PHARMACOGNOSTIC ANALYSIS AND BIOACTIVITY STUDIES IN *KAMETTIA CARYOPHYLLATA* (ROXB.) NICOLSON & SURESH

Thesis submitted to the University of Calicut

in partial fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY IN BOTANY

By

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Certificate

This is to certify that the thesis entitled, **"Pharmacognostic Analysis and Bioactivity Studies in *Kamettia caryophyllata* (Roxb.) Nicolson & Suresh"** submitted to the University of Calicut, for the award of the degree of **Doctor of Philosophy** in **Botany**, is a bonafide record of the original research work carried out by **Ms. JIJI P.G.**, at the PG & Research Department of Botany, Sree Narayana College, Nattika, Thrissur, Kerala, affiliated to the University of Calicut, Kerala under my supervision and guidance and no part of the present work has formed the basis for the award of any other degree/diploma to any candidate of any University previously.

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Dedicated to...

My Beloved Daughter

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Contents

ABSTRACT

Accurate recognition, uniformity, and quality assurance of medicinal plants are essential for ensuring the safety and effectiveness of herbal remedies. This study aims to establish a standard for *Kamettia caryophyllata* (Roxb.) Nicolson & Suresh, a climber belonging to the Apocyanaceae family, recognised in Indian alternative medicine for its efficacy in managing ailments like arthritis, scabies, and epilepsy, as detailed in Hortus Malabaricus. Until now, there has been no detailed scientific study on the pharmacognostic or chemical or bioactive properties of this plant. The objective of the study is to evaluate the quality, purity, identity, and bioactive principles of *K. caryophyllata* through pharmacognostic, physicochemical, phytochemical, and bioactivity analyses of the plant components. The study also seeks to validate the scientific evidence supporting traditional medicinal uses by isolating, purifying, and characterising a specific bioactive compound. Authentication of *K. caryophyllata* was achieved through morphological features and molecular methods using DNA barcoding with *rbcL* and *matK* gene sequences. Pharmacognostic features, including macroscopic, powder microscopic and anatomical characteristics and fluorescence colour behavioural patterns were documented. The physicochemical analysis revealed variations in total ash, water-soluble ash, acid-insoluble ash contents and extractive yields among different plant components, with the leaves showing superior characteristics, indicating greater concentration of phytoconstituents, suggesting their significant therapeutic potential than other components. The physicochemical characteristics combined with pharmacognostic, morphological, and molecular characteristics have contributed to create valuable reference markers for monograph preparation of the plant, which can serve as a reference standard for identification and detecting adulteration in crude drugs and guide traditional medicine practitioners in selecting the appropriate plant part of *K. caryophyllata* in herbal medicine. The preliminary phytochemical screening using various reaction tests and GC-MS and HR LC-MS screening for specific bioactive compounds revealed wide range of phytochemical groups with more diverse bioactive compounds in the methanolic extract of leaf, stem and root components with highest predominance in methanolic leaf extract. This inference was further substantiated by the quantitative estimation of secondary metabolites in the methanolic extracts which revealed that majority of these compounds, including terpenoids, alkaloids, and phenolics, were most concentrated in the leaf component compared to the stem and root. The analysis of various bioactivities such as antioxidant, antimicrobial, cytotoxic and anti-inflammatory properties of different component extracts revealed that the leaf methanolic extract has more promising potent activities than the stem and root extracts. The *in-vivo* anti-inflammatory studies using Carrageenan-induced and Formalin-induced inflammation models revealed, methanolic extract is very much effective in animal model. The study revealed that the phytochemical group terpenoids was one of the most abundant with highest dominance in leaf component. The phytochemical screening revealed the consistent occurrence of a terpenoid compound, squalene, in all the component extracts, which has diverse pharmacological properties including anti-inflammatory, antioxidant and antimicrobial. Through the isolation of this potential bioactive compound, squalene, scientific evidence and confirmation on the effectiveness of using *K. caryophyllata* in traditional medicine has been achieved. The study concludes that *K. caryophyllata* holds great potential as a natural medicine for preventing and managing various diseases. It provides a solid foundation for future research, which could lead to pioneering treatments derived from natural sources. However, further clinical studies are needed to validate the efficacy and safety of *K. caryophyllata* extracts for human use, paving the way for evidence-based therapeutic interventions.

Key words: *Kamettia caryophyllata*, Pharmacognostic, physicochemical, phytochemical, bioactivity, anti-inflammatory, antioxidant, antimicrobial, GC-MS, HR LC-MS, HPLC, FTIR

Chapter 1

General Introduction

CONTENTS

INTRODUCTION

Since ancient times, people globally have effectively used medicinal plants to treat various illnesses. The Roman, Greek, Chinese, Indian, and Egyptian civilisations provide evidence of the therapeutic use of herbs. Phytopharmacological evaluation of the therapeutic potential of the plants found in the Neanderthal grave of a Shanidar IV individual (Iraq)conducted by Jan Lietava indicates that herbal medicine may have been practised in the Iraq region for 60,000 years (Lietava, 1992; Petrovska, 2012). The Vedas, an ancient Indian scripture, refer to the healing properties of plants that are plentiful in the land. Numerous spice plants like nutmeg, pepper, clove, etc., that are used even today have their origins in India (Prakash et al., 2017). In the seventeenth century, Hendrik van Rheede, the Dutch Governor of Malabar (present-day Kerala, India), documented indigenous knowledge about medicinal plants of local healers and practitioners in Kerala in the book Hortus Malabaricus. Even today, use of medicinal plants remain popular due to their sustainable nature in effectiveness and safety aspects as it has the ability to eliminate the side effects associated with the predominance of a single synthetic compound in the body (Tyler, 1999). Further, while the synthetic drugs mostly exert their effects based on single xenobiotic compounds, the pharmacological action of plant-based medicine is often based on the additive or synergistic action of several phytocompounds acting at single or multiple target sites associated with a physiological process. This kind of synergistic or additive pharmacological effect of natural medicine is not only effective in

eliminating various ailments and pathogenic organisms but also reduces the chances of these organisms' developing resistance or adaptive responses (Parekh, 2007). Herbal medicine became widely recognized when the World Health Organization (WHO) encouraged the use of traditional remedies, particularly in developing countries, to address gaps in healthcare not covered by modern medicine.

Now a days, the demand for medicinal plants as natural resources has multiplied several times due to the knowledge of their advantages over synthetic drugs. The World Health Organization estimates that around 80% of people in developing countries depend on traditional herbal medicines for their primary healthcare needs (WHO, 2013). Around 9,000 licensed units in India produce traditional herbal formulations. Currently, over 1,100 medicinal plants from Indian systems of medicine are predominantly collected from the wild (Agarwal & Goyal, 2021). However, the major issues encountered in this field are identical plant names, visual similarities, lack of knowledge about the correct sources due to the absence of proper standardization procedures and monographs, careless collection practices and absence of strict quality control measures. Together with the above issues, the increasing demand for medicinal plants is leading to unguided and unprincipled commercial practice of adulterating, substituting and misidentifying the genuine herbal drugs which is posing a big obstacle in ensuring reproducible quality of herbal-based traditional medicines (Farnsworth & Loub, 1980).

Collecting and analysing the pharmacological research data, including traditional and ethnomedicinal knowledge, is important in developing plant-based drugs. Ensuring the standardisation and quality of medicinal plants is also crucial. The future of herbal medicine research is based on accurate identification, standardisation, and quality control through various scientific methods (Ravishankar & V.J, 2007). Medicinal plants need to be properly evaluated and standardised using various pharmacognostic and phytochemical techniques. These include macroscopy, microscopy, powder microscopy, analysis of fluorescence property, histochemical, physicochemical and phytochemical screening, followed by testing the bioactivity of the plant and isolating specific active compounds of interests (Kokate, 1997). To ensure the purity of herbal medicine, World Health Organisation (2013) has provided some guidelines and good manufacturing practices. WHO has also published monographs of medicinal

plants to serve as a model, aiding countries in developing their own national or regional monographs on medicinal plants or national formulations on herbal medicines (WHO, 2002). In India, the AYUSH department gives instructions and recommendations to ensure the standard, purity, and efficacy of traditional medicine.

The morphological and taxonomic examination has a long-standing history of authenticating and assessing the quality of traditional medicine products and such observation is typically the initial stage in identifying plant species or organs (Muyumba et al., 2021). Unlike taxonomic identification, pharmacognostic studies are crucial in confirming and authenticating medicinal plants by examining their physical, chemical, and anatomical features, supplemented by organoleptic elements such as colour, smell, and taste, which is crucial especially when medicinal plants lose their morphological identity. These studies ensure the consistency and purity of plant-based products, preventing misidentification and adulteration (Chanda, 2014), and are important in establishing reference standards for creating pharmacopeial monographs and ensuring that the herbal products meet the regulatory guidelines (Alamgir, 2017).

In traditional medical application, the herbal medicine is mostly used in powdered form or in any processed form and it is difficult to ensure the plant identity. So, in this context DNA based molecular marker analysis is another widely accepted strategy in the field of identification and authentication of herbal medicine. Moreover, these markers are not tissue specific and hence can be used for identification at any stage of development (Sinha et al., 2012).

Ayurvedic Pharmacopoeia of India (API, 2010) recommend the determination of physicochemical parameters, including loss on drying, ash content, pH values, water and alcohol soluble extractive, etc., as essential steps to be carried out for medicinal plants. Understanding physicochemical characteristics and their documentation is essential for determining the identity, purity, and quality standards of herbal plants; further, it is one of the crucial steps in drug discovery and development of medicinal plants (WHO, 2002).

Phytochemical analysis is mandatory for regulating the effectiveness of herbal products, which helps to fix the dosage of plant medicine and minimise the side

effects. Secondary metabolites or phytochemicals are the organic compounds in the plant body that help it fight against various pathogens, insects and herbivores as part of self-protection (Badria & Aboelmaaty, 2019). These metabolites have a crucial role in medicine and act as therapeutic agents. Phytochemical analysis validates the use of traditional medicine by identifying the potential therapeutic agents present in the plants. It bridges traditional knowledge with modern science, ensuring that herbal medicines are safe, effective, and of high-quality (Gupta et al., 2023). There are various qualitative and quantitative phytochemical screening techniques such as different chemical tests, GC/MS, and HR LC-MS analyses which are employed to profile these compounds, providing insights into the pharmacological potential of the plant. Terpenes, Phenolics, and Alkaloids are the three major classes of secondary metabolites in plants, reported to have diverse biological properties which offer significant pharmacological effects, such as antimicrobial, antioxidant, anti-inflammatory, or anticancer properties (Dalir & Safarnejad, 2017). This knowledge provides scientific backing for medicinal claims and pave the way for developing new drugs by isolating and enhancing active compounds from medicinal plants.

Bioactivity assays play a crucial role in phytochemical research by connecting different phytochemicals with various biological effects. Hence, these assays are necessary to evaluate plant extracts for different pharmacological properties, including antimicrobial, antioxidant, anti-inflammatory, and cytotoxic effects (Barba-Ostria et al., 2022). The bioactivity of the plant can be assessed trough in vitro and in vivo studies, which play a crucial role in drug development and validation of traditional plant-based treatments (Khan et al., 2013; Patil et al., 2019). *In vitro* studies provide preliminary insights into bioactivity, while *in vivo* research helps confirm the physiological significance of the bioactive compounds (Dorfer et al., 2019). Many plants in traditional medicine are not scientifically validated, and bioactivity studies give a scientific foundation on the efficacy of these traditional medicines, which supports their continuous use and aid the production of new pharmaceutical medicine. Bioactivity examination also reveals potential side effects that ensure the safe and effective use of herbal drugs (Muyumba et al., 2021).

Traditional plant-based treatments can be scientifically validated by linking pharmacognostic and phytochemical data with bioactivity. This approach can lead to

the discovery of new therapeutic agents or the development of novel plant-derived compounds for pharmaceutical applications. Hence, in order to harness the power of specific phytocompounds of interest, as well as for the scientific validation of traditional use, it is necessary to isolate these compounds from the plant as it is essential for acquiring a refined and characterised version of the compound from the plant. The major steps in the process of isolation are extraction of phytochemicals, screening of compounds, isolation, purification, structuration and toxicology, as well as clinical evaluation of the compound. This process involves the applications of various analytical techniques, including Saponification, Silica Gel column chromatography, Thin-layer chromatography (TLC), High-performance liquid chromatography (HPLC), Fourier-transform infrared spectroscopy (FTIR), Nuclear Magnetic Resonance spectroscopy (NMR) and Gas chromatography-Mass spectrometry (GC-MS) (Sasidharan et al., 2011).

The present investigation aims to assess the pharmacological applications of the plant *Kamettia caryophyllata* (Roxb.) Nicolson & Suresh, a climber belonging to the family Apocyanaceae, documented in the Indian traditional system of medicine for treating leprosy, arthritis, itches, scabies, Spasm, epilepsy and antidote for poison as documented in Hortus Malabaricus (Manilal & Remesh, 2010; Manilal, 2003). This study attempts to standardise the raw plant drug *Kamettia caryophyllata*, to reveal the details of quality, purity, identity, and bioactive principles of pharmacological interest. Further, the study aims to validate the scientific evidence, if any, in support of traditional medicinal use of the plant via isolation, purification and structuration of specific bioactive compounds of interest.

RESEARCH PROBLEM

Traditional medicine systems are undeniably a subject of global significance. Many plants in our collection of botanical diversity possess medicinal and nutritional benefits because of the various primary and secondary metabolites found within them. Numerous plant species are currently being utilised by traditional healers to create successful treatments for different illnesses. Nevertheless, few plant species with medicinal benefits have been rigorously assessed and confirmed for potential medical uses, while majority still lack scientific exploration. Analysing, identifying and extracting bioactive compounds with diverse biological activities from medicinal

plants enable pharmacological researchers to develop promising medicinal drugs which have less side effects and less expensive (Cragg & Newman, 2002). In this context, the present investigation is proposed with an attempt to assess the pharmacological applications of the plant *Kamettia caryophyllata* (Roxb.) Nicolson & Suresh, a climber belonging to the family Apocyanaceae, known to have uses in the Indian traditional system of medicine for treating leprosy, arthritis, itches, scabies, Spasm, epilepsy, antidote for poison as documented in Hortus Malabaricus (Manilal & Remesh, 2010; Manilal, 2003). To the best of knowledge, no detailed pharmacognostic and scientific chemical analysis has been previously reported on this plant. There is a need to fill this research gap through systematic studies and create a robust scientific knowledge that supports its traditional medicinal use. Therefore, it is of great interest to carry out the standardisation of this unexplored raw plant drug to validate its pharmacological potentialities through scientific analysis.

OBJECTIVES

In an attempt to address the research problem, the objective of the present study was to standardise the raw plant drug *Kamettia caryophyllata*, to reveal the details of quality, purity, identity, and bioactive principles of pharmacological interest. The major criteria for standardising the raw drug are pharmacognostic, physicochemical, phytochemical and bioactivity analysis. Further, the study aims to validate the scientific evidences if any, in support of traditional medicinal use of the plant via isolation, purification and structuration of specific bioactive compound of interest. The study includes,

1) Morphological & Molecular Characterisation of *Kamettia caryophyllata*
2) Pharmacognostic analysis
3) Physicochemical analysis
4) Phytochemical analysis
5) Bioactivity analysis
6) Isolation, purification and structuration of specific bioactive compound

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Chapter 2

Morphological & Molecular Characterisation of *Kamettia caryophyllata*

CONTENTS

INTRODUCTION

Natural medicine's synergistic, combined, or additive pharmacological effect proves highly effective to eliminate a wide range of pathogenic organisms and minimising the development of resistance or adaptive responses in these organisms (Parekh, 2007). Moreover, it successfully addresses the problematic side effects of relying predominantly on a single synthetic compound within the body (Tyler, 1999). Due to these advantages, the utilisation of medicinal plants as raw materials in drug production has been steadily increasing. It is estimated that a quarter of the world's population relies on traditional medicine to treat various ailments (Ekor, 2014). However, despite their advantages over synthetic medicines, natural plant-based medicines suffer from adulteration, substitution, and misidentification issues during drug production (Ekar & Krest, 2019). Medicinal plants used for preparation might be collected from the wild or contaminated areas by local collectors and this consequently may adversely affect the efficiency of herbal medicines. A major hindrance to the use of medicinal plants by herbal medicine industries lies in the non-availability of official monographs and incomplete documentation regarding the validation of many plant materials. Correct characterisation and quality assurance of

starting material are essential steps to ensure the reproducible quality of herbal medicine, which will help to justify its safety and efficacy (Akbar et al., 2014).

To address this concern, the World Health Organization (1999) has emphasised the study of morphological and microscopic characteristics of medicinal plants to ensure correct authentication and identification, thereby reducing adulteration and ensuring the quality, purity, and efficacy of medicinal preparations (Mustafa et al., 2016). Further, the DNA-based marker analysis using a short, standardized DNA region called DNA barcode (Hebert et al., 2003) has emerged as a widely accepted strategy in herbal medicine identification and authentication. These markers are not tissue-specific, allowing identification at any stage of development (Zubakov et al., 2018).

The diverse plants within the Apocyanaceae family bear substantial ecological, economic, and medicinal significance (Islam & Lucky, 2019). The plant *Kamettia caryophyllata* (Roxb.) Nicolson & Suresh, belongs to the family Apocynaceae and has a history of use in the Indian traditional system of medicine for treating various conditions such as leprosy, arthritis, itches, scabies, spasms, epilepsy, and poisoning, as documented in Hortus Malabaricus (Manilal & Remesh, 2010; Reede, 1689). The plant *K. caryophyllata* and its taxonomic/morphological descriptions were given in Hortus Malabaricus (Vol IX) in 1689. In Hortus Malabaricus, details are described under the name 'Kametti Valli, and its other name is mentioned as 'Ulu Valli.' The available literature reveals that no pharmacopoeia standards or DNA barcoding studies have been previously conducted and reported on this plant. Therefore, this chapter aims to conduct a detailed analysis of morphological and molecular-level characteristics of *K. caryophyllata* to ensure correct authentication and identification.

REVIEW OF LITERATURE

Traditional medicines are readily available and culturally acceptable, and from ancient times, we have used plants as medicine for the treatment of various diseases. This traditional knowledge is passed from generation to generation, mainly through verbal means. They provide easily accessible and less expensive health care management and serve as an essential source of livelihood for indigenous rural populations (Karunamoorthi et al., 2013). However, a critical obstacle that has hindered the

promotion of alternative medicines in developed countries is the absence of documentation and stringent quality control measures (Akbar et al., 2014).

Preparations of monographs are intended primarily to promote harmonisation in the use of herbal medicines concerning safety, efficacy, and quality control (WHO, 2009). Sometimes, the same vernacular names are given to closely related species; therefore, these plants are substituted during traditional medicine preparation in certain situations leading to lack of sustainability in the quality of herbal medicine. Correct identification and quality assurance of the starting materials are essential prerequisites to ensure the reproducible quality of herbal medicine, which will contribute to its safety and efficacy. In such cases, morphological and molecular characterisation studies will ensure plant identity, and will help to prevent adulteration. According to Thomas and Chandra (2008), the analysis of macroscopic and microscopic characters, chemical and physical parameters, and genetic information are the confirmatory tests for standardisation. These studies help in authenticate and ensure the reproducible quality of herbal products (Amponsah et al., 2014).

Hortus malabaricus is the most important and oldest published book about the medicinal plants of Malabar. Twelve volumes with 794 plant illustrations were published, and they contain plant descriptions, illustrations, the healing properties of the plant, herbal formulation methods and their applications are also given in the book (Manilal, 1984). The book included contributions to Ayurvedic medicine by Itty Achuthan, a respected traditional healer, along with other renowned physicians such as Ranga Bhattan, Vinayaka Pandithan, and Appu Bhattan, all known for their expertise in indigenous plants used in medicine and cooking (Sastry, 1965). Scientists and researchers have used the data found in Hortus Malabaricus to study medicinal characteristics of plants and uncover novel pharmaceuticals (Mohan, 2005). The plant *Kamettia caryophyllata* and its taxonomic/morphological descriptions were given in Hortus Malabaricus (Vol IX) in 1689. (Manilal & Ramesh, 2010; Manilal, 2003; Reede, 1689). In Hortus Malabaricus, details are described under the name ‘Kametti Valli, and its other name is mentioned as ‘Ulu Valli’. The name of the plant *Kamettia caryophyllata* was first published in the journal Taxon in the year of 1986 (Suresh & Nicolson, 1986).

According to Sattler (1994), morphological characterisation is a valuable method for plant identification, especially when combined with other techniques like molecular analysis. Botanists can classify and identify plant species based on similarities and morphological differences through observation and comparison. However, identifying and authenticating the plant's identity in dried and processed herbal products is often challenging. Molecular identification methods play a crucial role in overcoming these difficulties. According to Iroka et al. (2015), it is impossible to identify the plant using our conventional method in herbal medicine from the market in the form of dried crushed or slurry plant parts.

There is a potential need to develop robust DNA-based markers for plant identification and authentication at the commercial level with accuracy (Mishra et al., 2016). Among the prevailing genome-based approaches to overcome the difficulties of traditional taxonomy, DNA barcoding proposed by Hebert et al. (2003) has successfully identified existing species and discovered unknown species. DNA barcoding is an oversimplified solution to a complex problem, which provides a way to confirm the authentication of raw plant material and establish quality assurance within the marketplace (Sinha et al., 2012).

The study conducted by Pukhrambam et al. (2023), provided valuable insights into the molecular identification of *Polygonum posumbu*. It highlighted the potential of ITS marker-based DNA identification methods in botanical research. The study conducted by Rejitha et al. (2020) highlighted that the leaf and floral traits of four *Plumeria* species help to distinguish between the different species of *Plumeria*, ensuring the proper use of the plant as a drug. Iroka et al. (2015) was conducted a study on the morphological characteristics of *Stachytarpheta* species in Awka, a region in South Eastern Nigeria, which have traditionally been used for treating diabetes, sedation, hypertension, asthma, and fever. Physical observation and measurement of fresh floral and vegetative parts of the plants were used to study the morphological characteristics of *Stachytarpheta* species. Shamim (2021) conducted a morphological evaluation of some medicinal plants found in Bangladesh for correct authentication and identification. Taxonomical markers were used to identify valuable medicinal plants like, *Acorus calamus, Achillea millefolium, Arnebia nobilis, Gymnema sylvestre, Fumaria indica, Origanum vulgare, Peganum harmala,*

Paeonia emodi, Psoralea corylifolia, Rauwolfia serpentina, and *Vetiveria zizanioides* and their quality, safety, and standardization were evaluated through characterization and palynological studies, with botanical and crude drug descriptions ensuring quality assurance throughout collection, commerce, manufacturing, and production (Fazal et al., 2013).

Abdalla et al. (2016) reported, the morphology and anatomy of the leaves of two popular medicinal plants in Sudan, *Nerium oleander* and *Catharanthus roseus*, finding that both have green leaves with smooth edges, pointed tips, network-like veins, irregularly shaped stomata. However, *N. oleander* has more surface hairs, four layers of cells in the epidermis, calcium oxalate crystals, sunken stomata, and leaves that are the same on both sides, while *C. roseus* has fewer surface hairs, only one layer of epidermal cells, leaves with top and bottom sides and both plants have vascular bundles arranged in pairs. To evaluate the morphological and molecular genetic diversity among 18 *Coleus forskohlii* genotypes from various locations in central India, Tripathi et al. (2013) utilized RAPD (Random Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeat), and AFLP (Amplified Fragment Length Polymorphism) marker systems.

Safhi et al. (2023) in their study, nuclear ITS and chloroplast rbcL DNA regions of *Rumex nepalensis , Oxygonum sinuatum , Withania somnifera , Withania coagulans, Trichodesma africanum , Lindenbergia sp. ,Barleria prionitis , Hibiscus sabiensis , Myrtus communis , Sageretia thea , Sageretia paucicostata , Sageretia lucida, , Tribulus terrestris, Crotalaria sp. Crotalaria incana Vachellia tortilis* and *Vachellia reficiens* were subjected to sequencing and analysis using identification methods based on BLAST and phylogeny for authentication of these plants. Their study successfully helped in distinguishing ten out of the fourteen species through DNA barcoding, five of which were identified through physical examination and three of which were identified morphologically. The analysis by Safhi et al. (2023) clearly highlighted the importance of combining morphological observation with DNA barcoding to precisely identify wild plants, especially medicinal ones, to guarantee public health and safety. Saslis et al. (2012) explained that comparing phylogenetic data can help target screening actions on a selection of commonly utilised plants with higher levels of bioactive substances and might rejuvenate the application of

traditional wisdom in bioprospecting. Bare et al., (2024) conducted DNA barcoding on *Withania coagulans* using five genetic markers to identify species and investigate phylogeny with maximum likelihood method. Results indicated that *psbA* and *rbcL* are superior barcodes for studying *W. coagulans*, showing 100% conservation across various locations. The *rpoB* marker had the highest likelihood value (-889.38), with rbcL coming in second place (-967.83) among the various genetic markers. These results are important for the pharmaceutical industry as they allow for the use of DNA-based species identification to detect adulteration in *W. coagulans* during plant harvesting.

MATERIALS AND METHODS

Habitat & Distribution

Kamettia caryophyllata (Roxb.) Nicolson & Suresh., commonly called "Narumarathivu" or “Kammettivalli” in Malayalam, is predominantly found in evergreen and semi-evergreen forests and sacred groves. It thrives in a seasonally dry tropical forest. This plant is endemic to India's Western and Southern Ghats at altitudes ranging from 400 to 750 meters. It is found in many districts of kerala, and its IUCN status is not defined (https://powo.science.kew.org ; https://www.eflorakerala.com/)

Systematic position of the plant :

Kingdom	Plantae
Phylum	Streptophyta
Class	Equisetopsida
Subclass	Magnoliidae
Order	Gentianales
Family	Apocynaceae
Genus	Kamettia
Species	*Kamettia caryophyllata* (Roxb.) D.H. Nicolson & C.R. Suresh

Selection of Sampling Site

A significant concentration of *Kamettia caryophyllata* was discovered and observed to be flourishing within the Sankulangara sacred grove at S N Puram and its localities

which situated in the coastal belt of the Thrissur district, Kerala (figure 2.1). This site is characterised by coordinates of about 10°16'39.240N latitude and 76°09'58.690E longitude.

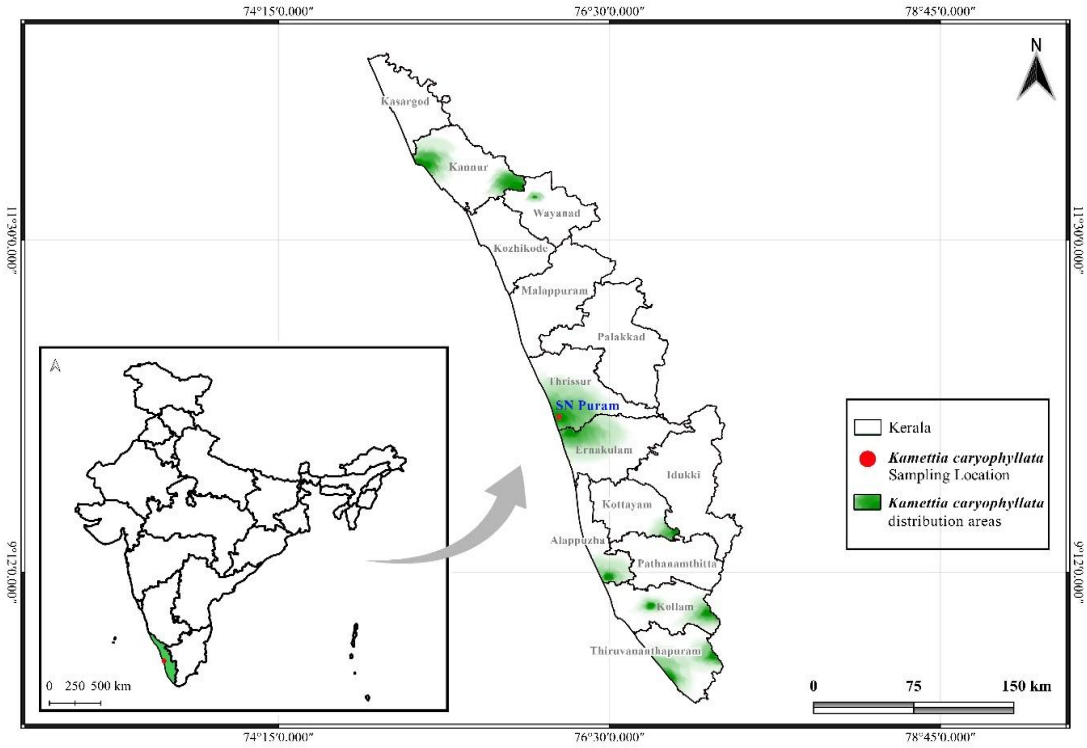

Figure 2.1: Study site and distribution map of *Kamettia caryophyllata*

Figure 2.2: Sampling site - S N Puram, Thrissur District, Kerala

The sampling site was selected based on the prevalence and thriving nature of *Kamettia caryophyllata* within the S N Puram area (figure 2.2). This yielded a

significant number of specimens suitable for further study and analysis, ensuring that the collected samples represent the plant's population in its most favourable habitat.

Authentication of plant material

The taxonomic identity of the plant was confirmed by Dr Sunil Kumar, an eminent taxonomist and Associate Professor in the Department of Botany, SNM College, Maliankara, Ernakulum District. The plant voucher specimen has been deposited in the SNMH! International Herbarium Collection, Department of Botany, SNM College, Maliankara which is accredited by the New York Botanical Garden and is assigned accession numbers 2002 and 2003 for future reference.

Morphological Analysis

Morphological studies were carried out with freshly collected whole plants. All the plant parts, such as the leaves, stem, root, flowers and seeds, were observed thoroughly with the bare eye and magnifying lenses. The detailed data collection of the plant materials was done with the Flora of the Presidency of Madras (Gamble & Fischer, 1923), Flora of Alappuzha (Sunil & Sivadasan, 2000), and from literatures available on the websites such as E-flora Kerala (https://www.eflorakerala.com/), Plants of the World Online (https://powo. science. kew.org), and India biodiversity Portal).

The molecular level of identification by DNA barcoding

DNA barcoding analysis is an efficient tool for identifying plant species. Plant chloroplast regions (*matK, rbcL*, and *trnH-psbA*) are specific to the species level identification (Asahina et al., 2010). *MatK* and *rbcL* genes were selected for barcoding analysis in the present study for the molecular level identification of *Kamettia caryophyllata*.

Genomic DNA extraction

Genomic DNA extraction was carried out using freshly collected leaf samples. Approximately 50 mg of tissue was homogenised using liquid nitrogen and one ml of CTAB extraction buffer, and the resulting powdered tissue was transferred to a microcentrifuge tube. The tube was incubated for one hour at 65^0C and shaken intermittently every ten minutes. One ml of chloroform: Iso-amyl alcohol (24:1) was

added and mixed gently, then centrifuged at 10000 rpm for ten minutes and transferred clear aqueous phase to fresh centrifuge tubes, added 160µl of 6 M NaCl and 1 ml of Absolute ice-cold ethanol refrigerated at -20^0 C for two hours. This solution spun at 13000 rpm for 2 minutes at 4^0C and poured off supernatant, and the pellet was washed clearly with one ml of 70% ethanol. Then, ethanol was removed clearly by vacuum drying at room temperature. To remove RNA, 2 µl(10mg/ml) of RNAse enzyme added and incubate at 37^0C for 1 hour. The DNA samples were stored at 4^0C for further use.

Analysis of DNA Purity and Quantity

The DNA stock samples were quantified using a UV spectrophotometer at 260 and 280 nm, with the assumption that 50 µg of DNA per ml is equivalent to one absorbance unit at the 260 nm wavelength. The concentration and purity of DNA were assessed by measuring the absorbance ratio at 260 and 280 nm. A purity level between 1.8 and 2.0 is considered favourable.

The concentration of DNA was estimated using the formula.

Concentration of DNA (mg/ml) = OD 260 x 50 x Dilution factor

Agarose gel electrophoresis was performed to evaluate the quality of the isolated DNA. For gel electrophoresis, 5µl of DNA was mixed with 1µl of 6X gel-loading buffer composed of 0.25% bromophenol blue and 30% sucrose in TE buffer, pH 8.0. The sample DNA was loaded onto a 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was conducted using 0.5X TBE as the electrophoresis buffer at 75 V until the bromophenol dye front migrated to the bottom of the gel. The gels were visualised in a UV transilluminator (Genei).

Polymerase Chain Reaction

rbcL gene fragment and *matK* gene fragment were amplified by PCR from plant genomic DNA using *rbcL*-PCR and *matK*-PCR universal primers.

Details of primers used for PCR

Forward Primer Name: *rbcL* F: **5'ATGTCACCACAAACAGAGACTAAAGC3'**

Reverse Primer Name: *rbcL* R: **5'GTAAAATCAAGTCCACCRCG3'**

Forward Primer Name: *matK* F: **5'TAATTTACGATCAATTCATTC3'**

Reverse Primer Name: *matK* R: **5'ACAAGAAAGTCGAAGTAT 3'**

(White et al., 1990; Järvinen et al., 2004). PCR was conducted using 25 µl PCR mixture in a thin wall PCR tube with a 200 µl capacity.

