 2021). Leaves of *Z. caribaeum* have secretory structures like glandular trichomes that can function as extra floral nectaries and cavities (Andrade *et al*., 2020).

Anatomical studies on the leaves of *Citrus maxima* (Burm.) Merr. carried out by Kharjul *et al*., (2012) showed the presence of palisade cells, spongy parenchyma cells, vascular bundles, oil glands, abundant anisocytic stomata on both surfaces of leaf and trichomes were uniseriate, thin-walled, multicellular and unlignified. Calcium oxalate crystals were also present in the

parenchymatous cells. Starch grains were present except vascular bundle. Similarly, macro and micro-morphological features of different parts of the plant were investigated by Sedeek *et al*., (2017) wherein transverse sections and powdered forms were used in the study.

Microscopic analysis of *Aegle marmelos* (L.) Correa was analyzed by Bhatti *et al*., (2013) wherein the transverse section of leaves showed the presence of circular, wide secretory cavities sparsely distributed in the ground parenchyma and had lysigenous secretion of volatile compounds. Some of the epidermal cells showed the presence of secretory cells, which produces aromatic oil compounds and non-glandular epidermal trichomes.

2.2. PHYTOCHEMISTRY

Phytochemical constituents are known for their pharmacological and medicinal activities in plant species. The bioactive molecules are studied for various medicinal and pharmacological properties which include anti-cancer, anti-malarial, anti-larval, anti-microbial activities etc. Phytochemical screening of some *Citrus* fruits namely, *C. aurantifolia, C. hystrix, C. maxima, C. reticulata, and C. medica* was carried out, study showed that methanolic and hexane solvents had more phytonutrients being extracted. *C. reticulata* showed the least amount of phytochemicals in all the solvents used (Balamurugan *et al*., 2014).

Acheampong *et al*., (2018) revealed the presence of alkaloids, flavonoids, saponins, tannins and glycosides as phytochemical constituents in the roots of *C. limon.* Bhatti *et al*., (2013) reported the presence of alkaloids, anthraquinones, coumarins, glycosides, tannins and triterpenoids in the ethanol extract of leaves of *A. marmelos*, found to be the most active against the cancer cell lines.

Two alkaloids, N-2-hydroxy-2-(4-methoxyphenyl)-ethylcinnamamide (aegeline), and 4,7,8-trimethoxyfuroquinoline (skimmianine) were isolated from *A. marmelos* and the bioassays of the isolated compounds were carried out against some microbes and cancer cell lines (Riyanto *et al*., 2001).

Phytochemical constituents and antimicrobial activity of an ethanolic extract of leaves *A. marmelos* were investigated (Venkatesan, 2009; Kothari *et al*., 2011). Quantitative phytochemical analysis of the fruit pulp of *A. marmelos* was carried out by Rajan *et al*. (2011).

Ethanolic extract of *C. maxima* leaves were investigated for phytochemical analysis by Showmiya and Ananthi (2018) revealed the presence of alkaloids, steroids, flavonoids, phenolic compounds, proteins, carbohydrates, cardiac glycosides and saponins also FTIR spectroscopic studies were carried out. Preliminary phytochemical screening and in vitro antibacterial activity of leaf extract of *C. maxima* in Odisha was studied by Prusty and Patro (2014). Similarly, tests with peels of the fruit were carried out by Mehmood *et al*. (2015) in Malaysia.

2.3. ANTIOXIDANT ACTIVITY

Rajan *et al*. (2011) worked on *A. marmelos* fruit pulp alcoholic extract and water extract. Both the extract showed good antioxidant power with IC_{50} value ranges for 37.11± 3.50 to 158.99 ± 59.46 µg/mL for aqueous extract; 35.02 ± 8.10 to 283.06 ± 135.80µg/mL for alcoholic extract

A. marmelos pulp ethanolic extract showed higher DPPH free radical scavenging activity followed by the methanolic and aqueous extract. This high scavenging property of ethanolic extract may be due to the hydroxyl group existing in the phenolic compound's

chemical structure that can provide the necessary component as a radical scavenger (Tagad *et al*., 2018).

Rajan (2011) stated that fruit pulp extract of *A. marmelos* showed good antioxidant power with IC_{50} value ranges from 37.11± 3.50 to 158.99±59.46 µg/mL for aqueous extract and 35.02±8.10 to 283.06±135.80 µg/mL for alcoholic extract. Likewise, Perumal *et al*. (2018) evaluated the antioxidant activity of the ethanolic extract of leaves; the IC_{50} of the DPPH radical scavenging assay was 78.36 µg/mL.

2.4. ISOLATION AND ANALYSIS OF ESSENTIAL OIL

Osanloo *et al*. (2017) extracted the essential oil of *Kelussia odoratissima* Mozaff., its major component Z-ligustilide was evaluated against *Anopheles stephensi,* total of thirty compounds of essential oil were identified by GC-MS analysis. At a concentration of 20 ppm or higher perfect larvicidal activity was observed.

Essential oils from leaves and fruits of *Alseodaphne semecarpifolia* Nees. from the Western Ghats of India showed variation in their chemical composition. Comparative results showed the presence of Camphene, exo-fenchol, borneol, α -terpineol, (E)- β-farnesene, β -copen-4- α -ol, and muurola-4,10(14)-dien-1-ol, were not detected in leaf oil but were present in the fruit essential oil, while α -thujene, α -elemene, germacrene D, (E)-nerolidol, and (E)-phytol were absent in fruit oil but present in the leaf oil (Verma *et al*., 2016). A similar comparative study between leaf and fruit rind essential oil of *C. maxima* was carried out in Coastal Karnataka, India by Prasad *et al*. (2016) wherein citronellol was the major component in leaves and D- limonene in the fruit rind, the two different parts found to have distinguished chemical composition, though both are from the same botanical origin.

Extraction and chemical characterization of essential oil from fruits of *Toddalia asiatica* (L.) Lam. and leaves of *Eucalyptus camaldulensis* Dehnh. was carried out by Gakuubi (2016), results obtained revealed that both oils were rich in monoterpenes and sesquiterpenes, both oils contained α-Pinene, β-Pinene, Myrcene, (E)- β-Ocimene and (E)-Caryophyllene.

Das and Swamy (2013) compared the volatile oil composition of three *Atalantia* species, namely *A. monophylla, A. wightii and A. racemose.* It was seen that the main compound in all three species was Asarone which has a neuroprotective effect and hypolipidemic activity.

Jiang *et al*. (2011) compared GC-MS analysis of essential oils extracted by three methods from the buds of *C. aurantium.* From Steam distillation extraction the extract yield was 0.16% and the main compounds were terpinen-4-ol (20.98%), dipentene (11.67%), terpinene (9.24%). From reflux extraction 2.18% yield was obtained having palmitic acid (20.61%), 2-chloroethyl linoleate (14.54%), tetracosane (12.26%), and α-linolenic acid (11.24%) was main compounds and the third method ultrasound-assisted extraction showed 2.34% yield having tetracosane (11.32%), heneicosane (11.06%), and palmitic acid (8.76%) as main compounds. It is concluded that different extraction methods may lead to different yields of essential oils; the choice of the appropriate method is very important to obtain more desired components with higher physiological activities.

Mahomoodally *et al*. (2018) reported the phytochemical composition of the essential oil of *A. marmelos* using GC-MS analysis. Anti-bacterial, anti-oxidant and enzyme-inhibitory activities were also carried out. Results showed promising inhibitory activities against vital enzymes related to diabetes, neurological and skin-related problems and mild anti-oxidant ability, thus highlighting the quenching abilities of surplus reactive oxygen species related to these disorders. Similar studies were carried out by Singh *et al*. (2010) wherein essential oils of

leaves of *C. maxima* and *C. sinensis* exhibited anti-oxidant activity; it was found that both were non-toxic to mammals.

Sun *et al*. (2014) stated that UV and sunlight could significantly affect the aroma of the *C. maxima* essential oil, providing valuable information that will benefit the production and storage of essential oil-based aromatic products. It was observed that sunlight-exposed essential oil was off-flavour, and off-odour due to the existence of linalool oxides and limonene oxides as well as lack of neral and geranial. UV significantly affected the aroma of essential oil.

2.5. MOSQUITO LARVICIDAL ACTIVITY

Das *et al*. (2015) reported a comparative account of the larvicidal efficiency of the plants of the genus *Atalantia*. Leaf essential oils of *A. monophylla, A. racemose and A. wightii* was tested and chemical composition was analyzed by GC-MS method. *A. racemosa* showed maximum activity against the three selected mosquito species namely *Culex quinquefasciatus, Anopheles stephensi* and *Aedes aegypti.* A similar study was conducted by Govindarajan (2010) using essential oils from the leaves of *Clausena anisate* which exhibited significant larvicidal activity, and was most effective against *Anopheles stephensi* followed by *Aedes aegypti* and *Culex quinquefasciatus*.

Peel and leaf essential oil of *Citrus aurantifolia* possess more ovicidal activity of LC_{50} value of 5.26 ppm and 17.71 ppm for leaf and peel oil respectively at 72 hrs than larvicidal activity. As larvicide, the essential oil from the peel of *C. aurantifolia* showed an immediate effect with LC_{50} value of 128.81 ppm at 24 hrs which reduced to 106.77 ppm at 72 hrs while the leaf oil showed a slow effect with LC_{50} value of 188.59 ppm, 107.37 ppm and 104.59 ppm at 24 hrs, 48 hrs and 72 hrs respectively (Sarma *et al*., 2019).

Similar results were seen in the case of essential oil of peel and leaves of *C. maxima* where it was most effective as ovicides having LC_{50} value 14.02 ppm in the case of essential oil of leaves and 17.06ppm in the case of that of peel at 72 hrs followed by larvicidal activity with LC_{50} value 18.53ppm and 40.59ppm at 72 hrs respectively (Mahanta *et al*., 2017).

CHAPTER 3: OBJECTIVE

OBJECTIVES

Mosquitoes are known to cause more diseases than any other arthropod group. They are controlled by the continued use of traditional, toxic, synthetic insecticides worldwide. This has resulted in the investigation of natural insecticides as an alternative control, focused on plant-derived compounds, including volatile chemical constituents (essential oils), as potentially bioactive substances against mosquito larvae. Essential oils exhibit broad-spectrum anti-insect activity and have received much attention as potentially useful bioactive compounds that play an important role in protecting plants from pathogens and herbivores. The majority of plants in the Rutaceae family are economically valuable. While *Aegle marmelos* and *Citrus maxima* are members of the Rutaceae family, many are unaware of their economic value. *Aegle marmelos* possesses great mythological importance. Both herbs have considerable therapeutic value in Ayurveda and other traditional medicine systems, but are not being used to their full potential. The present study was carried out to understand the anatomical characterization of the plant species along with the phytochemical investigation and antioxidant activity. The chemical composition of essential oils was studied to know the main compounds responsible for the aromatic nature, along with the larvicidal investigation to understand their biological activity.

The specific objectives of this study are as follows:

1. Anatomical characterization of selected plant species.
2. Preliminary Phytochemical analysis and antioxidant activity of selected plant species.
3. Extraction and Screening of chemical profile of leaf essential oils from selected plants.
4. Determination of larvicidal activity against larvae of *Anopheles stephensi* and *Culex quinquefasciatus*.

CHAPTER 4: MATERIALS AND METHODS

MATERIALS AND METHODS

4.1. COLLECTION OF PLANT SAMPLES

The Fresh plant samples of *Aegle marmelos* and *Citrus maxima* were collected from the natural habitat of Anjuna and Caranzalem, Goa, in early August 2022 during fruiting periods. Detail of collection site is mentioned in table 1. Mature and healthy leaves, stems and fruits were collected each in separate zip-lock polythene bags and brought to the laboratory for further studies. Plants were authenticated using Flora.

Table .1 Plant Collection Sites and their GPS Coordinates

Sr. No.	Name of the Plant	Collection Site	GPS Coordinates
1.	*Aegle marmelos* (L.) Corrêa	Anjuna, Bardez, Goa	15°35'19.1" N 73°45'41.5" E
2.	*Citrus maxima* (Burm.) Merr.	Caranzalem, Taleigao, Goa	15°27'59.0" N 73°48'31.8" E

4.2. ANATOMICAL STUDIES

Anatomical studies of plant species were carried out to understand the structure of the leaf, petiole and stem. Fresh leaf, petiole and stem of *A. marmelos* and *C. maxima* were brought to the laboratory. Free-hand thin sections of all the above parts were taken and stained with 0.1% safranin stain. Then the sections were thoroughly washed with distilled water and mounted on the glass slide using 10% glycerine. Sections were observed and photographed using bright-field microscopy. The following standard procedure was adopted for the preparation of stains.

Safranin: 0.1% w/v Safranin was prepared by dissolving 0.1g of safranin in 100 mL of distilled water. Thin free-hand sections were placed in distilled water and then placed in the stain for 2-

3 minutes and the excess stain was removed by washing the sections in distilled water. Then the sections were mounted on a clean stain-free glass slide using 10% glycerine and observed using a bright-field Nikon Eclipse E200 microscope under 4X, 10X and 40X magnification.

4.3 QUALITATIVE PHYTOCHEMICAL ANALYSIS

4.3.1 Extraction of plant samples for analysis (sample preparation)

Fresh and healthy, leaves and stems of *A. marmelos* and *C. maxima* were thoroughly washed with distilled water. The stem was chopped into small pieces and, both the leaves and stem were shade dried at room temperature for 14 days and later powered using a grinder and stored in an airtight container for further analysis. The fruits were washed separately then peeled off carefully with the help of a sharp knife to avoid any damage to oil glands.

Ten g of leaf and stem power of each plant sample was extracted in 150mL solvent for 24 hours using the Soxhlet extraction method using four different solvents: methanol, ethyl acetate, chloroform and water. The extract was later filtered using Whatman No. 1 filter paper and concentrated using a Rotary evaporator. The dried solvent-free extracts were stored at 4^{o}C for further analysis.

4.3.2. Phytochemical tests

The extracts obtained with different solvents were used for the prelim

inary qualitative estimation, which was carried out according to the methods described by Raman (2006).

1. Detection of alkaloids

50mg extract is stirred with a few mL of dilute HCl and filtered. The filtrate is tested carefully with various alkaloidal reagents as follows:

a. Mayer's test

To a 1mL of filtrate, add a drop of Mayer's reagent by the side of the test tube. A white or creamy precipitate indicates the test as positive.

Mayer's reagent: 1.358g mercuric chloride is dissolved in 60mL of water and 5.0g potassium iodide is dissolved in 10mL of water. The two solutions are mixed and made up to 100mL with water.

b. Wagner's test

To a 1mL of filtrate, a few drops of Wager's reagent are added by the side of the test tube. A reddish-brown precipitate confirms the test as positive.

Wager's reagent: 1.27g iodine and 2g potassium iodide are dissolved in 5mL of water and made up to 100mL with distilled water.

c. Hager's test

To a 1mL of filtrate, 2mL of Hager's reagent (saturated aqueous solution of picric acid) is added. A prominent yellow precipitate indicates the test is positive.

2. Detection of carbohydrates and glycosides

100mg extract is dissolved in 5mL of water and filtrated. The filtrate is subjected to the following tests.

a. Molish's test

2mL of filtrate, two drops of alcoholic solution of α-naphthol are added, the mixture, the mixture is shaken well and add 1mL of concentrated sulphuric acid is added slowly along the sides of the test tube and allowed to stand. A violet ring indicates the presence of carbohydrates.

b. Barfoed's test

To 2mL of filtrate, 1mL of Barfoed's reagent is added and heated on a boiling water bath for 2 minutes. Red precipitate indicates the presence of sugar.

Barfoed's reagent: 1.33g copper acetate is dissolved in 20mL of distilled water and 0.18mL glacial acetic acid is added.

c. Benedict's test

To 0.5mL of filtrate, 0.5mL of Benedict's reagent is added. The mixture is heated in a boiling water bath for 2 minutes. A characteristic coloured precipitate indicates the presence of sugar.

Benedict's reagent: 4.325g sodium citrate and 2.5g sodium carbonate are dissolved in 20mL of distilled water and boiled to make it clear. 3.46g copper sulphate dissolved in 20mL distilled water is added to it.

For detection of glycosides

50mg of the extract is hydrolyzed with concentrated HCl for 2 hours on a water bath, filtered and hydrolysate is subjected to the following tests.

d. Borntrager's test

To 2mL of filtrated hydrolysate, 3mL of chloroform is added and shaken, the chloroform layer is separated and 10% ammonia solution is added to it. Pink colour indicates the presence of glycosides.

e. Legal's test

50mg of the extract is dissolved in pyridine, sodium nitroprusside solution is added and made alkaline using 10% sodium hydroxide. The presence of glycoside is indicated by pink colour.

3. Detection of saponins

50mg of the extract is diluted with distilled water and made up to 20mL. the suspension is shaken in a graduated cylinder for 15 minutes. A 2 cm layer of the form indicates the presence of saponins.

4. Detection of proteins and amino acids

100mg of the extract is dissolved in 10mL of distilled water and filtered through Whatman No. 1 filter paper and the filtrate is subjected to tests for proteins and amino acids.

a. Biuret test

2mL of filtrate is treated with one drop of 2% copper sulphate solution. To this 95% ethanol is added, followed by excess of potassium hydroxide pellets. Pink colour in the ethanolic layer indicates the presence of proteins.

b. Ninhydrin test

2 drops of ninhydrin solution (10mg of ninhydrin in 200mL of acetone) are added to 2mL of aqueous filtrate. A characteristic purple colour indicates the presence of amino acids.

5. Detection of phytosterol

Libermann-Burchard's test

50mg extract is dissolved in 2mL acetic anhydride. To this, 2 drops of concentrated sulphuric acid are added slowly along the side of the test tube. An array of colour changes shows the presence of phytosterols.

6. Detection of sterol

To 1mL of the filtrate add 2mL of chloroform and 3mL of concentrated sulphuric acid. A Blue-green ring appears at the junction showing the presence of sterol.