The PCR mixture comprised the following components:

Deionised water - 17.7 µl, Taq buffer without MgCl2 (10X) - 2.5 µl, MgCl2 (15 mM) - 1.0 µl, Forward Primer (10 pm/µl) - 0.5 µl, Reverse Primer (10 pm/µl) - 0.5 µl, Taq DNA Polymerase (5U/µl) - 0.3 µl, dNTPs (2 mM each) - 0.5 µl, and Template DNA (20 ng/µl) - 2.0 µl.

The tubes with the mixture were lightly tapped and quickly spun at 10,000 rpm. Afterwards, the PCR tubes, along with all the ingredients, were moved to a thermal cycler (Gene Amp PCR System 9700, Applied Biosystems) for amplification. The PCR amplification profile depicted in table 2.1

Table 2.1: PCR amplification profile

Step	Process	Temperature	Time
1	Initial denaturation	95°C	5 minutes
2	Denaturation	94°C	30 seconds
3	Annealing	55°C	30 seconds
4	Extension	72°C	45 seconds
Go to step 2 for 29 times			
5	Final Elongation	72°C	10 minutes
End			

The PCR products were analysed using 1.2% agarose gels, prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. A commercially available 100bp ladder was utilised as a standard molecular weight DNA marker for reference. Next, the concentration of the purified DNA was determined, and the samples were subjected to automated DNA sequencing using the ABI3730xl Genetic Analyzer from Applied Biosystems, USA. Sequence Scanner Software v1 from Applied Biosystems was employed to assess the quality of the obtained sequences. Following the sequencing, the obtained sequences were aligned and edited as necessary with BioEdit

version 7.2.5 (Hall, 1999). The edited sequences (*rbcL* gene and *matK*) were then used for similarity searches via the BLAST (Basic Local Alignment Search Tool) program in the NCBI GenBank (www.ncbi.nlm.nih.gov) DNA database to identify the plant sample.

Evolutionary relationships of taxa

The Molecular Evolutionary Genetics Analysis Version 11 (MEGA 11) software program was used to construct phylogenetic trees (Tamura et al., 2021; Kim et al.,2016). The first five species from the BLAST results of *rbcL* and *matK* were chosen to construct a phylogeny tree. It helps to identify closely related species over time as they have evolved.

RESULTS AND DISCUSSIONS

Morphological analysis

Habit

Kamettia caryophyllata, a woody climbing plant with exceptional climbing capabilities, allows it to attain impressive heights of 13-15 meters within its ecological habitat (figure 2.2 & 2.4).

Figure 2.3: Seedlings of *Kamettia caryophyllata* (A. seedling; B. seedling with adventitious root)

This description of its growth habit outlines the essential features and strategies contributing to the remarkable vertical growth of *K. caryophyllata* to enhance light acquisition (Gianoli, 2015). The plant contains white latex in its leaves and young stem parts. Young plants of *K. caryophyllata* feature narrow, lanceolate leaves arranged oppositely, with leaves lacking any red colouration on the midrib (figure 2.3).

Figure 2.4: Morphological characteristics of *Kamettia caryophyllata* plant (A. habit; B. climbing stem; C. Shoot-root junction)

Root

The plant's roots comprise of a non-tuberous taproot system that often becomes somewhat woody with age. Due to secondary thickening, irregular splits or cracks can be observed on the outer layer of the roots, while the mature root typically has a cylindrical shape, measuring approximately 1.5-2.0 cm in diameter (figure 2.5).

Figure 2.5: Root characteristics of *K. caryophyllata* (A. root system; B. mature and young root; C. root bark)

Stem

The stem of the plant is smooth and hairless (glabrous), displaying a reddish-brown colour. It possesses a terete or cylindrical shape, with mature stems varying in diameter from 1 to 2.2 cm. The lenticels, which usually begin as small and pale spots on younger stems, develop into large corky pores or narrow lines as the stem matures (figure 2.6).

Figure 2.6: Stem characteristics of *K. caryophyllata*

Leaf

The leaves are simple, with a reticulate vein pattern that is closed in nature. Leaves are primarily arranged in opposite pairs, consisting of two leaves at each node. However, in many mature stems, it is observed that three leaves occur at each node, typically in the upper 4 or 5 nodes of the stem. The size of the leaves varied, with length ranging from 8 to 12 cm and width from 4 to 7 cm. The leaf blade is oblong, with an entire margin, a sharply pointed apex, and either an attenuate or cordate base. The reddish-coloured leaf petiole, connecting the leaf to the stem, measures approximately 0.5 cm long. As the leaf matures, the midrib turns reddish-brown. (figure 2.7).

Figure 2.7: Leaf characteristics of *K. caryophyllata* (A. Adaxial surface; B. abaxial surface; C. enlarged abaxial surface showing midrib)

Flower

The flowers grow in terminal peduncled cymes and accompanied by reddish-coloured subulate bracts (figure 2.8). The calyx, the outermost whorl of the flower, is bell-

shaped (campanulate), glabrous, and about 0.2 cm long. The lobes of the calyx have an acuminate shape. The corolla tube is slightly curved, with visible reddish streaks on its outer surface, measuring 0.9-1.0 cm in length. The petals, arranged twistedly, are 0.5-0.7 cm long, with an acuminate, oblong, and obtuse shape. The inner surface of the petals is white, while the outer surface displays a combination of white and reddish colours. The stamens are gamopetalous (fused to the petals) with penicillate anthers at the apex. The carpels, the female reproductive structures, are free. The style, a slender projection from the carpel, measures 0.5-0.6 cm in length and has a filiform appearance with a beaked stigma.

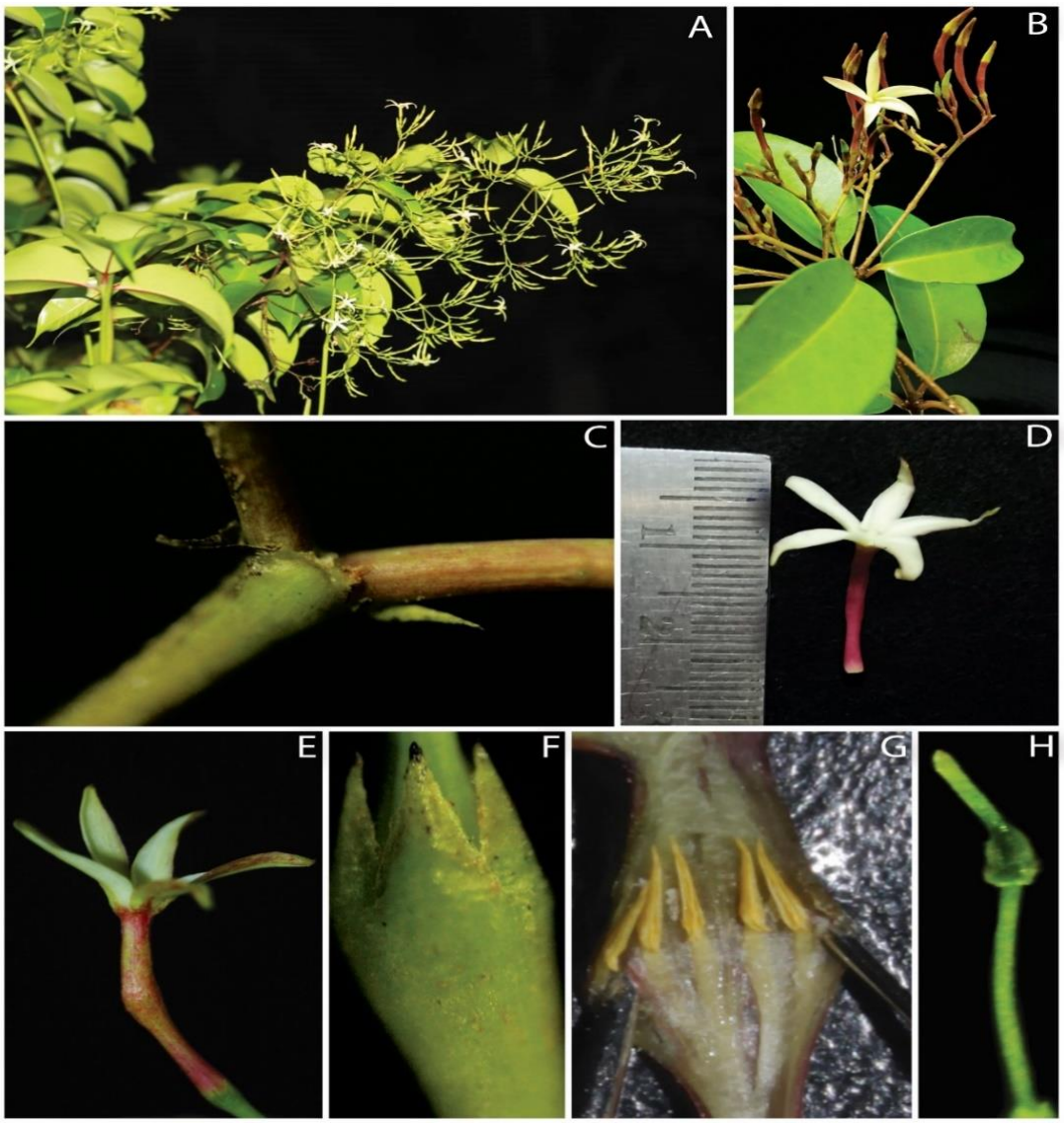

Figure 2.8: Flower characteristics of *K. caryophyllata* (A. & B. inflorescence; C. bracts; D. & E. flower; F. calyx; G. Corolla split –opened showing anthers; H. style with stigma)

Fruit

The fruit, known as follicles, measure 5-7 cm long, divaricate (widely spreading), linear, and terete or cylindrical with 0.5-0.7 cm diameter. The seeds are numerous, winged, measuring 2 cm long (figure 2.9).

Figure 2.9: Fruit and seed characteristics of *K. caryophyllata* plant (A.divaricate fruit; B. fruit surface having lenticels; C. fruit split-with seeds; D. seeds)

The major macroscopic characteristics of *K. caryophyllata* observed in the study are the leathery and glabrous nature of leaves with reddish coloured midrib, reddish-brown coloured stem with abundant lenticels distribution at maturity, presence of white coloured latex in leaves and young stems, gamopetalous flowers having white corolla tube on which reddish coloured streaks, epipetalous stamens and follicle fruits with winged seeds. The plant has no modified structures for climbing, though it is a climber. The study expects these prominent macroscopic features to be used as a first step towards establishing identity, which may be helpful in the qualitative control of the crude plant drug *K. caryophyllata* (Amponsah et al., 2014). The pharmaceutical benefits of this plant, along with the morphological traits crucial for successful breeding, improvement, domestication, and large-scale cultivation, require further establishment and exploration (Tripathi et al., 2013).

Molecular level Identification through DNA barcode

After obtaining nucleic acid sequences, each sequence was edited by manually correcting falsely identified bases and trimming to remove unreadable portions at the 3' and 5' ends, considering both peak and quality values for each base using the sequence analysis tools. The edited sequences of *rbcL* and *matK* were then subjected to similarity searches using the BLAST (Basic Local Alignment Search Tool) program in the NCBI GenBank (www.ncbi.nlm.nih.gov) to confirm their relation to sequences from other related genera or species.

***Kamettia caryophyllata* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene, partial cds; chloroplast**

Consensus Sequence of *rbcL* of *Kamettia caryophyllata* (498 bps)

AAGCGGGAGTTCCGCCCGAAGAAGCAGGGGCCGCGGTAGCTGCTGAA
TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGACTTACCAGCC
TTGATCGTTACAAAGGGCGATGCTACGGCATCGAGCCCGTTCCTGGAG
AAGAAGATCAATATATTGCTTATGTAGCTTACCCCTTAGACCTTTTTGAA
GAAGGTTCCGTTACTAACATGTTTACTTCCATTGTAGGTAATGTATTTGG
GTTCAAAGCCCTACGCGCTCTACGTCTGGAAGATTTGCGAATCCCTCCG
GCTTATATTAAAACCTTCCAGGGCCCGCCTCATGGCATCCAGGTTGAGA
GAGATAAATTGAACAAATATGGTCGTCCCCTGTTGGGATGTACTATTAA
ACCTAAATTGGGGTTATCCGCTAAAAACTACGGTAGGGCATGTTATGAA
TGTCTTCGCGGT

This *rbcL* sequence was then submitted to GenBank, specifically the National Centre for Biotechnology Information, where it was assigned the accession number MH201080.1. To further validate the results, The BLAST analysis was performed, and sequences were retrieved and compared ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene sequences from various plant species. The retrieved sequences included *Kamettia caryophyllata* (Accession: MH201080.1), *Kamettia chandeei* (Accession: DQ660649.1), *Tonduzia longifolia* (Accession: DQ660675.1), *Catharanthus roseus* (Accession: MN989917.1), and *Syzygium johnsonii* (Accession: KU564876.1). These BLAST results showed that the *rbcL* sequence exhibited a high similarity of 99.34% to *K. chandeei*. This

genetic resemblance indicates a phylogenetic relationship between these two *Kamettia* species, further supporting their taxonomic classification within the same genus. (table 2.2). There were no barcoding data for *K. caryophyllata* found in the NCBI database. Moreover, additional matches to other plant species were observed in the BLAST results, such as *T. longifolia* (98.43% identity), *C. roseus* (98.21% identity), which are coming under the Apocyanaceae family and *S. johnsonii* (98.21% identity) under the family Myrtaceae. These high percentages of sequence identity suggest potential evolutionary relationships with these species.

Table 2.2: Blast results Based on the *rbcL* –PCR analysis (NCBI database)

Description	Max Score	Total score	Query cover	E value	Percentage identity	Accession
Kamettia caryophyllata ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene, partial cds; chloroplast	835	835	100%	0.0	100.00%	MH201080.1
Kamettia chandeei ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene, partial cds; chloroplast	819	819	100%	0.0	99.34%	DQ660649.1
Tonduzia longifolia ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene, partial cds; chloroplast	787	787	98%	0.0	98.43%	DQ660675.1
Catharanthus roseus ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene, partial cds; chloroplast	782	782	98%	0.0	98.21%	MN989917.1
Syzygium johnsonii ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene, partial cds; chloroplast	782	782	98%	0.0	98.21%	KU564876.1

Kamettia caryophyllata **maturase K (*matK*) gene, partial cds; chloroplast**

Consensus Sequence of *MatK* of *Kamettia caryophyllata* (857 bps)

CAATTTTTCCATTTTTATTTTGTGTAGATATACTAATACCCCACCCCGTTC
ATCTGGAAATCTTGGTTCAAACCCTTCGCTATTGGGTAAAAGATGCCCCT
TCTTTGCACTTATTACGATTCTTTCTCCGCGAGTATTGGAATTGGAATAAT
CTTATTGCTACAAAGAACCCCTGTTTTGATTTTTTAACAAAAGGAAATCA
AAGATTCTTCTTTTTGTTATATAATTTTTATGTATGTGAATACGAATCCAT
TTTCGTCTTTCTCCATAACCAATCTTCTCATTTACGATCAACATCCTTTGG
GGTCCTTCTTGAACGAATCTATTTCTATGGAAAAATAGAATGTCTTGCCG
AAGTCTTTGCTAGGGATTTTCAGGCCAACTTGGGCTTGTTCAAAGATCCT
TTCATGCATTATGTTAGGTATCAAGGAAAATCCATTCTGGTTTCAAAGGG
CACGGCTCTTTTGATAAATAAATGGAAATCTTATCTTGTCAACTTTTGGC
AATGTCATTTTGACTTGTGGTTTCGCTCGGAAAGGGTCTATATAAAACAA
TTGTCCAATCATTCTCTTGACTTTATGGGTTATCTTTCAAGTGTGCGACTA
AACCCTTCAATCGTACGGAGTCAAATGCTAGAAAATGCATTTCTAATCA
ATAATGCTATTAAGCAATTTGATACCCTTGTTCCAATTCTTGCTCTGATTA
GATCATTGGCTAAAGCGAAATTTTGTAACCTATTAGGACATCCCATTAGT
AAGCCGGTTCGGACTGATTTATCGGATTCTGATATTATGGACAGATTTGG
GCGTATATGCAGAAACCTTTCTCATTATCATAGTGGATCTTCAAAAAAAA
GA

This *matK* sequence was then submitted to GenBank, the National Centre for Biotechnology Information, where it was assigned the accession number MH681795.1. The first five sequences obtained from BLAST analysis were retrieved, and maturase K (*matK*) gene sequences were compared to validate the results. The retrieved sequences included *Kamettia caryophyllata* (Accession: MH681795.1), *Kamettia chandeei* (Accession: DQ660522.1), *Ochrosia poweri* (Accession: KT955350.1), *Ochrosia kilneri* (Accession: KT955346.1), and *Neisosperma poweri* (Accession: DQ660527.1). These BLAST results showed that the *matK* sequence exhibited a high similarity of 99.4% to *K. chandeei*. This genetic resemblance indicates a phylogenetic relationship between these two *Kamettia* species, further supporting their taxonomic classification within the same genus. (table 2.3). No *matK* barcoding data for *K. caryophyllata* were found in the NCBI database.

Moreover, additional matches to other plant species are observed in the BLAST results, such as *O. poweri* (97.60% identity), *O. kilneri* (97.60% identity) and *N. poweri* (97.48% identity), which are coming under the Apocyanaceae family. These high percentages of *matK* sequence identity also suggest potential evolutionary relationships with these species.

Table 2.3: Blast results Based on the *matK* –PCR analysis (NCBI database)

Description	Max Score	Total score	Query cover	E value	Percentage identity	Accession
Kamettia caryophyllata maturase K *(matK)* gene, partial cds; chloroplast	1537	1537	100%	0.0	100.00%	MH681795.1
Kamettia chandeei maturase (*matK*)gene, partial cds; chloroplast	1509	1509	100%	0.0	99.40%	DQ660522.1
Ochrosia poweri voucher Forster 26594 (PRI) maturase K (*matK*) gene, partial cds; chloroplast	1426	1426	100%	0.0	97.60%	KT955350.1
Ochrosia kilneri voucher Forster 29925 (PRI) maturase K (*matK*) gene, partial cds; chloroplast	1426	1426	100%	0.0	97.60%	KT955346.1
Neisosperma poweri maturase (matK) gene, partial cds; chloroplast	1426	1426	100%	0.0	97.48%	DQ660527.1

Evolutionary relationships of taxa

The first five species from the BLAST results of *rbcL* and *matK* were chosen to construct a phylogeny tree. The retrieved sequences were then used as a foundation to construct a phylogenetic tree, shedding light on the evolutionary relationships among these species. These sequences were comprehensively analysed to discern their genetic relatedness and potential evolutionary connections. The BLAST results revealed high sequence similarity for all retrieved sequences, as evident from the exceedingly low E-values and high percentage identities (Pearson, 2014). These

similarities underscore the conserved nature of the *rbcL* and *matK* genes among the investigated species. The entire evolutionary analysis was conducted in MEGA11 (Tamura et al., 2021), a widely used software program for such analyses.

The evolutionary history was inferred using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). The percentage of trees in which the associated taxa clustered is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Likelihood (ML) approach and then selecting the topology with superior log likelihood value. The tree is accurately represented, with branch lengths indicated by the number of substitutions per site as shown in figure 2.10. (Scott & Baum, 2016). This examination included five sequences of nucleotides.

***rbcL* Evolutionary analysis by Maximum Likelihood method**

The figure 2.10 shows the phylogenetic tree of the first five species from the *Kamettia caryophyllata rbcL* blast result with the Maximum likelihood (-739.65). Species names with their accession numbers are included in the tree. Any positions with gaps or missing data were removed. The final dataset contained 452 positions. Utilising the Maximum Likelihood method and the Kimura 2-parameter model, a phylogenetic tree was constructed to illustrate the evolutionary relationships among these species. The tree was drawn to scale, with branch lengths representing the number of substitutions per site, effectively quantifying the genetic divergence between taxa (Tamura et al., 2021). The constructed phylogenetic tree displayed a coherent clustering pattern. *K. caryophyllata* and *K. chandeei* formed a closely related clade, aligning with their taxonomic proximity. 96 % of the bootstrap value between *K. caryophyllata* and *K. chandeei* indicates the accuracy of the result. Bootstrap values in a phylogenetic tree represent the frequency, out of 100, at which a particular branch is seen when generating the tree again using a different set of data (Kim et al., 2016). *Tonduzia longifolia* occupied a separate branch, indicating a distinct evolutionary trajectory, which means it evolved differently from the other species in the tree due to various factors, such as different environmental pressures, genetic mutations or periods of isolation (Hancock & Edwards, 2014; Ngou et al., 2024). *Catharanthus roseus* and *Syzygium johnsonii* were observed to share a branch, which suggests a common

genetic heritage between them, and a bootstrap value higher than 60% suggests the robustness of the inferred relationships.

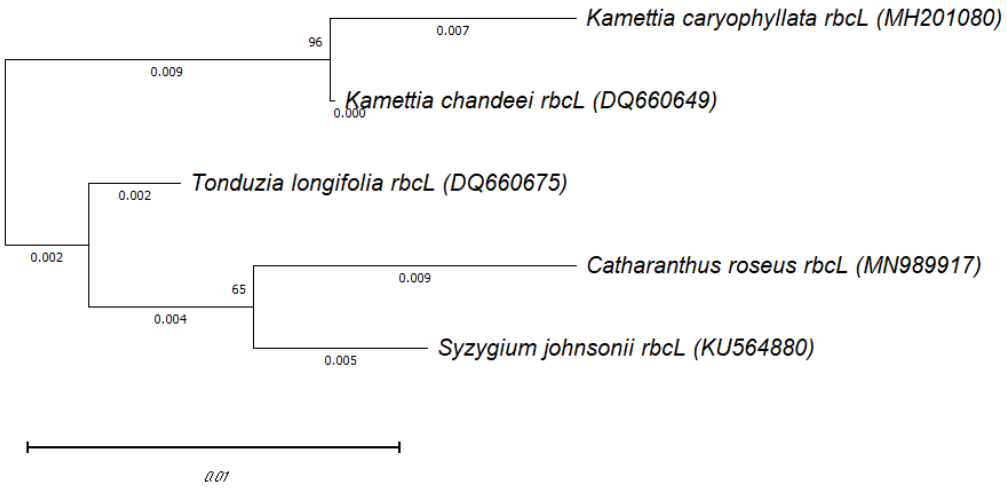

Figure 2.10: *rbcL* Phylogenic tree of *Kamettia caryophyllata* by Maximum Likelihood method (bootstrap values greater than 60% are shown above the branches)

matK **gene Evolutionary analysis by Maximum Likelihood method**

The evolutionary history of the *matK* Phylogenic tree of *Kamettia caryophyllata* was concluded using the Maximum Likelihood method and Kimura's 2-parameter model. The tree with the highest log likelihood (-1307.13) is shown in figure 2.11. The final dataset had 833 positions data with gap and missing was eliminated. The constructed phylogenetic tree based on the *matK* gene sequences provided the evolutionary relationships among five plant species obtained from the blast result of the *matK* gene of *K. caryophyllata*. The evolutionary tree topology revealed distinct patterns of divergence and genetic relatedness.

One of the significant findings was the close genetic relationship between *K. caryophyllata* and *K. chandeei*, similar to the *rbcL*-based phylogenetic tree. These two species shared a common ancestor, as indicated by their proximity in the phylogenetic tree. This genetic proximity aligned with their taxonomic classification suggested a recent divergence (Pozzi et al., 2014). In contrast, a separate tree branch encompassed *Ochrosia poweri*, *Ochrosia kilneri*, and *Neisosperma poweri*. This branch did not exhibit horizontal branches, indicating a lack of significant genetic divergence among these species (Krylov et al., 2003). The absence of horizontal branches in this clade implied that these three species, *O. poweri*, *O. kilneri*, and *N. poweri*, shared a

relatively recent common ancestor (Cavalier, 2010). These observations from the phylogenetic analysis revealed that they arose from the same ancestor and support a closer genetic relationship between *K. caryophyllata* and *K. chandeei*. Two species close in a phylogenetic tree suggest they recently diverged from common ancestors (Algarni, 2022).

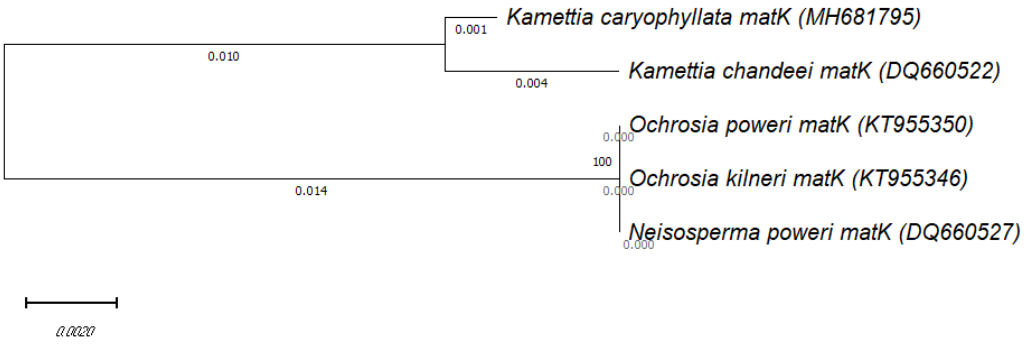

Figure 2.11: *matK* Phylogenic tree of *Kamettia caryophyllata* by Maximum Likelihood method (bootstrap values greater than 60% are shown above the branches)

Most of the rural population in developing countries has been utilising traditional medicines to treat various diseases since ancient times, particularly considering their availability and accessibility. However, now, as a result of scientific advancement in the field of medicine and understanding the significance and advantages of natural medicine over synthetic medicine like fewer side effects and sustainable efficiency against pathogens due to synergistic or additive action have led to its increasing demand among the rural as well as urban population not only in developing countries but also in developed countries(Anand et al., 2020; Gupta et al., 2023). This has resulted in massive commercialisation of plant resources and has emerged as one of the remarkable economic factors worldwide. In this scenario, the demand for plant resources has multiplied considerably, leading to misidentification of plant species during harvest and further over-exploitation, leading to scarcity, which gives way to substitution and adulteration (Chanda, 2014). In light of the above facts, the WHO has observed certain degradations in the quality and efficacy of plant-based traditional medicine and has put forth specific guidelines for documentation and monograph preparation of medicinal plant species to avoid such issues (World Health Organization, 2013). In the present study, the morphological characters of *K. caryophyllata* have been recorded for macroscopic and molecular-level identification.

Macroscopic examination of the plant species is an inexpensive method to identify and distinguish plant species from other similar plants (Amantayeva et al., 2023). According to Fazal et al. (2013), morphological traits are important for accurately identifying, describing, and delimiting taxa using existing markers. Taleb and Shamim (2021) explain that the taxonomic or botanical classification of medicinal plants is essential for their proper use in therapy and the accurate identification of raw drugs. Morphological characterisation of medicinal plants is inevitable in the herbal industry for identification, Quality control and pharmacognostic standardisation of medicinal plants.

DNA barcoding is an effective tool for the identification and discovery of species. Scientists can determine and find species by studying brief, uniform gene sequences extracted from an organism's DNA (Dorovskikh & Ivaneshkin, 1995).With the progress and improvements in molecular techniques, genetic analysis has been considered an effective and successful way to identify plant species. Recently, as proposed by Hebert et al. (2003), DNA barcoding analysis has emerged as a simple, economical and faster method with precision and accuracy for the identification and authentication of raw plant material in the preparation of traditional medicine to ensure the level of quality by finding adulteration, misidentification and substitution (Sinha et al., 2012).

In the present study, the molecular identification of *K. caryophyllata* was approached with a multi-gene strategy, utilising both the *matK* and *rbcL* genes. This comprehensive approach enhances the reliability of species identification and provides a more thorough understanding of the genetic characteristics of *K. caryophyllata*. The analysis of the *rbcL* gene, as previously discussed, highlighted the close genetic relationship between *K. caryophyllata* and *K. chandeei*, solidifying the identification of *K. caryophyllata*. The utilisation of the *matK* gene, another commonly employed marker in plant molecular systematics, adds an additional layer of evidence to our identification process. Moreover, it is worth noting that using multiple genes in species identification is a powerful approach, as it can help mitigate potential issues associated with genetic variation in specific regions. The findings from *matK* and *rbcL* data support the molecular identity of *K. caryophyllata.* This research employs *matK* and *rbcL* data to contribute to the accurate molecular

identification of *K. caryophyllata*. It showcases the effectiveness of a multi-gene approach in species identification and phylogenetic studies (Ho et al., 2021). Combining these molecular markers offer a robust foundation for future research *on K. caryophyllata*, including taxonomic and ecological investigations.

matK & *rbcL* phylogenic tree of *K. caryophyllata* by Maximum Likelihood method supports the close relatedness of *K. caryophyllata* and *K. chandeei.* These are the two species reported under the genus *Kamettia* under the family apocyanaceae, first reported in 1834. Suresh and Nicolson (1986) named the first species *Kamettia caryophyllata* found in the western ghats of India. The second species *Kamettia chandeei D. J. Middleton* discovered in Thailand by Middleton et al. (2005). Two species, close in a phylogenetic tree suggest they recently diverged from common ancestors (Algarni, 2022).

CONCLUSION

The increasing demand for natural medicines and effectiveness has highlighted the need to standardise and authenticate herbal materials. *Kamettia caryophyllata*, a plant with potential medicinal value, has been the focus of this study. Through a comprehensive approach involving morphological observations, molecular-level DNA barcoding, and evolutionary analysis by the maximum likelihood method, the identification and authentication of *K. caryophyllata* have been successfully established. The study revealed key macroscopic characteristics, such as reddish-coloured stems, white latex in leaves, and specific flower features, which serve as valuable indicators for the plant's identity. DNA barcoding using the *rbcL* and *matK* genes confirmed the plant's identity and revealed a close similarity to *Kamettia chandeei,* providing a robust authentication method. The BLAST analysis and subsequent phylogenetic reconstruction of the *rbcL* and *matK* gene sequences offer valuable insights into the evolutionary dynamics among these plant species. The congruence between genetic relatedness and taxonomic classifications suggests that the *rbcL* and *matK* genes can be reliable markers for elucidating molecular identification and evolutionary relationships within the plant kingdom.

This study contributes to our understanding of evolutionary processes and highlights the potential of utilising molecular markers in delineating species relationships, aiding

in biodiversity conservation and taxonomic studies. Further investigations using additional genes and broader taxonomic sampling could provide deeper insights into the intricate evolutionary history of these plant species. In conclusion, integrating the plant's macro and micro morphological and molecular characterisation has laid a solid foundation for the identification, authentication, and standardisation of *K. caryophyllata*. These findings are significant for promoting and accepting herbal-based traditional medicines and ensuring ethical and sustainable use of the plant in the healthcare system.

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Chapter 3

Pharmacognostic Characteristics of *Kamettia caryophyllata*

CONTENTS

INTRODUCTION

The therapeutic effectiveness of medicinal plants is determined by the quality and quantity of their chemical constituents. Misidentification and misuse of herbal medicines or natural products often arise from incorrect identification, as highlighted by Muyumba et al. (2021). A common error involves different species being referred to by the same common name. Pharmacognosy, the study of the physical, chemical, biochemical, and biological properties of plants, plays a crucial role in addressing these issues. Establishing pharmacognostic standards for medicinal plants is essential for ensuring the correct identification and effective use of these plants in various treatments. This field also involves the search for new drugs derived from natural sources. Examining the commercial varieties, substitutes, impurities and the quality control of pharmaceutical drugs is made easier by pharmacognostic evaluation. It is an easy-to-use and trustworthy tool for getting detailed information about the raw ingredients utilised in herbal medicine (Sharma & Kumar, 2016; WHO, 1998).

Pharmacognostic analysis, unlike taxonomic identification, involves specific parameters that aid in detecting adulteration in powdered form. This is necessary because when the plant is dried and turned into powder, it loses its morphological characteristics and is susceptible to being mixed with other substances (Tang et al., 2018). Pharmacognostic research confirms the identity of plants and establishes

guidelines for standardisation to prevent adulteration. Hence, these studies are important in verifying the authenticity of medicinal plants and guaranteeing the consistent quality of herbal products, ultimately ensuring the safety and effectiveness of natural products (Chidambaram & Aruna, 2013).

To check the identity and authenticity of medicinal plants in herbal drug preparation, most of the pharmacognostic studies have generally focused on several parameters such as organoleptic characters, macroscopic and microscopic studies, powder studies, histochemical analysis and fluorescence analysis (Chanda, 2014; Amponsah et al., 2014; Alam & Saqib, 2015; Kumar et al., 2012; EI Babili et al., 2021; Kokashi, 1958; Sultan et al., 2012). The current chapter focuses on the pharmacognostic assessment and standardisation of different plant components of the medicinal plant *Kamettia caryophyllata* with the aim of proper identification and collection of the plant in drug preparation and also for the detection of substitutes or adulterants for the plant in herbal preparations in future for ensuring quality.

REVIEW OF LITERATURE

Ancient remedies made from plants have been used for centuries to treat various illnesses, and they are easily accessible and culturally acceptable. This ancestral wisdom is transferred from one generation to the next, primarily through oral communication. They offer affordable healthcare services that are easily reachable and are a crucial income source for rural indigenous communities (Karunamoorthi et al., 2013). Nonetheless, the lack of documentation and strict quality control measures has impeded the advancement of alternative medicine usage in developed nations (Akbar et al., 2014).

Pharmacognosy comes from the Greek words 'Pharmacon' which means medicine and 'gnosis' meaning knowledge. The phrase was initially created by C.A. Seydler in his 1895 dissertation named 'Analectapharmacognosia' (Sarker et al., 2012). Across the world, a large population utilises traditional medicines for curing diseases. Therefore, the economic significance of herbal medicines is growing quickly, highlighting the importance of pharmacognosy in verifying the authenticity of commercial products in the market and identifying any adulterants or substitutes. The absence of standardised practices and unethical behaviour in adulterating and replacing genuine herbal

medicines hinders the promotion of traditional herbal-based medicines with proven efficacy (Sharma & Kumar, 2016).

The majority of studies in pharmacognosy have focused on identifying disputed plant species and confirming the authenticity of traditional medicinal plants using morphological, phytochemical, and physicochemical analysis (Alam & Saqib, 2015). According to Chanda (2014), pharmacognostic study differs from taxonomic identification by including parameters to detect adulteration in dried powder form. This is essential as the plant loses its physical characteristics and becomes susceptible to contamination when dried and turned into powder. These types of research will aid in verifying the identity of plants and guarantee consistent quality of herbal products, resulting in the safe and effective use of natural products. Patra (2011) reported, standardisation guarantees that all packets of medicine available for purchase contain the accurate dosage and will produce the intended therapeutic result. Pharmacognostic studies conducted in stages can lead to the standardisation process. These researches aid in determining and confirming the identity of plant materials.

Assessing plant material using macro-organoleptic methods is the first step in identifying its purity and identity, including characteristics like colour, shape, odour, taste, surface features, and texture (Amponsah et al., 2014). According to Alam and Saqib (2015), Pharmacognostic studies have mainly concentrated on identifying disputed plant species and verifying traditional medicinal plants using morphological, phytochemical, and physicochemical evaluations. Pharmacognostic studies go beyond taxonomic identification and also involve detecting adulteration in dried powder forms. This is important because plants lose their morphological characteristics after drying, making them vulnerable to adulteration. These investigations help verify plants and guarantee the consistent quality of herbal products, which is vital for their safety and effectiveness, as reported by Balekundri and Mannur in 2020.

Standardisation processes in pharmacognosy aim to ensure the reliability and efficacy of herbal medicines, accomplished through gradual pharmacognostic research that verifies plant materials (Patra, 2011). Precise recognition and quality control of initial ingredients are essential for maintaining consistent herbal medicine quality and improving safety and effectiveness (Thomas & Chandra, 2008). By utilising

macroscopic and microscopic features, in addition to chemical, physical, and genetic analyses, these studies function as confirmatory tests to establish standardisation (Onyambu et al., 2020). Basic pharmacognostic methods like macroscopic and microscopic powder microscopy are essential in this standardisation procedure.

The preparation of monographs is crucial in promoting standardised practices for herbal medicine usage, focusing on enhancing safety, efficacy, and quality control (WHO, 2009). Standardising the pharmacognostic and physicochemical characteristics of crude drugs is imperative to mitigate risks associated with adulteration, substitution, and misidentification (Saravanan, 2013). Pharmacognostic studies play a vital role in ensuring plant identity and establishing standardisation parameters where closely related species share vernacular names, leading to potential substitution. This study not only aids in preventing adulteration but also ensures the consistent quality of herbal products (Amponsah et al., 2014).

According to Kumar et al. (2012), microscopic techniques are essential for accurately identifying source materials and providing a cost-efficient solution. Pharmacognostic and anatomical information is essential for safeguarding traditional knowledge of herbal medicine and are now acknowledged as necessary instruments in both pharmacognosy and botany (Choundhury et al., 2015). Examining materials at a microscopic level is essential for determining the identity of substances that have been broken or turned into powder with the help of chemicals, even though it may not always give a definitive answer. This method provides crucial supporting evidence when combined with other analytical techniques (Bela & khale, 2011).

Histochemical localisation is a method in biology utilised to observe and identify the spatial arrangement of particular chemical substances within the cells and tissues of the organism. It includes using chemical reagents or stains that interact selectively with specific substances, causing noticeable colour changes or visible indicators (Dhale, 2012; Claudino et al., 2013). Histochemical localisation in plants helps to examine the distribution and presence of pigments, enzymes, metabolites, and ergastic substances in various plant structures, revealing the plant's unique nature (Hutzler et al., 1998; Kadam et al., 2012). Histolocalisation is a cost-effective and informative analytical method that enables simple tracing of metabolite distribution in newly researched medicinal plants. (EI Babili et al., 2021).

Fluorescence analysis and Powder study are vital factors for initial standardisation of raw drugs. Fluorescence is the property displayed by different chemical components found in plant material (Chanda, 2014). Different colour responses can be seen in various light wavelengths (visible light, UV 254nm, UV 366nm) during fluorescence analysis of Leaf, stem, and root powder treated with different reagents (Saravanan et al., 2013). Examining fluorescence using different wavelengths of light helps identify herbal medications once they are in powdered form. Certain ones display visible fluorescence during daylight hours (Sultan et al., 2012). Fluorescence is induced by light in various natural compounds, such as alkaloids like berberine, even though they do not appear fluorescent under normal daylight conditions (Evans, 2009). Fluorescent derivatives can be created from certain substances using various chemical reagents, even if the original substances are not fluorescent. Therefore, fluorescence can be a valuable parameter for qualitatively evaluating the crude drugs in some pharmacognostic assessment, according to Chase and Pratt in 1949 and Kokashi in 1958.

MATERIALS AND METHODS

Macroscopic studies

Macroscopic characteristics

Under macroscopic studies, various external characteristics of plants such as size, colour, shape, and surface were evaluated. This technique is used in traditional medicine to ensure that the correct plant species are used for a specific therapeutic purpose and to qualitatively control crude drugs as per WHO guidelines (Trease & Evans, 1983; Wallis, 1985; Tyler et al., 1977; Kokate, 1997).

Organoleptic characters

Various sensory parameters of plants were recorded for organoleptic evaluation. It provides the most straightforward and fastest means to establish identity and purity to ensure the quality of a drug. Organoleptic characters such as colour, texture, taste and odour were evaluated (Trease & Evans, 1983; API, 2001).

Microscopical studies

Stomatal studies

For stomatal studies, cut the fresh leaf obliquely to collect the adaxial and abaxial leaf epidermis, then peel off a piece from both the upper and lower surface and add to a watch glass that holds water. A few drops of safranin solution were added to the watch glasses and placed on each peel one at a time with the help of a brush on clear glass slides. A rectangular piece of peel was cut with the help of a blade. One drop of glycerine was added to each slide, and a cover slip was gently placed on the peel. The Labomed Lx 100 microscope was employed for these studies. Photographs were taken using the Micaps industrial digital Camera (Model no: PROHDMI1080CC) for documentation purposes. Stomatal measurements, including stomatal length, width, pore length, and pore width were conducted using a 10x objective lens. Various distinguishing characteristics were collected according to the WHO guidelines for quality control methods of herbal materials (WHO, 1998).

Quantitative microscopic study

Determination of the stomatal number and stomatal index

The stomatal number represents the average number of stomata per square millimetre of the leaf epidermis. The stomatal index is the percentage proportion of epidermal divisions that develop into stomata, calculated as $I = (S / E + S) \times 100$, where I is the stomatal index, S is the number of stomata per mm^2, and E is the number of ordinary epidermal cells per mm^2. To obtain the stomatal index, leaf sections were cleaned, and the upper and lower epidermis were separately peeled using forceps. These sections were mounted in glycerin on a slide and observed using a Labomed Microscope with 10X magnification and a Micaps industrial digital Camera. The mid-leaf area, away from edges, midrib, and veins, was chosen to minimise variations in results, following the methods of Poole and Kürschner (1999). Using Micaps software, 0.1 mm^2 grids were drawn, and all stomata and epidermal cells (including subsidiary cells) within these grids were counted and recorded. Eight images were counted for each leaf fragment, and the mean was calculated to determine the stomatal indices.

Determination of vein-islet and vein termination number

The vein islet refers to a small vascular bundle encircled by conducting tissues. The vein termination number represents the count of veinlet terminations per millimetre of the leaf surface. To determine the vein-islet and vein termination number, leaf piece was treated with Franklin solution for forty-eight hours to study these characteristics. Using a camera lucida and drawing board, a 1 mm line was drawn with the assistance of a stage micrometre. A square was constructed, and the slide was placed on the stage. Veins present within the square were traced to complete the outline of islets that overlap two adjacent sides of the square. The average number of vein islets within four adjoining squares was calculated to obtain the value for one square millimetre (Srinivasa et al*.,* 2008). Similarly, the number of veinlet terminations within the square was counted, and the average number of veinlet terminations from four adjoining squares was determined to obtain the value for 1 square millimetre, referred to as the vein termination number (Trease & Evans, 1989).

Determination of the palisade ratio

Leaf samples of *Kamettia caryophyllata* were boiled in Franklin solution for forty-eight hours and observed under a microscope. Using a camera lucida and drawing board, the outline of four epidermal cells was traced with a 4 mm objective. Subsequently, the focus was adjusted to the palisade layer, and enough cells were traced to cover the epidermal cell tracing. The outline of palisade cells intersected by the epidermal walls was completed. Palisade cells under the four epidermal cells (including cells covering more than half the area and excluding cells covering less than half within the epidermal cell area) were then counted. This determination was repeated for five groups of four epidermal cells from different leaf parts. The average number of cells beneath the epidermal cells was calculated, known as the palisade ratio (Trease & Evans, 1989).

Histological studies

Freshly collected plant samples were used for the study. Freehand sections of leaf, stem and root were taken using a fresh razor blade. Thin sections were selected and stained with safranin for 1-2 minutes. The stained sections were washed with clear

water. Safranin gives the cell wall, xylem cells, parenchyma cells, and stomata a pink colour (Ayurvedic Pharmacopoeia, 2016). The Labomed Lx 100 Microscope was used for observation, with 4X,10X and 40X magnification, and a Micaps industrial digital Camera (Model no: PROHDMI1080CC) was used for taking photographs of different magnification levels. Scale bars were set with a stage micrometre in Micaps software and engraved in photographs.

Histochemical localisation

Freshly collected samples were utilised for histochemical investigation. Thin freehand sections of the root, stem, and leaf were prepared and subjected to treatment with specific reagents to localise various phytochemical components, including starch, lipids, proteins, phenolics (anthraquinone), lignin, tannin, alkaloids and mucilage in the tissue. Detection of these components was achieved through the use of various reagents. (Ayurvedic Pharmacopoeia, 2016). Labomed lx 100 microscopes with Micaps industrial digital Camera were used for microscopic pictures.

Carbohydrate test

Periodic acid- Schiff's reagent

Freehand sections of leaf, stem and root were transferred to 1% periodic acid for 10 minutes. After that, they were rinsed with distilled water, and the sections transferred in the Schiff's reagent were kept in darkness for 15 minutes and then washed with distilled water for 10 minutes. The slides were fixed with glycerin. This technique involved the interaction between periodic acid and carbohydrates, causing the creation of carbonyl groups that can be detected with Schiff's reagent, producing a pink colour.

Starch

Iodine test

For the test, 0.3 grams of iodine and 1.5 grams of potassium iodide were diluted in 100 millilitres of distilled water. A drop of this solution was added to the sample section, which was then rinsed with water and examined under a microscope. A blackish-blue colour indicated the presence of starch in the sample (Johansen, 1940).

Lipids

Sudan III test

0.5 grams of Sudan III dissolved in 100 ml of 70% alcohol was used to stain plant sections. The sections were submerged in the Sudan III dye for 20 minutes. Then, the sections were transferred into the 50% alcohol and mounted in glycerin. The presence of blue, red, pink, or a precipitate indicated the presence of lipids in the cell (Sass, 1951).

Protein

Eosin test

The presence of protein in the plant sections was detected by eosin dye, which gave it a pink to red colour (James & Tas, 1984). The fresh plant sections were submerged in eosin dye, heated at about 60^0C for 2 minutes, washed off excess stain with distilled water, mounted with distilled water, and viewed.

Phenolics

Ferric Chloride Test

To highlight phenolic compounds, sections were treated with a 5% ferric chloride solution and left for 30 minutes. After thorough washing with fresh water, the sections were mounted with glycerin. This method revealed phenolic compounds through iron precipitation, resulting in a dark colour, typically black. Observations were made under a microscope to confirm the presence of phenolics (Badria & Aboelmaaty, 2019).

Potassium Dichromate Test

Sections were treated with a 10% potassium dichromate solution for 30 minutes and washed with water. Observation under a microscope revealed a blood-red to brown colouration, indicating the presence of phenolic compounds (Gabe, 1968; API, 2001).

Tannin

Vanillin–Hydrochloric Acid

Tannins, a subgroup of phenolic compounds, were detected using Vanillin–Hydrochloric Acid staining. Plant sections were immersed in a 0.5% vanillin solution for 20 minutes, rinsed with distilled water and then mounted with 9% hydrochloric acid. The presence of a red colour indicated the presence of tannins (Mace & Howell, 2011).

Lignin

Phloroglucinol - Hydrochloric test

Lignin, another group of phenolic compounds, was detected using Phloroglucinol Hydrochloric Acid. When exposed to phloroglucinol in an acidic medium, lignin in cell walls exhibited a pink to red staining. Fresh hand sections of plant parts were kept in 10 % phloroglucinol for 15 minutes and washed with distilled water. Then, 25% hydrochloric acid was added to the section, mounted, and observed under the microscope (Johansen, 1940).

Alkaloids

Dragendroff's Test

The sections were submerged in Dragendroff's reagent for 20 minutes, thoroughly washed in distilled water and then treated with 5% sodium nitrate for 5 minutes. After rinsing in fresh water, the sections were mounted in distilled water. The presence of an orange or reddish-brown staining indicated the presence of alkaloids (Svendsen & Verpoorte,1983)

Wagner's Test

Fresh plant sections were immersed in the Wagner's reagent for 20 minutes, washed off excess staining with distilled water and mounted in distilled water. The presence of a golden colour indicated the presence of alkaloids. Fresh plant material was recommended for this test (Furr & Mahlberg, 2004).

Mucilage

Tannic acid – Ferric chloride test

The methodology utilised in this research is founded on the interaction between tannic acid and mucilages, along with pectins, with subsequent visualisation facilitated by the introduction of ferric chloride, resulting in a colour change to grey or black. Firstly, the section was submerged in 5% tannic acid for 20 minutes, and excess tannic acid was rinsed in distilled water. Then, the section was transferred to a 3% ferric chloride solution for 5 minutes, washed with distilled water and mounted in glycerin (Pizzolato, 1977).

Microscopic Powder study

The dried powder of the plant leaf is examined microscopically to reveal details about cell types, characteristics and cell inclusions, following the methods described by Khandelwal et al. (1995) and Khandelwal (2006).

Fluorescence analysis

The fluorescence behavioural pattern of different phytoconstituents in the plant material of *K. caryophyllata* is standardised through fluorescence analysis of powdered leaves, stems and roots. This analysis followed the standard procedure that Chase and Pratt (1949) and Charles et al. (1958) described. Each powdered plant material, both in its natural state and treated with various acidic and basic reagents (1N NaOH in water, 1N NaOH in methanol, 50% KOH, 1N HCl, 50% H2SO4, Conc. H2SO4, 50% HNO3, Conc. HNO3, Iodine water, Ammonia 25%, Picric acid saturated, Acetic acid, and Water), was exposed to visible light and UV light at wavelengths of 254 nm and 366 nm within a UV/visible chamber.

RESULTS AND DISCUSSIONS

Macroscopic and organoleptic evaluation

The macroscopic and organoleptic evaluation of the leaf, stem, and root components provides valuable information about their physical properties and sensory attributes. Dried leaves were thin, olive green, and shrank; dried stems were dark brown with prominent white-coloured lenticels. The roots displayed a creamish-white colour with rough surfaces marked by longitudinal cracks. The powdered form of the leaf was smooth and olive green in colour, while the stem powder exhibited rough and brown colour due to its rich fibre content. The powder form of the root was rough and creamy brown(figure 3.1). In terms of taste and smell, the powdered leaves have a slightly bitter taste and a characteristic aroma, whereas no distinctive odour was detected from the stem and root components. These findings provide valuable insights into the physical appearance and sensory properties of the plant parts, which are essential considerations for their potential applications in various fields.

According to the WHO (World Health Organization, 2002), providing a detailed description of the macroscopic and microscopic levels of a medicinal plant is crucial in determining its identity and purity before conducting any tests. Organoleptic evaluations were conducted using sense organs to define some specific properties of the plant material and confirm its identity and degree of purity. The smell and odours of the dried plant parts were very subjective criteria dependent on personal preference. Thus, the explanation of this characteristic may occasionally lead to varying viewpoints (Praksia et al., 2016).

The smooth texture and olive-green colour of the leaf powder suggest a relatively fine and consistent structure and the presence of chlorophyll and other pigments, which are common in plant leaves (Sims & Gamon, 2002). In contrast, the rough texture of the stem powder and root powder is attributed to its high fibre content. Brown colour often signifies the presence of lignin and other cell wall components (Vermerris et al., 2010). This rough texture and colour can affect its use in various applications, as it may influence solubility and processing methods. Bitterness in the leaf is a notable organoleptic characteristic that could be caused by various phytochemicals, such as

alkaloids or flavonoids, which are often responsible for bitter tastes in plants (Coupland & Hayes, 2016).

Figure 3. 1: Macroscopic evaluation of *Kamettia caryophyllata* (A. Leaf; B. Stem; C. Root; D. Leaf powder; E. Stem powder; F. Root powder)

The colour, texture, and taste of these plant components can indicate their quality and authenticity. Deviations from the expected colour or texture might raise questions about the sourcing and processing of the material. These organoleptic characteristics can be used for quality control and authentication of products in herbal industries that use these plant components.

Microscopical studies

Stomata studies

In *Kametta caryophyllata* leaves, the stomatal distribution is hypostomatic. The upper (adaxial) epidermis is astomatiferous. On the other hand, the lower epidermis (abaxial epidermis) is characterized by more undulate cells than the adaxial cells, and it contains stomata, making it stomatiferous. The stomata in *K. caryophyllata* leaves are of the Anomocytic type, surrounded by 3-4 subsidiary cells that closely resemble the neighbouring epidermal cells (figure 3.2). Measurements of the stomata revealed that

the pore length was approximately 21.85 (±2.06) µm, and the pore aperture was approximately 8.24 (±0.42) µm. Additionally, the stomatal length and width were found to be approximately 27.88 (±2.38) µm and 18.65 (±1.74) µm respectively.

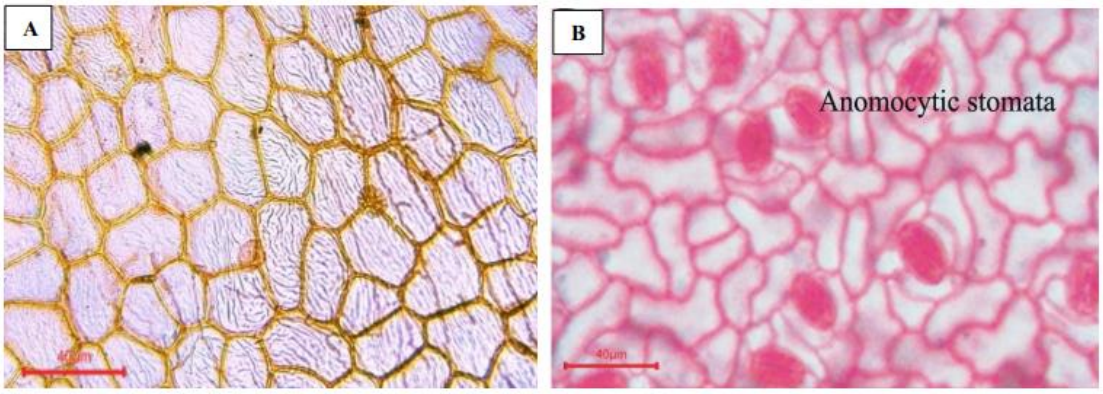

Figure 3.2: Leaf epidermal peelings of *Kamettia caryophyllata* (A. Astomatiferous adaxial epidermis; B. Abaxial epidermis with anomocytic stomatal distribution; scale-40µm)

Quantitative microscopic study

Quantitative microscopic studies such as stomatal number, stomatal index, vein islet number, vein termination and palisade ratio of the *K. caryophyllata* leaves are recorded in table 3.1

Table 3.1: Quantitative microscopy of the leaves of *K. caryophyllata*

Parameter	Value (Mean±SD)
Stomatal number	20 (±0.309)
Stomatal Index	10.22 (±0.474
Vein islet number	07 (±0.318)
Vein termination	05 (±0.408)
Palisade Ratio	1:6

Stomatal and quantitative microscopic studies such as stomatal number, stomatal index, vein islet number, vein termination and palisade ratio are the inexpensive ways generally used in the pharmacognostic evaluation of medicinal plants. Anomocytic and paracytic stomata are reported in the Apocyanaceae family (Sharma et al., 2012). Kannabiran and Ramassamy (1988) documented four types of stomata: anomocytic, anisocytic, tetracytic, and paracytic, along with various combinations, during their examination of 10 species within the Apocyanaceae family. Kumar (2011) and Alamgir (2017) reported that, the quantitative investigation of pharmacognostic

characteristics, such as stomatal length, stomatal index, vein islet, and vein termination numbers, can be used to distinguish closely related plant species to establish quality standards of herbal drugs.

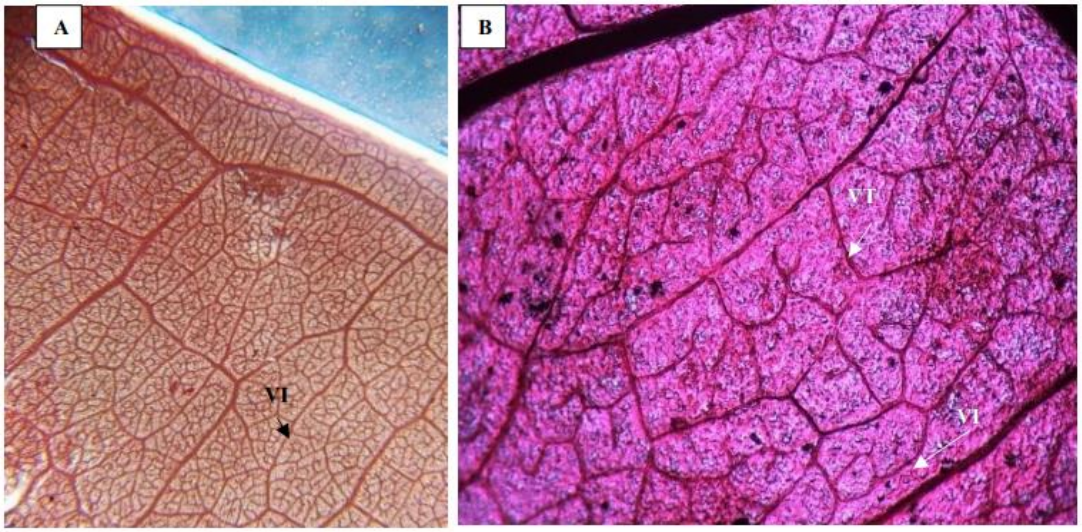

Figure 3. 3: Leaf venation system of the *Kamettia caryophyllata* showing vein islets and vein termination. (A. venation of leaf lamina; B. enlarged view, VI-Vein Islet, VT-Vein Termination).

The presence of anomocytic stomata as well as reticulate and closed venation recorded in *K. caryophyllata* leaves in the study can be considered a qualitative diagnostic character(figure 3.2 & 3.3). With respect to the distribution of stomata in the leaves, it is aggregated in the lower epidermis and is often associated with adaptations to reduce water loss. The quantitative microscopic average values of the leaves recorded such as stomatal number (20), stomatal index (10.22), vein islet number (07), vein termination (05) and palisade ratio (1:6) are also valuable and fundamental characteristics of plant to avoid improper handling during herbal drug preparation. Therefore, the details of stomatal type, their distribution and other physical constant values collected in the study can be considered as basic diagnostic characteristics of *K. caryophyllata* in the herbal industry for authentication, identification and quality control (Kavitha et al., 2014).

Histological studies

Leaf anatomy

The upper surface of the leaf is prominently coated with cuticle. The upper and lower epidermal cells are isodiametric shaped, giving a wavy outline for the epidermal

layers. In the midrib region, just beneath the upper epidermal layer, are 2-3 layered collenchyma followed by 3-4 layered chlorenchyma. This is followed by the cortical region of compactly arranged parenchymatous tissues. The vascular bundle is conjoint, collateral and closed, where the phloem surrounds the xylem, and the protoxylem is facing the upper epidermis. The xylem cells are arranged in parallel rows of 4-5 cells in the middle region; two are on the lateral ends. In the lamina region, following the upper epidermal layer is a single layer of elongated palisade parenchyma cells, which in turn is followed by isodiametric-shaped spongy parenchyma cells which become loosely arranged in the middle region of the lamina. The presence of secretory cavities and oil globules in the palisade and collenchymatous regions of the leaf are important features (figure 3.4).

Stem anatomy

The transverse section of the stem is circular and is covered by a thick cuticle. The periderm comprises two outer layers of thin-walled polygonal cells followed by phellogen and phelloderm. The periderm has structurally differentiated regions characterised by relatively loose arrangement of cells, which is a prominent characteristic called lenticels. The cortex is layered, is chlorophyllous in younger stems, and starts to disappear during maturity. Prior to endodermis, 4-5 layers of phloem fibres present as a distinct feature. The wide zone of the secondary xylem has large lignified vessels. The pith is made up of parenchymatous cells, and the central region is a hollow space. Stone cells or brachy scleride cells are found scattered in the pith region (figure 3.5).

Root anatomy

The transverse section of the root is circular but irregular (figure 3.6). The outer periderm layer consists of phellem, phellogen and phelloderm, which are made up of tangentially elongated cells arranged in regular rows. The cortical region is made of loosely arranged beaded-like parenchymatous cells. The presence of lacunar regions which characterises the cortex. The cortex and the inner core region of the root are separated by endodermis, followed by the pericycle. Immediately after, the pericycle is a multilayered phloem region having rich crystal cells and starch grains. This is followed by the wider zone of secondary xylem, which consists of xylem fibres and

large xylem vessels. Medullary rays are 2-4 layered and rich in starch grains. Pith is absent.

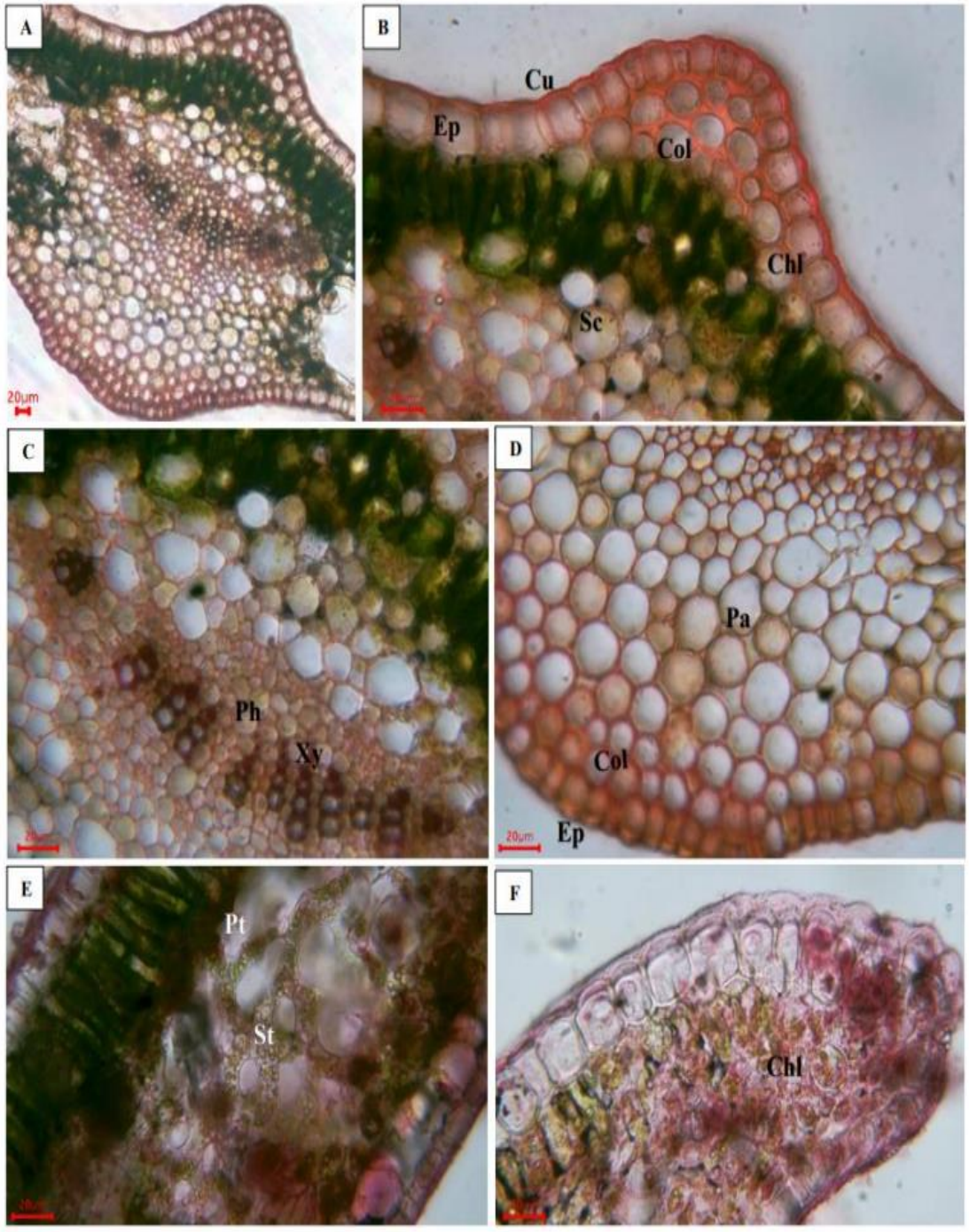

Figure 3.4: Anatomy of *Kamettia caryophyllata* – leaf. (A. T.S. of leaf midrib magnification 10X; B. magnification of upper portion of leaf midrib in 40X; C. magnified portion of xylem and phloem in 40X; D. magnified image of the lower portion of midrib in 40X; E. magnified portion of the leaf blade in 40X; F. magnified image of leaf edge in 40X; Cu-cuticle, Ep-epidermis, Col-collenchyma, Chl- chlorenchyma, SC- Secretary canal, Ph- phloem, Xy-xylem, Pa- parenchyma, , St- spongy tissue, Pt- Palisade tissue, Og- oil globules)(scale bar-20 µm).

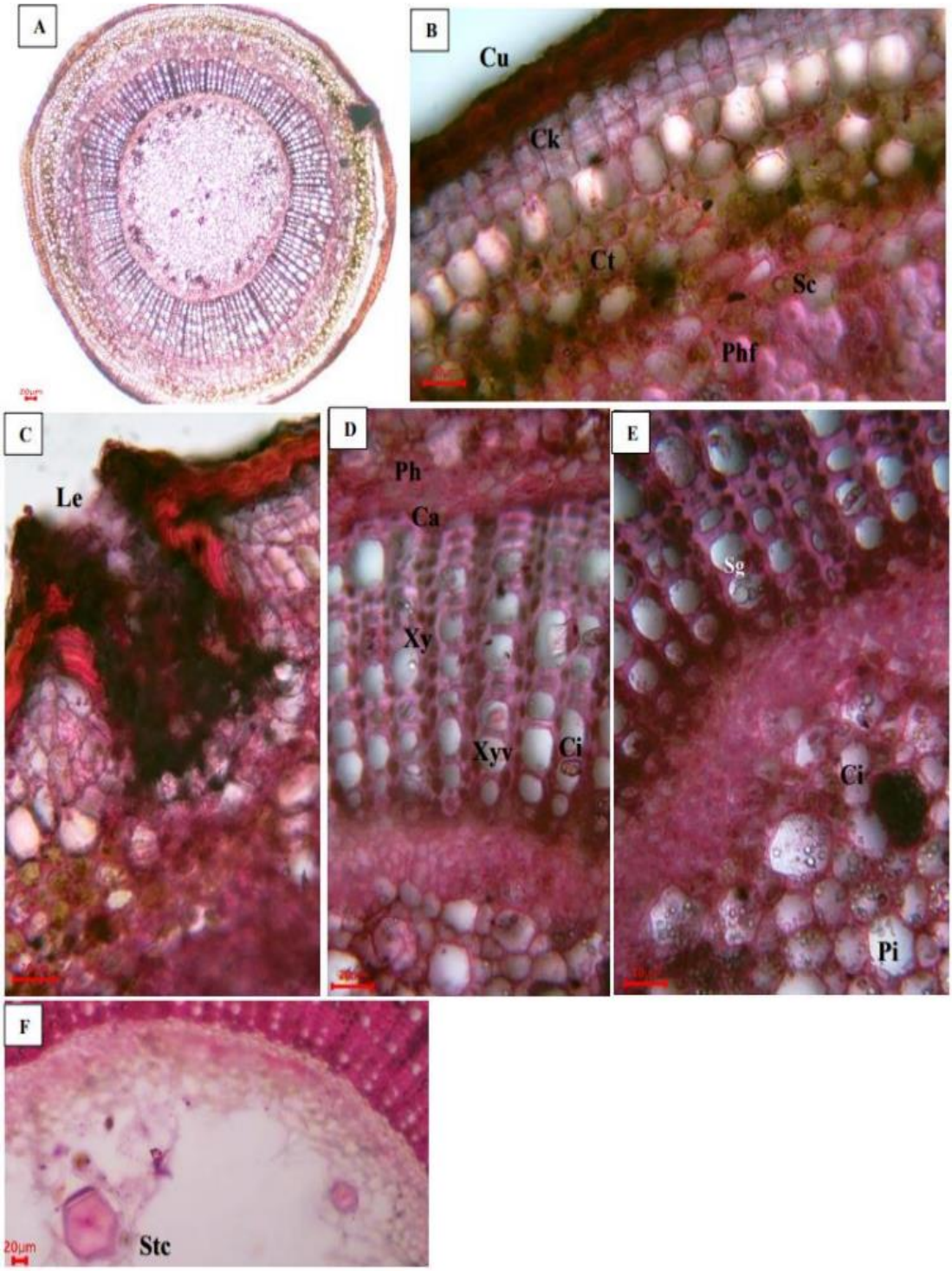

Figure 3.5: Anatomy of *Kamettia caryophyllata* - root. (A. T.S. of stem magnification 4X; B. magnification of cortex in 40X; C. magnification of lenticell in 40X; D. magnified image exhibits phloem and xylem in 40X; E. magnification of xylem and pith in 40X; F. magnification of pith and stone cells in 10X; Cu- cuticle, Ck-cork, Ct-cortex, Sc- Secretory cavity Phf-phloem fibres, Le-lenticels, Xy- xylem, Xyv- Xylem vessel, Ci- Cell inclusion, Sg- starch grain Pi-pith, Stc – stone cells (Brachy sclereids), (scale bar- 20 µm).