7. Detection of fixed oils and fats

Saponification test

Two drops of 0.5N alcoholic potassium hydroxide solution is added to a small quantity of extract along with a drop of phenolphthalein. The mixture is heated on the water bath for 2 hours. The formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

8. Detection of phenolic compounds and tannins

a. Ferric chloride test

50mg extract is dissolved in 5mL of distilled water. To this, a few drops of neutral 5% ferric chloride solution are added. A dark green colour indicates the presence of phenolic compounds.

b. Gelatin test

50mg extract is dissolved in 5mL of distilled water and 2mL of 1% solution of gelatin containing 10% sodium chloride is added to it. White precipitate indicates the presence of phenolic compounds.

c. Lead acetate test

50mg of the extract is dissolved in distilled water and to this; 3mL of 10% lead acetate solution is added. A bulky white precipitate indicates the presence of phenolic compounds.

9. Detection of Quinones

To 1mL of extract add 1mL of concentrated sulphuric acid. The red colour indicates the presence of Quinones.

4.4. ANTIOXIDANT ACTIVITY

The antioxidant capacity of the plant extract depends upon the composition of the extract and the conditions of the test system (Tagad *et al*., 2018). The antioxidant studies were carried out on leaves, stem, fruit pulp and rind of *A. marmelos* and *C. maxima* using α, α-diphenyl-β-picrylhydrazyl (DPPH) method.

α, α-diphenyl-β-picrylhydrazyl (DPPH) method: The antioxidant studies were assessed using α,α-diphenyl-β-picrylhydrazyl (DPPH) method on leaves, stem, fruit pulp and rind of *A. marmelos* and *C. maxima*.

4.4.1. Preparation of DPPH

24mg of DPPH was dissolved in 100mL of methanol to prepare the stock solution and stored in amber- coloured bottle in dark. The working solution was prepared by adding 10mL of stock solution in 45mL methanol.

4.4.2. Preparation of test solution

10mg of methanolic extract of stem, leaf fruit pulp and fruit rind were dissolved in 10mL of methanol. Serial dilutions were performed to prepare concentrations (12.5µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, and 200 µg/mL).

4.4.3. Preparation of L-ascorbic acid solution

10mg of ascorbic acid was dissolved in 10mL methanol. Serial dilution was carried out to prepare concentrations (12.5µg/mL, 25µg/mL, 50µg/mL, 100µg/mL, and 200µg/mL).

4.4.4. Preparation of control

In 3mL of DPPH, 250µl of distilled water was used as a control. In the reaction mixtures, 3mL of DPPH working solution was added to 250µL of leaf extract of different concentrations and incubated in the dark for 30 minutes at room temperature. The absorbance was measured at 517nm.

The following equation calculated the percent inhibition.

$$\textbf{Inhibition \%} = (A_0 - A_1)/A_0 \times 100$$

$\mathbf{A_0}$ is the absorbance of the control

$\mathbf{A_1}$ is the absorbance of the extract

The antioxidant assay was performed in triplicate, and the mean value was expressed. IC_{50} value was calculated by plotting percent (%) inhibition against the concentration (µg/mL) of the extract.

4.5. ISOLATION OF ESSENTIAL OIL

Fresh leaves of both aromatic plants were cut separately into small pieces with Secateur. 200g of each plant samples and 1500mL of distilled water were subjected to hydro-distillation method using a Clevenger- type apparatus with 5L capacity for 3 hours at 70°C. The extraction of essential oil from its aqueous phase was obtained using n-hexane. The extract was further dried over anhydrous sodium sulphate and stored in amber vials at 4°C until further analysis.

Essential oil yield was calculated using formula:

$$\textbf{Yield (\%)} = \frac{\textbf{Volume of oil extracted (mL)}}{\textbf{Weight of the sample taken}} \times 100$$

4.6. GAS CHROMATOGRAPHY-MASS SPECTROMETRY ANALYSIS

The essential oil was analysed by the GC-MS method. The sample was prepared by using 100µL of essential oil diluted in 2.5mL of n-hexane. Two Restek-Rxi columns were used as stationary phase. First column Restek-Rxi-5-MS (30m length x 250µ internal diameter x 0.25µ film thickness). Second column Restek- Rxi-17-silms (1.590m length x 250 µ internal diameter x 0.25µ film thickness). The carrier gas was helium at a flow rate of 1.0mL min^{-1}. The injection volume was 0.5µL in splitless injection mode. The temperature was programmed from

an initial 60°C fixed for 10 minutes, then, increased at 4°C/min till reach to 220°C held for 10 minutes to a final temperature of 240°C hold time for 5 minutes. The injection port temperature was set at 250°C. The detector temperature was maintained at 250°C with a solvent delay of 260 seconds. The ionizing voltage used was 70eV. Compound identification was accomplished by comparing the retention times with those of authentic compounds as well as the mass spectra in the NIST spectral library stored in computer software.

4.7. MOSQUITO LARVICIDAL ACTIVITY

4.7.1. Source of mosquito larvae

Mosquito larvae of *Culex quinquefasciatus* and *Anopheles stephensi* were screened for larvicidal activity. The ICMR-National Institute of Malaria Research, Campal, Panjim, Goa maintained the cyclic colony of these two mosquito species in their insectary. 3rd instar larvae were used for the bioassay. Fish flake was used in the laboratory as food for maintaining mosquito larvae.

4.7.2. Larval Bioassay

Detection of susceptibility of larvae to the essential oil of *Aegle marmelos* and *Citrus maxima* leaves- 25 larvae of each species were selected and placed in separate plastic bowls containing 250mL distilled water. The essential oil was dissolved in Dimethyl sulfoxide (DMSO) to prepare various concentrations (20ppm, 40ppm, 100ppm and 120ppm). The control was prepared using 25µL of DMSO in 250mL of distilled water. For each concentration, three replicates were maintained. Larval mortality was assessed by direct observation of larvae movements after 24 and 48 hours after exposure at room temperature.

The percentage mortality was calculated using the following formula:

$$\textbf{Percentage mortality} = \frac{\textbf{Average of dead larvae}}{\textbf{Total number of larvae exposed/treated}} \times 100$$

4.7.3. Calculation of LC_{50} and LC_{90} Value

The activity of the essential oil against test mosquito larvae in terms of LC_{50} and LC_{90} value was calculated using SPSS software and associated 95% fiducial limits i.e. upper and lower confidence limits.

CHAPTER 5:
RESULTS
AND
DISCUSSION

RESULTS AND DISCUSSION

5.1. ANATOMICAL STUDIES

5.1.1 *Aegle marmelos* (L.) Corrêa

5.1.1.1. Leaf Anatomy

The leaf was dorsiventral and smooth with a prominent midrib. The transverse section of the lamina showed the presence of a single-layered epidermis with a thin layer of cuticle. Epidermal cells were thick-walled and rectangular-shaped. The abaxial epidermis is comparatively thinner than the adaxial side. The lower side of the epidermis consists of anomocytic stomata. The mesophyll tissue is differentiated into palisade cells and spongy tissue. Below the adaxial epidermis, the palisade cells were in 3-4 layers, made up of compactly arranged elongated cells. The spongy tissue is loosely arranged in parenchyma cells. A large number of secretory cavities are seen in the mesophyll tissue. The vascular system consists of a plano-convex cylinder, which consists of an upper flat part and a lower bowl-shaped. The xylem elements were thick-walled and present in a radial arrangement similar to that observed by Bhatti *et al*. (2013) **(Plate 3).**

5.1.1.2. Petiole Anatomy

The transverse section of the petiole showed the presence of the epidermis layer, which is made up of rectangular parenchyma cells. The surface was thinly cuticularized. Trichomes were present on the surface of the petiole. Single-layered hypodermis was observed below which 3-5 layers of chlorenchymatous tissue were present. Secretory cavities (oil glands) are present along the cortical region close to the epidermis. Vascular bundles are collateral. Phloem patches are arranged in circular form whereas the xylem occurs as long parallel xylem elements. Pith is made up of parenchymatous cells **(Plate 4: a, b and c)**.

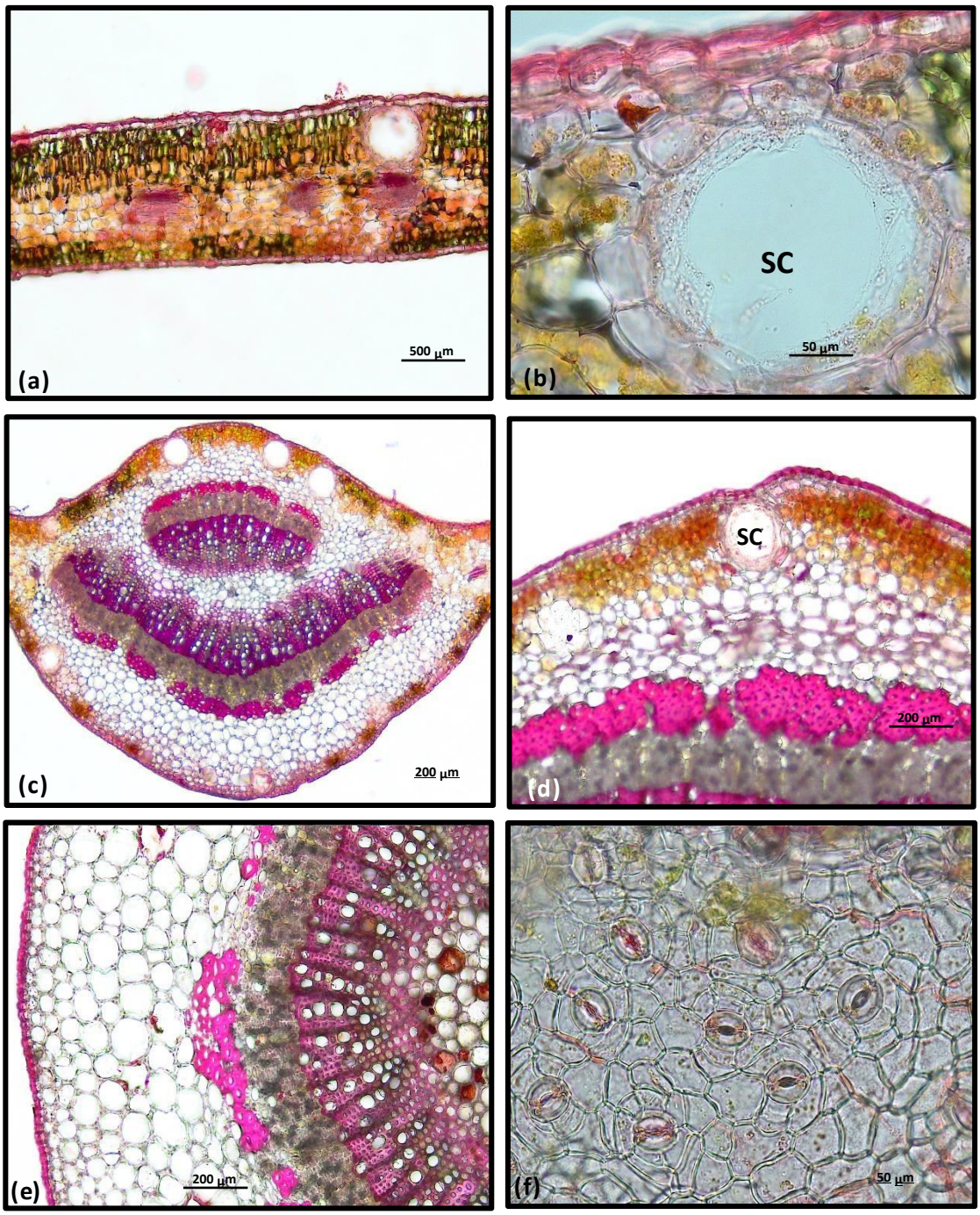


Plate 3: Anatomy of *A. marmelos* leaf. (a) Overview of leaf (4X), **(b)** T.S of leaf showing secretory gland and epidermal layer (40X), **(c)** Overview of leaf midrib (4X), **(d)** T.S of leaf mi drib showing presence of secretory gland (10X), **(e)** Section of leaf showing cortex and vascular cells (10X), **(f)** Leaf showing anomocytic stomata. SC=Secretory gland (40X).

5.1.1.3. Stem Anatomy

The stem of *A. marmelos* is elliptical in shape. Single-layered epidermis with parenchymatous rectangular cells was observed. Cuticle was absent; however, trichomes were present on the surface of the stem. Towards the periphery secretory glands embedded in the cortex region were noted wherein 8-10 layers of cortex were present. The arrangement of vascular bundles was similar to that of the petiole. A large parenchymatous pith was present in the center **(Plate 4: d, e and f)**.

5.1.2. *Citrus maxima* (Burm.) Merr.

5.1.2.1. Leaf Anatomy

The transverse section of the leaf of *C. maxima* showed an epidermis covered by a thick layer of cuticle. The lower layer of the epidermis shows the presence of trichomes and anisocytic stomata. Mesophyll tissue is differentiated into palisade tissue and spongy parenchymatous tissue. 3-4 layers of compact palisade parenchyma tissue showed the presence of oil glands arranged close to the epidermal layer. The spongy layer consisted of round, loosely arranged cells **(Plate 5)**.

5.1.2.2. Petiole Anatomy

The petiole of *C. maxima* has a winged outline. The anatomical structure is similar to that of the leaf however petiole showed a wider cortex region **(Plate 6: a, b and c)**.

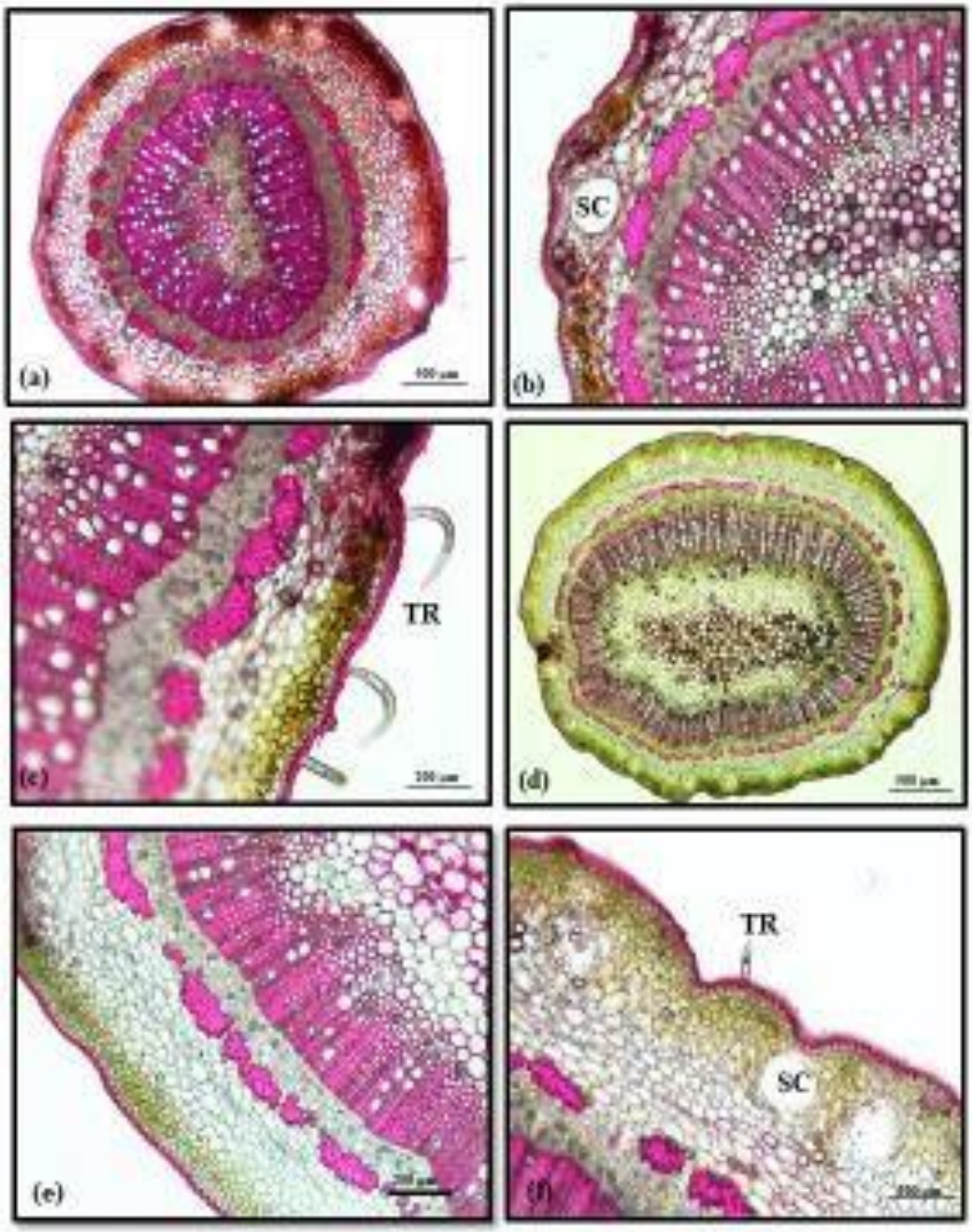


Plate 4: Anatomy of *A. marmelos* petiole and stem. (a) Overview of petiole (4X), (b) Section of petiole showing presence of secretory gland along with cortex and vascular cells (10X), (c) Petiole showing presence of trichome (40X), (d) Overview of stem (4X), (e) Section of stem showing cortex and vasculature (10X), (f) T.S of stem showing secretory glands and trichome (40X). SC=Secretory gland, TR=Trichome.