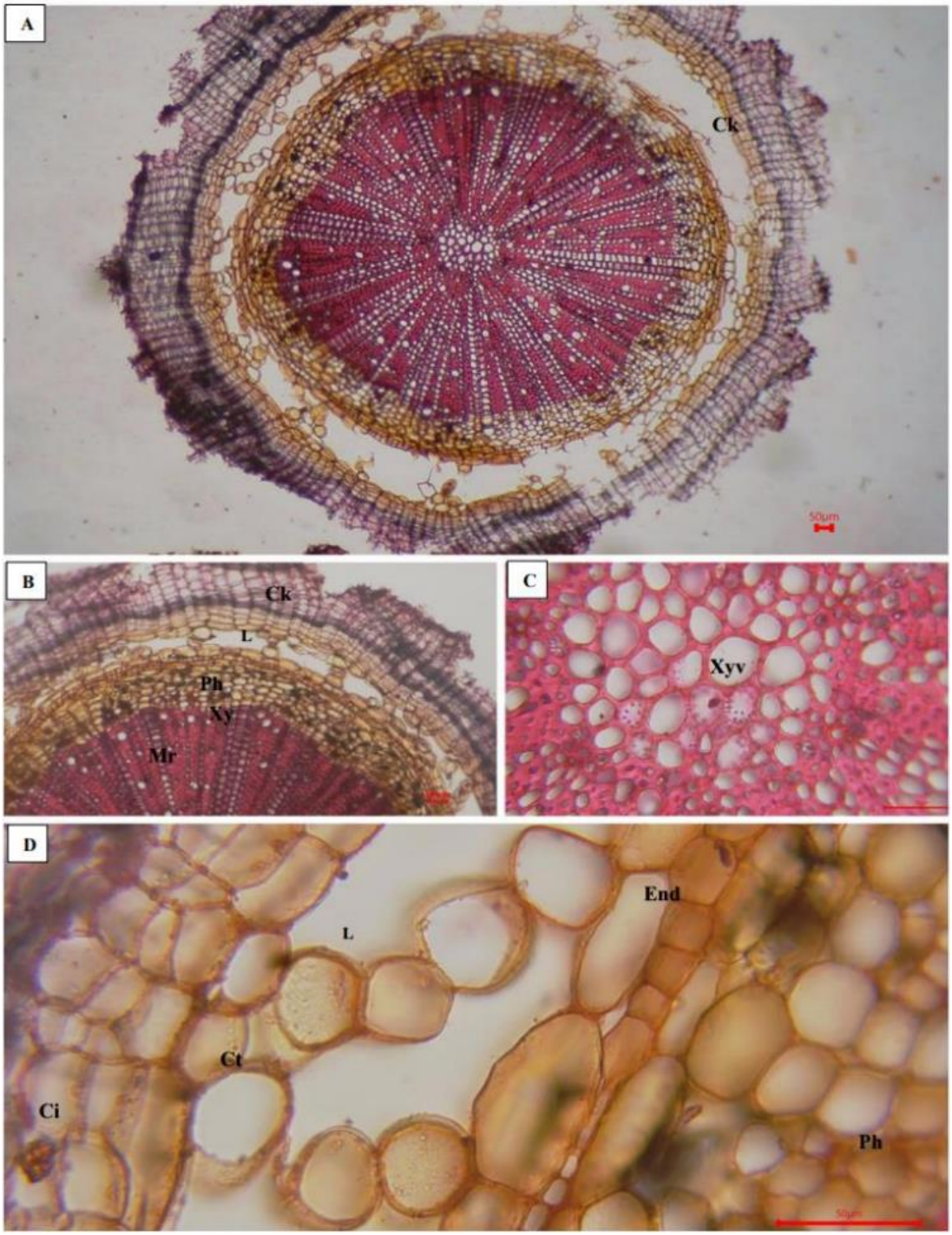

Figure 3.6: Anatomy of *Kamettia caryophyllata* - root. (A. T.S. of root magnification 4X; B. magnification in 10X; C. magnified portion of xylem in 40X; D. magnified image exhibits cortex and phloem in 40X; Ck-cork, Ct-cortex, L-lacunae, Ph-phloem, Xy-xylem, Mr-medullary rays, Xyv-xylem vessels, End-endodermis)(scale bar- 50 µm).

One of the simple and less expensive methods to establish proper identification of raw plant materials that are used in traditional drug preparation is the microscopic analysis of plant material by taking simple free-hand sections (Singh et al., 2010; Kumar et al., 2012; Choundhury et al., 2015). The microscopic studies of the leaf, stem and root of

K. caryophyllata revealed some distinct basic features which may be useful for the identification and harvesting of raw plant materials during drug preparation. The important anatomical features observed in the leaf component of the plant include hypodermal stomata, oil globules and secretary cavities, while the prominent features of the stem component include the presence of lenticels, 4-5 layers of phloem fibres prior to endodermis, hollow pith with scattered stone cells whereas the prominent features of root components are loosely arranged beaded cells in the cortex with lacunar regions and presence of crystal cells and starch grains in the phloem regions.

Histochemical localisation

Histochemical localisation is an important technique in pharmacognosy research, offering researchers valuable insights into the identification, distribution and roles of different compounds within plants. This information is of utmost importance for developing safe and efficacious herbal medicines and contributes significantly to the scientific validation of traditional remedies.

Histochemical analysis of the leaf, stem and root of Kamettia caryophyllata

The results of various histochemical tests conducted in the leaf, stem and root components of *Kamettia caryophyllata* is depicted in the table 3.2. Different reagents were used for the different tests, and the safranin stain was used for the control(figure 3.7).

The carbohydrate test with Schiff's reagent revealed pink colouration in the parenchyma and mesophyll cells of the leaf, in the cortex of the stem and root and in the pith of the stem. Xylem cells in the root also showed the presence of carbohydrates(figure 3.8). The iodine test indicated blackish-blue colouration in the cortex, phloem cells, and xylem cells of all the plant parts, ensuring the presence of starch grains (figure 3.9). Localisation of protein was conducted using the eosin stain, which gave a pink colour when the protein was present. In the leaf, some cells in the cortical region and epidermis were filled with pink colour, indicating the presence of the protein. All the leaf, stem, and root cells that absorbed the eosin stain showed this colour(figure 3.10). Localised substances were seen in the cortex, xylem and phloem cells of all the plant parts of *K. caryophyllata.* Oil and waxes were stained red with Sudan red. Lipid globules were observed in various leaf tissues, including the upper

and lower epidermis, cortex parenchyma cells, mesophyll, and spongy tissues. Similarly, the Sudan test also showed the presence of lipid globules in the cortex, phloem and xylem cells, in the pith parenchymatous cells of the stem and in the cork, cortex and phloem cells in the root(figure 3.11).

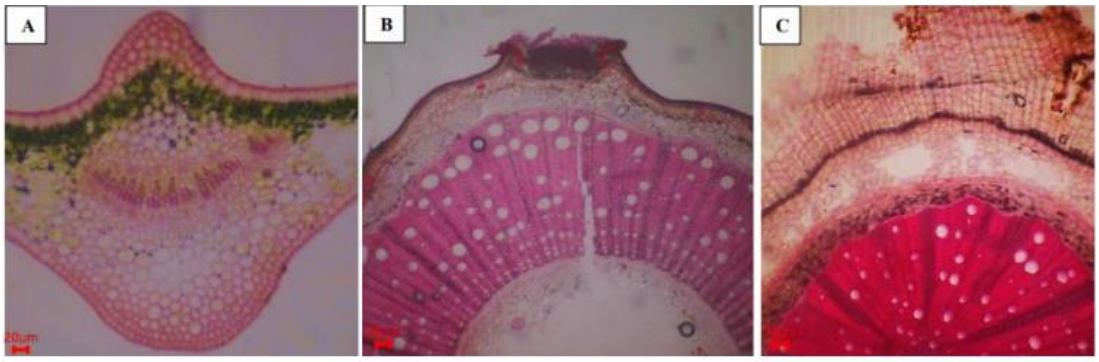

Figure 3.7: T. S of leaf (A), stem (B) and root (C) of *Kamettia caryophyllata* stained with saffranin as control (Scale bar-20 µm)

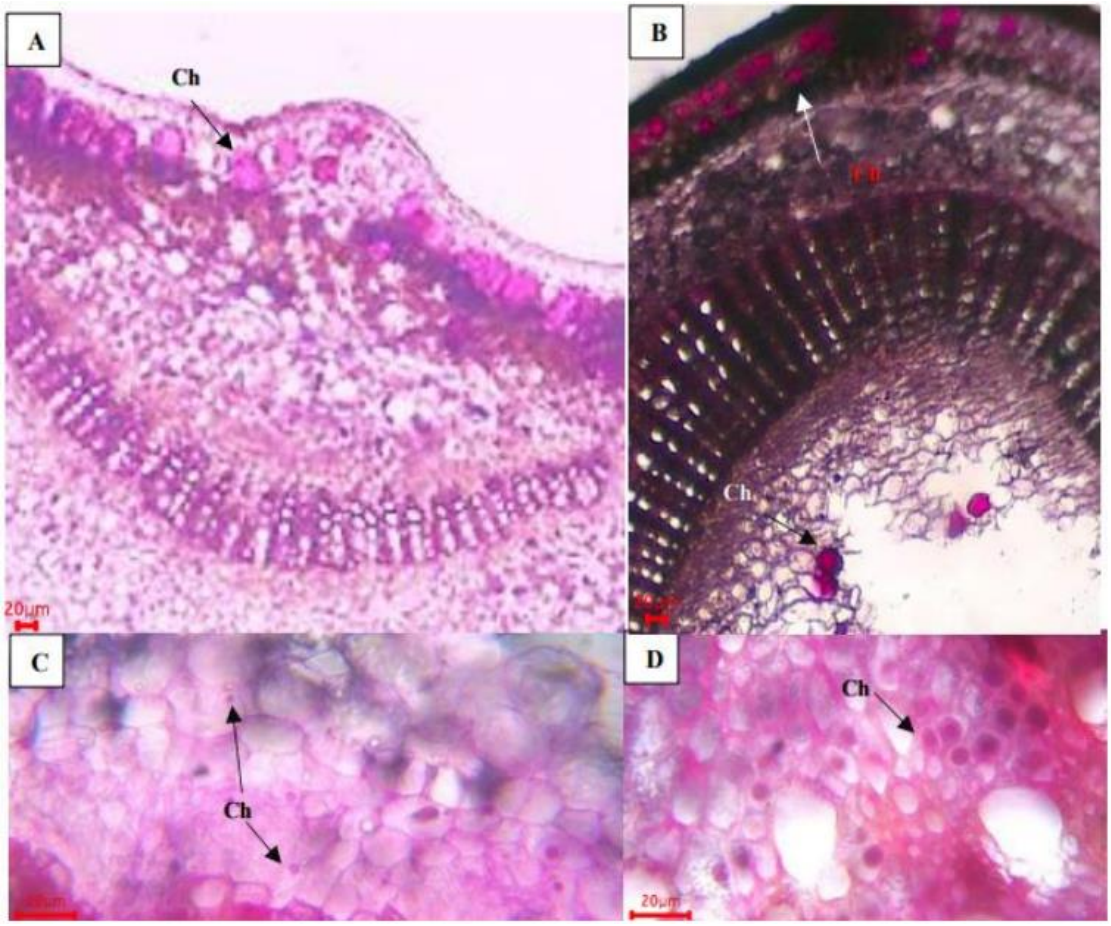

Figure 3.8: Histochemical localization of carbohydrate (Ch) in *K. caryophyllata*. (A. leaf - cortical region; B. stem- the cortex and pith); C-D. root (C. the cortex and phloem; D. xylem cells) Scale bar-20 µm)

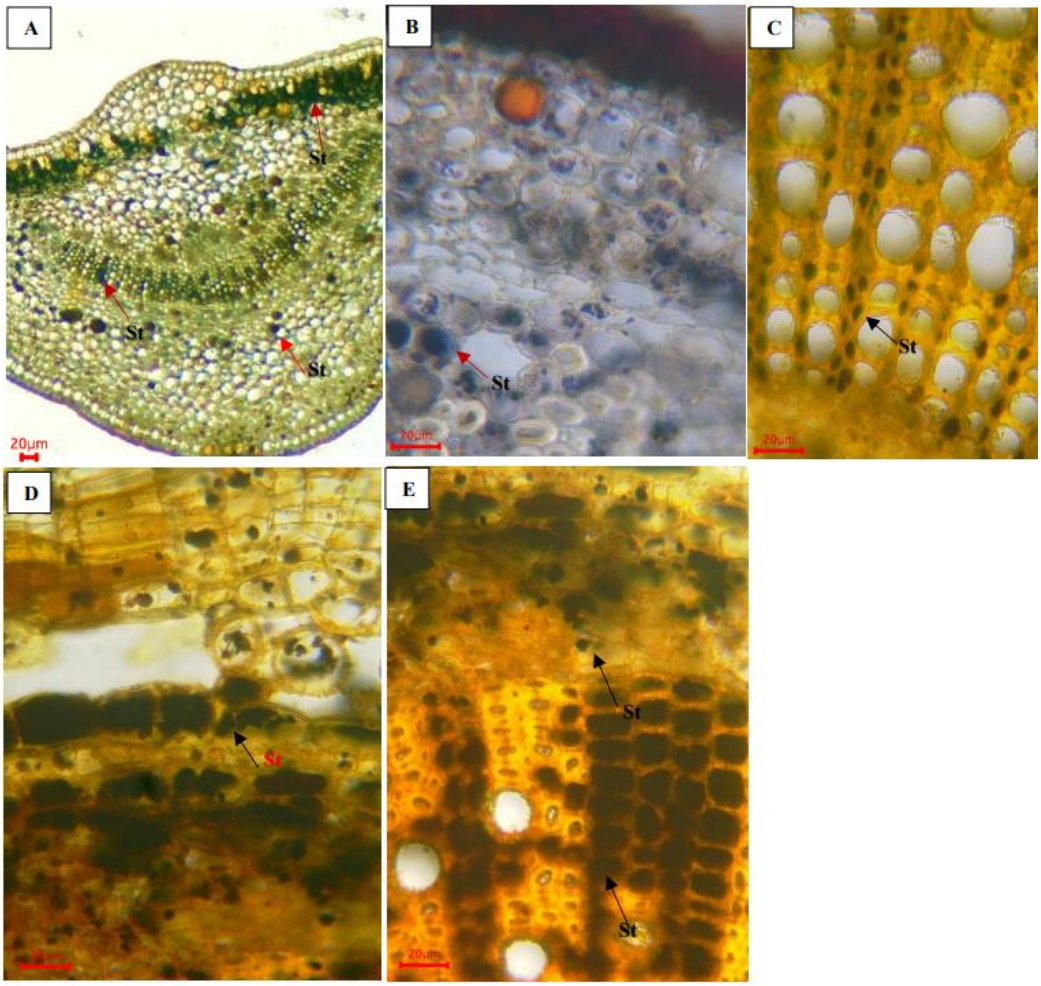

Figure 3.9: Histochemical localization of starch (St) in *Kamettia caryophyllata*. (A. leaf-cortex cells and xylem cells; B-C. stem(B. cortical region; C. phloem cells);D-E. root (D. Cork cells and cortical region; E. phloem and xylem cells) Scale bar-20 µm)

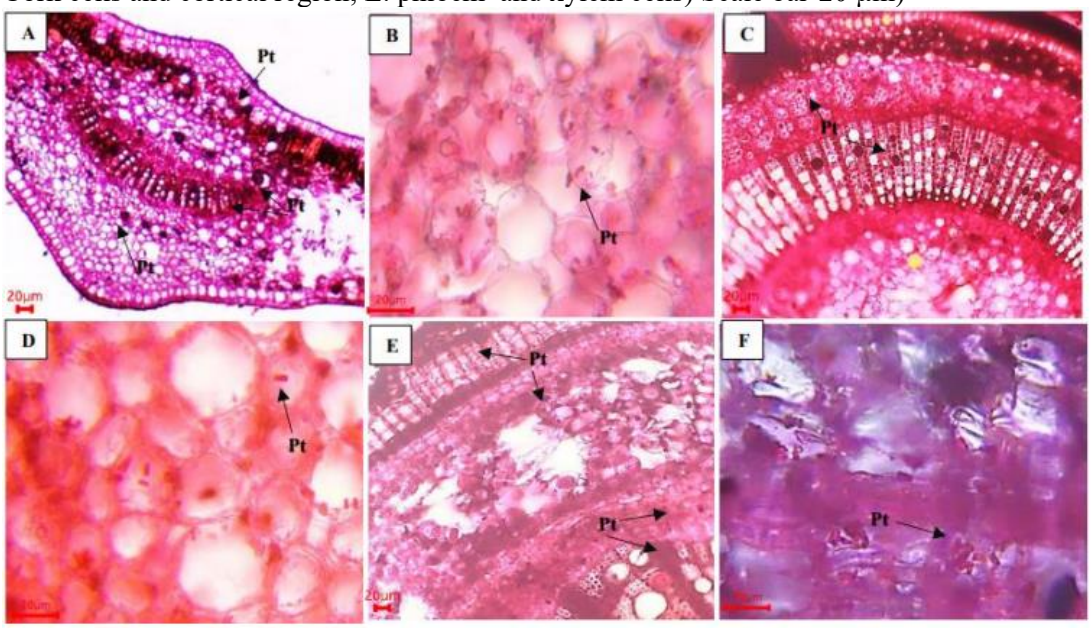

Figure 3.10: Histochemical localization of protein(Pt) in *Kamettia caryophyllata.* (A-B leaf(A. cortical region and xylem cells; B. cortical cells enlarged);C-D. stem(C. cortex, xylem and phloem; D. cortical cells enlarged);E-F. root (E. cork cells, cortex, phloem and xylem cells; F. cortical cells enlarged) Scale bar-20µm)

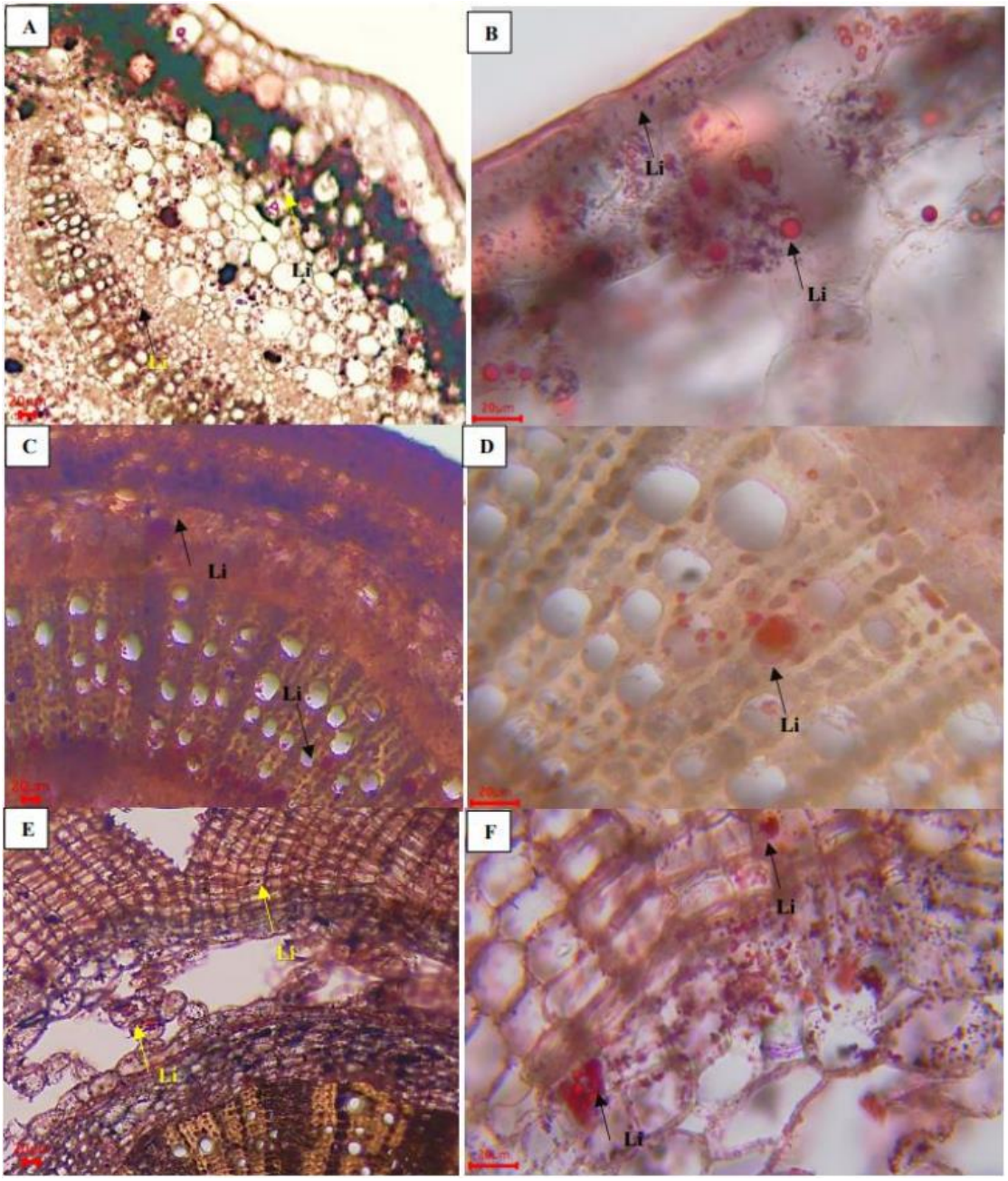

Figure 3.11: Histochemical localization of lipid (Li) in *Kamettia caryophyllata*- (A-B. leaf. (A. cortex and xylem; B. epidermis and cortex enlarged); C-D. stem (C. the cortex and xylem; D. xylem enlarged); E-F. root (E. The cork cells and the cortex region; F. The cork cells and the cortex region enlarged) Scale bar-20µm)

The Ferric chloride and Potassium dichromate tests displayed positive for the presence of phenolics in the cortex of all the plant parts of *K. caryophyllata*. Phloem and xylem cells showed significant levels of phenolic content in the stem(figure 3.12). The vanillin-hydrochloric acid staining test to identify tannins displayed a reddish-brown colour in the cells. This test in the study indicated the presence of tannins in parenchymatous and collenchymatous cells in the cortex of the *K. caryophyllata* leaves, stems and roots. Other parts of the *K. caryophyllata*, such as the phloem cells and xylem cells of the root, also exhibited tannin content(figure 3.13). Tannins are often used to defend against herbivores and pathogens, and their presence in the cortex may support this function.

Lignin was stained in a magenta colour with the phloroglucinol-hydrochloric acid test, indicating its presence in the parenchyma cells of the cortex and the xylem cells of both leaves and stems of *K. caryophyllata*. However, this test revealed the presence of lignin only in the xylem cells of the root(figure 3.14). Lignin adds rigidity to plant cell walls, providing strength and structural support in these tissues.

When tested with Dragendroff's and Wagner's reagents, the alkaloids present exhibited dark brown colour and golden yellow colour, respectively, in the cortex layer of all the plant parts tested in the study. Alkaloids were identified in the pith region of the stem and palisade and spongy tissues of the leaf and also in the phloem and xylem cells of the stem and root(figure 3.15). Alkaloids can have various functions, including defense mechanisms or medicinal properties. The tannic acid and ferric chloride tests showed black staining, representing mucilage, in the anatomical components of leaves, such as the cortex and xylem of *K. caryophyllata*(figure 3.16). However, these tests showed lack of mucilage in the stem and root components of *K. caryophyllata.*

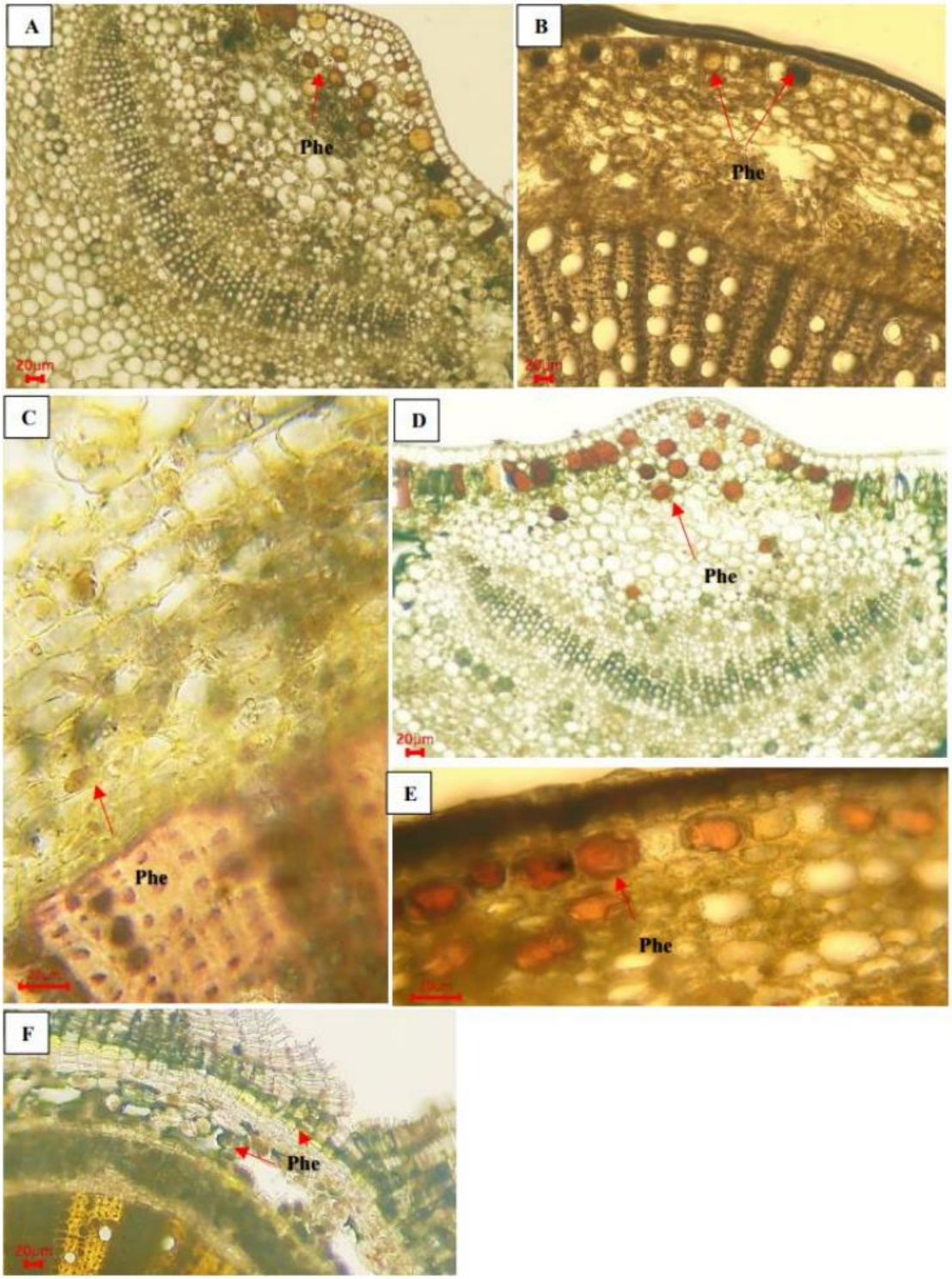

Figure 3.12: Histochemical localization of phenolics (Phe) in *Kamettia caryophyllata.* (A- C. Ferric chloride test(A. leaf-Cortical region; B. stem- cortex region; C. Root- cortex region) D-F. Pottassium dichromate test(D. leaf- cortical region; E. Stem-cortical region; F. root - cork and cortical region) Scale bar-20µm)

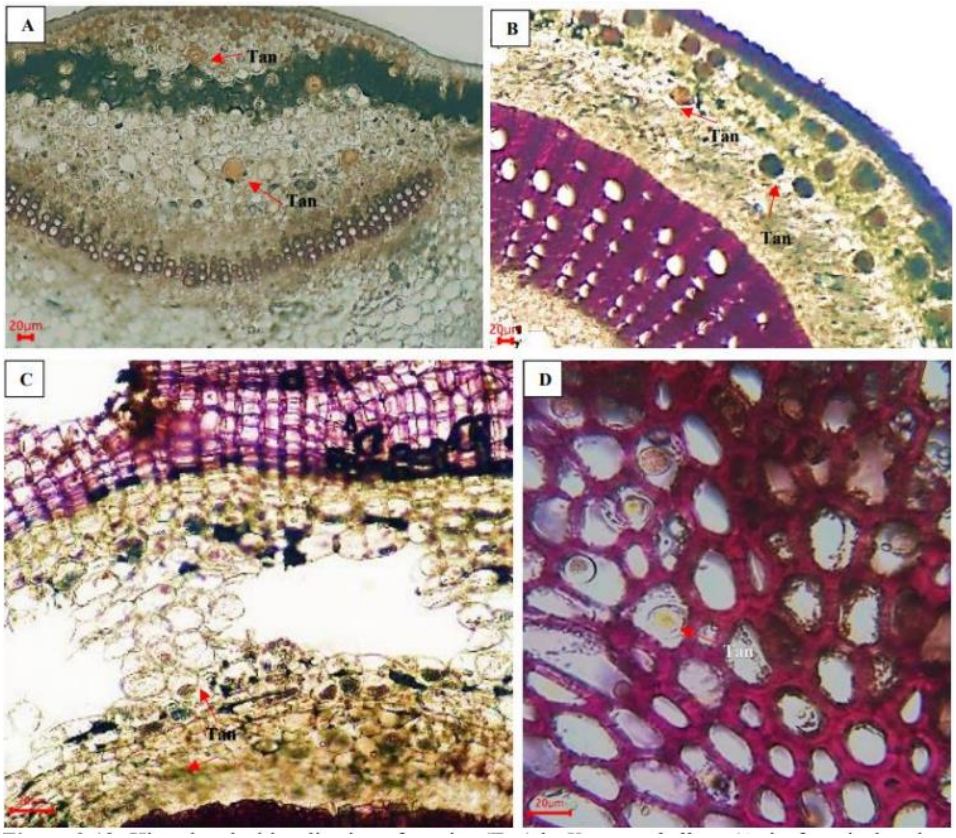

Figure 3.13: Histochemical localization of tannins (Tan) in *Kamettia caryophyllata.* (A. leaf-cortical region; B. stem-cortex region; C. root-cortex region; D. root-xylem) (Scale bar-20µm)

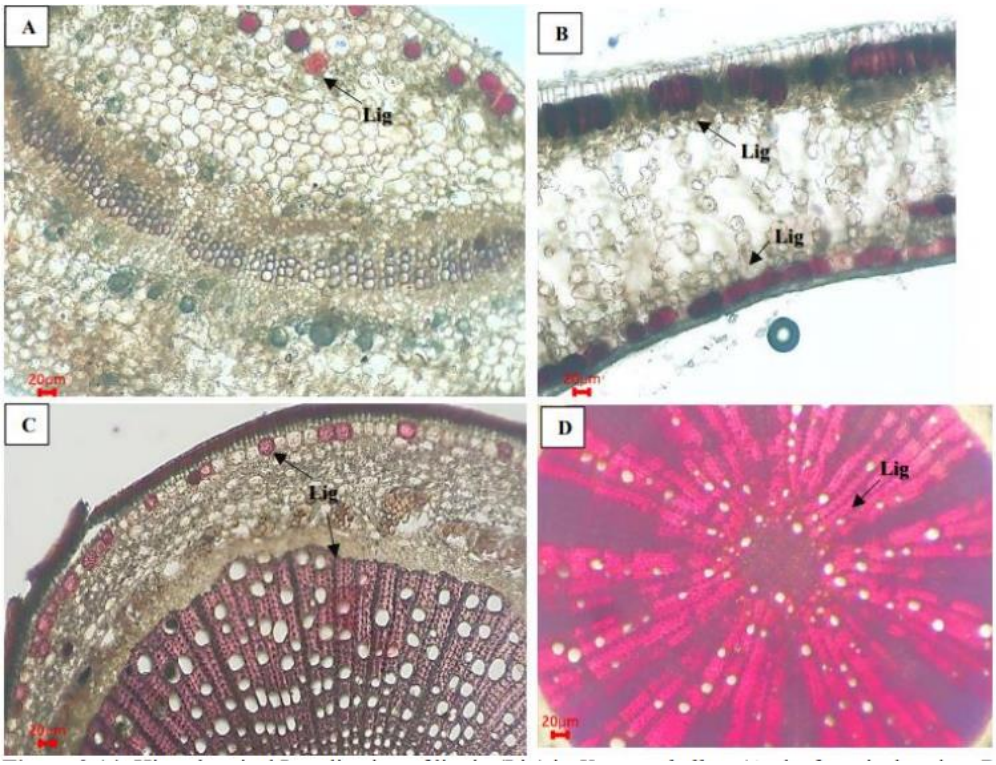

Figure 3.14: Histochemical Localization of lignin(Lig) in *Kamettia caryophyllata.* (A. leaf-cortical region; B. leaf-palisade and spongy cells; C. stem-cortex region and xylem cells; D. root-xylem cells) (Scale bar-20µm)