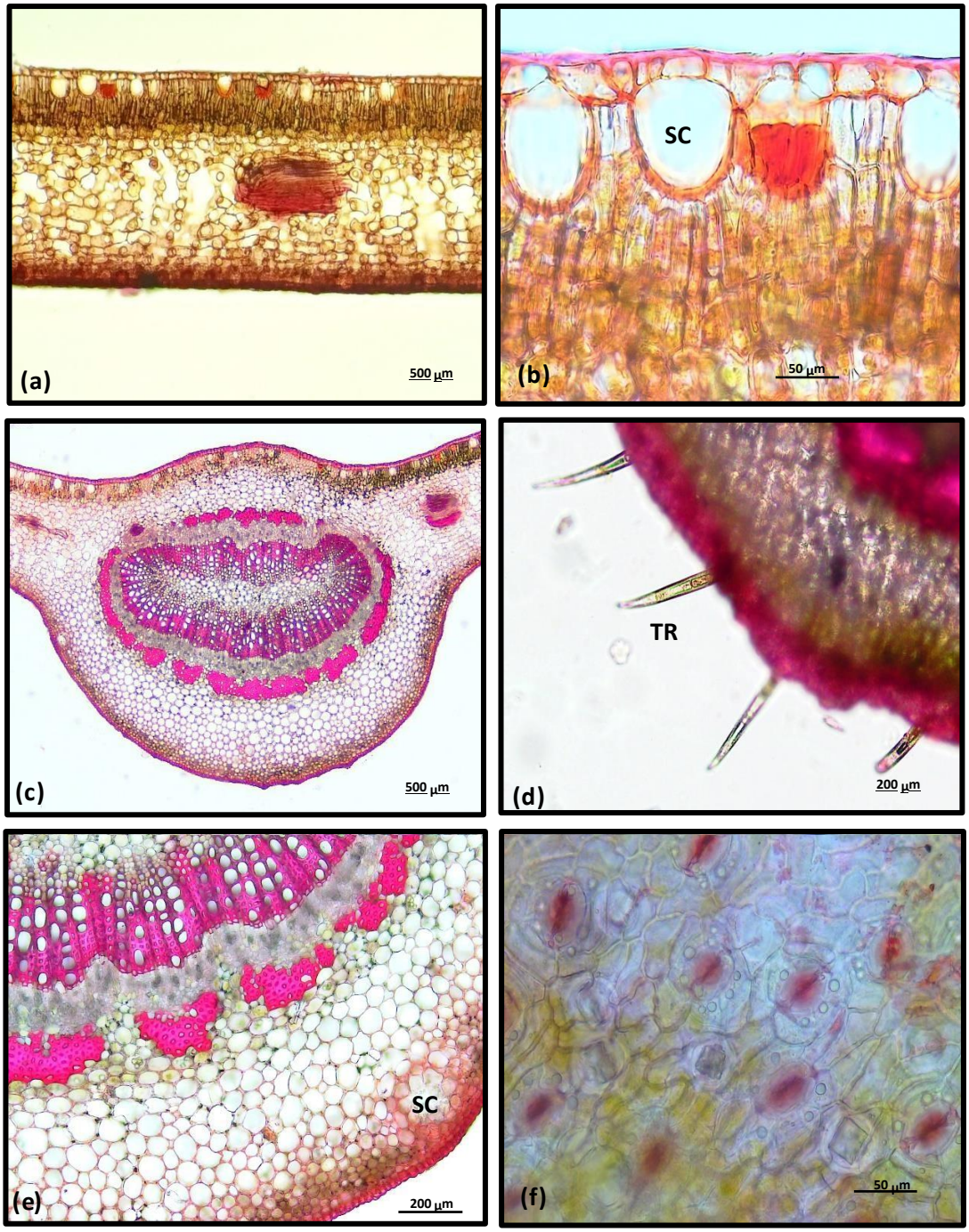


Plate 5: Anatomy of *C. maxima* leaf. (a) Overview of leaf (4X), **(b)** T.S of leaf showing secretory glands and epidermal layer (40X), **(c)** Overview of leaf midrib (4X), **(d)** Midrib region showing presence of trichomes (40X), **(e)** Section of leaf showing secretory gland and cortex cells (10X), **(f)** Leaf epidermis showing anisocytic stomata (40X). SC=Secretory gland, TR=Trichome.

5.1.2.3. Stem Anatomy

Stem is angular in shape. The single-layered epidermis consists of polygonal cells with straight thin cellulosic anticlinal walls, covered by a cuticle, showing unicellular, non-glandular hairs similar to the observations reported by Mohammed *et al*. (2016). The cortex consists of 10-12 rows of thin-walled parenchyma cells containing oil glands. Phloem was present in patches whereas the xylem was seen to be radially arranged. Pith consists of round parenchyma cells **(Plate 6: d, e and f)**.

Similar results were reported by Aswini *et al.*, (2012) where *C. maxima* leaf exhibited the presence of oil glands, trichomes, and an abundance of anisocytic stomata on both surfaces of the leaf. In addition, calcium oxalate crystals and starch grains were found in parenchyma cells. Similar results were also recorded by Mohammed *et al*., in 2017 in the anatomy of leaf, he further carried out anatomical studies on stem and petiole. Stem showed presence of schizolysigenous glands and crystals of calcium oxalate. He also observed unicellular, non-glandular hairs and anomocytic stomata in stem section. Petiole section was similar to that of leaf.

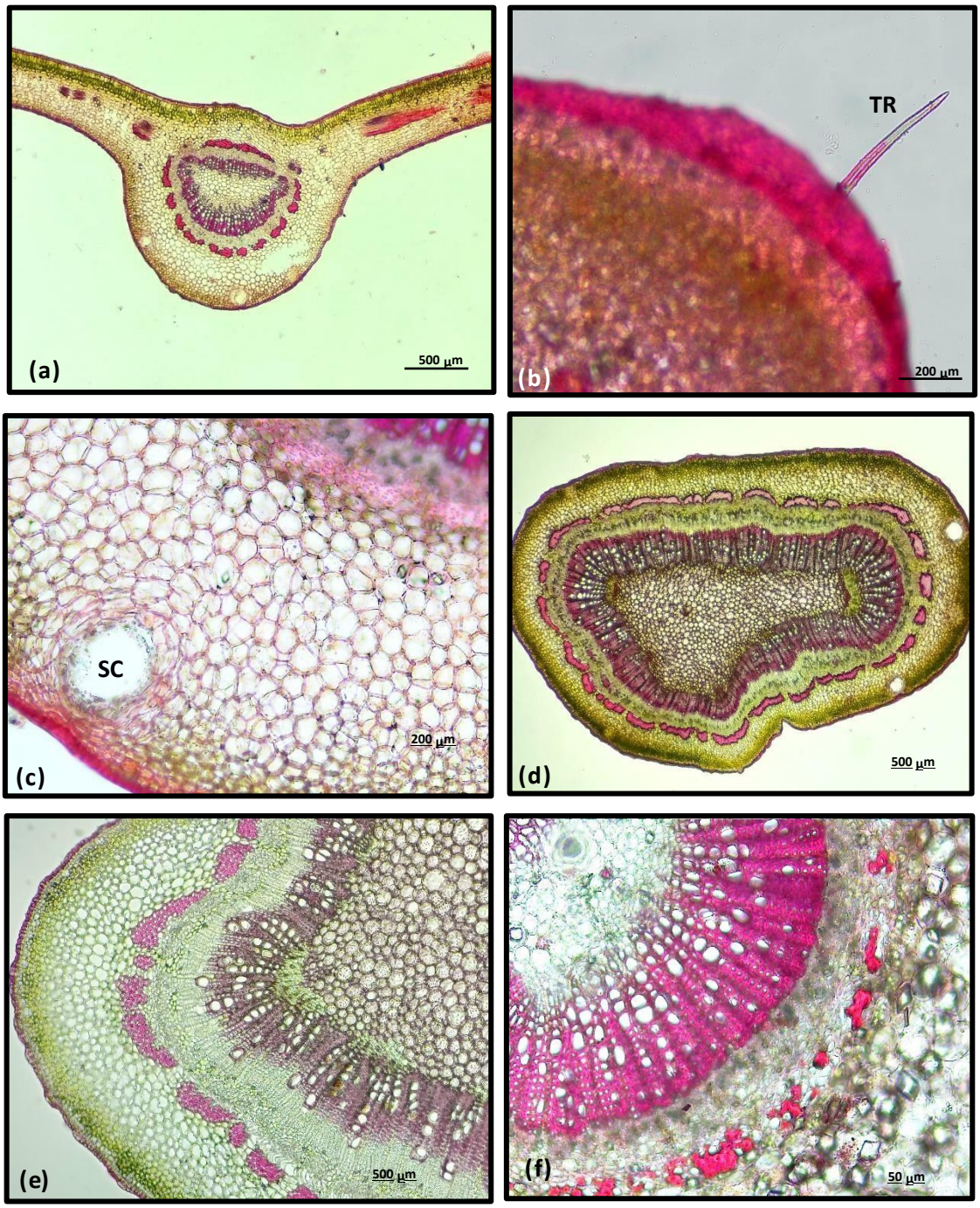


Plate 6: Anatomy of *C. maxima* petiole and stem (a) Overview of petiole (4X), **(b)** Petiole showing presence of trichome (40X), (**c)** Section of petiole showing presence of secretory gland (10X), **(d)** Overview of stem (4X), **(e)** Section of stem showing cortex cells and vasculature (10X), **(f)** T.S of stem showi ng vascular region (40X). SC=Secretory gland, TR=Trichome.

5.2. QUALITATIVE PHYTOCHEMICAL ANALYSIS

The stem and leaves of *A. marmelos* and *C. maxima* were subjected to qualitative phytochemical analysis, which confirmed the existence of bioactive components. Water, methanol, ethyl acetate, and chloroform were utilized as solvents in the extraction. The polarity of the solvent, in the order water>methanol >ethyl acetate>chloroform, is critical to extraction yield.

Alkaloids, carbohydrates, glycosides, saponins, proteins, sterols, phytosterols, fixed oils and fats, phenolic compounds and tannins, and quinones were all detected using various techniques. The use of various solvents resulted in various outcomes. The majority of the phytochemical contents were found in methanol extracts of the stem and leaves of *A. marmelos* and *C. maxima*, followed by water extracts. Ethyl acetate and chloroform extracts contained very few phytochemicals.

The presence of alkaloids, carbohydrates, glycosides, saponins, proteins, sterols, phytosterols, fixed oils and fats, phenolic compounds and tannins, and quinones was detected in the water and methanol extracts of *A. marmelos* stem. The ethyl acetate extract contained carbohydrates, sterols, phytosterols, and quinones. The chloroform extract of the *A. marmelos* stem contained only two phytochemicals, phytosterols and quinones **(Table 2; Plate 7)**. The water extract and methanol extract of *C. maxima* stem yielded similar results. The chloroform extract of the *C. maxima* stem contained sterols and phytosterols. In the case of the stems of both plants, all of the solvents yielded good findings for phytosterols **(Table 3; Plate 8)**.

Preliminary phytochemical analysis of *A. marmelos* and *C. maxima* leaves in water and methanol extract yielded positive results for alkaloids, carbohydrates, glycosides, saponins, proteins, sterols, phytosterols, fixed oils and fats, phenolic compounds and tannins, and

quinones. However, polysaccharides, saponins, sterols, fixed oils, and lipids were found in ethyl acetate extracts of *A. marmelos* and *C. maxima* leaves. The chloroform extract of *A. marmelos* leaves, on the other hand, revealed the presence of alkaloids and saponins **(Table 4; Plate 9)** whereas, the chloroform extract of *C. maxima* leaves revealed the presence of sterols, fixed oils, and fats. In the case of *C. maxima* leaves, all solvents yielded positive results for fats and oils **(Table 5; Plate 10)**.

According to the study, *A. marmelos* and *C. maxima* contain a diversity of phytochemicals, indicating that they can be employed for a number of medical uses. These plants have significant potential for usage in novel developments in the fields of ayurveda and contemporary pharmaceuticals (Akinmoladun *et al*., 2007). The presence or absence of particular phytochemicals can explain plant biological activity. These secondary metabolites contribute considerably to the biological actions of medicinal plants, such as anti-diabetic, antioxidant, anti-microbial, and so on (Sarkar *et al.*, 2020). Tannins have incredible rigorous characteristics. They are believed to speed the healing of wounds and irritated mucous membranes. The methanolic extracts of *A. marmelos* and *C. maxima* produced the largest number of primary and secondary metabolites. As a result, methanolic extract was chosen for the antioxidant study (Rajan *et al*., 2011).

Table 2: Qualitative Analysis of Phytochemical Constituents from Stem of *Aegle marmelos*

Phytochemical	Name of the test	Solvent used for extraction			
		Water	**Methanol**	**Ethyl acetate**	**Chloroform**
1) Alkaloids	a) Mayer's test	-	-	-	-
	b) Wagner's test	-	+	-	-
	c) Hager's test	-	-	-	-
2) Carbohydrates and Glycosides	a) Molish's test	+	+	+	-
	b) Barfoed's test	-	-	-	-
	c) Benedict's test	-	+	-	-
	d) Borntrager's test	-	+	-	-
	e) Legal's test	-	-	-	-
3) Saponins	Saponins test	+	+	-	-
4) Proteins and amino Acids	a) Buret test	-	+	-	-
	b) Ninhydrin test	-	-	-	-
5) Phytosterols	Liberman-Burchard Test	+	+	+	+
6) Sterols	Sterol test	+	+	+	-
7) Fixed oils and fats	Saponification test	-	+	-	-
8) Phenolic compounds and tannins	a) Ferric chloride test	-	+	-	-
	b) Gelatin test	-	-	-	-
	c) Lead acetate test	+	+	-	-
9) Quinones	Quinones test	+	+	+	+

Note: (+) Present; (-) Absent

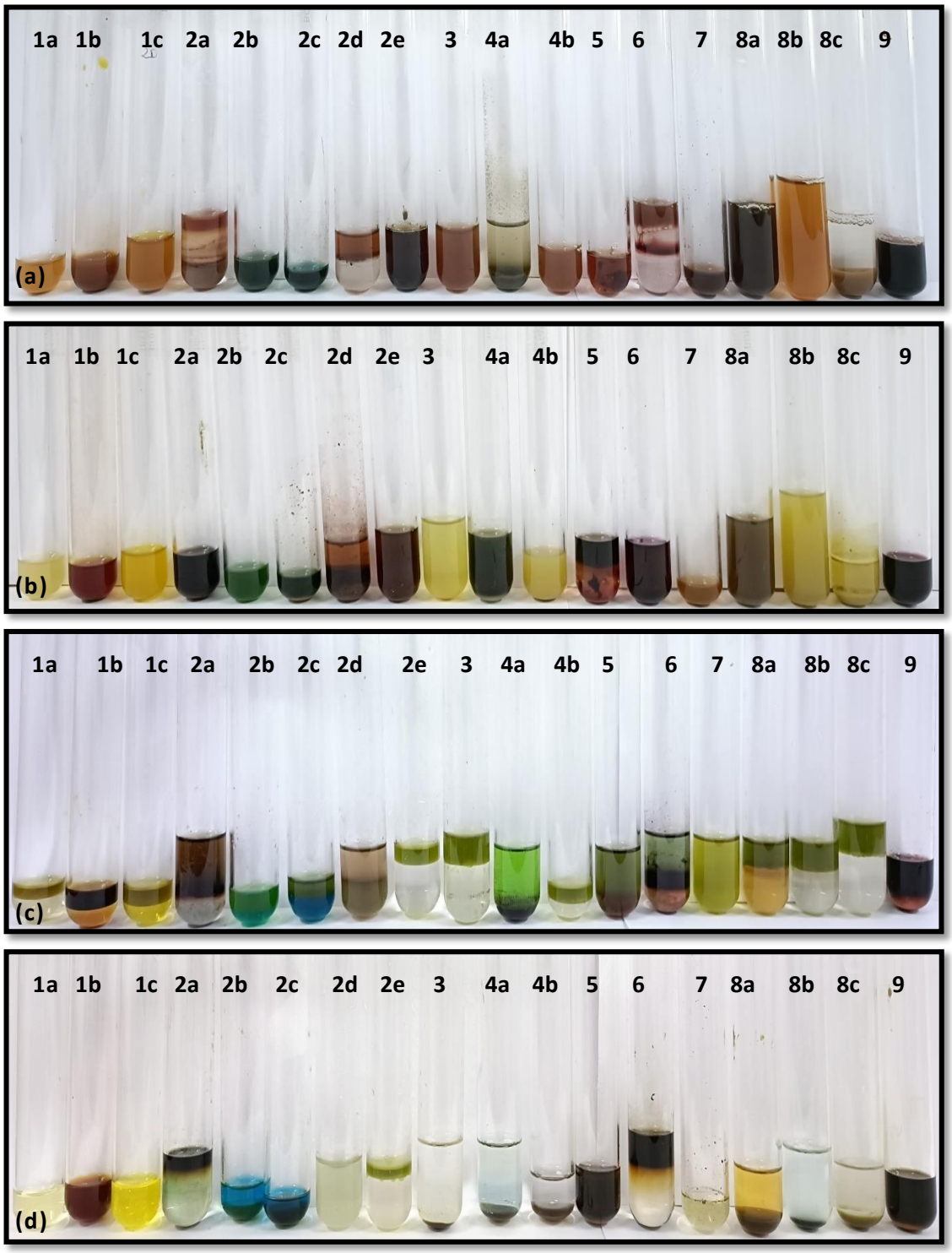


Plate 7: Qualitative phytochemical analysis of *A. marmelos* stem using various solvents. (a) Water, **(b)** Methanol, **(c)** Ethyl acetate, **(d)** Chloroform.

Table 3: Qualitative Analysis of Phytochemical Constituents from Stem of *Citrus maxima*

Phytochemical	**Name of the test**	**Solvent used for extraction**			
		Water	**Methanol**	**Ethyl acetate**	**Chloroform**
1) Alkaloids	a) Mayer's test	+	-	-	-
	b) Wagner's test	+	+	-	-
	c) Hager's test	+	-	-	-
2) Carbohydrates and Glycosides	a) Molish's test	+	+	+	-
	b) Barfoed's test	-	-	-	-
	c) Benedict's test	-	+	-	-
	d) Borntrager's test	-	+	-	-
	e) Legal's test	+	-	-	-
3) Saponins	Saponins test	+	+	-	-
4) Proteins and amino Acids	a) Buret test	-	+	-	-
	b) Ninhydrin test	-	-	-	-
5) Phytosterols	Liberman-Burchard test	+	+	+	+
6) Sterols	Sterol test	+	+	+	+
7) Fixed oils and fats	Saponification test	+	+	-	-
8) Phenolic compounds and tannins	a) Ferric chloride test	-	+	-	-
	b) Gelatin test	-	-	-	-
	c) Lead acetate test	+	+	-	-
9) Quinones	Quinones test	+	+	+	-

Note: (+) Present; (-) Absent

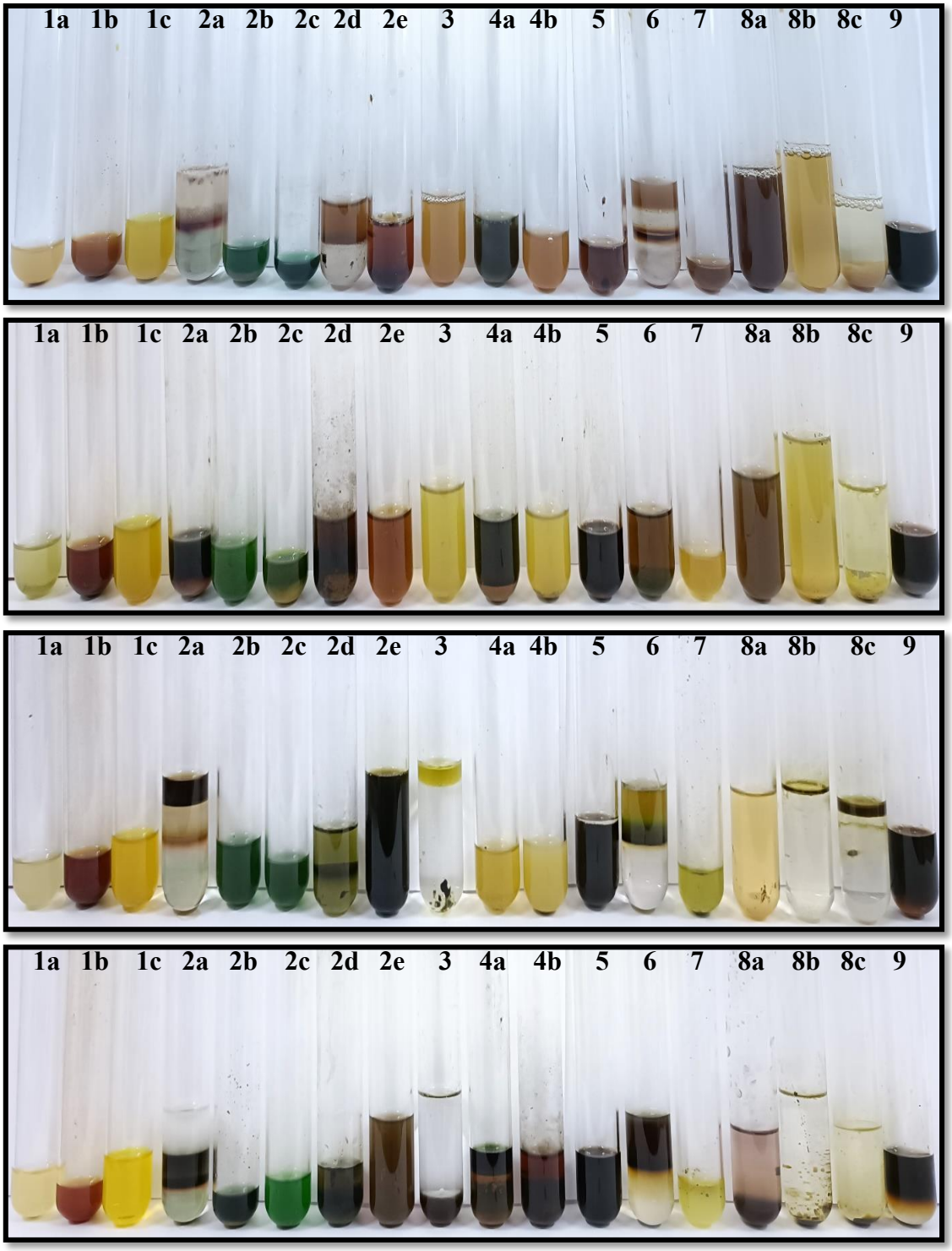


Plate 8: Qualitative phytochemical analysis of *C. maxima* stem using various solvents. (a) Water, **(b)** Methanol, **(c)** Ethyl acetate, **(d)** Chloroform.

Table 4: Qualitative Analysis of Phytochemical Constituents from leaves of *Aegle Marmelos*

Phytochemical	Name of the test	Solvent used for extraction			
		Water	**Methanol**	**Ethyl acetate**	**Chloroform**
1) Alkaloids	a) Mayer's test	-	+	-	-
	b) Wagner's test	+	+	-	+
	c) Hager's test	-	+	-	+
2) Carbohydrates and Glycosides	a) Molish's test	+	+	+	+
	b) Barfoed's test	-	-	-	-
	c) Benedict's test	+	-	-	-
	d) Borntrager's test	-	+	-	-
	e) Legal's test	-	+	-	-
3) Saponins	Saponins test	+	+	+	+
4) Proteins and amino Acids	a) Buret test	-	+	-	-
	b) Ninhydrin test	-	-	-	-
5) Phytosterols	Liberman-Burchard test	+	+	-	-
6) Sterols	Sterol test	+	+	+	-
7) Fixed oils and fats	Saponification test	+	+	+	-
8) Phenolic compounds and tannins	a) Ferric chloride test	-	+	-	-
	b) Gelatin test	-	-	-	-
	c) Lead acetate test	+	+	-	-
9) Quinones	Quinones test	+	+	-	-

Note: (+) Present; (-) Absent

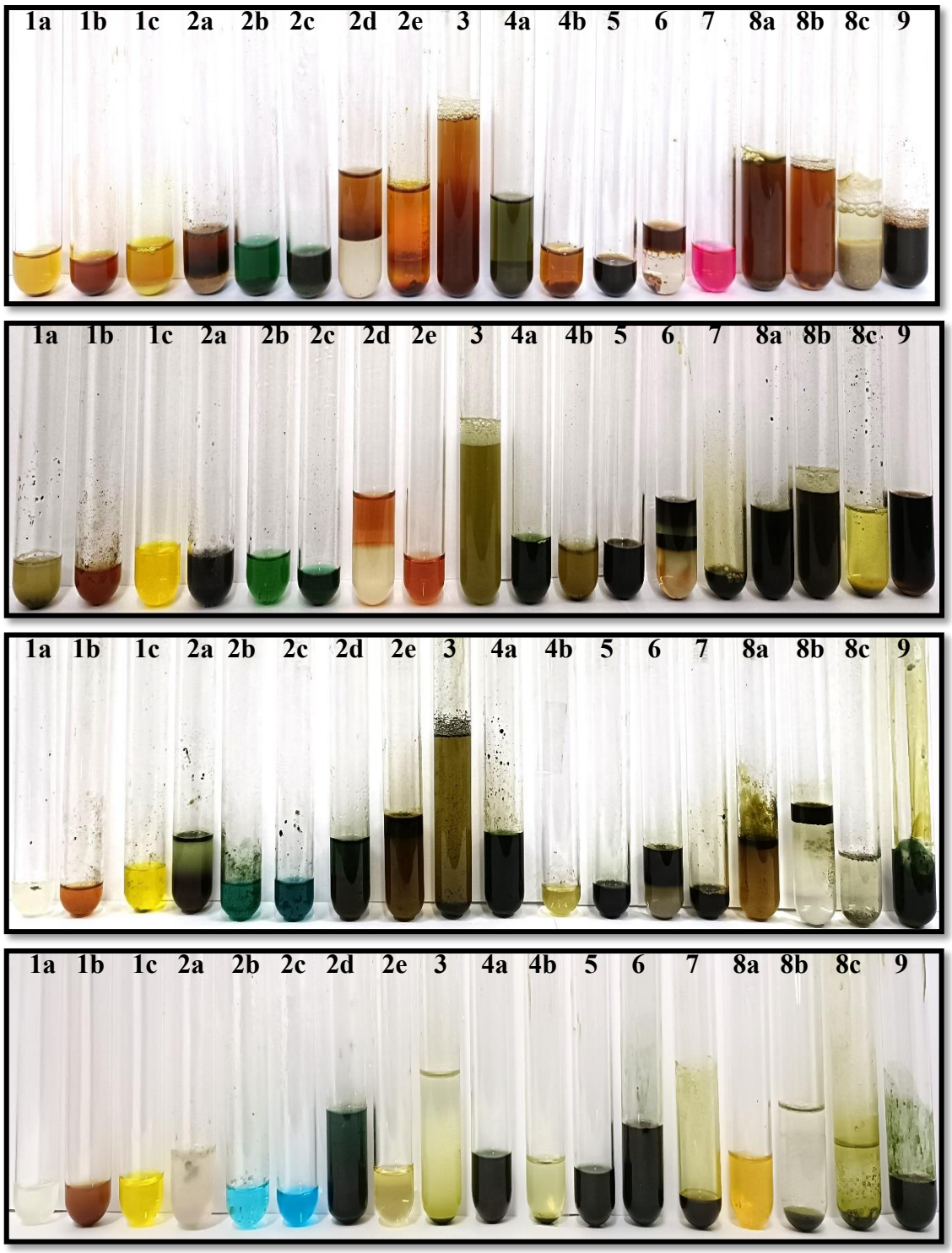


Plate 9: Qualitative phytochemical analysis of *A. marmelos leaves* using various solvents. (a) Water, **(b)** Methanol, **(c)** Ethyl acetate, **(d)** Chloroform.

Table 5: Qualitative Analysis of Phytochemical Constituents from leaves of *Citrus maxima*

Phytochemical	Name of the test	Solvent used for extraction			
		Water	**Methanol**	**Ethyl acetate**	**Chloroform**
1) Alkaloids	a) Mayer's test	-	+	-	-
	b) Wagner's test	+	+	-	-
	c) Hager's test	-	+	-	-
2) Carbohydrates and Glycosides	a) Molish's test	+	+	+	-
	b) Barfoed's test	-	-	-	-
	c) Benedict's test	+	-	-	-
	d) Borntrager's test	-	+	-	-
	e) Legal's test	-	+	-	-
3) Saponins	Saponins test	+	+	+	-
4) Proteins and amino Acids	a) Buret test	-	+	-	-
	b) Ninhydrin test	-	-	-	-
5) Phytosterols	Liberman-Burchard test	+	+	-	-
6) Sterols	Sterol test	-	+	+	+
7) Fixed oils and fats	Saponification test	+	+	+	+
8) Phenolic compounds and tannins	a) Ferric chloride test	-	+	-	-
	b) Gelatin test	-	-	-	-
	c) Lead acetate test	+	+	-	-
9) Quinones	Quinones test	+	+	-	-

Note: (+) Present; (-) Absent

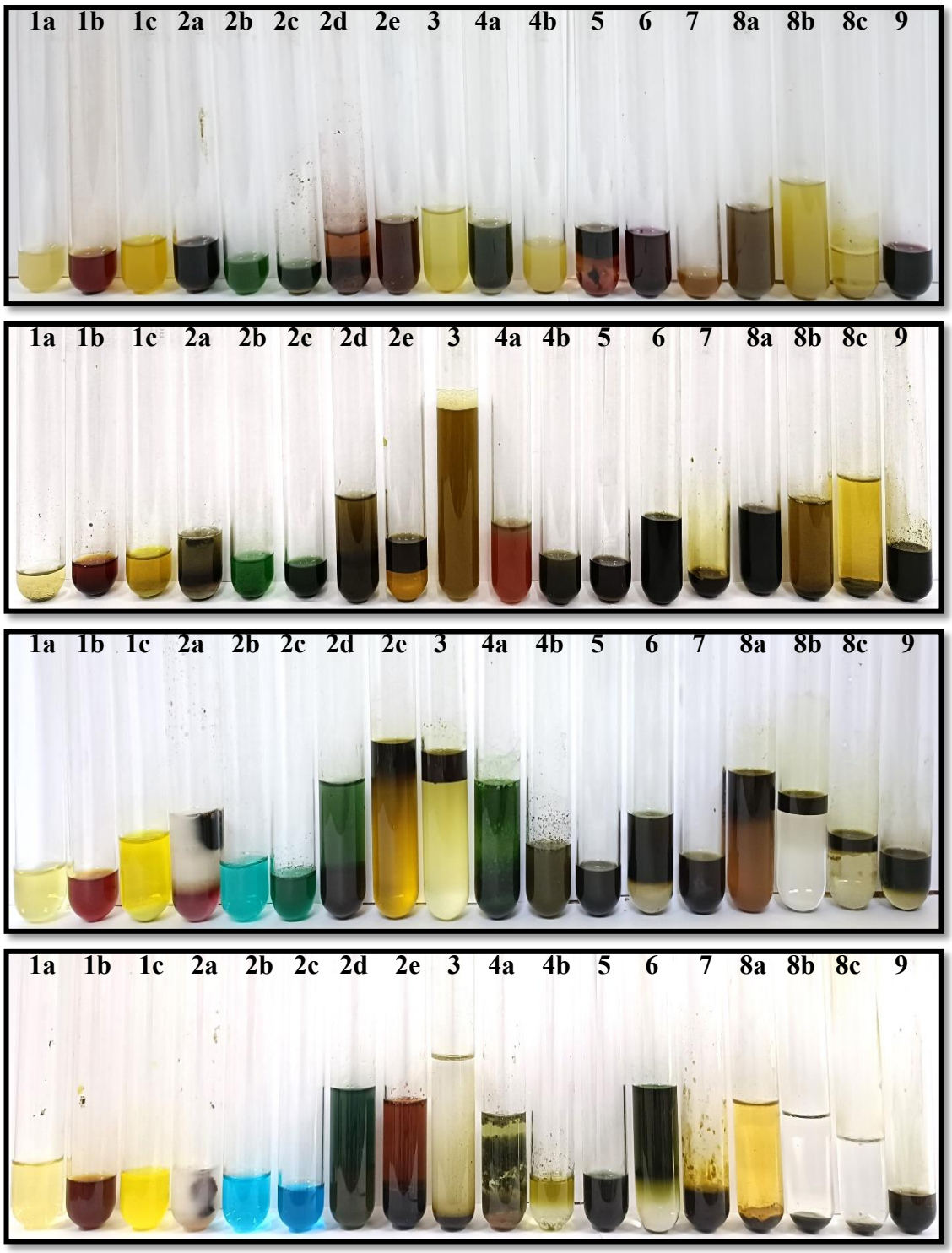


Plate 10: Qualitative phytochemical analysis of *C. maxima leaves* using various solvents. (a) Water, **(b)** Methanol, **(c)** Ethyl acetate, **(d)** Chloroform.

5.3. ANTIOXIDANT ACTIVITY

Antioxidants have a vital role in the prevention of human diseases. They can function as free radical scavengers, pro-oxidant metal complexes, reducing agents, and singlet oxygen quenchers (Raja and Khan, 2017). Free radicals have the ability to reduce the oxidative damage associated with a number of diseases such as AIDS, cataracts, cancer, and neurological ailments.

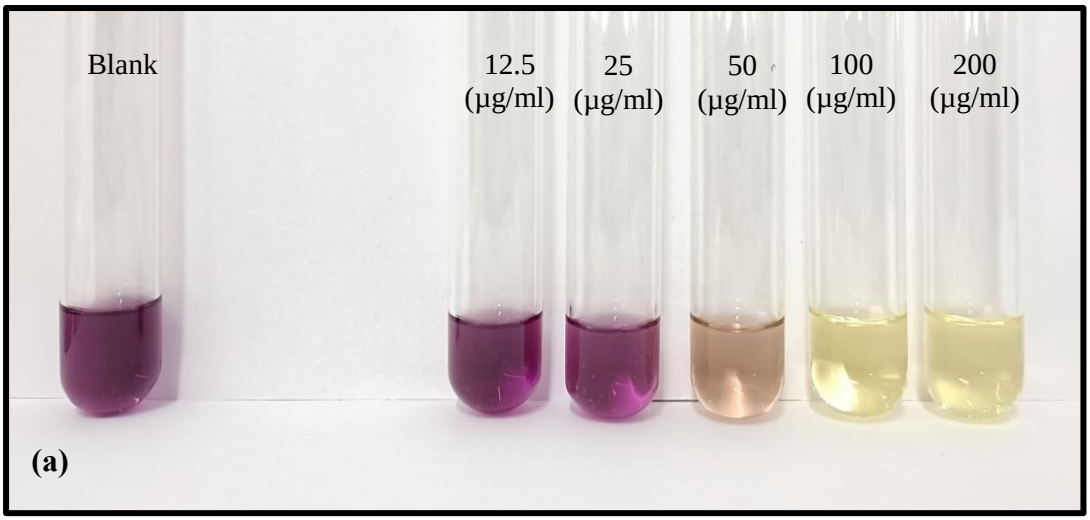
Methanolic plant extracts of diverse plant parts such as stem, leaves, fruit rind and, fruit pulp of *A. marmelos* and *C. maxima* were subjected to the 1, 1-diphenyl-2-picrylhyrazyl (DPPH) test to determine their potential as free radical scavengers. This method measures the decrease in the absorption of the DPPH solution after adding an antioxidant at 517 nm **(Table 6 and 7)**. The standard was ascorbic acid. The experiment was carried out in triplicate **(Plate 11)**.