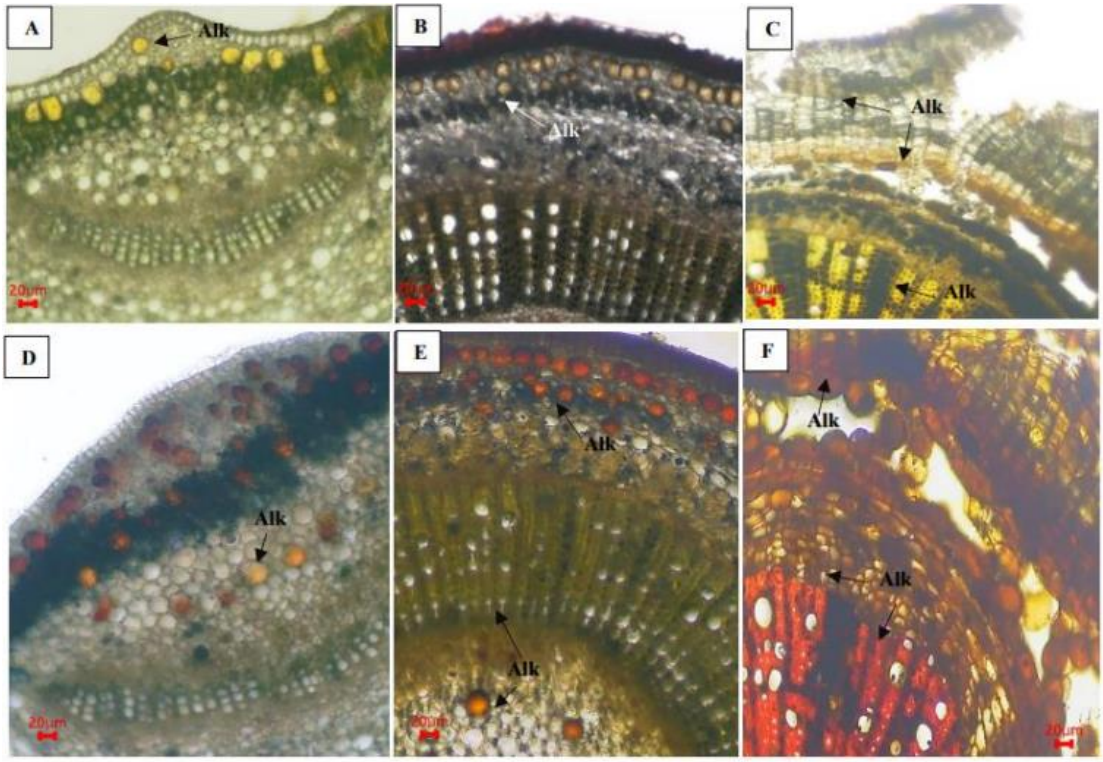

Figure 3.15: Histochemical localization of alkaloids (Alk) in *Kamettia caryophyllata*. (A- C. Dragendroff's reagent test (A. leaf- cortical region; B. stem- cortical region; C. Root- cork, cortex region and xylem cells) D-F. Wagner's reagent test (D. leaf- cortical region; E. stem- cortical region, xylem cells and pith; F. root - cortical region, phloem and xylem cells) Scale bar-20µm)

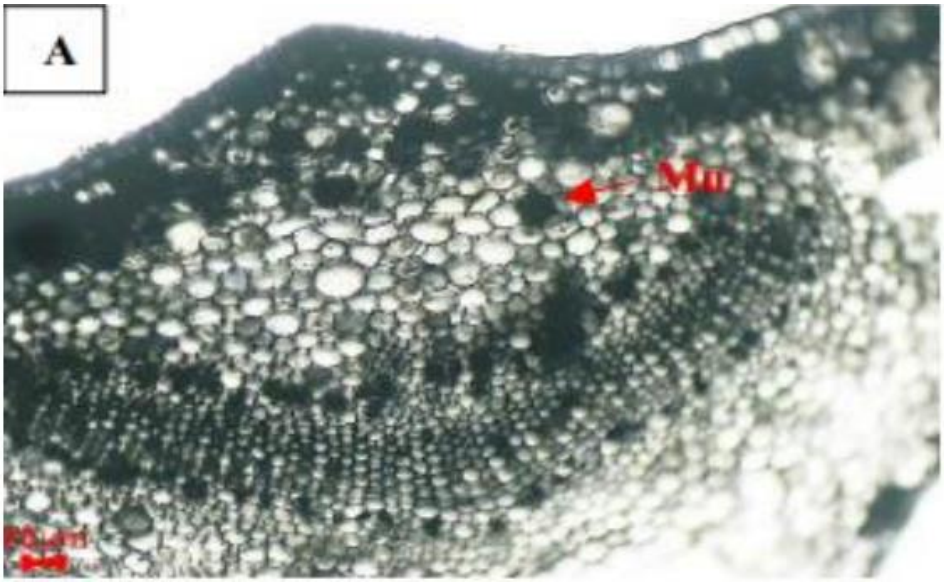

Figure 3.16: Histochemical localization of mucilage (Mu) in *K. caryophyllata* leaf (Scale bar-20µm)

Table 3.2: Histochemical tests conducted in different components of *K. caryophyllata*

SI. No.	Ergastic Content	Reagent	Colouration	Leaf	Stem	Root
1	Carbohydrate	Schiff's reagent	Pink	Cortex	Cortex, pith	Cortex, xylem
2	Starch	Iodine test	Blackish-blue	Cortex, phloem and xylem	Cortex, phloem, xylem	Cortex, phloem, xylem
3	Protein	Eosin test	Pink to red	Cortex	Cortex, xylem, pith	Cortex, phloem, xylem
4	Lipid/Oils and Fats	Sudan III test	Red, pink, light blue	Epidermis, Cortex, phloem, xylem	Cortex, phloem, xylem, pith	Cork cells, Cortex, phloem, xylem
5	Phenolics	Ferric chloride test	Dark to black	Cortex	Cortex	Cortex, phloem, xylem
		Potassium dichromate test	Red to brown	Cortex	Cortex	Cortex, phloem, xylem
6	Tannins	Vanillin-hydrochloric acid	Reddish brown	Cortex	Cortex	Cortex, phloem, xylem
7	Lignin	Phloroglucinol-hydrochloric acid	Darkish /Magenta-red	Cortex, xylem	Cortex, xylem	Xylem
8	Alkaloids	Dragendroff's reagent test	Orange/ Dark brown	Cortex, palisade and spongy tissues	Cortex, phloem, xylem, pith	Cortex, phloem, xylem
		Wagner's reagent test	Golden yellow	Cortex	Cortex, phloem, xylem, pith	Cortex, phloem, xylem
9	Mucilage	Tannic acid& Ferric Chloride test	Black/Grey	Cortex, xylem	Not detected	Not detected

The histological methods are important for analysing the qualitative and quantitative determination of various cellular components such as proteins, starch, carbohydrates, lipids or oils and other histochemical groups like phenolics, tannins, lignin, alkaloids, mucilage etc. found in cells (Yang et al., 2018; Krishnan et al., 2003).

The detection of carbohydrates in the leaves, stems, and roots of *K. caryophyllata* indicates their role in energy storage and metabolic activities within the tissue. Carbohydrates, not directly linked to therapeutic benefits, have been seen to improve the effectiveness of critical therapeutic components (Osadebe & Okoye, 2003). They have been utilised in the production of polysaccharides, serving as immunomodulators with significant therapeutic and vaccine implications (Mahendra et al., 2021). The

confirmation of starch in leaf, stem, and root sections through the iodine test signifies its status as the primary ergastic substance of the protoplast. Comprising long-chain molecules, starch is abundant in various plant parts such as fruits, seeds, and rhizomes and within chlorophyll-containing tissues like leaves (Kokate, 2019). The presence of protein is determined with eosin dye in specific regions of the leaf, stem and root of *K. caryophyllata*, suggesting their involvement in structural support and metabolic processes. Proteins constitute the primary component of living protoplasm and can exist as inactive, ergastic bodies within plant cells. Plant cell walls contain proteins that serve crucial functions. Among these functions, hydroxyproline-rich glycoproteins, resembling rods with connector sites, are notable (Cooper et al., 1987). This study observed eosin-stained intracellular components in the leaf and stem cortex cells. According to Van Loon et al. 2006, plants can produce Pathogenesis-related proteins (PR proteins) in response to attack by pathogens. These are grouped into 17 families according to biological activity and sequence similarity (Pusztahelyi, 2018). The Sudan III test demonstrated the presence of oils and fats in the cortex, phloem cells, and xylem cells in the leaf, stem and root. Lipid droplets were also present in the pith of the stem, cork cells of the root, and epidermis of the leaves. Fixed oils and fats can be found extensively throughout the vegetative and reproductive parts of plant (Danladi et al., 2015). Fixed oils are typically found as tiny oil globules, often found alongside aleurone grains (Kokate, 2019). Lipids often serve as energy reserves and are vital components of cell membranes. Carbohydrates, proteins, and lipids are the primary metabolites that are involved in the metabolic process to produce secondary metabolites (Gutzeit & Ludwig, 2014).

Phenolics, localised with ferric chloride and potassium dichromate tests, were marked as dark or black and dark brown, respectively, in all the plant parts tested in *K. caryophyllata*. Phenolics come under secondary metabolites, primarily to defend against pathogens and oxidative stress in plants (Bhattacharya et al., 2010). Phenolic localisation using reagents also indicated their presence mainly in cortex cells. Phenolic compounds are frequently generated and stored in the subepidermal layers of plant tissues when facing stress and pathogen invasion (Zhao et al., 2023). The level of a specific phenolic compound in plant tissue fluctuates seasonally and throughout various growth and development phases, and exposure to light also changes the production of phenolics in the plants (Chepel et al., 2020). Previous

research conducted by Yang et al. (2018) indicated that the length of exposure to light was a crucial factor in controlling the amounts of different phenolic phenylpropane derivatives in *Xanthium* plants.

The detection of reddish brown colour in the cortex elements of all the plant parts and the xylem elements of root in *K. caryophyllata* when subjected to Vanillin - hydrochloric acid test, indicates the presence of tannin and their role as defense compounds against herbivores and pathogens. This was Siqueira et al. in 2012 reported various pharmacotherapeutic effects of tannin in plants, such as wound healing and defense against microbial attack. Lignin, a polyphenolic polymer, is directly deposited in the cell wall of plants and is found to do different roles in plants like protective, sustaining or disruptive actions based on the specific situation like stresses (Islam & Lucky, 2019; Pradhan & Loque, 2014). Heavy lignin deposition was revealed in *K. caryophyllata* in the study, as Dark pink-red colouration observed in the xylem elements of all plant parts, in the parenchymatous cells and palisade mesophyll cells of leaves and in the cortex cells of stem, when stained with Phloroglucinol-HCl. The process of cell wall lignification takes place as cells develop into different types and when there are specific environmental changes, as stated by Barros et al. (2015).

Alkaloids are belonging to the broad category of secondary metabolites containing nitrogen with a mildly basic nature produced through amino acid biosynthesis (Hussain et al., 2018). The histochemical localisation of alkaloids in the leaf, stem, and root component of *K. caryophyllata* in the study showed, they are primarily located in the cortical cells, xylem and phloem cells of the leaf, stem and root and in the pith region of the stem. The alkaloids are phytochemical groups having important role as antimicrobial agents and promoters of haemoglobin in leukaemia cells, and they can also serve as active triggers, suppressors, and terminators of internal regulatory mechanisms (Mahendra et al., 2021). The histochemical localisation of alkaloids with Dragendorff's and Wagner’s reagents in the study revealed the importance and scope of *K. caryophyllata* for pharmaceutical industries.

The plant-based mucilage, known for its unique health benefits like anticancer properties, angiotensin-converting enzyme inhibition linked to diabetes, and immunity boost, is commonly utilised as a key component in developing

pharmaceuticals, functional foods, and nutraceutical products (Dybka et al., 2021; Amiri et al., (2021). The mucilage test with the tannic acid and ferric chloride showed black colour stained regions in the cortex and xylem of *K. caryophyllata* leaves, indicating presence of mucilage and chances of its health benefits and their utilization in the field of pharmaceutical developments.

The findings of the study provide an understanding of the plant's biochemical composition and reveal the existence, localization and spread of phytochemicals such as alkaloids, phenolics, tannins, oils or lipids, proteins, starch grains, carbohydrates etc. in different components like leaf, stem and root of *K. caryophyllata*. The study results show great potential for use in chemotaxonomy and for identification and authentication of plant *K. caryophyllata*, which will be useful in detecting the use of possible adulterants in herbal products in place of *K. caryophyllata* (Badria & Aboelmaaty, 2019). Further, understanding the distribution of these compounds is crucial for pharmaceutical purposes, as it helps to identify the parts of the plant that contain specific bioactive compounds. This information also contributes to the scientific validation of traditional medicinal uses of *K. caryophyllata* by providing concrete evidence of the presence and localisation of bioactive compounds which play significant roles in the plant's defence, structural support, and metabolic functions. These findings offer insights into potential applications of *K. caryophyllata* in the herbal industry.

Powder microscopy

The microscopic observation of leaf powder drug of *Kamettia caryophyllata* showed the presence of epidermis, stomata, oil globules, vessels with spiral thickening and chlorophyllous spongy parenchymatous cells (figure 3.17). The analysis of stem powder showed the presence of cork cells, lignified xylem fibres, non-lignified phloem fibers, spiral-shaped xylem vessels and parenchyma cells (figure 3.18). The microscopic observation of the root powder drug showed the presence of simple and compound starch grains, calcium oxalate crystals and pitted vessels (figure. 3.19).

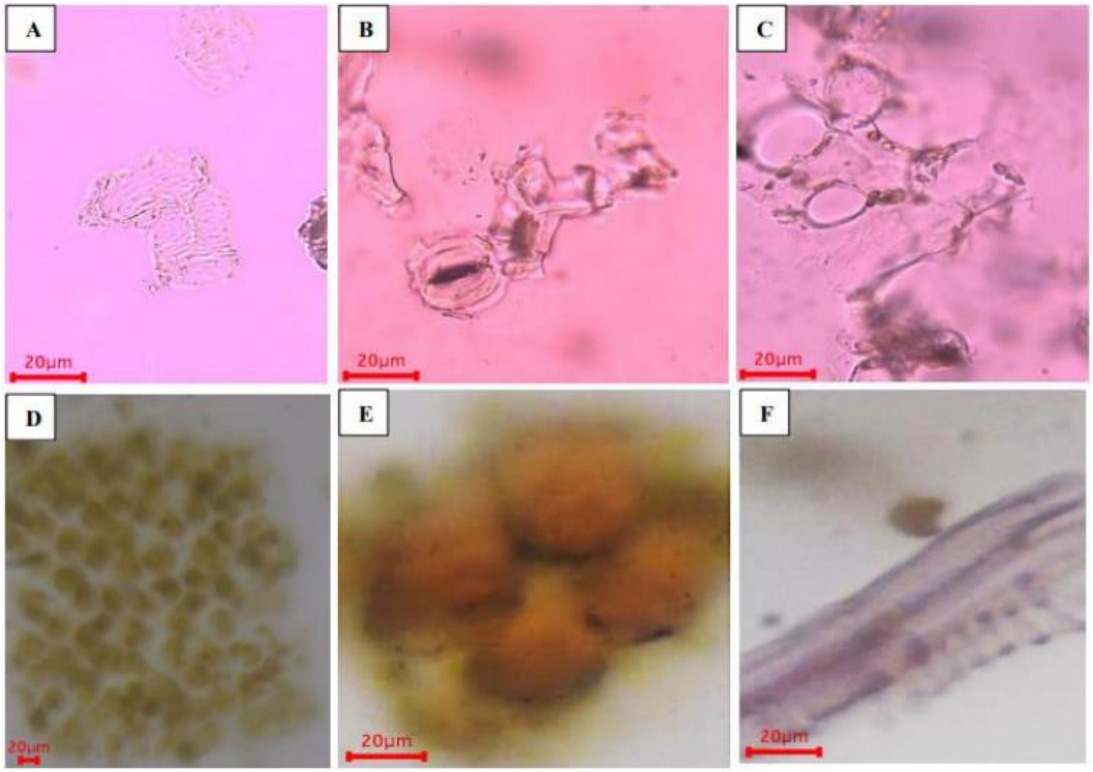

Figure 3.17: Leaf powder microscopy. (A. adaxial epidermis; B. stomata; C. mesophyll cells; D. chlorophyll cells; E. oil globules; F. xylem vessels -Scale bar 20µm)

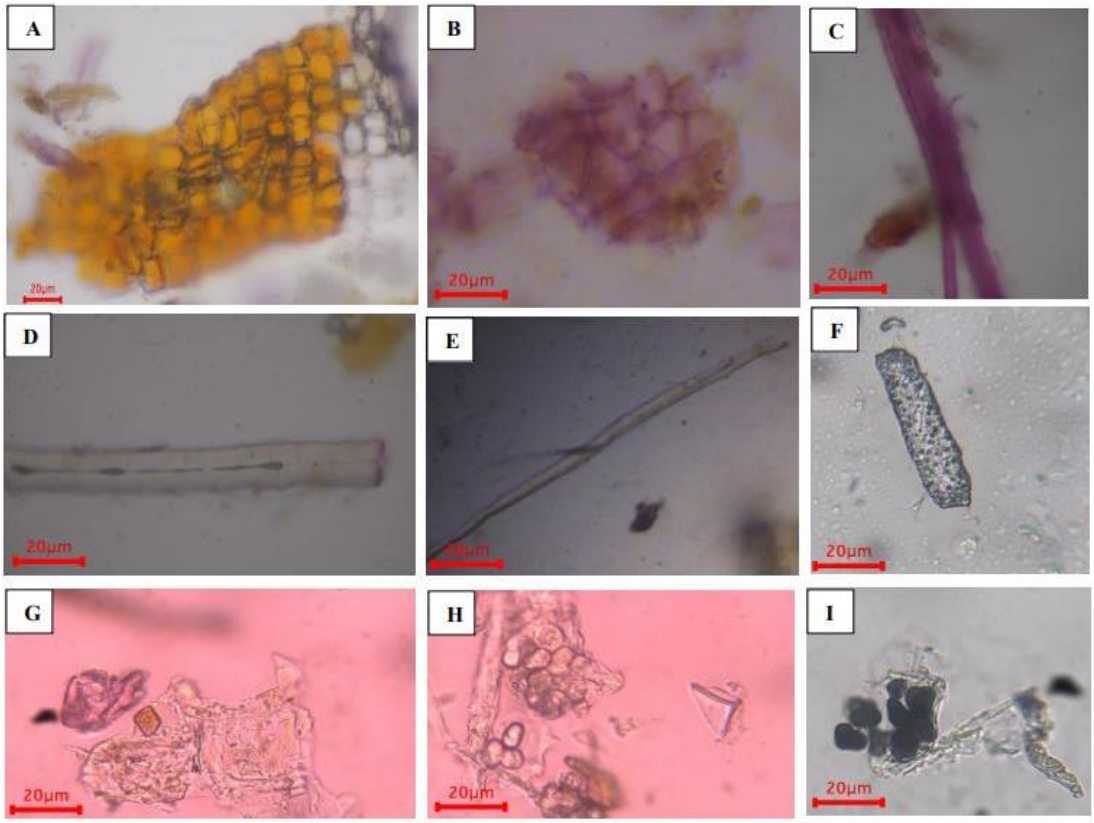

Figure 3.18: Stem powder microscopy. (A. cork cells; B. parenchyma; C. xylem fibres; D-E. phloem fibres; F. pitted vessels; G. crystal cells inside cortical cells; H. Starch grains; I. starch grain after iodine test- Scale bar 20µm).

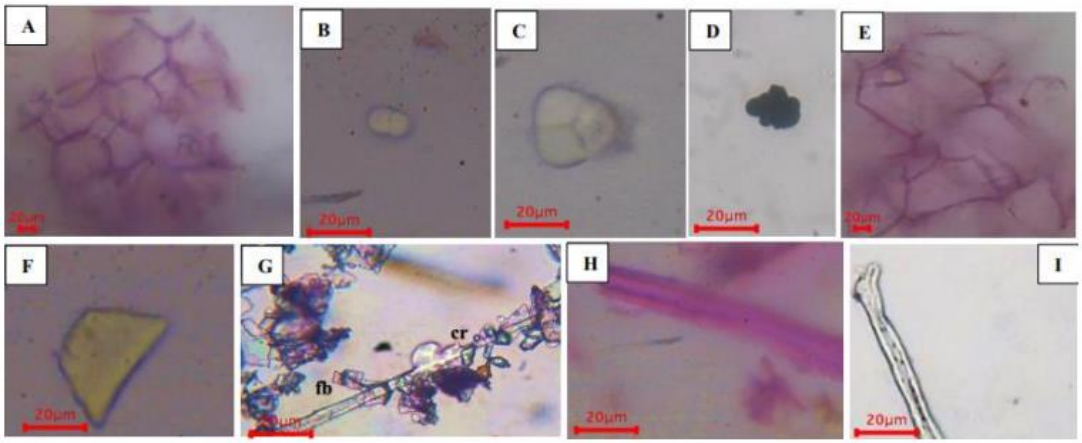

Figure 3.19: Root powder microscopy- (A. starch grains in parenchyma cells; B-C. starch grains; D. starch grain after iodine test; E. crystal cell in parenchyma cells; F. crystal cell; G. fibres and crystal cells; H. xylem fibres; I. tailed pitted vessels-Scale bar 20µm)

Powder microscopy is a simple, fast and inexpensive technique used in pharmacognostic studies to verify herbal medicines with similar physical characteristics and to identify and assess their quality (Singh et al., 2018). This technique ensures the purity of the herbal drug and detects any adulterants present in the sample (Dutta & Chaurasia, 2023). The powder microscopic observations of *K. caryophyllata* done in the study revealed several important features like occurrence of oil globules, vessels with spiral thickening and chlorophyllous spongy parenchymatous cells in leaf powder, while cork cells, lignified xylem fibres, non-lignified phloem fibres, spiral-shaped xylem vessels etc. in the stem powder, whereas the root powder recorded the occurrence of simple and compound starch grains, calcium oxalate crystals, and pitted vessels. The powder microscopy generally provides a glimpse into the structural characteristics of the plant or plant component in their powder form. Understanding these features can be crucial for determining the plant's identity, assessing its quality, and also gaining insights into potential therapeutic properties (Jelani et al., 1993).

Therefore, all the important features including the structural and anatomical indicators, specific cell types and tissues, the type, shape, size and arrangement of starch grains and calcium oxalate crystals observed in the powder microscopy of *K. caryophyllata* as their important characteristic which can be considered as a reference may be useful in confirming the identity as well as to check the purity or the adulteration if any within the herbal drug (Regina et al., 2014; Konyar et al., 2014). By comparing the observed microscopic characteristics within the herbal drug with

those reference characteristics of the authentic *K. caryophyllata* samples, potential adulterants can be identified, ensuring the quality and authenticity of herbal products.

Fluorescence Analysis

The fluorescence or other colour pattern obtained with different plant components of *Kamettia caryophyllata* when treated with various chemical reagents and exposed to different light wavelengths are depicted in the tables 3.3, 3.4 &3.5.

The powder of leaf, stem and root components of the plant treated with different reagents showed multifarious colour developments under visible and UV light and these colour patterns indicate the presence of certain specific compounds or chemical reactions occurring in the plant material. The various colour patterns recorded when leaf powder treated with different reagents include olive green, dark green, black, blackish green, blackish red, brown, yellow-brown, greenish-brown, orange and orange fluorescence, and in which leaf powder treated with 1N NaOH in methanol as well as treated with acetic acid showed fluorescent orange colouration when exposed to UV366nm (table 3.3 & figure 3.20).

Table 3.3: Fluorescence analysis in the leaf component of *K. caryophyllata*

Sl. No.	Reagents	Visible light	UV254nm	UV366nm
1	Powder	Olive green	Olive green	Blackish green
2	Water	Dark green	Dark green	Black
3	1N NaOH in water	Blackish green	Blakish green	Blackish red
4	1N NaOH in methanol	Blackish green	Black	Fluorescent orange
5	50%KOH	Blackish green	Black	Blackish green
6	1 N HCl	Brown	Greyish black	Black
7	50% H2SO4	Blackish green	Blackish green	Blackish red
8	Conc. H2SO4	Black	Black	Black
9	50%HNO3	Brown	Brown	Black
10	Conc. HNO3	Orange	Yellow brown	Black
11	Iodine water	Greenish brown	Black	Black
12	Aammonia 25%	Blackish green	Black	Green
13	Picric acid saturated	Olive green	Green	Black
14	Acetic acid	Black	Black	Fluorescent orange

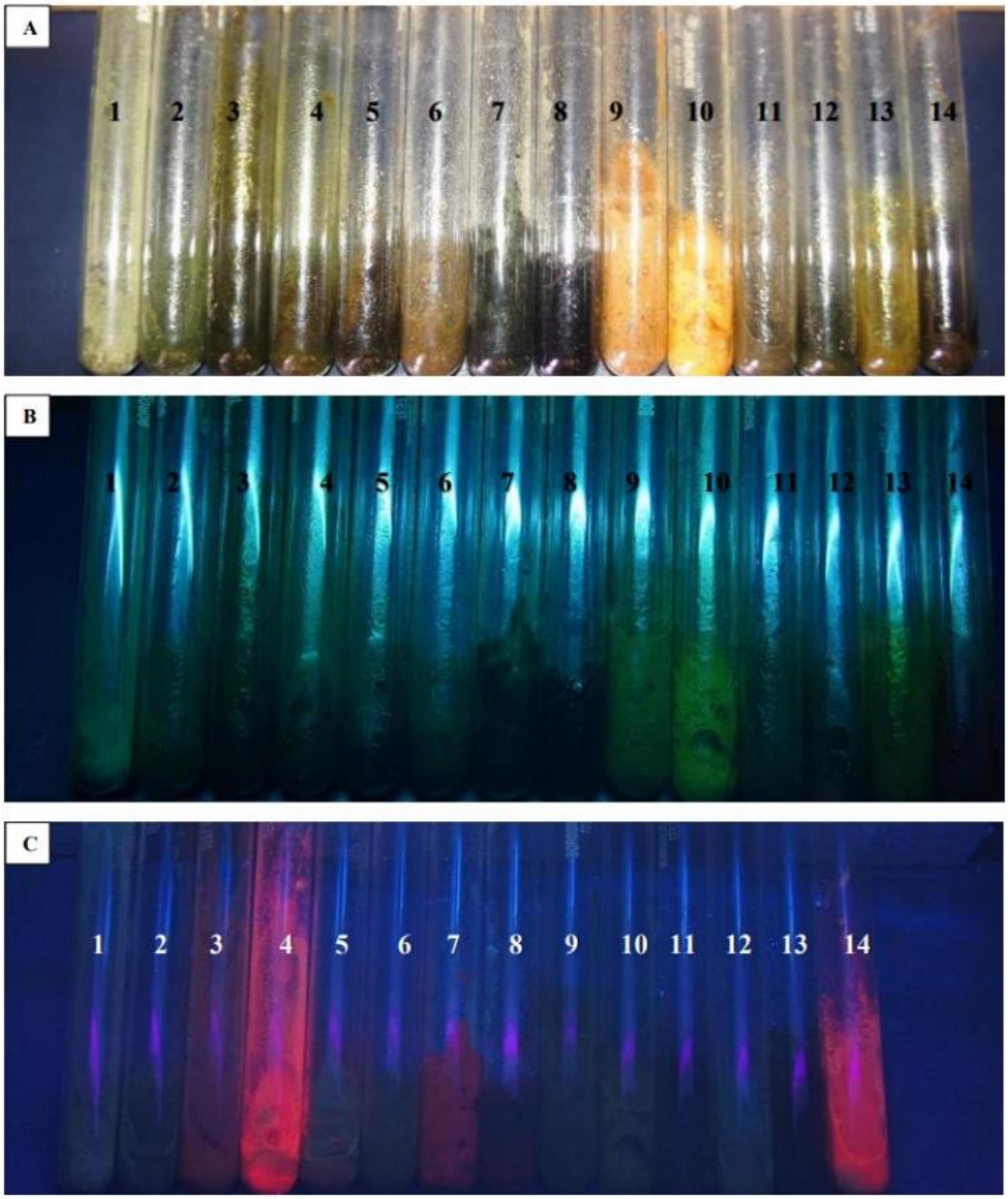

Figure 3.20: Fluorescence analysis of Leaf in *Kamettia caryophyllata*. (A. visible light; B. UV254nm; C. UV366nm; 1. leaf powder; 2. water ;3. 1N NaOH in water; 4. 1N NaOH in methanol; 5. 50%KOH; 6. 1 N HCl;7. 50% H_2SO_4; 8. Conc. H_2SO_4; 9. 50%HNO_3 ;10. Conc. HNO_3; 11. Iodine water; 12. Ammonia 25%;13. Picric acid saturated; 14. Acetic acid)

The fluorescence analysis of stem powder showed different colour developments, which include green, olive green, pale green, light yellow, yellowish green, creamy, creamy white, grey, brown, light brown, blackish brown, reddish brown, dark reddish brown, black, dark red, greenish black, yellow, pale yellow, fluorescent orange and fluorescent yellow. Here also, the orange fluorescence colour developed as in the case of leaf powder when it was treated with 1N NaOH in methanol as well as with acetic

acid, while the yellow fluorescence colour developed when the stem powder was treated with 25% Ammonia and exposed to UV366nm light (table 3.4 & figure 3.21).