When concentration increased, absorbance values decreased and percentage inhibition increased. The linear regression of the percentage of inhibition v/s antioxidant activity demonstrates an inverse relationship between the antioxidant potential and the IC_{50} value. IC_{50} value represents the concentration at which a substance exerts half of its maximal inhibiting a specific biochemical process **(Fig 2 and 3)**.

The maximum free radical scavenging activity in *A. marmelos* was observed in the methanolic extract of rind with an IC_{50} value of 107.31µg/mL, followed by leaves, fruit pulp, and stem with IC_{50} values of 112.829µg/mL, 160.959µg/mL, and 199.535µg/mL, respectively. The methanolic rind extract of *C. maxima* had the highest free radical scavenging activity, with an IC_{50} value of 71.71µg/mL, followed by the leaves, fruit pulp, and stem with IC_{50} values of 101.26µg/mL, 127.65µg/mL, and 194.054µg/mL, respectively **(Fig 4; Table 8)**. When the

antioxidant activity of both plants was compared, *C. maxima* outperformed *A. marmelos*. The fruit rind had the highest anti-oxidant activity in both species, followed by the leaves.

Nigam and Nambiar in 2015 concluded that unripe fruit had more antioxidant activity than ripe fruit. *A. marmelos* leaf aqueous extract had the highest radical scavenging activity compared to other solvents used namely, methanol and ethanol, with an IC_{50} value of 125µg/mL (Khan, 2017). Both aqueous and alcoholic extracts demonstrated similar DPPH anion scavenging power of 44.362.09% and 40.125.36%, respectively at 100µg/mL concentration, with 92.64830.68µg/mL of IC_{50} for aqueous extract, 106.1525.33µg/ml for alcoholic extract, and 63.99 25.24µg/mL for Ascorbic acid (Sharmila and Vasundra, 2011).



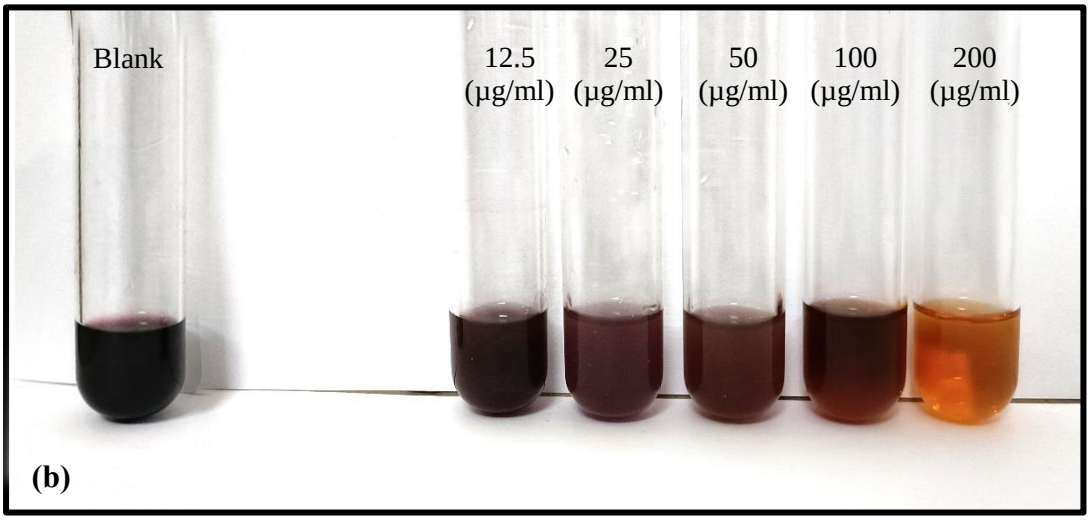


Plate 11: Antioxidant studies using DPPH method **(a)** L-Ascorbic acid as standard, **(b)** Plant extract.

Table 6: DPPH free radical scavenging assay: % scavenging activity of DPPH by ascorbic acid and methanolic extracts of various parts (stem, leaves, fruit rind and fruit pulp) of *A. marmelos*.

Sr. No.	Concentration (µg/mL)	L-Ascorbic Acid	Methanolic Extract of *Aegle marmelos*			
			Stem	Leaves	Fruit Rind	Fruit Pulp
1.	**12.5**	6.32 ± 0.039	5.18±0.004	18.88±0.008	5.8±0.005	5.64±0.002
2.	**25**	11.92 ± 0.037	14.93±0.003	27.87±0.004	16.9±0.004	9.61±0.009
3.	**50**	73.65 ± 0.065	24.17±0.003	42.59±0.016	36.5±0.009	25.99±0.004
4.	**100**	93.56 ± 0.018	32.05±0.003	56.65±0.004	59 ± 0.009	37.28±0.012
5.	**200**	95.85 ± 0.023	47.82±0.004	64.05± 0.003	77.2±0.004	57.79±0.004

Results are reported as % inhibition ± standard deviation of triplicate measurements

Table 7: DPPH free radical scavenging assay: % scavenging activity of DPPH by ascorbic acid and methanolic extracts of various parts (stem, leaves, fruit rind and fruit pulp) of *C. maxima*.

Sr. No.	Concentration (µg/mL)	L-Ascorbic Acid	Methanolic Extract of *Citrus maxima*			
			Stem	Leaves	Fruit Rind	Fruit Pulp
1.	12.5	6.32 ± 0.039	1.3±0.008	26.37 ± 0.011	19.1 ± 0.005	8.9 ± 0.087
2.	25	11.92 ± 0.037	2.68 ± 0.005	35.85 ± 0.038	35 ± 0.004	22.4 ± 0.002
3.	50	73.65 ± 0.065	19.83 ± 0.007	46.75 ± 0.010	56.4 ± 0.005	34.7 ± 0.004
4.	100	93.56 ± 0.018	30.5 ± 0.002	50.58 ± 0.011	66.8 ± 0.008	49 ± 0.004
5.	200	95.85 ± 0.002	48.6 ± 0.004	67.38 ± 0.004	81.2 ± 0.005	65.6 ± 0.004

Results are reported as % inhibition ± standard deviation of triplicate measuremen

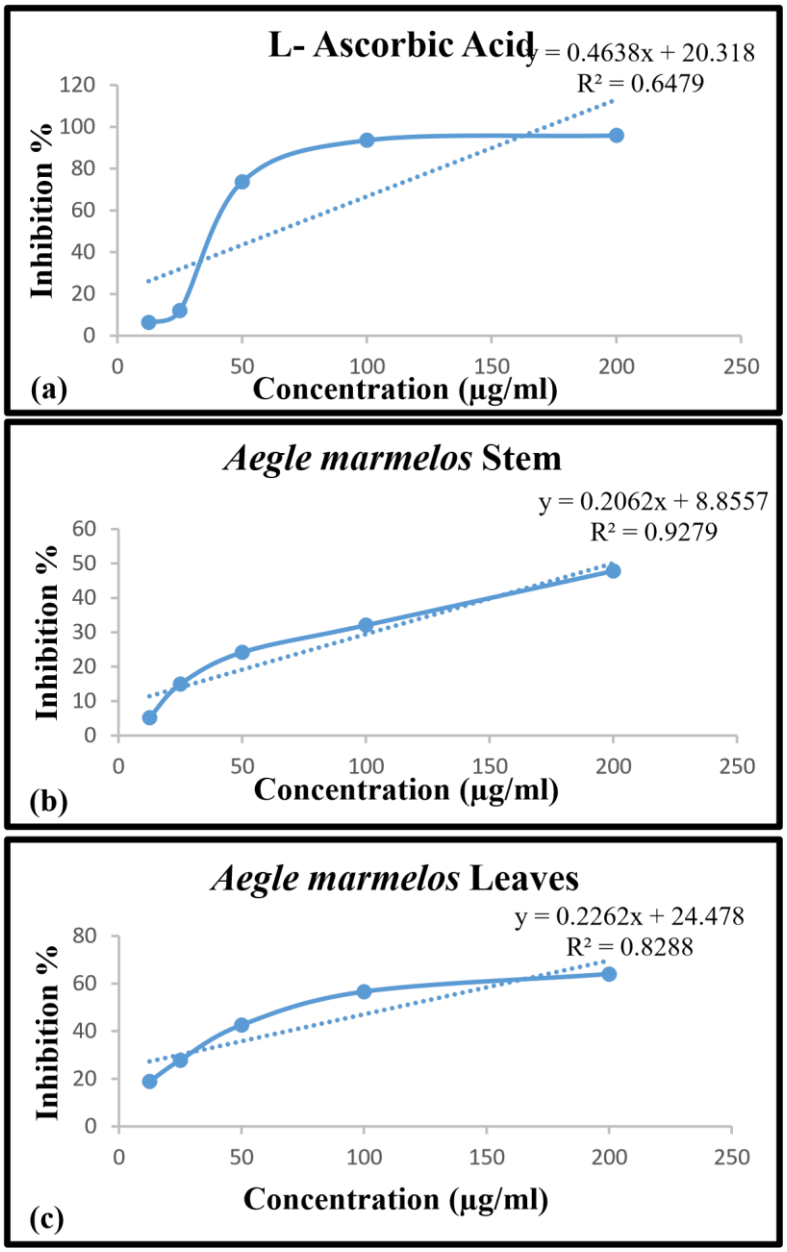


Figure 2: DPPH Radical Scavenging activity of **(a)** L- Ascorbic acid, **(b)** Methanolic extract of *A. marmelos* stem, **(c)** Methanolic extract of *A. marmelos* leaves.

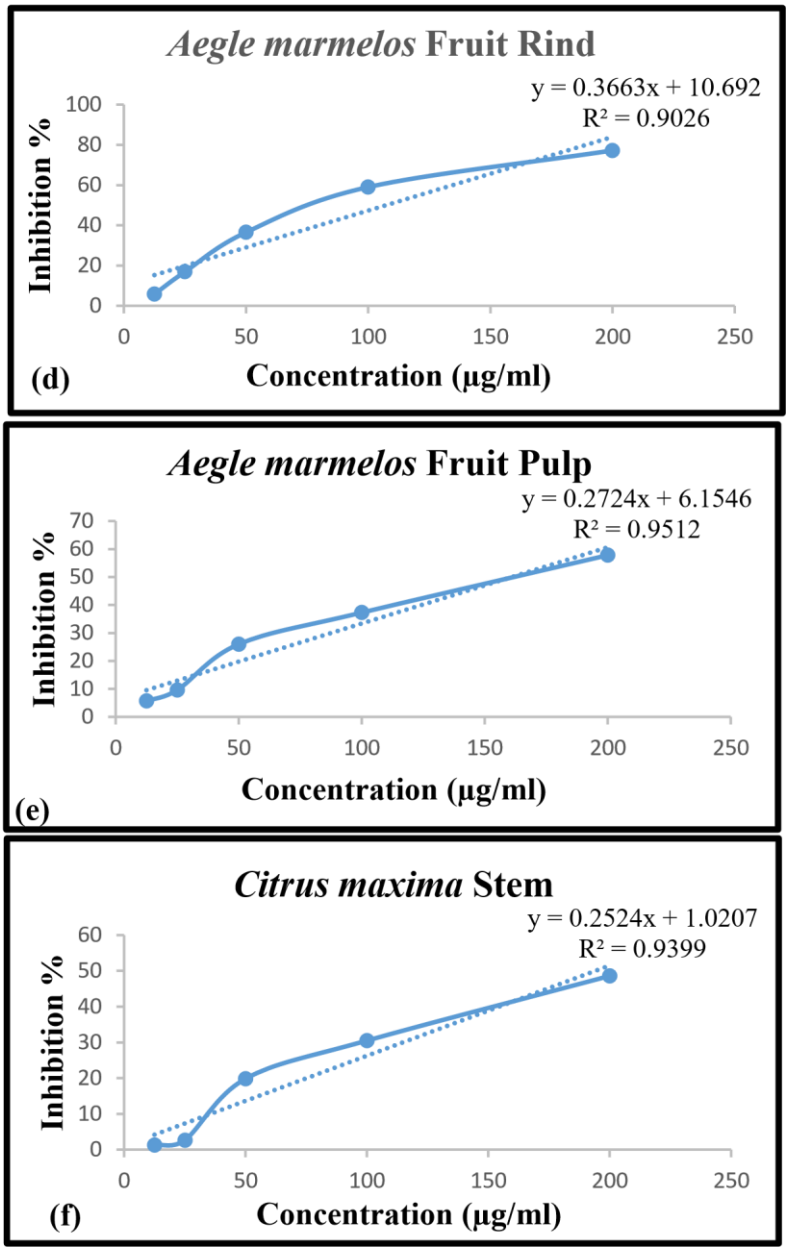
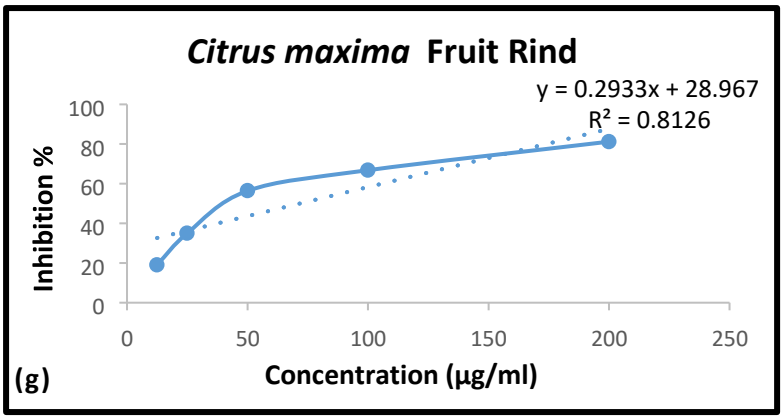
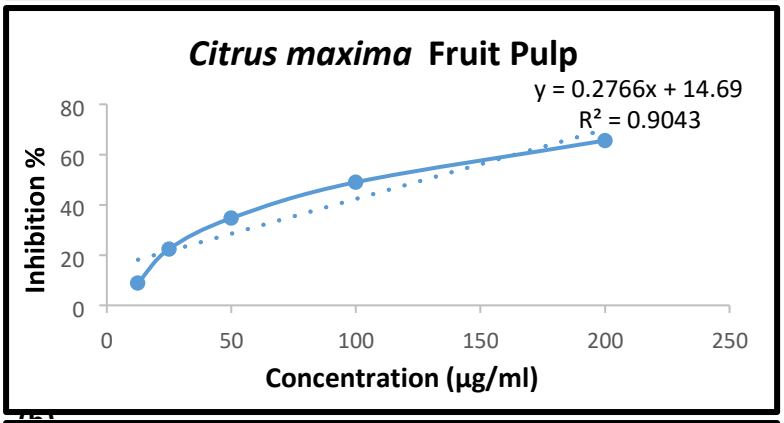


Figure 2: DPPH Radical Scavenging activity of **(d)** Methanolic extract of *A. marmelos* fruit rind, **(e)** Methanolic extract of *A. marmelos* fruit pulp, **(f)** Methanolic extract of *C. maxima* stem.





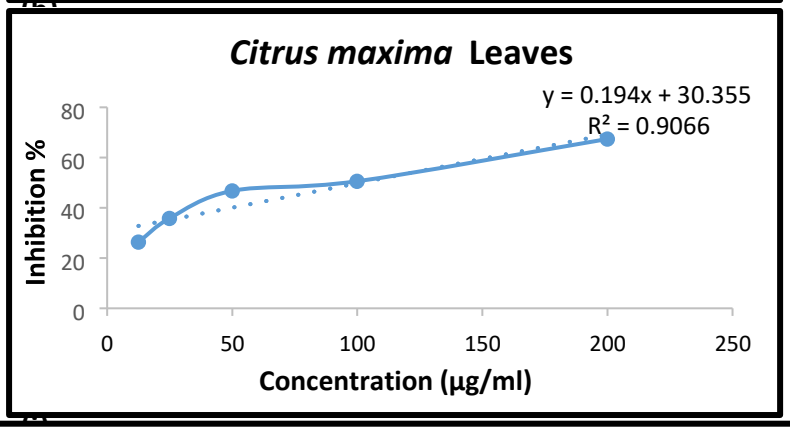


Figure 2: DPPH Radical Scavenging activity of **(g)** Methanolic extract of *C. maxima* leaves, **(h)** Methanolic extract of *C. maxima* fruit rind, **(i)** Methanolic extract of *C. maxima* fruit pulp.

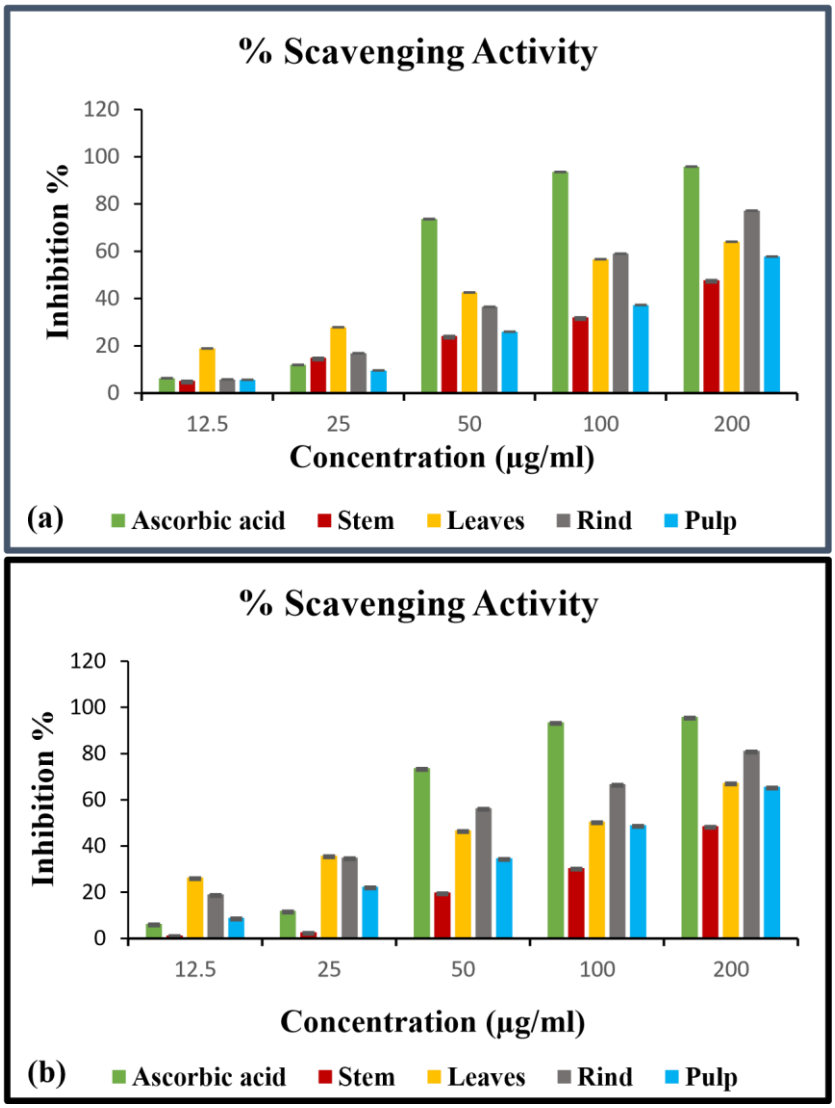


Figure 3: Percentage scavenging activity of **(a)** L- Ascorbic acid (Standard) with *A. marmelos* Stem, leaves, fruit rind and fruit stem, **(b)** L-Ascorbic acid with *C. maxima* Stem, leaves, fruit rind and fruit pulp.

Sr. No.	Plant Name	IC_{50} Value of Different Parts of the Plant			
		Stem (µg/mL)	Leaves (µg/mL)	Fruit Rind (µg/mL)	Fruit Pulp (µg/mL)
1.	*Aegle marmelos*	199.535	112.829	107.31	160.959
2.	*Citrus maxima*	194.054	101.26	71.711	127.65

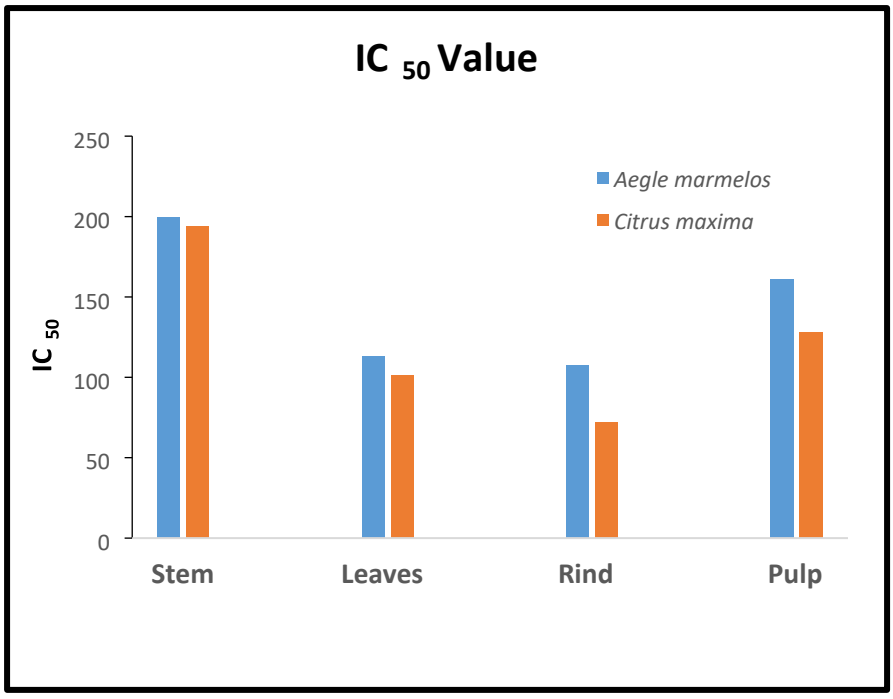


Figure 4: IC_{50} value of *A. mamelos* and *C. maxima*

5.4. ESSENTIAL OIL EXTRACTION

The essential oil of fresh *A. marmelos* and *C. maxima* leaves was extracted using a Clevenger hydro-distillation apparatus **(Plate 12 a)**. The essential oil yield of *A. marmelos* was 0.92%, whereas *C. maxima* had a yield of 1.04% **(Plate 12 b).** The essential oil isolated from the leaves of *A. marmelos* was colourless with a sweet fruity aroma while, *C. maxima* leaves essential oil was also colourless and had a strong, tangy and, refreshing aroma.

External conditions, harvesting stage, plant material, storage conditions, and isolation techniques, as well as certain intrinsic (genotype and ontogeny) and extrinsic (light, temperature, water, and nutrients) aspects, all have an impact on production of essential oil yield. Differences between yields may be explained considering whether younger or older leaves were collected, although extreme care was taken to for uniform collection.

Plant essential oils have several potential applications, including usage as food additives, preservatives, fragrances, and medications. Its utilisation is mainly owing to the medical and pharmacological activity of these oils, such as antibacterial, antioxidant, and anti-inflammatory properties (Poonkodi *et al*., 2019). Pale white essential oil was extracted from the leaves of *C. maxima* using Clevenger apparatus (Prasad *et al.,* 2016).

Plate 12: (a) Clevenger apparatus, **(b)** Essential oil extracted from the leaves of *A. marmelos* (**right)** and *C. maxima* **(left)**.

5.5. GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Gas chromatogram of essential oil extracted from *A. marmelos* leaves and *C. maxima* leaves along with the retention time (RT) and major compounds found are shown in **Fig. 5** and **Fig. 7** respectively. The compound name, chemical formula, retention time and area % of *A. marmelos* and *C. maxima* leaves essential oil are presented in **Table 9 and Table 10** respectively.

The chromatogram obtained was analysed and a total of fifty-eight compounds were identified in the essential oil of *A. marmelos* leaves. The major compounds were D-Limonene (28.02%), 1,3,6-Octatriene,3,7-dimethyl-,(Z) (9.86%), trans-β-Ocimene (4.95%), β-Myrcene (3.13%), (1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo[4.4.0.02,7]decane-rel- (2.82%), Caryophyllene (2.13%), Humulene (2.05%), α-Pinene (0.97%), Phytol (0.58%), Bicyclogermacrene (0.33%), Sabinene (0.32) and Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)- (0.25%). The D-Limonene MS graph was mentioned since it was abundant in the essential oil **(Fig. 6)**.

A total of fifty-five compounds detected from the essential oil extracted from the leaves of *C. maxima* and the major compounds were D-Limonene (45.44%), β-Myrcene (6.81%), α-Pinene(1.74%), (1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo [4.4.0.02,7] decane-rel (1.31%), Bicyclo [3.1.1] heptane, 6,6-dimethyl-2-methylene-, (1S)- (0.96%), 1,3,6-Octatriene, 3,7-dimethyl-, (Z)- (0.88%), Linalool (0.64%), Sabinene (0.58%), α-Terpineol (0.55%), Citral (0.42%), 2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)- (0.36%), Caryophyllene (0.26%), 1-Naphthalenol,1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl 4 (1methylethyl), [1R (1α,4β,4aβ,8aβ)]- (0.23%) and Bicyclogermacrene (0.21%). The D-Limonene MS

graph was noticed since it was predominant in *C. maxima* essential oil **(Fig. 8)**. α-Pinene, Camphene , Sabinene, β- Myrcene, 1,3,8-p-Menthatriene, Limonene, γ-Terpinene, Linalool, 2-Nonen-1-ol, Limonene oxide trans, cis-Verbenol, α-Terpineol, Carveol, Citral, γ-Elemene, Copaene, β-Guaiene, Caryophyllene, Humulene, Muurolene, Bicyclogermacrene, α-Farnesene and Phytol were identified in both the leaves essential oils though in varied concentration **(Table 11)**.

A study of the chemicals found that sesquiterpenes were the most prevalent type of compound in both. Earlier reports showed that terpenoid compounds were efficient as a repellent and larvicidal against various mosquito species (Das and Swamy 2015). The therapeutic qualities of essential oils may be attributed to the combined action of these bioactive components. From a taxonomy perspective, the chemical composition of plants containing essential oils is more important than the morphological features (Grayer *et al*., 1996). In a comparative analysis of the chemical composition of essential oils of *C. maxima* leaves and fruit rind, none of the detected chemicals were found occuring in both leaf and rind, even though both are of the same botanical origin. (Prasad *et al.,* 2016).

Table 9: Chemical composition of leaf essential oil of *Aegle marmelos* (https://pubchem.ncbi.nlm.nih.gov/)

Chemical Name	Area %	RT Sec	Molecular formula
5,5-Dimethyl-1-vinylbicyclo [2.1.1]hexane	0.001	442.783	$C_{10}H_{16}$
Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-	0.002	466.059	$C_{10}H_{16}$
α-Pinene	0.976	486.963	$C_{10}H_{16}$
Camphene	0.006	535.989	$C_{10}H_{16}$
Benzaldehyde	0.01	583.37	C_7H_6O
Sabinene	0.328	631.881	$C_{10}H_{16}$
β-Pinene	0.053	642.307	$C_{10}H_{16}$
β-Myrcene	3.13	703.827	$C_{10}H_{16}$
D-Limonene	28.029	848.915	$C_{10}H_{16}$
trans-β-Ocimene	4.956	875.003	$C_{10}H_{16}$
1,3,6-Octatriene,3,7-dimethyl-, (Z)	9.862	910.833	$C_{10}H_{16}$
γ-Terpinene	0.017	936.756	$C_{10}H_{16}$
cis-Linaloloxide	0.002	982.087	$C_{10}H_{18}O_2$
Linalool	0.035	1064.71	$C_{10}H_{18}O$
2-Nonen-1-ol, (E)-	0.002	1078.56	$C_9H_{18}O$
1,3,8-p-Menthatriene	0.002	1095.02	$C_{10}H_{14}$
Hotrienol	0.003	1115.57	$C_{10}H_{16}O$
2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethenyl)-, trans-	0.003	1120.93	$C_{10}H_{16}O$
Limonene oxide, trans-	0.014	1169.44	$C_{10}H_{16}O$
2,4,6-Octatriene, 2,6-dimethyl-, (E, E)-	0.007	1179.81	$C_{10}H_{16}$
α-Terpineol	0.009	1307.79	$C_{10}H_{18}O$
Carveol	0.006	1377.92	$C_{10}H_{16}O$
cis-3-Hexenyl-α-methylbutyrate	0.014	1411.54	$C_{11}H_{20}O_2$
cis-Verbenol	0.003	1431.54	$C_{10}H_{16}O$
(-)-Carvone	0.007	1437.08	$C_{10}H_{14}O$
Citral	0.005	1499.8	$C_{10}H_{16}O$
Naphthalene, 2-methyl-	0.004	1544.37	$C_{11}H_{10}$
Indole	0.007	1550.05	C_8H_7N
γ-Elemene	0.002	1623.18	$C_{10}H_{14}O$
2-Methoxy-4-vinylphenol	0.006	1627.24	$C_9H_{10}O_2$
Copaene	0.207	1724.54	$C_{15}H_{24}$
Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1α,2β,4β)]-	0.315	1757.45	$C_{15}H_{24}$
β-Guaiene	0.002	1793.56	$C_{15}H_{24}$
Naphthalene, 1,6-dimethyl-	0.001	1805.72	$C_{12}H_{12}$
Caryophyllene	2.138	1813.94	$C_{15}H_{24}$
Humulene	2.054	1879.45	$C_{15}H_{24}$
γ-Muurolene	0.008	1922.8	$C_{15}H_{24}$

(1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo [4.4.0.02,7]decane-rel-	2.827	1932.55	$C_{15}H_{24}$
Bicyclogermacrene	0.335	1961.24	$C_{15}H_{24}$
α-Farnesene	0.024	1977.62	$C_{15}H_{24}$
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	0.258	2009.07	$C_{15}H_{24}$
Nerolidol	0.02	2077.16	$C_{15}H_{26}O$
3-Hexen-1-ol benzoate	0.054	2091.96	$C_{13}H_{16}O_2$
Caryophyllene oxide	0.08	2118.3	$C_{15}H_{24}O$
β-Guaiene	0.012	2126.71	$C_{15}H_{24}$
14-Hydroxycaryophyllene	0.035	2202.98	$C_{15}H_{24}O$
11,11-Dimethyl-4,8-dimethylenebicyclo [7.2.0] undecan-3-ol	0.078	2210.17	$C_{15}H_{24}O$
β-Acorenol	0.024	2239.97	$C_{15}H_{26}O$
8-Heptadecene	0.009	2270.36	$C_{17}H_{34}$
Neophytadiene	0.034	2524.7	$C_{20}H_{38}$
2-Pentadecanone, 6,10,14-trimethyl-	0.003	2534.36	$C_{18}H_{36}O$
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.008	2562.43	$C_{20}H_{40}O$
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	0.004	2573.38	$C_{16}H_{22}O_4$
Isophytol	0.012	2685.87	$C_{20}H_{40}O$
1,6,10,14-Hexadecatetraen-3-ol, 3,7,11,15-tetramethyl-, (E, E)-	0.011	2804.29	$C_{20}H_{34}O$
Phytol	0.588	2916.63	$C_{20}H_{40}O$
2,4-Bis(dimethylbenzyl)-6-t-butylphenol	0.023	3722.46	$C_{28}H_{34}O$
Benzene, 1,1'-(1,1,2,2-tetramethyl-1,2-ethanediyl) bis-	0.004	3768	$C_{20}H_{26}$

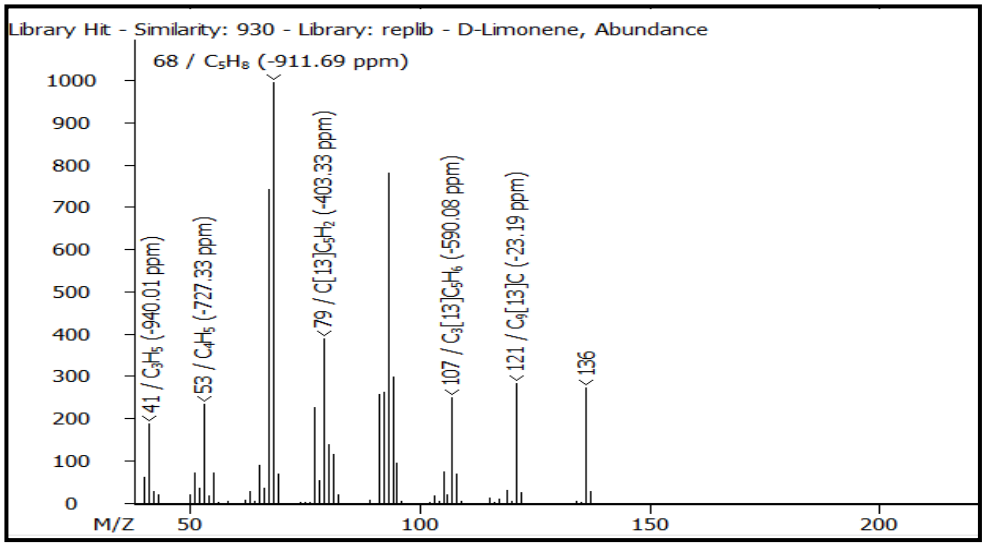


Figure 6: MS graph of D-Limonene (abundance) from leaf essential oil of *Aegle marmelos*

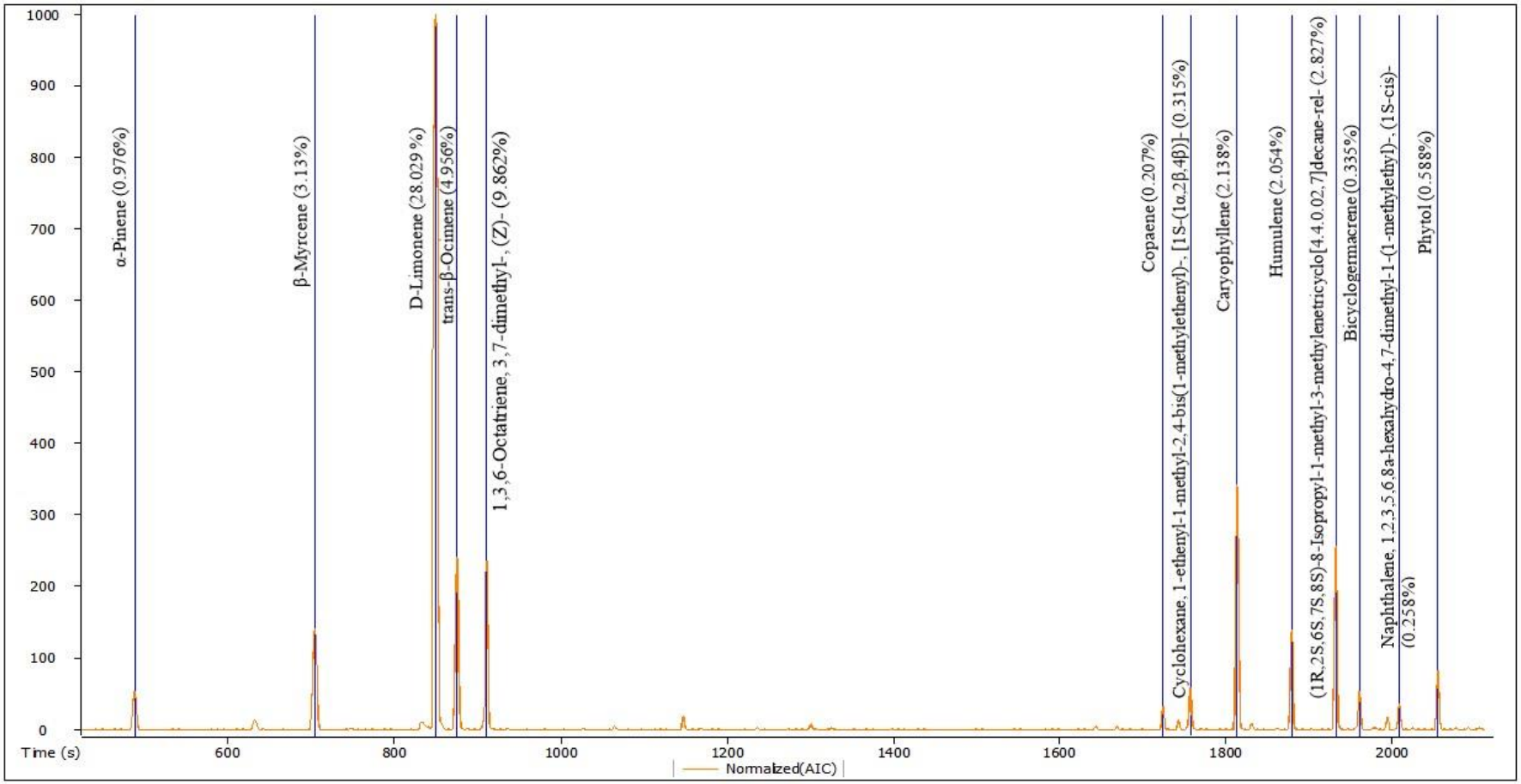


Figure 5: GC spectrum of essential oil extracted from the leaves of *Aegle marmelos*