Table 3.4: Fluorescence analysis in the stem component of *K. caryophyllata*

Sl. No.	**Reagents**	**Visible light**	**UV 254 nm**	**UV366 nm**
1	Powder	Olive green	Creamy white	Grey
2	Water	Olive green	Creamy	Pale green
3	1N NaOH in water	Brown	Black	Blackish brown
4	1N NaOH in methanol	Green	Yellowish green	Fluorescent orange
5	50%KOH	Brown	Blackish brown	Yellowish green
6	1 N HCl	Light brown	Grey	Dark red
7	50% H2SO4	Green	Greenish black	Reddish brown
8	Conc. H2SO4	Black	Black (red tinch)	Dark reddish brown
9	50%HNO3	Brown	Pale yellow	Blackish brown
10	Conc. HNO3	Reddish brown	Light yellow	Blackish brown
11	Iodine water	Blackish brown	Blackish brown	Black
12	Aammonia 25%	Blackish brown	Yellowish green	Fluorescent yellow
13	Picric acid saturated	Yellow	Yellow	Black
14	Acetic acid	Blackish brown	Blackish brown	Fluorescent orange

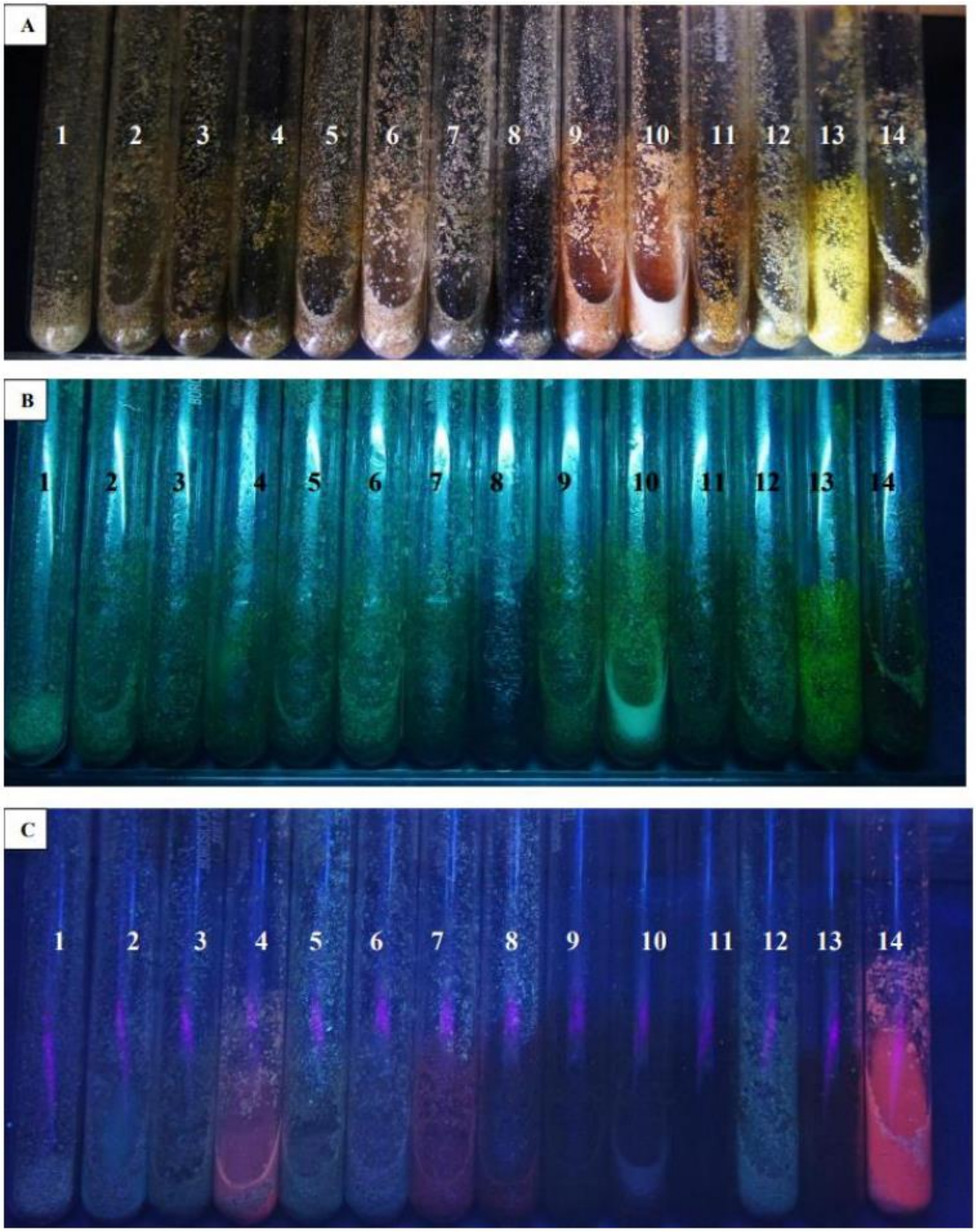

Figure 3.21: Fluorescence analysis of Stem in *Kamettia caryophyllata*. (A. visible light; B. UV254nm; C. UV366nm; 1. stem powder; 2. Water ;3. 1N NaOH in water; 4. 1N NaOH in methanol; 5. 50%KOH; 6. 1 N HCl;7. 50% H_2SO_4; 8. Conc. H_2SO_4; 9. 50%HNO_3 ;10. Conc. HNO_3; 11. Iodine water; 12. Ammonia 25%;13. Picric acid saturated; 14. Acetic acid)

In the fluorescence analysis of root powder, the different colour variations produced are cream, creamy white, creamy brown, brown, light brown, yellow-brown, yellowish-white, whitish yellow, whitish brown, dark brown, light brown, pale brown, orange-brown, black, light black, blackish red, dark grey, fluorescent green,

fluorescent white and fluorescent yellow. The fluorescence green colouration developed when root powder was treated with 50% KOH and exposed to UV366nm light, while fluorescence white colouration developed when treated with Acetic acid under the UV366nm light, whereas the fluorescent yellow colouration was recorded when root powder treated with 25% Ammonia and exposed to UV254nm light (table 3.5 & figure 3.22).

Table 3.5: Fluorescence analysis in the root component of *K. caryophyllata*

SI. No.	Reagents	Visible light	UV 254 nm	UV366 nm
1	Powder	Cream	Creamy white	Creamy white
2	Water	Creamy brown	Pale brown	Creamy brown
3	1N NaOH in water	Brown	Yellow brown	Yellow brown
4	1N NaOH in methanol	Brown	Yellow brown	Yellow brown
5	50% KOH	Dark brown	Light brown	Fluorescent green
6	1 N HCL	Light brown	Pale brown	Light brown
7	50% H2SO4	Light black	Dark grey	Dark grey
8	CONC. H2SO4	Black	Black	Black
9	50%HNO3	Orange brown	Yellowish brown	Brown
10	CONC. HNO3	Orange brown	Yellowish white	Brown
11	Iodine water	Blackish red	Black	Black
12	Ammonia 25%	Creamy	Yellowish white	Whitish yellow
13	Picric acid saturated	Yellow	Fluorescent yellow	Black
14	Acetic acid	Light brown	Whitish brown	Fluorescent white

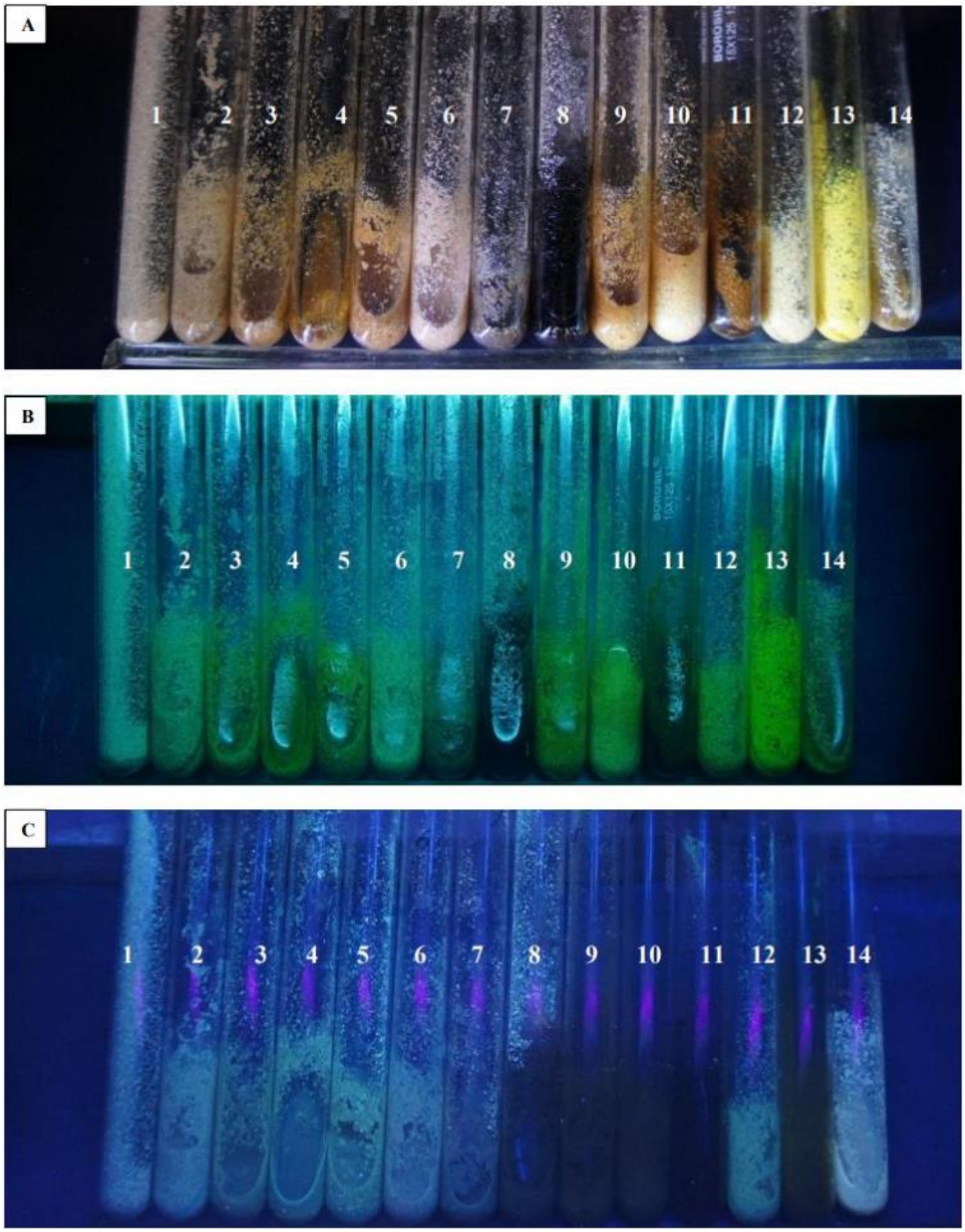

Figure 3.22: Fluorescence analysis of Root in *Kamettia caryophyllata*. (A. visible light; B. UV254nm; C. UV366nm; 1. root powder; 2. Water ;3. 1N NaOH in water; 4. 1N NaOH in methanol; 5. 50%KOH; 6. 1 N HCl;7. 50% H_2SO_4; 8. Conc. H_2SO_4; 9. 50%HNO_3 ;10. Conc. HNO_3; 11. Iodine water; 12. Ammonia 25%;13. Picric acid saturated; 14. Acetic acid)

The fluorescence analysis of leaf, stem, and root powders indicates and provides a comprehensive overview of the diverse chemical compositions and reactions in each plant part. This is a fast and accurate method for identifying different components in the sample (Amponsah et al., 2014). Identifying the medicinal plant and qualitatively assessing crude drugs by the fluorescence analysis approach can be considered an

essential step for pharmacognostic evaluation (Chanda, 2014). This is based on the fluorescence behavioural pattern exhibited by a particular plant powder, which may be unique to that plant in some aspect (Kamble & Gaikwad, 2019).

The fluorescence analysis carried out in the present study observed certain fluorescence colour development by different components of *K. caryophyllata* under UV 366nm and UV 254nm wavelength. An orange fluorescence colour was found to develop when the leaf powder treated with acetic acid and 1N NaOH in methanol and followed by exposure to UV light of 366nm wavelength. A similar observation was also found to develop for the stem powder when treated with acetic acid and with 1N NaOH in methanol; however, in addition to this, a yellow fluorescence colour was also found to develop when stem powder was treated with 25% ammonia, under the UV366nm light. However, in the case of root powder, green fluorescence colour was developed when treated with 50% KOH and white fluorescence when treated with acetic acid, both under UV-light of 366nm wavelength, and further a yellow fluorescence colour when treated with 25% ammonia and exposed to UV254nm light has also been observed. Besides various fluorescence colour developments, several non-fluorescence colours were also found to be produced when powders of different components of *K. caryophyllata* treated with various chemical reagents, and this may be in accordance with the nature and type of constituents present in the plant materials (Preetham *et al*., 2015). The fluorescence and the non-fluorescence colour behavioural pattern exhibited by *K. caryophyllata* against the selected chemical reagents in the study can be used as a reference to identify and qualitatively assess the crude plant drug in the future.

Advancements in medicine and the recognised benefits of natural remedies over synthetic remedies, such as fewer side effects and better pathogen resistance, have significantly increased the demand for natural medicines globally (Ekor, 2014). This surge has led to the commercialization of plant resources, creating economic opportunities and thereby causing frequent misidentification, scarcity, substitution, and adulteration of medicinal plant species (Agarwal & Goyal, 2021). In response, the WHO has observed a decline in the quality and efficacy of plant-based medicines and issued guidelines for proper documentation and monograph preparation of medicinal plants to address these issues. The pharmacognostic standardisation of the plant

material *K. caryophyllata* has been carried out in the present study with the help of microscopic and organoleptic observations; histochemical localisation and powder studies may be the first step in the monograph preparation of the *K. caryophyllata*, and no other studies have been reported on this plant.

CONCLUSION

The macroscopic and organoleptic evaluation of the leaf, stem, and root components offers valuable information about their physical and sensory characteristics, which have implications for their potential applications, quality assessment, and use in various products. Histochemical localisation and the fluorescence analysis of the plant powder may help to understand the specific compounds responsible for the observed characteristics and their potential benefits. Although herbal-based traditional medicines offer an efficient, accessible and affordable health care regime, the lack of standardisation and stringent quality control measures are the major obstacles that have hindered the promotion of these alternative medicines. The lack of said factors and increasing demand have led to many unethical commercial practices of adulterating and substituting genuine herbal medicines. The plant *Kamettia caryophyllata* (Roxb.) Nicolson&Suresh. is a highly valued plant in traditional medicine, as mentioned in Hortus Malabaricus. However, at present, the plant does not have any pharmacopoeia standards for proper identification and authentication. The present investigation results obtained from various macroscopic and microscopic studies, powder microscopy and fluorescence analysis can be considered as reference or an identification tool for authentication and standardisation of the plant *K. caryophyllata*.

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Chapter 4

Physicochemical Properties of *Kamettia caryophyllata*

CONTENTS

INTRODUCTION

According to the World Health Organization (1998), understanding physicochemical characteristics and its documentation is essential for determining the identity, purity, and quality standards of herbal plants; further it is one of the crucial steps in drug discovery and development from medicinal plants. Ayurvedic Pharmacopoeia of India (API, 2016) recommended the determination of physicochemical parameters including loss on drying, ash content, pH values, water and alcohol soluble extractive etc. as essential steps to be carried out for medicinal plants. Researchers and quality control professionals can evaluate the purity and safety of plant materials and can identify potential contaminants by examining these parameters.

The moisture content of the herbal material is one of the important aspects to be considered with respect to their storage span and quality, as too much moisture content is more favourable for harmful bacteria to grow and cause break down of important compounds (Jeevitha et al., 2021; Siraj et al., 2020). By testing the ash content that include the acid-insoluble ash, water-soluble ash and total ash content, it is useful to understand the level of inorganic substances and contaminants present in the herbal material, which play a key role in assessing the purity and quality of plant material (Saravanan et al., 2013; Prakasia et al., 2016). While assessing the medicinal value of a plant, the most important aspect is to get a comprehensive detail of its phytochemical

constituents and their extraction values. Based on the solubilization property of different phytochemical substances like glycosides, phenols, tannins, alkaloids, flavonoids and sugars in various solvents like alcohol, water etc., they can be extracted and extraction values can be determined. The extractive values represent the solubility of the compounds present in the plant or plant parts (Siraj et al., 2020). The acidic or alkaline nature of the plant material which can be determined by measuring the pH value of the plant extract is another important factor to be considered and assessed with respect to a medicinal plant, as it may exert an influence on its stability, quality and medicinal use (Silas et al., 2012; Chanda, 2014).

On understanding the relevance of knowing various physicochemical properties for determining the identity, purity and quality standards of herbal plants, the present chapter aims to assess different physicochemical parameters of *Kamettia caryophyllata* using API (2016) recommended methods. The study expects that by revealing information on moisture content, ash content, pH level, phytochemical groups, solubility property of phytoconstituents and their extraction values, it may contribute significantly to a deeper understanding about quality and purity of *K. caryophyllata* and its potential use in herbal industry.

REVIEW OF LITERATURE

Various factors, including physical, chemical, and geographical factors, influence the quality of herbal materials (Balekundri & Mannur, 2020), and these aspects contribute to the overall efficacy and performance of herbal products (Kumbhar & Godghate, 2015). In 1998, the World Health Organization (WHO) established guidelines for standardizing herbal medicines, focusing on key evaluation factors such as sensory characteristics, ash values, water content, microbial impurities, and chromatographic and spectroscopic assessments. These standards help assess authenticity, purity, and safety, ensuring the quality and potency of herbal ingredients. Analysing the physical and chemical components of a substance is important for establishing the quality of natural drugs (Fazal et al., 2011). Analysing physicochemical factors like moisture content and ash values is beneficial because it indicates the condition of ash, which then allows for assessing microbial growth potential and the presence of contaminants or impurities. Correctly drying herbs is crucial in preserving the drug's shelf life and key compounds such as essential oils

and bioactive compounds in the plant drug. As per API standards, herbal matter should have a moisture content of 8 % to 14 %, as higher levels can increase their vulnerability to microbial attack. Jeevitha and colleagues (2021) found that the moisture content of the bark of *Acacia ferruginea* is 7.61%, indicating a higher resistance to degradation. Siraj et al. (2020) found that *Barleria argentea* had a moisture content of 21.063% w/w, while *Euphorbia abbyssinica* had a moisture content of 16.347% w/w. Both plant species exceeded the recommended standard for moisture content, making them susceptible to quality degradation from microbial activity. Increased levels of moisture in the drug sample can lead to the degradation of the active ingredients and reduce the quality and effectiveness of the drug (Garg & Dwivedi, 2021). According to Venkatachalam et al. (2018), when the water content of the plant extract is within the limits of International Pharmacopoeia, there is a greater chance of preventing oxidation and fermentation reactions, as well as a decreased chance of microbial growth and drug contamination.

The total ash content refers to the minerals and inorganic substances like carbonates, phosphates, and silicates remaining after burning a raw drug. Plant extract ash values can indicate purity by detecting foreign materials or contaminants (Barus et al., 2018). The acid-insoluble ash is the calcium oxalate found, while the water-soluble ash is the total ash minus water-insoluble ash in the herbal drug. According to Humphries (1956), the acid-insoluble ash value reflects the inorganic residue resistant to acid decomposition, potentially containing impurities such as silica or minerals. Hence, the ash content of the herbal drug indicates the level of contamination, substitution, or adulteration present. Therefore, it is necessary to evaluate ash values like total ash, water-soluble ash, acid-insoluble ash, sulphated ash, etc. in order to determine the quality of herbal medicines (Ajazuddin & Shailendra, 2010). Krishna and colleagues (2016) conducted physical and chemical analysis on medicinal plants and found that the bark of *Rhododendron arboreum* had a higher total ash content, consisting of both physiological and non-physiological ash (12.22%). The bark of *Toona ciliata* has a higher level of water-soluble ash, measuring 4.05 %. It was discovered that the leaves of *Lantana camara* contained a higher level of acid-insoluble ash at 2.28%.

The value extracted from plant materials is beneficial in assessing a crude drug as it provides insight into the chemical components present and aids in estimating the

soluble chemical constituents in the extraction solvent used (Joseph & George, 2011). Water-soluble extractives show sugars, acids, and inorganic compounds, while alcohol-soluble extractives reveal polar substances such as phenols, alkaloids, steroids, glycosides, flavonoids, and other secondary metabolites. Phytochemical constituents play a crucial role in reproduction and growth, as well as providing protection against predators and pathogens. Therefore, it is essential to conduct a quantitative analysis of these compounds to ensure the quality of herbal drugs (Eleazu et al., 2012). The existence of different phytochemicals, such as terpenoids, steroids, proteins, carbohydrates, alkaloids, flavonoids, tannins, resin, saponins, etc., in herbal substances validates the historical medicinal use of this plant by rural communities of India (Garg & Dwivedi, 2021). A greater extract yield percentage suggests a higher concentration of different phytoconstituents in the sample. The traditional usage and preparation of conventional remedies can be impacted by the solubility of plant compounds in various solvents. Regupathi and Chitra (2015) reported that the water extract value of *Eclipta alba* was determined to be 17.56%, suggesting that water is able to penetrate the cells of its aboveground parts effectively, making it a more superior extract compared to the alcohol-soluble extract value of 10.27%. The water-soluble extractive value for *Lippia nodiflora* is only 9.42%, whereas the alcohol-soluble extractive value is much higher at 18.63%. This implies that water does not efficiently penetrate the cells of the aerial parts of *L. nodiflora*, unlike in *E. alba*. Similarly, alcohol does not effectively permeate the cells of *L. nodiflora*, unlike in *E. alba.*

The acidity or alkalinity of the plant extracts is indicated by their pH, showing the levels of acidic and basic components in the extract. Research has shown that the pH level of a plant extract can significantly impact its medicinal properties (Silas et al., 2012). In 2018, Gonelimali and colleagues stated that plant extracts with acidic pH levels display antibacterial properties. This review showed how important pH is when analyzing the quality of plant material or extracts from plants. Saad and colleagues (2015) performed pH analysis on ten herbal extracts and found that all of them had a pH below 5, with *Androghophis panculata* having the highest pH of 6.22 and curry leaves having the lowest pH of 2.66. The pH of a plant extract is not just a measure of its acidity or alkalinity, but a key determinant of its medicinal properties, making it a crucial factor in the quality assessment of herbal materials.

MATERIALS AND METHODS

The different physicochemical parameters of *Kamettia caryophyllata* plant (leaf, stem and root) such as moisture content (loss on drying), total ash content, acid-insoluble ash content, water-soluble ash content, pH value in 10% solution, aqueous and alcoholic extractive values were carried out according to the methods recommended by API (2016).

Loss on drying

Without preliminary drying, 10 g of the plant parts -leaf, stem and root of *K. caryophyllata* was accurately weighed and placed in a tarred evaporating dish, which was pre-weighed. It was then dried at 105°C for 5 hours. The Petri dish and the sample were weighed, and the drying and weighing process was continued at one-hour intervals until the difference between two successive weighing corresponded to not more than 0.25 per cent. The point of constant weight was reached when two consecutive weighing, after drying for 30 minutes and cooling for 30 minutes in a desiccator, showed no significant change in weight. The percentage of moisture in the air-dried sample was then calculated.

Total Ash Content

To determine the total ash content, the silica crucible was heated to 350°C for 30 minutes; after the heating period, the crucible was allowed to cool down and weighed. A quantity of 1g of leaf, stem, and root powder of *K. caryophyllata* was measured and spread evenly across the crucible, dried at a temperature of 100 ^{0}C to 105^{0}C for an hour and subsequently ignited in a muffle furnace at a temperature of 600±25^{0}C for five hours. The process continued until the ash turned white, indicating the absence of carbon. If carbon-free ash was not obtained, the crucible was allowed to cool, and then 15 ml of 95% ethanol was added and broken up to ash with a glass rod. The ethanol was burned off, and the mixture was heated to a temperature of 675± 25^{0}C. Following this, the crucible was allowed to cool in a desiccator, the ash was weighed, and the percentage was calculated.

Acid-Insoluble Ash Content

The total ash of the plant powder was subjected to boiling in 25 ml of 2 N HCl for a duration of 5 minutes. The insoluble matter was collected on an ashless filter paper

and thoroughly washed with hot water. The filter paper containing the insoluble matter was then transferred into a pre-dried crucible and subjected to ignition in a muffle furnace at a temperature of 600±25°C for 3 hours until a constant weight was achieved. The residue was then allowed to cool in a desiccator and measured. The percentage of acid-insoluble ash was then calculated with respect to the weight of the dried plant powder.

Water-soluble ash content

The estimation of the total ash content was performed, and then it was boiled with 25ml of distilled water for 5 minutes. The insoluble ash was gathered on a filter paper devoid of ash and thoroughly rinsed with hot water. The material containing the insoluble ash and the ashless filter paper was then moved into a crucible. This was ignited at a temperature between 450^0C and 250^0C for a period of 15 minutes until a constant weight was achieved. The percentage of the ash content that was water-insoluble was then calculated, based on the dried drug.

pH Value at 10% Dilution

A precise weight of 10g of the plant parts leaf, stem and root powder of *K. caryophyllata* was dissolved in an exact measurement of 100ml of water each. This solution was then filtered. The pH of the resulting filtrate was assessed using a standardized glass electrode.

Determination of Alcohol-soluble Extractive

5 g of the air-dried, coarsely powdered *K. caryophyllata* plant parts were macerated with 100 ml of alcohol of specified strength in a sealed flask for a period of 24 hours. Then, the mixture was quickly filtered, and precautions were taken to prevent solvent loss. 25 ml of the filtrate was evaporated to dryness in a tared flat-bottomed shallow dish and dried at 105^0 C until a constant weight was achieved. The dried extract was then weighed. The percentage of alcohol-soluble extractives was calculated concerning the air-dried drug.

Determination of Water-soluble Extractive

To determine the water-soluble extractive value of the *K. caryophyllata* plant, 5g of the dried powder was macerated with 100 ml of chloroform water (prepared by adding

2.5 ml of chloroform to purified water to produce 1000 ml). The mixture was placed in a sealed conical flask for a period of 24 hours. During this time, the contents were frequently agitated for a duration of 6 hours, and then they were allowed to settle for the remaining 18 hours, then filtered using Whatman No. 1 filter paper. 25 ml of the filtrate was taken and allowed to dry in a tared flat-bottomed shallow dish in a water bath. Finally, the dish was transferred to an oven and dried at 105^0C until a constant weight was achieved.

Statistical analysis

The observations/data presented were reported as mean± standard deviation. Statistical analysis was done using SPSS software and One-Way ANOVA with Duncan analysis was performed.

RESULTS AND DISCUSSIONS

The results of various physicochemical characteristics such as loss on drying, total ash content, acid-insoluble ash content, water-soluble ash content, pH value in a 10% solution, and water-soluble and alcohol-soluble extractive values of *K. caryophyllata* analysed in the study is depicted in table 4.1.

Table 4.1: Physicochemical parameters in the leaf, stem and root of *K. caryophyllata*

Test parameters	Leaf (%)	Stem (%)	Root (%)
Loss on drying 105^0C	11.76 (±0.59)	7.62 (±0.50)	11.49 (±0.44)
Total ash	8.86 (±0.18)	3.03 (±0.40)	3.93 (±0.40)
Acid insoluble ash	0.076 (±0.09)	0.02 (±0.04)	0.33 (±0.04)
Water soluble ash	4.291 (±0.27)	1.89 (±0.22)	1.16 (±0.15)
pH value of 10 % solution	5.39 (±0.07)	6.32 (±0.02)	5.92 (±0.02)
Alcohol Soluble extractive	9.67 (±2.08)	7.98 (±0.78)	7.11 (±0.73)
Water Soluble extractive	12.32 (±0.53)	11.76 (±0.38)	10.97 (±0.68)

Loss on drying (LOD)

Loss on drying or moisture content is an important parameter with respect to herbal materials as it affects the stability and shelf life of herbal products. In this study, the

moisture content was highest in the leaf powder component (11.76%) and lowest in the stem powder component (7.62%), while the moisture content of root powder was 11.49%, which is falling in between (figure 4.1). This inference suggested that the leaf component contains more water content than stem and root components. The statistical analysis using Duncan's multiple range test (table 4.2) reveals the difference in moisture content was significantly lower in the stem component compared to root and leaf while the differences were not significant between the moisture content of root and leaf components

Table 4.2: One-Way ANOVA and Duncan analysis of moisture content in the stem, root and leaf components of *K. caryophyllata*

One-Way ANOVA:

F value	**P value**	**η^2_p (Effect size)**
$F_{(2,15)}$= 152.801	P<0.001	η^2_p(Effect size)=0.953

Duncan's Multiple Range Test:

Subset for alpha=0.05		
Plant parts	1	2
Stem	7.6179	
Root		11.4865
Leaf		11.7601
Sig	1.000	0.318

Means for groups in homogeneous subsets are displayed

According to Ayurvedic Pharmacopoeia of Indian standard (2016), the recommended moisture content of vegetable matter is 8 % to 14 %. The moisture content above this level may easily make them susceptible to microbial attack. Here, the moisture content of all the plant parts of *K. caryophyllata* fell under the API standard. Differences in moisture content among the plant parts may align with the physiological roles of different plant parts. Leaves, actively involved in transpiration and photosynthesis, naturally retain more water (Zhou et al., 2021). Roots, responsible for water absorption and storage, exhibit a moderate moisture level essential for their functions (Ievinsh, 2023). The lowest LOD in the stem powder may indicate relatively lower water content than in leaves and roots. Stems typically serve as conduits for water transport and structural support, potentially explaining their comparatively lower moisture levels (Driesen et al., 2021). The level of water in plant material is an important consideration for the durability and shelf life of herbal products prepared from it. High moisture levels may stimulate microbial growth, higher enzymatic

activity, and chemical degradation, which can cause decrease in the longevity of products made from such plant components (Saraf & Ajazuddin, 2010). Thus, it is crucial to maintain the right amount of moisture level to uphold the quality and effectiveness of herbal products. Traditional medicine often utilizes specific plant parts depending on the composition and content of bioactive compounds of medicinal interest. Therefore, monitoring moisture content in herbal materials is highly relevant to determine suitable preparation methods to retain potency in the herbal industry so as to ensure product quality by preventing microbial contamination and deterioration of bioactive principles.

Total Ash Content

The total ash content represents the inorganic substances in the plant samples, including minerals and other inorganic matter. In this study, the highest total ash content was recorded in the leaf component (8.86%), followed by the root (3.93%) and the lowest content recorded in the stem component (3.03%). This indicated that the leaf component contains higher amount of inorganic matter compared to stem and root. The statistical analysis reveals that the variation in total ash content among different components is significant (table 4.3).

Table 4.3: One-Way ANOVA and Duncan analysis of total ash content in the stem, root and leaf components of *K. caryophyllata*

One-Way ANOVA:

F value	**P value**	**η^2_p (Effect size)**
F(2,15) = 645.033	P < 0.001	η^2p (Effect size) = 0.99

Duncan's Multiple Range Test:

Subset for alpha=0.05			
Plant parts	**1**	**2**	**3**
Stem	3.0167		
Root		3.9267	
Leaf			8.8623
Sig	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed

Water-soluble ash Content

Water-soluble ash content is a subset of the total ash content that dissolves in water. The leaf component of *K. caryophyllata* has recorded the highest water-soluble ash content (4.29%), followed immediately by stem (1.89%) and then root (1.16%). This inference suggested that, the water-soluble inorganic matter content was higher in the leaf component while the lower content was noticed in the root. The variations in water-soluble ash content among different components significantly differed from each other (table 4.4).

Table 4.4: One-Way ANOVA and Duncan analysis of water-soluble ash content in the stem, root and leaf components of *K. caryophyllata*

One-Way ANOVA:

F value	P value	η^2_p (Effect size)
F (2,15) = 405.857	P < 0.001	η^2p (Effect size) = 0.99)

Duncan's Multiple Range Test:

Subset for alpha=0.05			
Plant parts	**1**	**2**	**3**
Stem	1.1633		
Root		1.8942	
Leaf			4.2910
Sig	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed

Acid-Insoluble Ash

Acid-insoluble ash represents silicious matter present in the plant samples. It measures the non-dissolvable inorganic matter content. In this study, the acid-insoluble ash content was relatively low in all the component parts, with the highest value obtained in the root (0.33%), followed by the leaf (0.076%), and the lowest acid-insoluble ash value in the stem component (0.02%). The difference in the acid-insoluble ash content recorded between the stem and leaf components insignificantly differed while their difference over the content of root component was significantly lower (table 4.5).

Table 4.5: One-Way ANOVA and Duncan analysis of acid-insoluble ash content in the stem, root and leaf components of *K. caryophyllata*

One-Way ANOVA:

F value	P value	η^2_p (Effect size)
F(2,15) = 205.57	P<0.001	η^2p (effect size) = 0.97

Duncan's Multiple Range Test:

Subset for alpha=0.05		
Plant parts	**1**	**2**
Stem	0.02	
Leaf	0.076	
Root		0.33
Sig	0.082	1.000

Means for groups in homogeneous subsets are displayed

The analysis of total ash, water-soluble ash and acid-insoluble ash content in different component parts of *K. caryophyllata* revealed the presence of inorganic materials in the plant powder. Leaves showed the highest total ash and water-soluble ash content, indicating the presence of comparatively higher amounts of inorganic substances like carbonate, oxalate, phosphate etc., compared to stems and roots. These observations done in the study was in similar lines with the observation done by Ray et al. (2018) in *Solanum glaucophyllum*. Tambe and Kadam (2010) in *Madhuca indica* reported more acid-soluble ash content in the leaves than in the stems, while the amount of acid-insoluble ash is considerably more in the stems than in the leaves.

The variations in total ash content observed in the leaf, stem, and root component of *K. caryophyllata* in the study may be due to differences in accumulation of mineral and inorganic matter among components. Leaves with higher total ash content compared to stem and root could be the result of increased accumulation of inorganic substances in the leaf component, possibly because of their vigorous photosynthetic and transpiration activities (Balekundri & Mannur, 2020). Similarly, the results indicate the amount of water-soluble ash content is much higher in the leaf component compared to the content of stem and root and this reveals that a considerable part of the inorganic matter mostly phosphate salts, carbonate salts and some oxalate in leaves is soluble in water (Humphries, 1956). These differences further reveal variations in the solubility of inorganic matter among different plant components of *K. caryophyllata*. Recognizing differences in inorganic composition and their property in the herbal material is essential for evaluating the quality and for standardizing

herbal products. The acid insoluble-ash content recorded in the study was in minimal level in all the plant components with comparatively higher value obtained from root powder compared to leaf and stem powders and this indicates presence of slight contamination caused by impurities from sand or soil during preparations (Chandel et al., 2011).

pH (10% Solution)

pH is a measure of the acidity or alkalinity of a solution. 10% solution of each component part of *K. caryophyllata* was prepared and their pH values were measured. The results indicate, all the plant parts are acidic nature with more or less variations, which may be related to the differences in organic acids or acidic compounds present in the plant parts(figure 4.1). The leaf component recorded the lowest pH value of 5.39 (±0.07), immediately followed by root component with a pH value of 5.92 (±0.024) and this followed by the stem component which is only slightly acidic and recorded an average pH value of 6.32 (±0.024). The variation in pH values of different component parts of *K. caryophyllata* recorded in the study may be due to the differences in composition or concentration or both of organic acids, alkaloids, or other acidic/basic substances present in these plant components (Silas et al., 2012). The stability and efficacy of bioactive chemical extraction in herbal medicines or extractions depend on pH values. Understanding the pH range of plant extracts is crucial for refining extraction methods and determining potential applications in pharmaceutical sectors. The traditional or therapeutic uses of plants may be impacted by the varying pH values in their component parts. Before being utilized in conventional medicine, certain plant components are chosen based on their chemical content and other characteristics like pH.

Alcohol-Soluble and Water-Soluble Extractive values

The result of the alcohol-soluble and water-soluble extractive values in different components of *K. caryophyllata* is depicted in the table 4.1 The alcohol-soluble extract represents the portion of the plant material that dissolves in alcohol. The data indicates, the highest extractive value recorded in the leaf component (9.67%), followed immediately by the stem component (7.98%) and the lowest extractive value in the root component (7.11%). Statistical analysis reveals the alcohol-soluble

extractive values of leaf components differed significantly over the values of root and stem components while differences in the values between root and stem components were insignificant (table 4.6). This inference in the study reveals the presence as well as the concentration of various polar constituents (may be like alkaloids, flavonoids, phenols, steroids, glycosides etc.) and other constituent materials which are having solubility property in alcohol was significantly higher in the leaf component compared to root and stem components (Chandel et al., 2011).

Table 4.6: One-Way ANOVA and Duncan analysis of alcohol-soluble extractive values in the stem, root and leaf components of *K. caryophyllata*

One-Way ANOVA:

F value	**P value**	**η^2_p (Effect size)**
$F_{(2,15)} = 22.249$	P<0.001	η^2p (effect size) = = 0.78

Duncan's Multiple Range Test:

Subset for alpha=0.05		
Plant parts	**1**	**2**
Root	7.11	
Stem	7.98	
Leaf		9.67
Sig	0.232	1.000

Means for groups in homogeneous subsets are displayed

The water-soluble extract represents the portion of the plant material that dissolves in water. The highest water-soluble extractive value was recorded in the leaf component (12.32%), followed immediately in the stem (11.76%) and the lowest value recorded in the root component (10.97%)(figure 4.1). The Duncan's analysis of the data confirmed significant variation in the water-soluble extractive values among leaf, stem and root components of *K. caryophyllata*. The extractive value of leaf component differed significantly higher over the stem and root components (table 4.7). The inference of the study indicates that the types and concentrations of material like sugar, acids, glycosides, tannins, inorganic compounds and other constituents having solubility property in water was highest in the leaf component while lowest concentration in the root component (Chandel et al., 2011; Siraj et al., 2020).

Table 4.7: One-Way ANOVA and Duncan analysis of water-soluble extractive values in the stem, root and leaf components of *K. caryophyllata*

One-Way ANOVA:

F value	P value	η^2_p (Effect size)
$F_{(2,15)} = 18.012$	P<0.001	η^2p (effect size) = 0.71

Duncan's Multiple Range Test:

	Subset for alpha=0.05		
Plant parts	**1**	**2**	**3**
Root	10.97		
Stem		11.76	
Leaf			12.32
Sig	1.00	1.00	1.00

Means for groups in homogeneous subsets are displayed

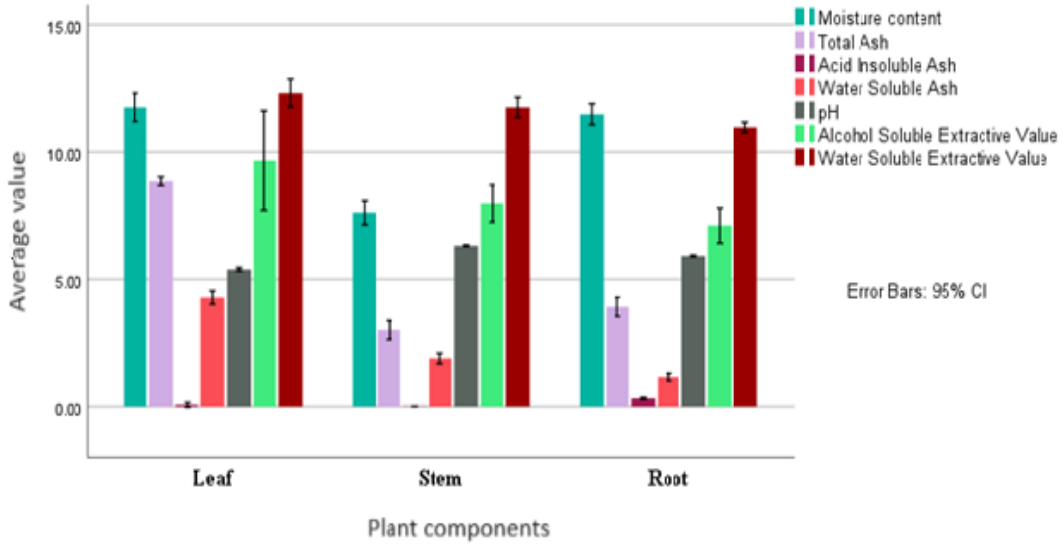

Figure 4.1: Comparative evaluation of physicochemical properties of leaf, stem and root

The alcohol-soluble as well as the water-soluble extractive value plays an important role in evaluation of crude drugs. Comparing the water-soluble extractive values with alcohol-soluble values of the crude drugs, it was clear that the percent of water-soluble extractive values were higher in the leaf, stem and root components over the alcohol-soluble extractive values of the same. This indicates presence of more amounts of water-soluble contents than alcohol-soluble contents in the plants. The variations recorded in extractive values of different plant components further indicate differences in types and concentrations of water-soluble as well as alcohol-soluble compounds in different parts of *K. caryophyllata*. The inference of the study clearly revealed higher extractive yield in the leaf component compared to stem and root components, both in water-soluble and alcohol-soluble extraction. The insights

obtained from the above information can guide traditional medicine practitioners in selecting appropriate plant parts for medicinal purposes. The most important aspect of this study is, the data and information collected on physicochemical parameters can be used as a set of standards which are important for quality assessment and standardizing crude drug and products made from *K. caryophyllata* available in the market. For instance, less extractive values of crude drug collected from the market in comparison to the standard values indicate addition of poor or waste material, adulteration, contamination or incorrect processing during drying, storage or during formulating drugs (Hait et al., 2011).

CONCLUSION

The information and the data obtained in the study from various physicochemical characteristics of *Kamettia caryophyllata* such as the water-soluble and alcohol-soluble extractive values, water-soluble ash, acid-insoluble ash and total ash content, moisture content and pH can be effectively utilized in proper identification and authentication of the plant. This will help in avoiding or detecting its adulteration in herbal preparations and thereby useful for quality control and standardization of herbal products derived from this plant. The variation in moisture content among leaves, stems and roots of *K. caryophyllata* highlights their physiological differences and potential implications for herbal product stability. The differential levels of total ash, water-soluble ash, and acid-insoluble ash among plant components reflect their diverse inorganic composition. The differences in pH reflect potential differences in their chemical compositions, which can have implications for their use in traditional medicine and industries engaged in herbal preparations where pH plays a significant role in applications and formulations. The differences in water-soluble as well as acid-soluble extractive value among leaves, stems and roots reveal variations in the type and composition of phytoconstituents. Therefore, the study concludes that the findings can be used as a set of physicochemical reference charecteristics for *K. caryophyllata*, which could be effectively utilized for standardization and quality control to ensure consistency of the plant product with guaranteed constituents. Further, the findings of the study can also offer insights into their potential uses in traditional medicine and pharmacology.

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Chapter 5

Preliminary Phytochemical Screening and Quantification in *Kamettia caryophyllata*

CONTENTS

INTRODUCTION

Plant phytochemicals are vital bioactive compounds that defend against microbial threats and herbivores. Beyond plant protection, these constituents offer health benefits to humans, potentially mitigating the risk of various diseases. Traditional herbal medicine remains pivotal in healthcare in numerous developing nations, a sentiment underscored by the World Health Organization (Barus et al., 2018; WHO, 1998). An extensive array of low-molecular-weight organic compounds is produced within the plant kingdom. Scientists categorise these compounds into three groups based on their metabolic roles. Primary metabolites directly contribute to plant growth, while secondary metabolites facilitate plant-environment interactions and protection whereas the hormones, the third group, regulate organismal processes and metabolism (Erb & Kliebenstein, 2020). The therapeutic potential of plant species primarily resides in their diverse secondary metabolic constituents such as alkaloids, flavonoids, saponins, tannins, terpenoids and phenolics, present in them with distinct pharmacological effects on the human body and can serve as valuable and safe alternative to artificial drugs (Krishnaiah, 2007; Naseem et al., 2014).

The requirement of medicinal plant as raw material for manufacturing drugs is increasing considerably due to their potentials in combating different kinds of diseases

including the issue of drug resistance in micro-organisms against synthetic drugs. Therefore, the interest of scientists for screening plant extracts has been enhanced greatly for discovering new drugs which are effective for the treatment of various diseases (Dimayuga & Garcia, 1991). The most important strategy and decisive step in the selection of a plant for pharmacological interest is by collecting the traditional knowledge if any available regarding the use of natural plant resources in folk medicine, followed by phytochemical screening to identify specific bioactive compounds with known medicinal activities. The isolation of crucial components from medicinal plants involves extraction methods by utilising various solvents, such as ethanol, methanol, chloroform, acetone, hexane, petroleum ether, ethyl acetate, and water, to determine the phytochemical composition (Agidew, 2022; Venkatesh et al., 2017). The present chapter is an attempt to detect major phytochemical groups using qualitative phytochemical screening tests and to establish a profile of important specific bioactive compounds of interest in the leaf, stem and root components of *Kamettia caryophyllata* using methanol, chloroform and petroleum ether extracts, with the aid of GC-MS and HR LC-MS analysis to evaluate the medicinal efficacy of the plant.

REVIEW OF LITERATURE

Preliminary phytochemical screening methods are crucial and initial step in identifying and quantifying bioactive compounds within plants. Plants produce various secondary metabolites, such as alkaloids, steroids, cyanogenic glycosides, phenolics, flavonoids, saponins, and terpenoids. These compounds function as a defence mechanism against diseases, pests, and environmental stressors, showcasing their multifaceted roles in promoting plant survival(Guerriero et al., 2018; Harborne, 1974). According to Agidew (2022), phytochemicals are highly efficient bioactive compounds present in medicinal plant parts, serving as precursors for the synthesis of valuable drugs.

Extraction and purification of active compounds from plant resources are essential for identifying and harnessing the medicinal potential of these phytochemicals. Maceration, infusion, soxhlation, percolation, or digesting methods have been widely used for the extraction and isolation of bioactive compounds from plants (Giacometti et al., 2018). Preliminary screening of phytochemicals is a vital step towards

identifying bioactive principles in medicinal plants, contributing significantly to drug discovery and development (Singh et al., 2022).

The members of Apocynaceae family are reported to have excellent medicinal properties for cancer, inflammation, diabetes, etc. Bhadane et al. (2018) has observed that Apocynaceae plants contain phytochemicals such as alkaloids, terpenoids, steroids, flavonoids, glycosides, simple phenols, lactones, and hydrocarbons. Kumari et al. (2013) reported that the root and rhizome of *Rauvolfia serpentina* which are rich in alkaloids, carbohydrates, flavonoids, glycosides, phlobatannins, phenols, resins, saponins, sterols, tannins, and terpenes have been employed in Ayurvedic medicine to treat numerous ailments, including high blood pressure, mental agitation, epilepsy, traumas, anxiety, excitement, schizophrenia, sedative insomnia, and insanity.

Agrahari et al. (2010) screened the occurrence of secondary metabolites in methanolic, petroleum ether, ethylacetate and chloroform extracts of *Curculigo orchioides* and they reported the detection of saponins, carbohydrates and glycosides. Orabueze et al. (2016) analysed methanolic extract of *Olax subscorpioidea* and reported the occurrence of alkaloids, saponins, tannins, cardiac glycosides, phenols, steroids, carbohydrates, etc. It was Rhoads (2008) who reported that the phytochemicals are primary and secondary compounds of which chlorophyll, proteins and common sugars are included in primary constituents while compounds such as terpenoid, alkaloids and phenolics are included in secondary constituents. The screening of methanolic extract of *Epipremnum aureum* revealed presence of alkaloids, flavonoids, tannins, terpenoids, anthraquinones, sterols and phenols (Sonawane et al., 2011). Kalairasan et al. (2011) reported that the phytochemical screening of petroleum ether and ethanol extracts of *Bulbophyllum kaitense* stem component revealed the occurrence of alkaloids, glycosides, carbohydrates, flavonoids, phenols, saponins and tannins.

Secondary metabolites in plants are categorized into 3 major groups such as terpenoids, alkaloids and phenolics and each of these groups includes a large number of phytocompounds which are very effective for the treatment of a variety of diseases (Savithramma et.al., 2011). Some of the important phytocompounds coming under these groups include - atropine, nicotine, morphine, and codeine belonging to the group alkaloids while the compound linalool belonging to terpenoids whereas the

compounds like flavonoids, proanthocyanidins, lignans etc. belonging to the group phenolics. The phenolic group includes diverse types of phytochemicals containing one or more phenol groups. Because of various relevant pharmacological characteristics of phenolic compounds like anti-microbial, anti-cancer, antioxidant properties, they are used in the preparation of several drugs. Flavonoids are one of the major phenolic compounds, its interest in the field of pharmaceuticals is increasing mainly due to their antioxidant, anti-allergic as well as anti-thrombotic activities. The tannins are another important phenolic compound having medicinal properties such as anti-diarrhoeal, antidotes in poisoning, whereas the phenolic compound coumarins are having anticoagulant and anti-Alzheimer properties, while phenolic compound lignans are having antimicrobial activities (Kaushik et al., 2021).

Tannins, previously considered antinutritional, have shown promise in medicinal applications in antimicrobial activities including antiviral and antibacterial and as wound-healing agents (Jayaraj et al., 2019; Momin & Kadam, 2011; Tong et al., 2022). Flavonoids, known for their multifaceted benefits encompassing antibacterial, anti-inflammatory, and antioxidant properties, play an essential role in shielding organisms from free radical damage and exhibit strong anticancer activities (Phuyal et al., 2020; Siqueira et al., 2012; Yang et al., 2018). According to Guerrieo et al. (2018), phenolic compounds are widespread in plants, possess antioxidant qualities and demonstrate antimutagenic effects, which are crucial in preventing oxidative cell damage and disease onset. Their presence often signifies potential antimicrobial defences, protecting against predators and pathogens. Combining carbohydrates and non-carbohydrate elements, glycoside compounds have significant medicinal implications, particularly in cardiac insufficiency, they also contribute to increased bodily strength and serve as dietary supplements (Bhadane et al., 2018; Florence et al., 2015).

The plant secondary metabolite terpenoids primarily contain a five-carbon isoprene unit. James et al. (2008) reported diverse pharmacological activities of terpenoids which include anti-inflammatory, antimalarial, anti- cholesterol synthesis, anticancer, anti-bacterial and anti-viral activities. It was Franklin et al. in 2001 who reported, the plant Cannabis is one of the important terpene sources with medicinal properties such as antimicrobial, anti-hyperglycemic, antifungal, anticancer, anti-inflammatory,

antiviral, analgesic and antiparasitic activities and further they also reported higher skin penetration property of terpenes. Alvarez et al. (2021) reported that terpenes have antimicrobial properties because of their capacity to inhibit DNA and protein synthesis and also due to their capacity to rupture cell and hence terpenes are useful against antibiotic-susceptible as well as antibiotic-resistant bacteria. Recently a number of studies have reported that terpenes/terpenoids are important in supporting human health. These bioactive compounds have significant role in treating different kind of diseases, in various studies *in vitro* as well as *in vivo* using them as antimicrobial, anti-inflammatory, anti-cancer agents, antioxidants, anti-coagulation, antiallergic, anti-coagulation and analgesic through the activity of sesquiterpenes, mono- di- tri- and tetraterpenes and glycoside compounds (Zhao et al., 2016). Terpenoids, renowned for their diverse medicinal properties such as anticarcinogenic, antimalarial, antimicrobial, and diuretic effects, have demonstrated efficacy against mosquito larvae and pathogenic microorganism (Lukowski et al., 2022; Mahizan et al., 2019).

Alkaloids are secondary metabolite with nitrogen-containing compounds and are found widely occurring in considerable number of plant families such as Apocynaceae, Solanaceae, Fabaceae, Papaveraceae, Rubiaceae, Rutaceae, and Asteraceae (Yang & Stockigt, 2010), where these phytochemicals are found distributed either in the whole plant or in certain specific plant parts. Alkaloids are found exhibiting many medicinal properties like antidepressant, analgesic, sedative, and hypnotic properties (Codeine); acute pulmonary edam and reduce the shortness of breath (Morphine); insecticide, anti-inflammatory, antiherbivore (Nicotine) and the alkaloid compound atropine exhibit properties like anti-cholinergic, anti-myopia etc. (Kaushik et al., 2021). In modern medicine, plant-derived alkaloids are getting more attention because of their pharmacological scopes in the therapeutic area such as analgesic properties of the alkaloid morphine, antiasthmatic properties of ephedrine, anticancer activities of vincristine, antihypertensive effect of reserpine, antipyretic effects of quinine, antihyperglycemic activities of piperine etc. (Ng et al., 2015).

Alkaloids are a group of phytochemical constituents identified for their effect on the central nervous system, appetite regulation and control, diuretic action, and high antioxidant capabilities (Kamble & Gaikwad, 2019; Hussain et al., 2018; Zohra, 2015; Zhu et al., 2014). Although alkaloids are highly valued for their therapeutic

importance, their natural toxicity requires careful attention when used in medicinal settings (Harborne, 1974; Yang et al., 2018). Kamble & Gaikwad (2019) found that these substances have various biological impacts, mainly working as antioxidants to remove reactive oxygen species. Despite being valued for their therapeutic importance, the natural toxicity of alkaloids requires careful attention in medical situations (Bakir et al., 2022). Jayaraj et al. (2019) noted that *Vinca rosea*, belonging to the Apocynaceae family is distinguished for its medicinal significance, possessing over 70 alkaloids and chemotherapeutic substances recognised for fighting against different types of diseases including melanomas, Hodgkin, and non-Hodgkin's lymphoma and cancers affecting breast, lung and uterine. As per the research conducted by Mohammed et al. (2021), *Catharathus roseus* demonstrates anti-cancer properties due to vinblastine, an alkaloid that adds to its pharmaceutical value.

Gas chromatography coupled with mass spectrometry (GC-MS) analysis of various ethnobotanical plants have unveiled an array of secondary metabolites with diverse biological activities. Compounds like phytol, dodecanoic acid, Tetradecanoic acid, n-hexadecenoic acid, copane, caryophyllene, Cardinol, and others, identified through these analyses, demonstrate antimicrobial, anti-inflammatory, antioxidant, and anticancer properties (Olagunju et al., 2006; Hasan et al., 2014; Lamia, 2015; Raja et al., 2011). The GC-MS analysis conducted in the ethanolic extract of *Calotropis gigantea* flower revealed the presence of 14 major compounds (Dhivya & Manimegalai, 2013). According to Allen and Mc Whinney (2019), QTOF HR LCMS (Quadrupole Time-of-Flight High-Resolution Liquid Chromatography Mass Spectrometry) represents a technology transforming phytochemical analysis. This hybrid device integrates high-resolution mass spectrometry with liquid chromatography, delivering exact mass measurements, precise identification, and detailed understanding of various bioactive compounds found in plant samples (Petras et al., 2017). Its exceptional sensitivity and specificity empower the identification of trace phytochemicals in intricate matrices, allowing for a comprehensive assessment of phytochemical groups like alkaloids, flavonoids, terpenoids, phenolic compounds and other constituents concurrently (Marulasiddaswamy et al., 2021). So far, many researchers and scientists have already explored the details of specific bioactive compounds of large number of plants. Chen et al. (2017) observed that the metabolomic analysis conducted on two plants from Apocynaceae family,

Catharanthus roseus and *Vinca minor*, employing a combination of GC-MS and LC-MS techniques revealed the presence of 58 compounds in the leaf of both plants, and the examination of nine common alkaloids in both *V. minor* and *C. roseus* through LC-MS analysis revealed elevated concentrations of important terpenoid indole alkaloid (TIA) intermediates such as loganin, serpentine, and tabersonine in *V. minor* compared to *C. roseus*. The findings from these studies underscore the high potential of plant constituents as a source for novel therapeutic agents with diverse pharmacological activities. Further exploration and research into these compounds hold promise for developing new drugs and treatments.

Al-Azzawi et al. in 2012 analysed and prepared the phytochemical profile of *Sesuvium portulacastrum* in different extracts of ethanolic, dichloromethane and aqueous solvents and reported the detection of triterpenoids, phenols, tannins, flavonoids, saponins and glycosides. The study further reported that the GC-MS analysis of the ethanolic extract revealed the presence of specific bioactive compounds 3,4,5-trihydroxy- (Gallic acid), Benzoic acid, Capsaicin and (2R,3R)- (-)-Epicatechin. Ezhilan and Neelamegam in 2012 conducted GC-MS analysis of *Polygonum chinense* ethnolic whole plant extract and recorded the presence of antimicrobial compounds like squalene (47.01%), mono[2- ethylhexyl]ester (40.30%) and 1,2-benzenedicarboxylic acid. The GC/MS analysis done in the hexane leaf extract of *Juniperus phoenicea* by Henda et al. (2017) reported the detection of 36 phytocompounds which had 56.5% of sesquiterpene hydrocarbons and the major compounds in this fraction includes: α-humulene (16.9%), pentadecane (10.2%), α-cubebene (9.7%) and β-cadinene (4.9%). The study reports that the bioactive principles identified in the GC/MS analysis were having antimicrobial, antioxidant anti-inflammatory, and antidiabetic activities. Konappa et al. (2020) reported the detection of 25 bioactive compouds in the GC–MS analysis of ethyl acetate as well as methanol leaf and rhizome extracts of *Amomum nilgiricum* and they further noted that these compounds are having remarkable antibacterial, antioxidant, antifungal, antidiabetic and antiviral properties. Serverogenin acetate, 2,4-dimethyl-1,3-dioxane and (1,3-$^{13}C_2$)propanedioic acid were the major phytoconstituents.

The LC-MS analysis conducted by *A*rumugam et al. (2022) in the methanolic leaf extracts of *Saraca asoca* revealed presence of many phytocompounds belonging to

flavonoids, terpenes, fatty acids, and alkaloids in which phytocompounds of flavonoid group like naringenin, quercetinand and epiafzelechin are recorded as higher than any other phytocompounds responsible for the increased response to several diseases and damage. Macedo et al. (2020) conducted a LC-MS-based untargeted analysis in the leaves, pulp and seed of e *Eugenia calycina* and revealed the identification of about 153 phytocompounds belong to various chemical groups, while the targeted LC-MS analysis of same for the phenolics group revealed that epicatechin gallate, ellagic acid and myricitrin are the major bioactive compounds identified.

MATERIALS AND METHODS

Preparation of extract

Fresh and healthy *Kamettia caryophyllata* plants were collected, and the leaves, stems, and roots were meticulously separated and washed extensively under running tap water. They were then air-dried in shaded conditions and grounded into a fine powder. About 100 g of the powdered plant material was placed into a Soxhlet apparatus and subjected to extraction using various solvents such as methanol, chloroform, and petroleum ether (figure 5.1). After extraction, the resulting extract underwent filtration, and the filtrate obtained was then evaporated using a vacuum evaporator under reduced pressure at a temperature not exceeding 40°C (figure 5.2). This process continued until a consistent weight was achieved, indicating the attainment of dryness. The crude dried extract, obtained post-evaporation, was carefully stored in desiccators for subsequent and further studies and analysis.

Figure 5.1: Soxhlet extraction of plant material using the solvent

Figure 5.2: Vacuum evaporation of the extract using rotary evaporator

Preliminary phytochemical analysis

Various biochemical tests were carried out to create a basic and qualitative overview of different bioactive phytochemical groups in the methanol, chloroform and petroleum ether extracts of the leaves, stems, and roots of *K. caryophyllata*. The standard procedures outlined in Experimental Phytopharmacognosy were followed for the qualitative phytochemical tests. (Khadabadi et al., 2013).

Detection of alkaloids

Alkaloid detection involves specific tests conducted on the plant extract dissolved in 2 N HCl, followed by subsequent filtration. Two distinct tests, namely Dragendorff's and Mayer's tests, were performed:

Dragendorff's Test: A portion of the solution was combined with 2 ml of Dragendorff's reagent. The formation of a reddish-brown precipitate upon mixing indicates the probable presence of alkaloids.

Mayer's Test: Another portion of the solution was mixed with 2 ml of Mayer's reagent. The observation of a creamish precipitate following the combination suggests the potential presence of alkaloids.

Detection of tannins and phenolic compounds

The Ferric chloride test and the Lead acetate test were employed to reveal the presence of tannins and phenolic compounds in the plant extract:

Ferric Chloride Test: Three drops of 5% ferric chloride solution were added to the plant extracts of *K. caryophyllata*. Blue-green to blue-black colour change indicates the presence of tannins and phenolic compounds.

Lead Acetate Test: In to the plant extracts 3 ml of lead acetate (10%) solution was added. White precipitate formation after adding lead acetate suggests the existence of tannins and phenolic compounds.

Detection of flavonoids

Shinoda Test: Two or three drops of concentrated HCl or H_2SO_4 and magnesium powder were added to 2 ml of the test solution, giving colours such as orange, red, purple, pink, or magenta indicating the potential presence of flavonoids.

Alkaline Reagent Test: The presence of flavonoids was detected by adding a few drops of sodium hydroxide solution to the test solution. A yellow hue was observed, which turned colourless upon adding a few drops of dilute acid, indicating the presence of flavonoids.

Detection of glycosides

Legal's test: 2 ml of pyridine and sodium nitroprusside was combined with 1 ml of plant extract. The appearance of a pink or red colouration indicated the potential presence of glycosides.

Detection of terpenoids

Salkowski test: A solution of 1-2 mg of the test sample in 1 ml of $CHCl_3$ (chloroform) was prepared, followed by adding 1 ml of concentrated H_2SO_4 (Sulfuric acid) to the mixture. The red colour in the chloroform layer and green fluorescence in the acid layer suggested the possible existence of terpenoids.

Detection of saponins

Foam test: Shaking the test sample's water solution vigorously resulted in a stable foam lasting at least 15 minutes, signifying the presence of saponins.

Detection of carbohydrates

Molisch's Test: 1 ml of Molisch's reagent mixed with 2 ml of the extract solution, followed by adding 1 ml of concentrated H_2SO_4.The appearance of a red-to-violet ring at the junction of the two liquids indicated the presence of carbohydrates.

Benedict's Test: 2 ml of Benedict's reagent was added to 2 ml of the extract solution and boiled in a water bath. A red, yellow, or green colour or precipitate indicated the presence of carbohydrates.

Detection of Proteins

Biuret Test: 2 ml of plant extract solution mixed with 2 ml of biuret reagent and the appearance of a violet-to-pink colour indicated the presence of proteins.

Millon's Test: A mixture was prepared by combining 2 ml of extract solution with 2 ml of Millon's reagent and boiling. The red colour formation indicated the presence of proteins.

Quantitative determination of secondary metabolites

Considering the diversity of phytochemical compounds detected and identified in the qualitative phytochemical screening of different solvent extracts of leaf, stem and root components in *K. caryophyllata*, the methanolic extract of plant components was selected for further studies.

Total estimation of alkaloids

The determination of total alkaloid content was carried out using the Acid Dye Colorimetric Method as described by Trease and Evans (2019). Alkaloids, under acidic conditions, form coloured complexes by combining with an acid dye. Methanolic extract from the leaf, stem, and root components (1mg) were dissolved in methanol, and 1 ml of 2 N HCl was added to the solution. This solution was then filtered and transferred to a separating funnel. Subsequently, 5 ml each of bromocresol green solution and phosphate buffer were added. The mixture was vigorously shaken with 1ml, 2ml and 3ml, and then finally 4 ml chloroform. The chloroform layers collected were pooled into a 10 ml volumetric flask and diluted with chloroform to reach the volume. Sets of reference standard solutions ranging from 2 to 12 µg/ml (2,4,6,8,10, and 12 µg/ml) of atropine were prepared. Absorbance readings for both test and standard solutions were measured against the reagent blank at 470 nm using a UV-visible spectrophotometer. The total alkaloid content was determined as atropine equivalents per gram of extract using the linear regression equation derived from the atropine standard curve.

Total estimation of phenolics

The total phenolics in the extracts were quantitatively analysed with the Folin-Ciocalteu reagent (Singleton &Rossi, 1965). The Folin-Ciocalteu reagent, made from a blend of phosphotungstic acid and phosphomolybdic acid, is reduced to produce blue tungsten and molybdenum oxides, when phenols are oxidised. This process takes place in alkaline environments with the help of sodium carbonate. The ease of electron

removal from the phenol molecule in these conditions leads to a blue colouration, with maximum absorption around 760 nm, proportional to the initial total quantity of phenolic compounds, often expressed as gallic acid equivalents. Analysis was conducted using a gallic acid standard with concentrations ranging from 2 to 12 µg/ml. Solutions were made by extracting at a concentration of 1 mg/ml in methanol. 0.05 ml of every sample was combined with 2.5 ml of a Folin-Ciocalteu reagent diluted 10 times and 2 ml of sodium carbonate at 7.5% in test tubes. The tubes were sealed and allowed to sit at room temperature for half an hour before measuring absorbance at 760 nm with an absorption spectrometer. The spectrophotometric measurement was used to quantify the blue colour produced when the Folin-Ciocalteu reagent reacted with reducing compounds like polyphenols. A linear regression equation was determined by creating a calibration curve with the concentration of gallic acid plotted against absorbance. The total phenol content was determined with this formula and reported as milligrams of gallic acid equivalent per gram of extract (mg GAE/g).

Total estimation of tannin

The Folin-Denis method was used to estimate tannins. There are two main categories of tannins: hydrolysable tannins have polyhydric alcohol, while condensed tannins are made up of flavanols. This calculation technique relies on the non-stoichiometric oxidation of compounds with phenolic hydroxyl groups. Tannins can reduce phosphotungstomolybdic acid in an alkaline solution, producing a highly coloured blue solution. The strength of this colour is directly related to the quantity of tannins and is quantified using a spectrophotometer at 700 nm. Roughly 0.05 ml of methanolic extracts from the leaf, stem, and root of *K. caryophyllata* were transferred into test tubes for examination. Following this, 0.5 ml of the Folin-Denis reagent and 0.8 ml of distilled water were included. The tubes were left untouched for a quarter of an hour. Afterwards, 1 ml of sodium carbonate solution was added, and the volume was topped up to the rest using 7.5 ml of distilled water. Absorbance readings at 700 nm were recorded after shaking the tubes for 30 minutes. Tannic acid was prepared in different concentrations (2, 4, 6, 8, 10, and 12 µg/ml) in separate test tubes, following the same steps. Absorbance was correlated with concentration to create a calibration curve for tannic acid. An equation based on the calibration curve was developed for

linear regression, which was then utilised to calculate the quantity of total tannins represented as milligrams of tannic acid equivalent per gram of extract (mg TAE/g).

Total estimation of flavonoids

The estimation of flavonoid compounds was conducted using the modified Aluminium chloride Colorimetric Method based on the procedure outlined by Woisky and Salatino (1998). This method relies on aluminium chloride's ability to form acid-stable complexes in the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavanols. Additionally, it forms acid-labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids. For standardisation, quercetin was employed to establish a calibration curve. Ten milligrams of quercetin were mixed with 1 ml of methanol and subsequently thinned to levels of 2, 4, 6, 8, 10, and 12 µg/ml. The standard solutions (20 µl) were combined with methanol (1.5 mL), aluminium chloride (0.1 ml), sodium acetate (0.1 ml), and distilled water (2.8 ml) in different preparations. After incubating at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm using a spectrophotometer. Methanol served as the blank. Likewise, 1.5 ml of methanol extracts were treated with aluminium chloride after the same procedure to measure the flavonoid content.

Total estimation of terpenoids

The terpenoid content was calculated using the procedure outlined by Ghorai et al (2012). 1 mg/ml stock solution was prepared using the leaf, stem, and root methanol extract of *K. caryophyllata*. In a test tube, 1.5 ml of chloroform was added, and 100 µl of the sample extract was mixed thoroughly. The mixture sample was given a three-minute time to settle. Following that, 100 µl of concentrated sulphuric acid was included. If heat was produced, the system was cooled with an ice pad for up to 15 minutes. Next, the combination was left in the dark at room temperature for 1.5 to 2 hours. Different concentrations of linalool (2 to 12 µg/ml) in methanol were used to create the standard curve. The standard solutions required an incubation time of not more than five minutes. The solutions remained undisturbed during the incubation period and a reddish-brown precipitate was formed. The supernatant was carefully decanted, and the precipitate was collected. Subsequently, 2 ml of 95% methanol was added and

thoroughly vortexed until the precipitate was completely dissolved in methanol. The absorbance was read at 538 nm using a spectrophotometer. The total terpenoid concentration of the unknown plant sample was calculated by utilising the regression equation obtained from the standard curve.

Chromatographic analysis

GC-MS (Gas Chromatography-Mass Spectrometry) screening for volatile bioactive compounds

The GC-MS analysis of methanolic extracts prepared from leaf, stem, and root of *K. caryophyllata* was carried out using an Agilent Technologies GC system (Model – 5975C) interfaced with an MS 7890A mass spectrometer. Here the specific parameters used: The analysis utilised a DB5-MS fused silica capillary column (30 X 0.25 mm ID X 0.25 mm film thickness) consisting of 5% Phenyl and 95% Dimethyl Polysiloxane. Operating in electron impact mode at 70 eV, helium (99.999%) served as the carrier gas at a constant 1ml/min flow rate. The injector temperature was set at 250°C, while the ion-source temperature was maintained at 150°C. The oven followed a programmed temperature sequence, starting at 40°C for 5 minutes and increasing by 5°C per minute until reaching 280°C, which was held for 10 minutes. Mass spectra were collected at 70 eV, with a scan interval of 0.2 seconds, scanning fragments from 50 to 550 Da. The entire GC process ran for a total duration of 57 minutes. Compound identification was accomplished by comparing constituents with available compounds in the attached Computer Library (NIST ver. 2.1), and subsequent reporting was based on these identifications.