Table 10: Chemical composition of leaf essential oil of *Citrus maxima* (https://pubchemncbi.nlm.nih.gov/)

Chemical Name	Area %	R. T. Sec	Molecular formula
α-Phellandrene	0.007	465.999	$C_{10}H_{16}$
α-Pinene	1.74	486.933	$C_{10}H_{16}$
Camphene	0.01	535.981	$C_{10}H_{16}$
Sabinene	0.584	631.794	$C_{10}H_{16}$
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	0.964	642.014	$C_{10}H_{16}$
β-Myrcene	6.814	703.77	$C_{10}H_{16}$
1,3,8-p-Menthatriene	0.005	821.276	$C_{10}H_{14}$
D-Limonene	45.441	848.65	$C_{10}H_{16}$
1,3,6-Octatriene, 3,7-dimethyl-, (Z)-	0.888	906.176	$C_{10}H_{16}$
γ-Terpinene	0.056	936.103	$C_{10}H_{16}$
Linalool	0.645	1064.34	$C_{10}H_{18}O$
2-Nonen-1-ol, (E)-	0.025	1078.34	$C_9H_{18}O$
Limonene oxide, cis-	0.004	1156.92	$C_{10}H_{16}O$
cis-p-Mentha-2,8-dien-1-ol	0.017	1161.45	$C_{10}H_{16}O$
Limonene oxide, trans-	0.017	1169.41	$C_{10}H_{16}O$
1,5,7-Octatrien-3-ol, 2,6-dimethyl-	0.004	1223.63	$C_{10}H_{16}$
cis-Verbenol	0.011	1243.52	$C_{10}H_{16}O$
Terpinen-4-ol	0.056	1272.65	$C_{10}H_{18}O$
α-Terpineol	0.554	1307.08	$C_{10}H_{18}O$
Carveol	0.037	1332.38	$C_{10}H_{16}O$
Decanal	0.02	1346.45	$C_{10}H_{20}O$
(-)-Carvone	0.027	1436.65	$C_{10}H_{14}O$
2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)-	0.366	1462.42	$C_{12}H_{20}O_2$
Citral	0.428	1498.84	$C_{10}H_{16}O$
p-Mentha-1,8-dien-7-ol	0.026	1542.07	$C_{10}H_{16}O$
γ-Elemene	0.002	1623	$C_{15}H_{24}$
α-Cubebene	0.014	1669.63	$C_{15}H_{24}$
4-Hexen-1-ol, 5-methyl-2-(1-methylethenyl)-, acetate	0.008	1678.56	$C_{10}H_{18}O$
Copaene	0.063	1724.33	$C_{15}H_{24}$
Hexanoic acid, 3-hexenyl ester, (Z)-	0.005	1733.62	$C_{12}H_{22}O_2$
Bicyclosesquiphellandrene	0.013	1753.46	$C_{15}H_{24}$
Dodecanal	0.004	1787.5	$C_{12}H_{24}O$
β-Guaiene	0.003	1800.72	$C_{15}H_{24}$
Caryophyllene	0.265	1812.56	$C_{15}H_{24}$
Alloaromadendrene	0.004	1849.9	$C_{15}H_{24}$
5,9-Undecadien-2-one, 6,10-dimethyl-, (Z)-	0.011	1873.64	$C_{13}H_{22}O$
Humulene	0.092	1878.74	$C_{15}H_{24}$
cis-Muurola-4(15),5-diene	0.012	1896.69	$C_{15}H_{24}$

γ-Muurolene	0.017	1922.24	$C_{15}H_{24}$
(1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo[4.4.0.02,7]decane-rel-	1.318	1931.59	$C_{15}H_{24}$
Bicyclogermacrene	0.215	1960.96	$C_{15}H_{24}$
α-Farnesene	0.013	1977.58	$C_{15}H_{24}$
α-Calacorene	0.007	2045.12	$C_{15}H_{20}$
Nerolidol	0.023	2077.04	$C_{15}H_{26}O$
4-Isopropyl-6-methyl-1-methylene-1,2,3,4-tetrahydronaphthalene	0.002	2081.63	$C_{15}H_{20}$
(-)-Globulol	0.022	2118.99	$C_{15}H_{26}O$
Isoledene	0.007	2154.98	$C_{15}H_{24}$
1-Naphthalenol, 1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl)-, [1R-(1α,4β,4aβ,8aβ)]-	0.236	2225.03	$C_{15}H_{26}O$
trans-Farnesol	0.013	2344.66	$C_{15}H_{26}O$
2,6,10-Dodecatrienal, 3,7,11-trimethyl-, (E,E)-	0.007	2378.46	$C_{15}H_{24}O$
Nootkatone	0.002	2485.04	$C_{15}H_{22}O$
1-Iodo-2-methylundecane	0.003	2758.88	$C_{12}H_{25}I$
Nonadecane	0.013	2896.73	$C_{19}H_{40}$
Phytol	0.004	2916.52	$C_{20}H_{40}O$
Methyl dehydroabietate	0.002	3274.02	$C_{21}H_{30}O_2$

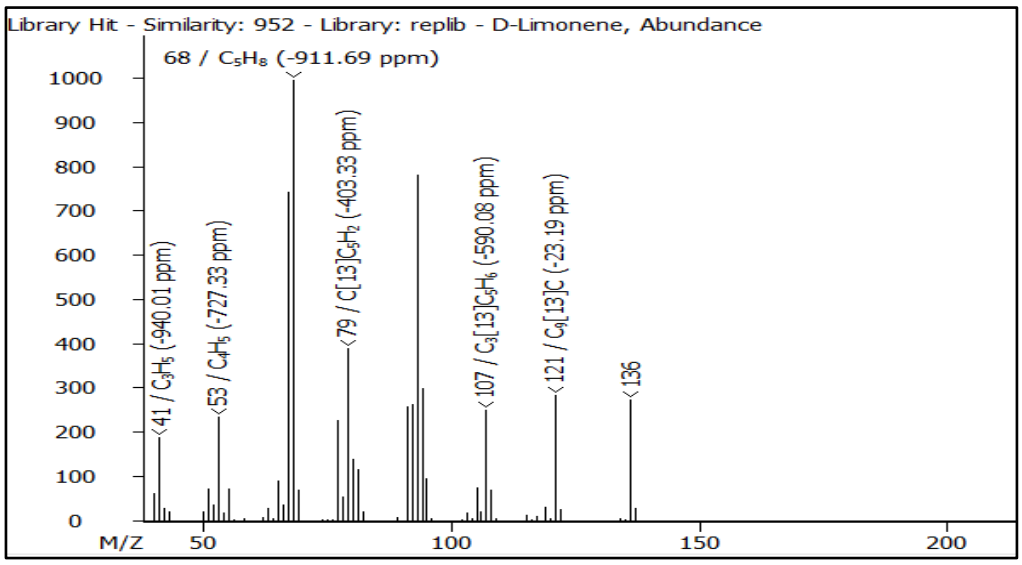


Figure 8: MS graph of D-Limonene (abundance) from leaf essential oil of *Citrus maxima*

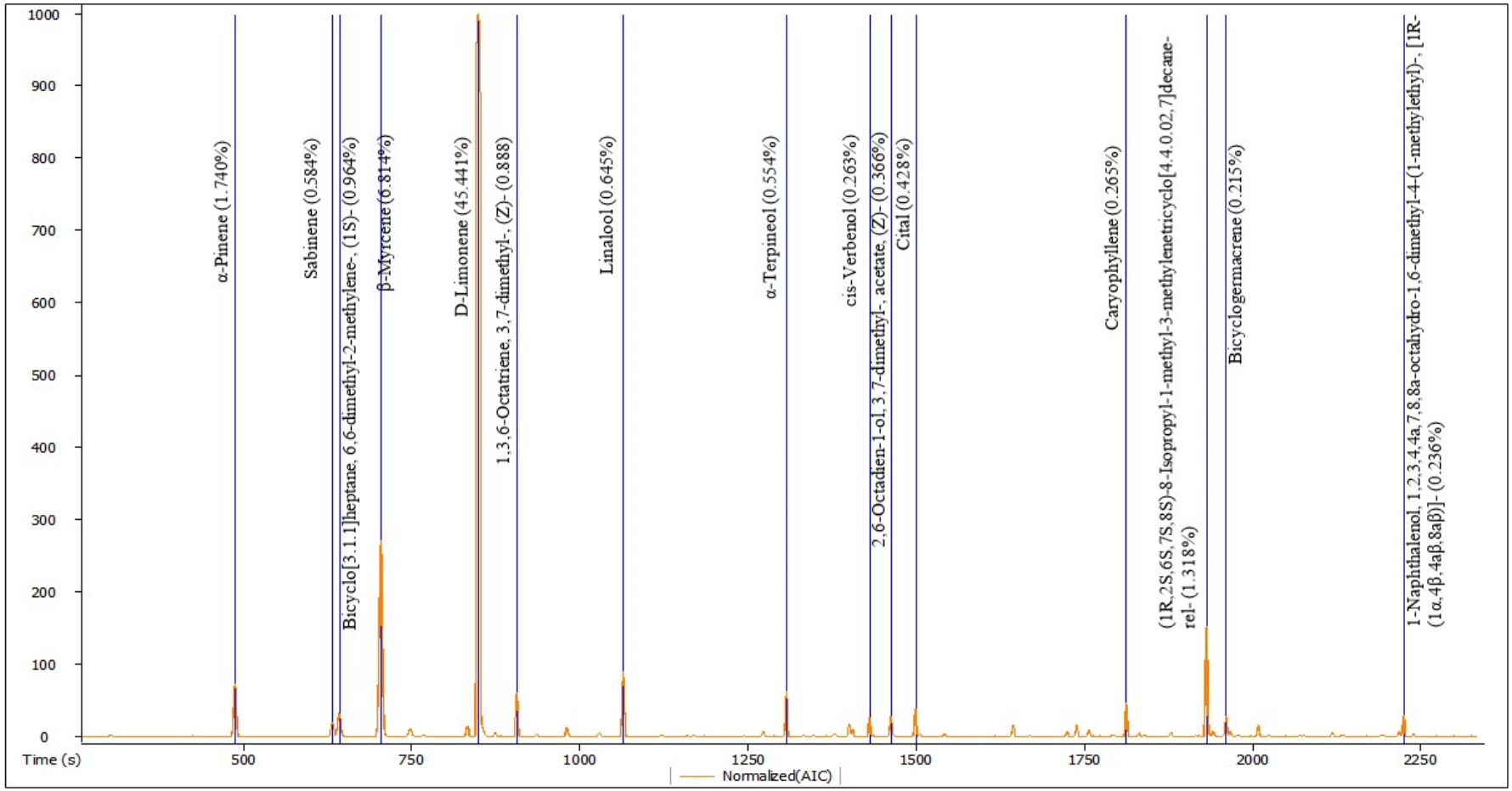


Figure 5: GC spectrum of essential oil extracted from the leaves of *Citrus maxima*

5.6. MOSQUITO LARVICIDAL ACTIVITY

Plants are high in bioactive substances, they may be used to control a wide range of harmful insects. Natural insecticides derived from plants are mostly composed of extracts or essential oils, and they are an excellent alternative to synthetic pesticides for decreasing adverse effects on human being and the environment (Mahanta *et al*., 2017). . In our study mosquito larvicidal activity was performed using the essential oils extracted from the leaves of *A. marmelos* and *Citrus maxima*. Four different concentrations of oils were prepared and tested against 3rd instar larvae of *Culex quinquefasciatus* and *Anopheles stephensi.* 25 larvae of 3rd instar larvae were selected for the bioassay as they grow properly and have a healthy build-up and are in the best feeding stage **(Table 12 and 18).** 1st and 2nd instar larvae are too small and 4th instar larvae can form into pupae within a span of 24 hrs. The exposure time and concentration of the oil were shown to be directly associated to larval mortality in the study. After 24 hours **(Table 13, 15, 19 and 21)** and 48 hours of exposure, larval mortality was measured **(Table 14, 16, 20 and 22)**.

The LC_{50} value of essential oil extracted from the leaves of *A. marmelos* against *Culex quinquefasciatus* was 61.916ppm and 56.797ppm at 24 and 48 hours, respectively **(Fig 9a)**, while the LC_{90} value was 193.947ppm and 180.606ppm at 24 and 48 hours, respectively **(Fig 9b)**. In the case of *Anopheles stephensi*, the LC_{50} values for 24 and 48 hours were 57.352ppm and 54.913ppm, respectively, while the LC_{90} values for 24 and 48 hours were 186.834ppm and 193.828ppm, respectively **(Table 17)**. It was discovered that essential oil derived from the leaves of *A. marmelos* found to be better against *Anopheles stephensi* than *Culex quinquefasciatus* larvae. LC_{50} value of essential oil extracted from the leaves of *C. maxima* against *Culex quinquefasciatus* was 52.773ppm and 50.233ppm **(Fig 9a)** and their LC_{90} value was 153.712 ppm and 150.024 ppm at 24 hours and 48 hours respectively **(Fig 9b)**. In the case of *Anopheles stephensi* LC_{50} values were 48.995ppm and 46.277ppm and their LC_{90} were

147.405ppm and 145.188ppm at 24 hours and 48 hours respectively **(Table 23)**. Leaves essential of *C. maxima* showed better toxicity towards *Anopheles stephensi* as compared to the *Culex quinquefasciatus.*

Essential oil of leaves of *C. maxima* was found more toxic to *Culex quinquefasciatus* and *Anopheles stephensi* as compared to the *A. marmelos* essential oil. Variations in the composition of essential oil may be one of the responsible factors for their variation in larvicidal activity. The essential oils derived from *Citrus* genes produced monocyclic monoterpenoids, the primary component of which is D-Limonene, which is responsible for the insecticidal function (Mahanta and Sarma, 2017). Sarma and Mahanta's 2017 report revealed that the peel of *C. maxima* has the highest activity as larvicides, followed by adulticidal and ovicidal activity. The variations in sensitivity to two distinct essential oils as larvicides are related to variances in intake rate, membrane penetration, detoxification, and so on. Mosquitoes respond differently to essential oils, and it has previously been demonstrated that the sensitivity of various developmental stages of the same species of mosquito to the same compound can differ. Again, essential oils can be inhaled, ingested, or absorbed through the skin of insects **(Plate 13)**.

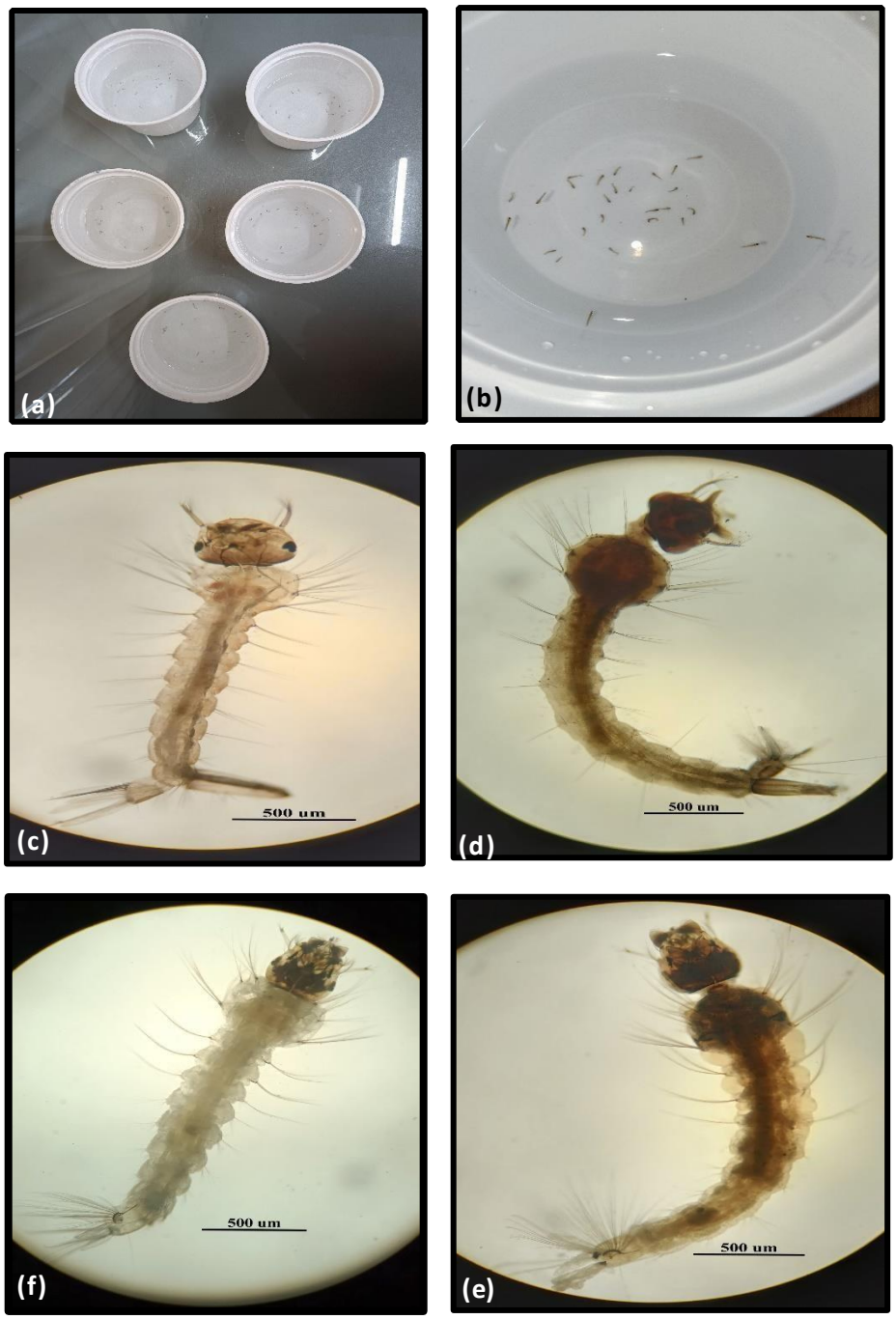


Plate 13: Larvicidal activity (a) larvae in different concetrations of essential oil, **(b)** larvae in 250ml of distilled water, **(c)** 3rd instar *Culex quinquefasciatus* (untreated) (4X), **(d)** 3rd instar *Culex quinquefasciatus* exposed to essential oil (4X), **(e)** 3rd instar *Anopheles stephensi* (untreated) (4X), **(f)** 3rd instar *Anopheles stephensi* exposed to essential oil (4X).

Table 12: Effect of leaf essential oil of *A. marmelos* against 3rd instar *Culex quinquefasciatus* and *Anopheles stephensi* larvae.

Name of sample	***Aegle marmelos***
Number of larvae exposed in each bowl	25
Volume of water	250 mL
Replicates	3

Table 13: Larvicidal activity of leaf essential oil of *A. marmelos* against 3rd instar of *Culex quinquefaciatus* larvae after 24 hours of exposure

Sr. No.	**Dose (ppm)**	**Number of dead larvae**			**% mortality**
		R_1	**R_2**	**R_3**	
1.	**20**	3	2	3	10.4
2.	**40**	9	7	7	30.4
3.	**100**	17	18	14	65.2
4.	**120**	20	21	20	81.2
5.	**control**	0	0	0	0

Table 14: Larvicidal activity of leaf essential oil of *A. marmelos* against 3rd instar of *Culex quinquefaciatus* larvae after 48 hours of exposure

Sr. No.	**Dose (ppm)**	**Number of dead larvae**			**% mortality**
		R_1	**R_2**	**R_3**	
1.	**20**	4	3	3	13.2
2.	**40**	10	8	7	33.2
3.	**100**	17	18	15	66.4
4.	**120**	22	22	20	85.2
5.	**control**	0	0	0	0

Table 15: Larvicidal activity of leaf essential oil of *A. marmelos* against 3rd instar of *Anopheles stephensi* larvae after 24 hours of exposure

Sr. No.	Dose (ppm)	Number of dead larvae			% mortality
		R_1	R_2	R_3	
1.	**20**	3	3	4	13.2
2.	**40**	11	6	9	34.4
3.	**100**	13	17	17	62.4
4.	**120**	23	21	21	86.4
5.	**control**	0	0	0	0

Table 16: Larvicidal activity of leaf essential oil of *A. marmelos* against 3rd instar of *Anopheles stephensi* larvae after 48 hours of exposure

Sr. No.	Dose (ppm)	Number of dead larvae			% mortality
		R_1	R_2	R_3	
1.	**20**	5	3	4	16
2.	**40**	11	7	10	37.2
3.	**100**	13	17	17	62.4
4.	**120**	23	21	21	86.4
5.	**control**	0	0	0	0

Table 17: LC_{50} and LC_{90} values of leaf essential oil of *A. marmelos* against the 3rd instar larvae of *Culex quinquefasciatus* and *Anopheles stephensi* after 24 and 48 hrs of exposure.

Mosquito species	Exposure period	LC_{50} (ppm) (LCL-UCL)	LC_{90} (ppm) (LCL-UCL)	Regression equation	Slope (± SE)	X^2
Culex quinquefasciatus	**24 hrs**	61.916 (42.042-91.185)	193.947 (131.694-285.628)	Y=2.595x + 0.354	2.595±0.385	0.931
	48 hrs	56.797 (38.479-83.837)	180.606 (122.356-266.586)	Y=2.576x + 0.4876	2.576±0.086	0.866
Anopheles stephensi	**24 hrs**	57.352 (38.707-83.071)	186.834 (126.095-276.832)	Y=2.544x + 0.540	2.544±0.087	0.799
	48 hrs	54.913 (36.299-83.071)	193.828 (128.127-293.221)	Y=2.387x + 0.861	2.387±0.092	0.793

LC_{50} = lethal concentration killing 50% of the exposed larvae, LC_{90} = lethal concentration killing 90% of the exposed larvae, LCL: Lower confidence limit, UCL: Upper confidence limit, SE = Standard error, X^2 = Chi-squar

Table 18: Effect of leaf essential oil of *C. maxima* against 3rd instar *Culex quinquefasciatus* and *Anopheles stephensi* larvae

Name of sample	***Citrus maxima***
Number of larvae exposed	25
Volume of water	250 mL
Replicates	3

Table 19: Larvicidal activity of leaf essential oil of *C. maxima* against 3rd instar of *Culex quinquefasciatus* larvae after 24 hours of exposure

Sr. No.	**Dose (ppm)**	**Number of dead larvae**			**% mortality**
		R_1	**R_2**	**R_3**	
1.	**20**	3	2	4	12
2.	**40**	9	9	11	38.4
3.	**100**	18	19	18	73.2
4.	**120**	23	21	21	86.4
5.	**control**	0	0	0	0

Table 20: Larvicidal activity of leaf essential oil of *C. maxima* against 3rd instar of *Culex quinquefasciatus* larvae after 48 hours of exposure

Sr. No.	**Dose (ppm)**	**Number of dead larvae**			**% mortality**
		R_1	**R_2**	**R_3**	
1.	**20**	4	2	5	14.4
2.	**40**	9	9	11	38.4
3.	**100**	19	19	18	74.4
4.	**120**	23	22	21	88
5.	**control**	0	0	0	0

Table 21: Larvicidal activity of leaf essential oil of *C. maxima* against 3rd instar of *Anopheles stephensi* larvae after 24 hours of exposure

Sr. No.	Dose (µL)	Number of dead larvae			% mortality
		R_1	R_2	R_3	
1.	**20**	4	2	5	14.4
2.	**40**	11	12	9	42.4
3.	**100**	19	18	18	73.2
4.	**120**	22	23	22	89.2
5.	**control**	0	0	0	0

Table 22: Larvicidal activity of leaf essential oil of *C. maxima* against 3rd instar of *Anopheles stephensi* larvae after 48 hours of exposure

Sr. No.	Dose (µL)	Number of dead larvae			% mortality
		R_1	R_2	R_3	
1.	**20**	5	2	6	17.2
2.	**40**	12	12	10	45.2
3.	**100**	19	18	18	73.2
4.	**120**	22	23	23	90.4
5.	**control**	0	0	0	0

Table 23: LC_{50} and LC_{90} values of leaf essential oil of *C. maxima* against the 3rd instar larvae of *Culex quinquefasciatus* and *Anopheles stephensi* after 24 and 48 hrs of exposure.

Mosquito species	Exposure period	LC_{50} (ppm) (LCL-UCL)	LC_{90} (ppm) (LCL-UCL)	Regression equation	Slope (±SE)	X^2
Culex quinquefasciatus	**24 hrs**	52.773 (36.486-76.331)	153.712 (106.272-222.329)	Y=2.771x + 0.229	2.772±0.082	0.955
	48 hrs	50.233 (34.492-73.158	150.024 (103.012-218.491)	Y=2.712x +0.39	2.713±0.083	0.958
Anopheles stephensi	**24 hrs**	48.995 (33.603-71.439)	147.405 (101.096-214.928)	Y=2.707x + 0.432	2.707±0.084	0.933
	48 hrs	46.277 (31.404-68.194)	145.188 (98.525-213.59	Y=2.623x +0.642	2.623±0.086	0.926

LC_{50} = lethal concentration killing 50% of the exposed larvae, LC_{90} = lethal concentration killing 90% of the exposed larvae, LCL: Lower confidence limit, UCL: Upper confidence limit, SE= Standard error, X^2 = Chi-square

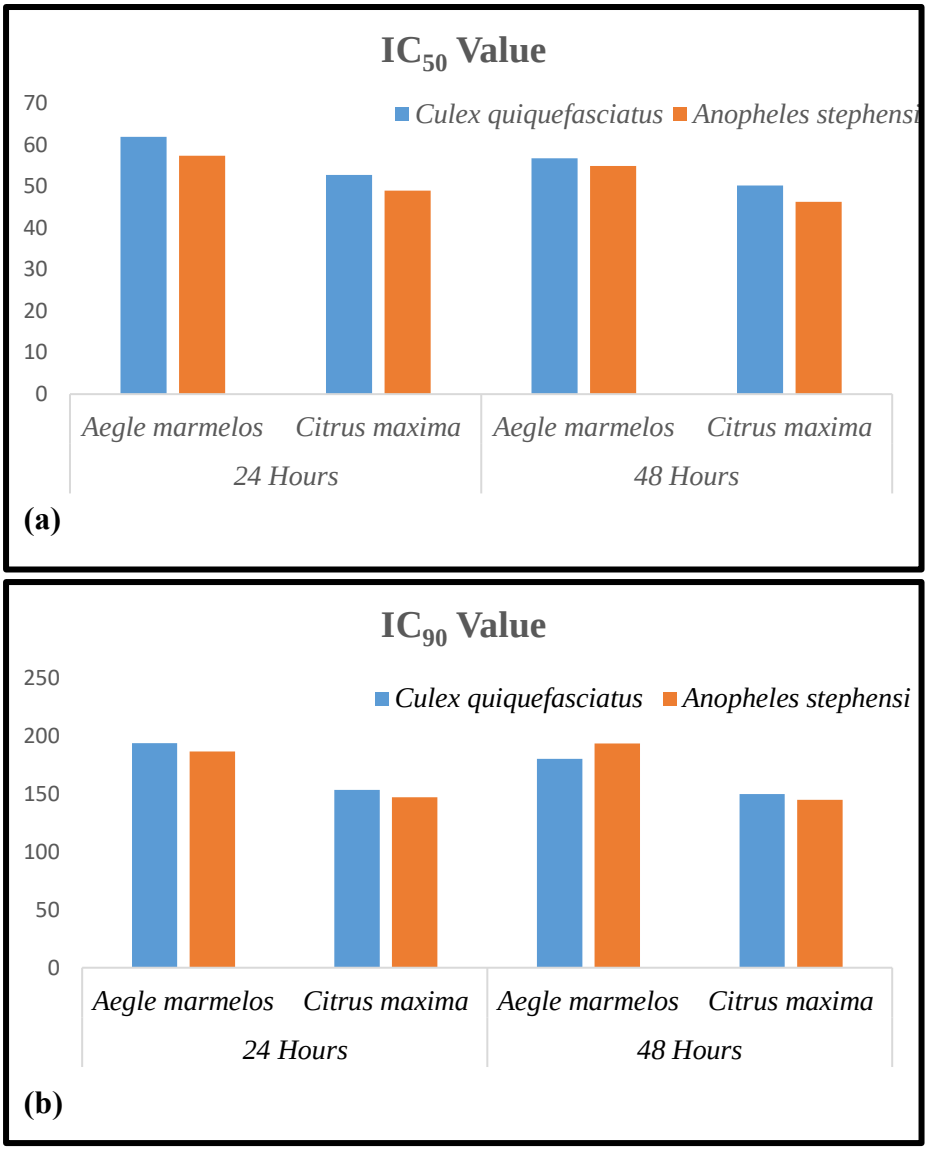


Figure 9: Mosquito larvicidal activity **(a)** IC_{50} value, **(b)** IC_{90} value.

CHAPTER 6: CONCLUSION

CONCLUSION

Citrus maxima and *Aegle marmelos*, two plants from the Rutaceae family, were studied for anatomical, phytochemical, and biological activity. The essential oil was extracted from fresh leaves of both plants. The anatomical characterization of *A. marmelos* and *C. maxima* indicated the existence of distinct anatomical traits. Oil glands were abundant on the surface of the leaves, stem and petiole. This opens up the possibility of utilizing the plants for commercial gain. *A. marmelos* and *C. maxima* belong to the Rutaceae family and are not well know for their commercial uses.

Both plant extracts contained a variety of phytoconstituents, indicating their use for a variety of medicinal purposes. Four different solvents were used among which methanol showed the best results. Alkaloids, carbohydrates, glycosides, saponins, proteins, sterols, phytosterols, fixed oils and fats, phenolic compounds and tannins, and quinones were all detected using various phytochemical techniques.

Citrus fruits are the most common and extensively eaten fruits. It has several therapeutic qualities and has low toxicity. Compound identification may be useful in researching and connecting various therapeutic characteristics in *C. maxima* extract of leaves and fruit rind (Balamurugan *et al*., 2014). The antioxidant activity of the fruit rind in both plant species was higher than in other parts of the plant analysed, boosting its utility as a food additive and aiding in the prevention of food degradation.

Many studies have documented the isolation of volatile chemicals from the leaves of *A. marmelos* grown in various parts of the world. Additionally, earlier publications literature data indicated that essential oil composition is highly influenced by its geographical location. The chemical profiles of the two essential oils were different, according to the GC-MS data. Nonetheless, both oils were high in monoterpenes and sesquiterpenes. *C. maxima* essential oil

extraction yielded more than *A. marmelos*. D-Limonene concentrations were higher in both oils.

During the last two decades, there has been increasing interest in the use of essential oils for the prevention of mosquito-transmitting vector illness since they are target specific and ecologically safe when compared to synthetic products. The essential oils extracted from the *Aegle marmelos* and *Citrus maxima* leaves were effective against the larvae of *Culex quinquefasciatus* and *Anopleles stephensi*. *C. maxima* essential oils have the maximum effectiveness as larvicidal activity against *Anopheles stephensi*. The different responses of the same mosquito species to the same plant essential oils at different developmental stages were linked to physiological and morphological variations. The major constituent compounds of essential oils are usually one of the important elements responsible for particular essential oils insecticidal effect (Das *et al*., 2015).

However, more research into the method of action and field testing are required before they can be considered a potential choice for mosquito control.

CHAPTER 7: SUMMARY

SUMMARY

The Rutaceae family, commonly known as the "Rue family" or "Citrus family," consists mostly of perennial trees, shrubs, and plants containing aromatic volatile oils held in glands visible on the surface of leaves, new branches, inflorescences, flowers, fruit, or seeds. Members of this family have a high amount of vitamin C and various alkaloids. The Rutaceae family is used commercially in the pharmaceutical, medical, cosmetic, and food sectors. The anatomical examination of the stem, leaf, and petiole of *Aegle marmelos* and *Citrus maxima*, highlighted the plants distinct anatomical traits in their cellular structure. Sections were stained using 0.1% Safranin stain and observed under bright field microscopy. Oil glands were abundant in the leaf, stem, and petiole, increasing its economic value. Anamocytic stomata were present in *A. marmelos* leaves. Whereas *C. maxima* had anisocytic stomata. Phytochemical characteristics were investigated. The influence of different solvents on extraction yield varied according to solvent polarity. Methanol outperformed the other solvents tested for phytochemical analysis. Both plants contained alkaloids, carbohydrates, glycosides, saponins, proteins, sterols, phytosterols, lipids and oils, phenolic compounds and tannins, and quinones, as per phytochemical testing.

Antioxidant studies were carried out using DPPH Method. Methanolic extract of Stem, leaves, fruit rind and fruit pulp of both the plants were analysed for antioxidant activity. *Citrus maxima* extracts were better than *A. marmelos* in terms of antioxidant activity. In both species, the fruit rind showed the highest anti-oxidant activity, followed by the leaves, fruit pulp, and stem. The rising therapeutic use of plant essential oils in aromatherapy has prompted research into their biological potency. The essential oils were extracted from the leaves of both plants using a Clevenger apparatus. GC-MS analysis of leaves essential oil revealed the presence α-Pinene, Camphene, β-Pinene, β-Myrcene, D-Limonene, γ-Terpinene, Linalool, Carveol, Citral,

γ-Elemene, Caryophyllene, Humulene, γ-Muurolene, α-Farnesene, Phytol and Octacosane as common chemical compounds in both the plants. D-Limonene was found to be abundant in both the plant species. Essential oils that are rich in monoterpenes have been recognized as food preservatives and natural anti-oxidants. The major constituent components of essential oils are often one of the most important factors responsible for the larvicidal activity of the specific essential oils. *C. maxima* leaf essential oil was shown to be more harmful to *Culex quinquefasciatus* and *Anopheles stephensi* than *A. marmelos* leaf essential oil. Differences in the content of essential oils may be one of the causes of their varying larvicidal effectiveness.

Further research may be conducted using the chemical makeup of essential oils and plants obtained from various geographic locations. This will become an important tool for detecting different chemotypes and confirming traditional usage and bio-prospecting

CHAPTER 8: REFERENCES

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This is to certify that Dr./Mr/Ms Flory Venie Nunes has attended/ ~~Delivered Lead Lecture~~/ Presented a paper (~~Oral~~/Poster) entitled "Comparative phytochemical analysis of leaves and extraction - - - - - Citrus maxima (Burm.) Merr.

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