HR LC-MS(High Resolution Liquid Chromatography-Mass Spectrometry) screening for non-volatile bioactive compounds

The analytes underwent chromatographic separation and detection via Liquid Chromatography coupled with a quadrupole time-of-flight mass spectrometry system (ESI-QTOF-LC/MS) from Agilent Technologies (model G6550A). Control of the instrument and data acquisition was managed using Mass Hunter LC/MS Data Acquisition software (version B.06.01). In contrast, the evaluation of acquired data was performed using Mass Hunter Qualitative and Quantitative Analysis software (version B.07.00). All samples underwent filtration using 0.2µm nylon membrane

filter before injection. The process of chromatographic separation involved utilising a Hypersil GOLD C18 column that was 100 x 2.1mm in size, containing particles with a size of 3µm, and running at a constant flow rate of 0.3ml/min. An acetonitrile solvent system containing 0.1% formic acid was used for analysis, running at a flow rate of 0.31ml/min and a constant pressure of 1,200 bar. The LC system was linked to the quadrupole time-of-flight mass spectrometer that had an electrospray ionisation (ESI) interface and could work in both positive and negative ionisation modes. A volume of 5µl was designated for the injection of samples. Mass spectrometry data was collected across a range of m/z values from 50 to 1000. Obtaining mass spectra required employing collision energy ranging from 5 to 30 electron volts. The whole procedure lasted for 35 minutes.

Statistical analysis

The observations/data were reported as mean± standard deviation. Statistical analysis was done using SPSS software and One-Way ANOVA with Duncan analysis was performed.

RESULTS AND DISCUSSIONS

Preliminary phytochemical screening

The qualitative phytochemical analysis of extracts from various component parts of *Kamettia caryophyllata*, such as leaves, stems, and roots, indicated the presence of a broad range of compounds, showcasing the diverse chemical compositions achieved through extraction with various solvents like methanol, chloroform, and petroleum ether. The table 5.1 summarises the diversity of phytochemical compounds detected in different solvent extracts of *K. caryophyllata* leaf component obtained through different phytochemical screening tests. The methanol leaf extract displayed the detection of wide range of substances such as alkaloids, phenolics, tannins, flavonoids, glycosides, terpenes, saponins, carbohydrates and proteins. However, several compounds were found not detected in both chloroform as well as petroleum ether extracts compared to methanol extract. This includes absence of several compounds like alkaloids, phenolics, tannins, saponins and proteins in chloroform extract while with respect to petroleum ether extract, in addition to the above-mentioned compounds, flavonoids and carbohydrates were also absent.

Table 5.1: Preliminary phytochemical screening of *Kamettia caryophyllata* leaf component

SI No	Constituents	Test	Methanol			Chloroform			Petroleum ether		
			R1	R2	R3	R1	R2	R3	R1	R2	R3
1	Alkaloid	Dragendorff's test	+	+	+	-	-	-	-	-	-
		Mayers test	+	+	+	-	-	-	-	-	-
2	Phenolics	Ferric chloride test	+	+	+	-	-	-	-	-	-
		Alkaline reagent test	+	+	+	-	-	-	-	-	-
3	Tannins	Ferric chloride test	+	+	+	-	-	-	-	-	-
		Lead acetate	+	+	+	-	-	-	-	-	-
4	Flavonoid	Alkaline reagent Test	+	+	+	+	+	+	-	-	-
		Shinoda Test	+	+	+	+	+	+	-	-	-
5	Glycosides	Legal test	+	+	+	+	+	+	+	+	+
6	Terpenes/ Steroids	Salkowski test	+	+	+	+	+	+	+	+	+
7	Saponins	Foam test	+	+	+	-	-	-	-	-	-
8	Carbohydrates	Benedict's test	+	+	+	+	+	+	-	-	-
		Molisch's Test	+	+	+	-	-	-	-	-	-
9	Proteins	Biuret Test	+	+	+	-	-	-	-	-	-
		Millon's Test	+	+	+	-	-	-	-	-	-

As in the case of stem component, the methanolic extract of stem component recorded detection of higher number of phytocompounds while lower number of compounds detected in both chloroform and petroleum ether extracts. The details are depicted in table 5.2. The phytocompounds recorded from the methanolic extract include alkaloids, phenolics, tannins, flavonoids, glycosides, terpenes, saponins and carbohydrates, while compounds recorded from chloroform extracts are flavonoids, glycosides, terpenes and carbohydrates whereas only glycosides, terpenes and carbohydrates with respect to petroleum ether extract.

Table 5.2: Preliminary phytochemical screening of *K. caryophyllata* stem component

SI No	Constituents	Test	Methanol			Chloroform			Petroleum ether		
			R1	R2	R3	R1	R2	R3	R1	R2	R3
1	Alkaloid	Dragendorff's test	+	+	+	-	-	-	-	-	-
		Mayers test	+	+	+	-	-	-	-	-	-
2	Phenolics	Ferric chloride test	+	+	+	-	-	-	-	-	-
		Alkaline reagent test	+	+	+	-	-	-	-	-	-
3	Tannins	Ferric chloride test	+	+	+	-	-	-	-	-	-
		Lead acetate	+	+	+	-	-	-	-	-	-
4	Flavonoid	Alkaline reagent Test	+	+	+	+	+	+	-	-	-
		Shinoda Test	-	-	-	-	-	-	-	-	-
5	Glycosides	Legal test	+	+	+	+	+	+	+	+	+
6	Terpenes/ Steroids	Salkowski test	+	+	+	+	+	+	+	+	+
7	Saponins	Foam test	+	+	+	-	-	-	-	-	-
8	Carbohydrates	Benedict's test	+	+	+	+	+	+	+	+	+
		Molisch's Test	+	+	+	-	-	-	-	-	-
9	Proteins	Biuret Test	-	-	-	-	-	-	-	-	-
		Millon's Test	-	-	-	-	-	-	-	-	-

The screening of phytochemicals in the methanolic root extract revealed the presence of alkaloids, phenolics, tannins, flavonoids, terpenes and carbohydrates, while only terpenes and carbohydrates in the case of chloroform root extract and only terpenes detected with respect to petroleum ether extract. The details of compounds detected in the root component are summarised in the table 5.3.

Table 5.3: Preliminary phytochemical screening of *K. caryophyllata* root component

SI No	Constituents	Test	Methanol			Chloroform			Petroleum ether		
			R1	R2	R3	R1	R2	R3	R1	R2	R3
1	Alkaloid	Dragendorff's test	+	+	+	-	-	-	-	-	-
		Mayers test	+	+	+	-	-	-	-	-	-
2	Phenolics	Ferric chloride test	+	+	+	-	-	-	-	-	-
		Alkaline reagent test	+	+	+	-	-	-	-	-	-
3	Tannins	Ferric chloride test	+	+	+	-	-	-	-	-	-
		Lead acetate	+	+	+	-	-	-	-	-	-
4	Flavonoid	Alkaline reagent Test	+	+	+	-	-	-	-	-	-
		Shinoda Test	+	+	+	-	-	-	-	-	-
5	Glycosides	Legal test	-	-	-	-	-	-	-	-	-
6	Terpenes/ Steroids	Salkowski test	+	+	+	+	+	+	+	+	+
7	Saponins	Foam test	-	-	-	-	-	-	-	-	-
8	Carbohydrates	Benedict's test	+	+	+	+	+	+	-	-	-
		Molisch's Test	+	+	+	+	+	+	-	-	-
9	Proteins	Biuret Test	-	-	-	-	-	-	-	-	-
		Millon's Test	-	-	-	-	-	-	-	-	-

The results obtained from the qualitative phytochemical analysis revealed that there is a variation in the phytochemical profile obtained from different component parts of leaf, stem and root and the inference of the study revealed the maximum diversity of compounds in the leaf component compared to stem and root components and this may be due to variation in the distribution of chemical constituents in different component parts. The inference of the study also revealed, there is variation in the diversity of phytochemical groups from different plant component into different extraction solvents and this may be due differential affinity or solubility properties of different phytochemical groups in different solvents. In this respect, the study results

also indicate, the methanolic extract of plant components, whether it is leaf or stem or root, recorded maximum number of diverse groups compared to chloroform and petroleum ether extracts. The methanolic extract of leaf, stem and root recorded highest diversity of 9, 8 and 6 chemical groups respectively while chloroform extract of leaf, stem and root recorded a diversity of 4, 4 and 2 and petroleum ether extract of leaf, stem and root recorded a diversity of 2, 3 and 1 groups respectively.

All the component parts leaf, stem and root of *K. caryophyllata* exhibited common positive results for the phytochemical groups alkaloids, phenolics, tannins, flavonoids, terpenes, and carbohydrates. However, in addition to these, the compounds glycosides, saponins and proteins were also detected in the leaf component while only glycosides and saponins in the case of stem component but any of these compounds were not detected with respect to the root component. The **alkaloid** compounds are known for their pharmacological effects which often exhibit analgesic, anti-inflammatory, antimicrobial, and potentially cytotoxic properties (Fadhil et al., 2007). Some alkaloids can also act as neurotransmitter modulators or it can affect the central nervous system (Hussain et al., 2018).

The **phenolic** compounds are reported to have shown various health-promoting properties including antioxidant and anti-inflammatory effects, but their direct role as antidiabetic agents might not be well-established as other compounds are specifically designed or identified for their anti-diabetic properties (Kisiriko et al., 2021). **Tannins** are polyphenolic compounds detected in the plant parts of *K. caryophyllata* that possess antioxidant properties, aiding in neutralizing free radicals and reducing oxidative stress within the body. Besides, tannins exhibit antimicrobial effects, potentially inhibiting the growth of various microorganisms (Pizzi, 2021). Their astringent nature allows them to bind to proteins, offering potential benefits in traditional medicine for conditions such as minor bleeding and diarrhoea, but excessive intake may interfere with nutrient absorption (Fraga-Corral et al., 2021; Tong et al., 2022). The compound flavonoids detected are also polyphenolic compounds recognised for their robust antioxidant effects, combating oxidative stress by scavenging free radicals in the body (Ullah et al., 2020). Their potential health benefits reported also include supporting cardiovascular health, reducing

inflammation, lowering the risk of chronic diseases (Dhalaria et al., 2020; Panche et al., 2016).

Glycosides are another important compound detected in *K. caryophyllata,* which contains a sugar molecule (glycone) linked to a non-sugar compound (aglycone). These compounds are reported to have diverse biological activities including antimicrobial, antiviral, or antioxidant properties and hence these compounds are significant in traditional medicine and pharmaceutical research due to their varied bioactive potential and therapeutic applications. The carbohydrates detected in the phytochemical test serve as vital energy stores in plants, which when consumed, combine with oxygen to provide energy for essential bodily functions. Plants rich in carbohydrates and glycosides are recognized for their positive impact on the immune system, potentially enhancing overall body strength, and are therefore valued as beneficial dietary supplements (Shahriar et al., 2012).

The phytochemical group terpenoids detected in the study are playing essential role in plant defence mechanisms and have significant applications in traditional medicine and pharmaceuticals (Saravanan et al., 2013; Wu et al., 2020). This phytochemical group encompass a wide range of compounds which include essential oils, sterols and hormones, and exhibit diverse pharmacological properties such as anti-inflammatory, antimicrobial, antioxidant, and anticancer effects (Gutierrez-Delrio et al., 2021). The occurrence of saponins recorded in the study is highlighting the significance of *K. caryophyllata* in traditional medicine and pharmaceutical research, as these compounds are reported to have diverse biological properties like cholesterol-lowering and antimicrobial actions and further they have been studied for their immune-modulating capabilities and potential anticancer effects (Juang & Liang, 2020; Timilsena et al., 2023).

GC-MS analysis of methanolic extracts of plant components

GC-MS analysis performed preferably targeted for volatile compounds and the chromatogram profile obtained from the methanolic extracts of leaf, stem and root in *K. caryophyllata* altogether identified 31 specific bioactive compounds with respect to leaf extract (figure 5.3), while 12 compounds with respect to stem extract (figure 5.4), whereas 24 compounds identified with respect to root extract (figure 5.5).

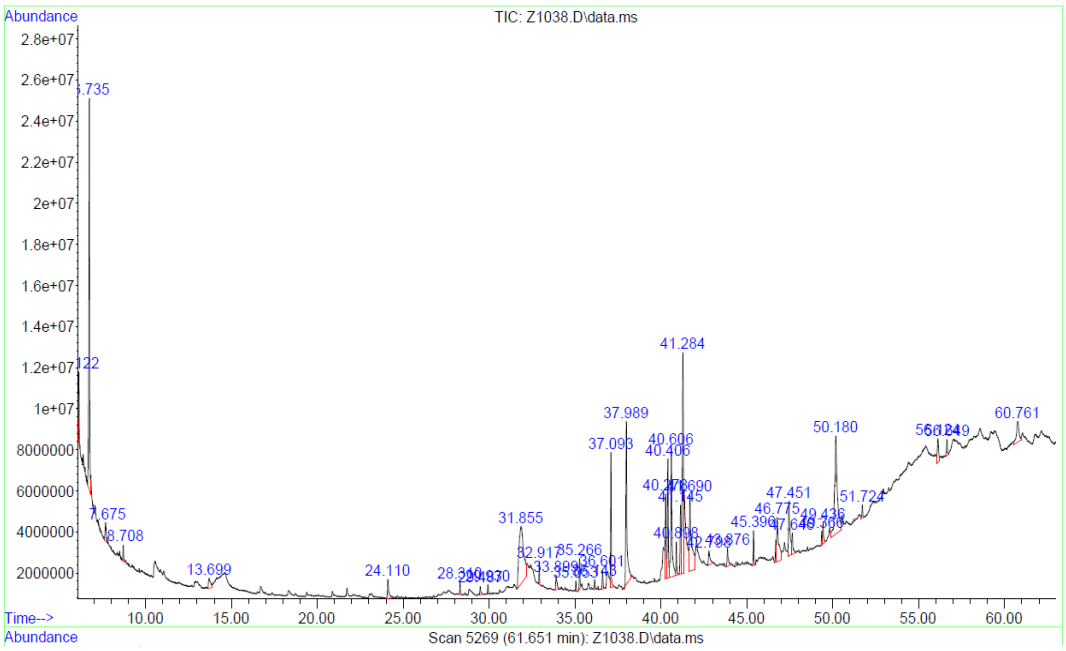

Figure 5.3: GC-MS chromatogram of methanolic leaf extract of *K. caryophyllata*

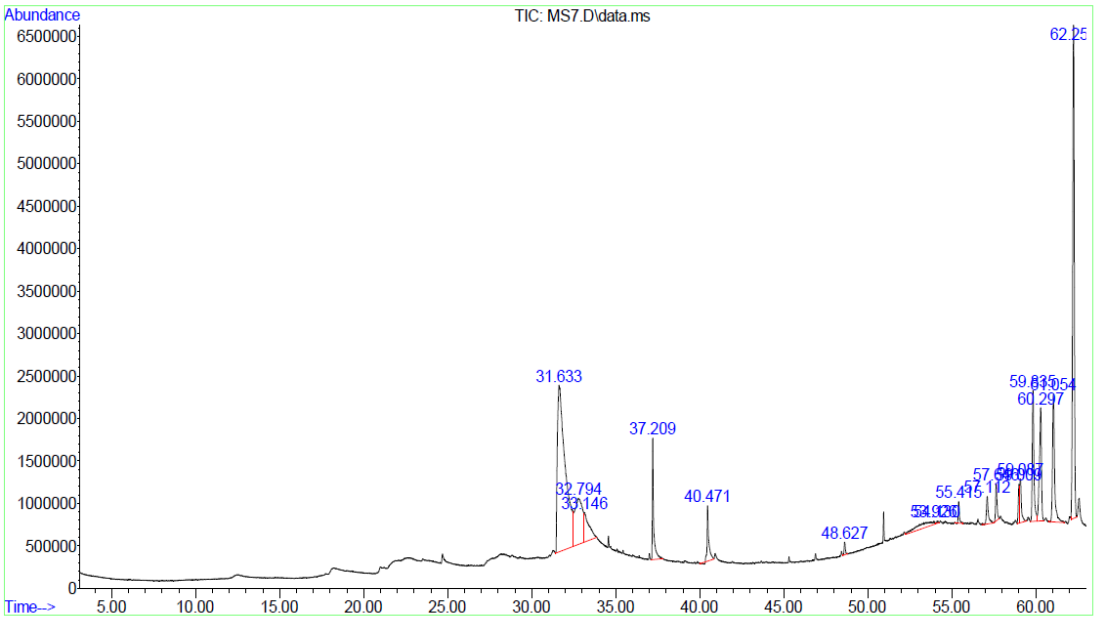

Figure 5.4: GC-MS chromatogram of methanolic stem extract of *K. caryophyllata*

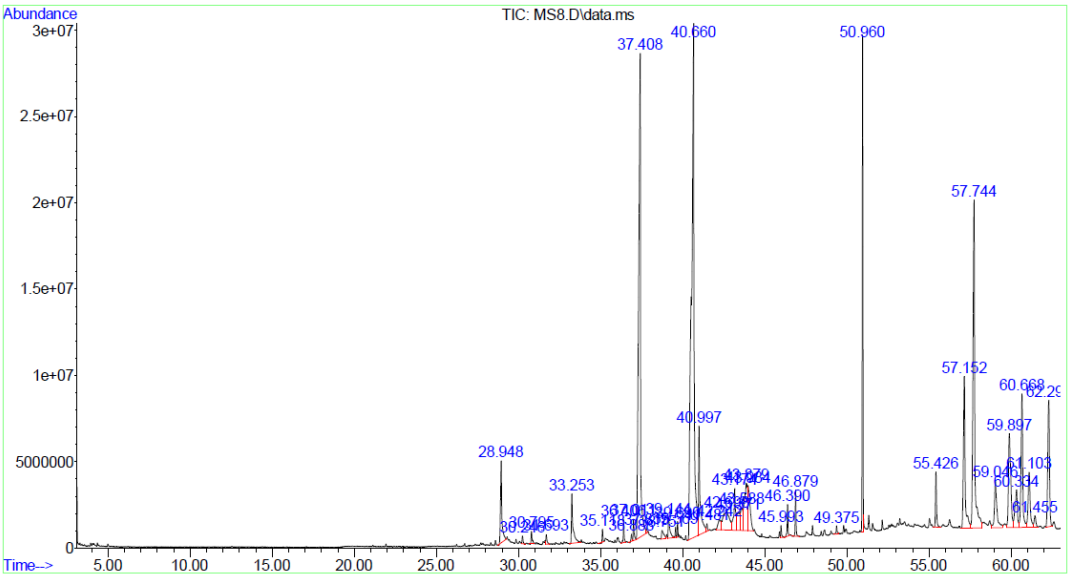

Figure 5.5: GC-MS chromatogram of methanolic root extract of *K. caryophyllata*

The details of compounds identified such as peak number, name of compound, retention time (RT), percent peak area, molecular formula and molecular weight are shown in the table 5.4, table 5.5 and table 5.6 respectively for the leaf, stem and root methanolic extracts.

Table 5.4: Details of bioactive compounds identified in the methanolic leaf extract

Peak	Retention Time (min)	Compound name	Molecular formula	Molecular weight (g/mol)	Peak area%
1	6.122	Benzene, [(methyl sulfinyl)methyl]-	$C_8H_{10}OS$	154.23	1.504
2	7.675	Propanoic acid, 2-oxo-, methyl ester	$C_4H_6O_3$	102.09	0.746
3	8.708	2-Pentanone, 4-hydroxy-4-methyl-	$C_6H_{12}O_2$	116.16	0.420
4	13.699	2-Hydroxy-gamma-butyrolactone	$C_4H_6O_3$	102.09	0.753
5	24.110	Eugenol	$C_{10}H_{12}O_2$	164.201	1.027
6	28.310	Dodecanoic acid, methyl ester	$C_{13}H_{26}O_2$	214.349	0.293
7	29.487	Dodecanoic acid	$C_{12}H_{24}O_2$	200.322	0.318
8	32.917	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242.397	0.500
9	33.899	Tetradecanoic acid	$C_{14}H_{28}O_2$	228.371	0.685
10	35.053	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	256.424	0.271
11	35.266	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296.539	0.820
12	36.601	9-Hexadecenoic acid, methyl ester, (Z)-	$C_{17}H_{32}O_2$	268.435	0.615
13	37.093	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.45	3.477
14	37.989	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.539	7.284

15	40.278	9,12-Octadecadienoic acid (Z, Z)-, methyl ester	$C_{19}H_{34}O_2$	294.479	3.012
16	40.406	trans-13-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296.495	3.957
17	40.606	Phytol	$C_{20}H_{40}O$	296.539	5.818
18	40.898	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298.504	0.953
19	41.145	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280.445	2.807
20	41.284	cis-Vaccenic acid	$C_{18}H_{34}O_2$	282.468	9.873
21	41.690	Octadecanoic acid	$C_{18}H_{36}O_2$	284.477	5.983
22	43.876	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	$C_{35}H_{68}O_5$	568.924	0.813
23	45.396	Hexanedioic acid, bis(2-ethylhexyl) ester	$C_{22}H_{42}O_4$	370.574	0.900
24	46.775	9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{21}H_{40}O_4$	356.547	3.346
25	47.451	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	330.509	3.017
26	49.366	9-Octadecenoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester	$C_{27}H_{56}O_4Si_2$	500.902	0.344
27	49.436	Heptanoic acid, docosyl ester	$C_{29}H_{58}O_2$	438.789	0.730
28	51.724	Squalene	$C_{30}H_{50}$	410.739	1.431
29	56.124	Tetratetracontane	$C_{44}H_{90}$	619.184	1.298
30	56.649	dl-α-Tocopherol	$C_{29}H_{50}O_2$	430.717	0.623
31	60.761	γ-Sitosterol	$C_{29}H_{50}O$	414.707	2.036

Table 5.5: Details of bioactive compounds identified in the methanolic stem extract

Peak	Retention Time (min)	Compound name	Molecular formula	Molecular weight (g/mol)	Peak area%
1	31.63	3-O-Methyl-d-glucose	$C_7H_{14}O_6$	194.18	33.505
2	37.21	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.43	3.766
3	40.47	trans-13-Octadecenoic acid	$C_{18}H_{34}O_2$	296.495	2.212
4	48.62	Squalene	$C_{30}H_{50}$	410.73	0.385
5	55.42	dl-α-Tocopherol	$C_{32}H_{52}O_2$	430.00	0.628
6	57.11	Campesterol	$C_{28}H_{48}O$	400.691	1.363
7	57.64	Stigmasterol	$C_{29}H_{48}O$	412.702	1.333
8	59.01	γ-Sitosterol	$C_{29}H_{50}O$	414.718	1.164
9	59.84	β-Amyrin	$C_{30}H_{50}O$	426.72	5.638
10	60.29	Lup-20(29)-en-3-one	$C_{30}H_{48}O$	424.7015	5.956
11	61.05	Lupeol	$C_{30}H_{50}O$	426.729	6.704
12	62.25	12-Oleanen-3-yl acetate, (3α)-	$C_{32}H_{52}O_2$	468.766	20.732

Table 5.6: Details of bioactive compounds identified in the methanolic root extract

Peak	Retention Time (min)	Compound name	Molecular formula	Molecular weight (g/mol)	Peak area%
1	28.95	Dodecanoic acid	$C_{12}H_{24}O_2$	200.322	1.910
2	30.24	Globulol	$C_{15}H_{26}O$	222.372	0.133
3	30.79	2-Naphthalene methanol, decahydro-α,α,4a-trimethyl-8-methylene-, [2R-(2α,4aα,8aβ)]-	$C_{15}H_{26}O$	222.3663	0.221
4	33.25	Tetradecanoic acid	$C_{14}H_{28}O_2$	228.379	1.317
5	36.41	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.457	0.322
6	36.88	6-(p-Tolyl)-2-methyl-2-heptenol	$C_{15}H_{22}O$	218.34	0.092
7	37.41	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.43	15.889
8	38.75	cis-10-Heptadecenoic acid	$C_{17}H_{32}O_2$	268.441	0.330
9	39.57	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294.479	0.118
10	39.69	C9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296.4879	0.186
11	40.66	cis-13-Octadecenoic acid	$C_{18}H_{34}O_2$	282.468	23.527
12	40.99	Octadecanoic acid	$C_{18}H_{36}O_2$	284.477	2.992
13	42.24	Urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)-	$C_{31}H_{48}O_3$	468.722	2.103
14	43.98	Lup-20(29)-en-3-ol, acetate, (3β)-	$C_{30}H_{48}O$	424.7015	1.038
15	50.96	Squalene	$C_{30}H_{50}$	410.73	6.044
16	55.47	Tocopherol (Vitamin E)	$C_{29}H_{50}O_2$	430.00	1.027
17	57.15	Campesterol	$C_{28}H_{48}O$	400.691	4.638
18	57.74	Stigmasterol	$C_{29}H_{48}O$	412.702	10.415
19	59.04	γ-Sitosterol	$C_{29}H_{50}O$	414.718	2.022
20	59.89	β-Amyrin	$C_{32}H_{52}O_2$	468.766	3.812
21	60.33	Lup-20(29)-en-3-one	$C_{30}H_{48}O$	424.713	1.569
22	60.67	4,22-Stigmastadiene-3-one	$C_{29}H_{46}O$	410.686	3.995
23	61.10	Lupeol	$C_{30}H_{50}O$	426.729	2.029
24	62.29	12-Oleanen-3-yl acetate, (3α)-	$C_{32}H_{52}O_2$	468.766	3.930

The study analysed the biological activities of phytocompounds detected in the GC-MS chromatogram with the help of bioactivity sources like Dr.Duke's Phytochemical and Ethnobotanical Databases and other scientific literatures. The nature of compounds and bioactivity details of phytocompounds identified in the methanolic extracts of leaf (table 5.7), stem (table 5.8) and root (table 5.9) are summarised below:

Table 5.7: Nature and biological activity of compounds detected in the leaf methanolic extract

Peak	Name of compound	Nature of compound	Bioactivity**
1	Benzene, [(methylsulfinyl)methyl]-	Phenolic compound	Antiallergic, antiparasitic, antibacterial, antiasthmatic.(Dr.Duke's phytochemical and Ethnobotanical Database)
2	Propanoic acid, 2-oxo-, methyl ester	Pyruvic acid methyl ester	Acidifier, acidulant, inhibit production of uric acid (Dr.Duke's Phytochemical and Ethnobotanical Database)
3	2-Pentanone, 4-hydroxy-4-methyl-	Beta-hydroxy ketone	Inhibitor of 17-beta-hydroxysteroid dehydrogenase, inducer of Testosterone-Hydroxylase (Dr.Duke's Phytochemical and Ethnobotanical Database)
4	2-Hydroxy-gamma-butyrolactone	Carbonyl compounds	analgesic, antibacterial and anti-diabetic (Moorthy & Boominathan, 2011)
5	Eugenol	Terpene-phenylpropanoids	Antioxidative property, sexual attractants (Gupta et al., 2002; Silva et al., 2003).
6	Dodecanoic acid, methyl ester	Fatty acid ester	Antioxidant activity (Lalitharani, 2009)
7	Dodecanoic acid	Saturated fatty acid	Antioxidant activity (Lalitharani, 2009)
8	Methyl tetradecanoate	Fatty acid ester	Antioxidant, cancer-preventive, hypercholesterolemic, lubricant, nematicide (Ravikumar et al., 2012)
9	Tetradecanoic acid	Saturated fatty acid	Antioxidant activity, anticancer, hypocholesterolemic, nematicide (Lalitharani, 2009; Santhosh et al., 2014)
10	Pentadecanoic acid, methyl ester	Fatty acid ester	Antimicrobial, antifungal (Belakhdar et al., 2015)
11	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Fatty acid	Antimicrobial, anti-inflammatory (Lalitha et al., 2014)
12	9-Hexadecenoic acid, methyl ester, (Z)-	Palmitic acid ester	Antibacterial and antifungal (Dr. Duke's Phytochemical and Ethnobotanical Database)
13	Hexadecanoic acid, methyl ester	Palmitic acid Ester	Antioxidant, hypercholesterolemic, antiandrogenic, flavor, nematicide (Lalitha et al., 2014)
14	n-Hexadecenoic acid	Palmitic acid	Antioxidant activity, nematicide, hypocholesterolemic, antiandrogenic, Hemolytic (Lalitharani, 2009; Santhosh et al., 2014)
15	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	Fatty acid ester	Anti-cancer (Yu et al., 2005)
16	Trans-13-Octadecenoic acid, methyl ester	Fatty acid ester	Anti-inflammatory, antileukotriene-D4, hypocholesterolemic, cancer

			preventive (Krishnamoorthy & Subramaniam, 2014)
17	Phytol	Acyclic diterpene	Antimicrobial, anti-inflammatory, diuretic, anticancer (Lalitha et al., 2014; Santhosh et al., 2014)
18	Octadecanoic acid, methyl ester	Fatty acid ester	Antimicrobial (Belakhdar et al., 2015)
19	9,12-Octadecadienoic acid (Z,Z)-	Fatty acid	Anti-inflammatory and antiarthritic (Jones, 2002)
20	Cis-Vaccenic acid	Omega-7fatty acid	Antivirus substance and inactivation of T5 phage (Hirotani et al., 1991), Anti-inflammatory (Haider et al., 2016)
21	Octadecanoic acid	Saturated fatty acid	Antimicrobial activity (Rahuman, 2000)
22	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	Fatty acid ester	Antioxidant, hypocholesterolemic, antiandrogenic, hemolytic, Alpha reductase inhibitor (Markkas & Madhuramozhi, 2015)
23	Hexanedioic acid, bis(2-ethylhexyl) ester	Fatty acid ester	Acidifier, acidulant, antiuric acid production (Dr. Duke's Phytochemical and Ethnobotanical Database)
24	9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	Fatty acid ester	Inhibition of proliferative effect in keloid fibroblast(Godswill et al.,2014)
25	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	Fatty acid ester	Hemolytic, pesticide, antioxidant (Gnanavel & Mary Saral, 2013).
26	9-Octadecenoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester	Oleic acid propyl ester	Urine acidifier, urinary acidulant, antidote, anti-HIV integrase, improve cerebral hypoxia (Dr. Duke's Phytochemical and Ethnobotanical Database)
27	Heptanoic acid, docosyl ester	Acid methyl ester	Acidifier, acidulant, arachidonic acid inhibitor, inhibit production of uric acid (Dr. Duke's Phytochemical and Ethnobotanical Database)
28	Squalene	Triterpene	Antitumors, cancer prevention, Antimicrobial, anti-inflammatory, antioxidant, pesticide, immunostimulants, chemopreventive (Santhosh et al., 2014; Huang et al., 2009; Cardino et al., 2015)
29	Tetratetracontane	Long chain alkane	Hypoglycaemic, antioxidant activities (Sivakumar & Gayathri, 2015)
30	dl-α-Tocopherol	Fat-soluble vitamin	Analgesic, antidiabetic, antitumor, antiinflammatory, antioxidant, antidermatitic, antiaging, anticancer, antileukemic, hypocholesterolemic (Rajalakshmi & Mohan,2016)
31	γ-Sitosterol	Terpenoids	Anti-inflammatory effect (Dr. Duke's Phytochemical and Ethnobotanical Database)

**Bioactivity source: Dr.Duke's Phytochemical and Ethnobotanical Databases

Table 5.8: Nature and biological activity of compounds detected in the stem methanolic extract

Peak No.	Compound name	Nature of compound	Bioactivity**
1	3-O-Methyl-d-glucose	Sugar derivative	Preservative
2	n-Hexadecanoic acid	Palmitic acid	Antioxidant activity, nematicide, hypocholesterolemic, antiandrogenic, Hemolytic (Lalitharani, 2009; Santhosh et al., 2014)
3	Trans-13-Octadecenoic acid	Fatty acid ester	Anti-inflammatory, antileukotriene-D4, hypocholesterolemic, cancer preventive (Krishnamoorthy & Subramaniam, 2014)
4	Squalane	Triterpene	Antitumor, cancer preventive, Antimicrobial, anti-inflammatory, antioxidant, pesticide, immunostimulant, chemo preventive (Santhosh et al., 2014; Huang et al., 2009; Cardino et al., 2015)
5	dl-α-Tocopherol	Fat soluble vitamin	Analgesic, antidiabetic, antitumor, anti-inflammatory, antioxidant, antidermatitic, antiaging, anticancer, antileukemic, hypocholesterolaemia (Rajalakshmi & Mohan,2016)
6	Campesterol	Sterol 3-beta-D-glucoside	Anti-inflammatory, antidiabetic, and anticancer activities(Miras-Moreno et al., 2016)
7	Stigmasterol	Triterpene	Anti-inflammatory, antidiabetic, and anticancer activities(Miras-Moreno et al., 2016)
8	γ-Sitosterol	Pentacyclic triterpenoids	Anti-inflammatory effect (Dr. Duke's Phytochemical and Ethnobotanical Database)
9	β-Amyrin	Triterpene	Antibacterial and antifungal (Kwun et al.,2021)
10	Lup-20(29)-en-3-one	Triterpene	Antiproliferative activity(Boryczka et al., 2013)
11	Lupeol	Triterpene	Anti-inflammatory, anti-virus, anti-diabetes, anti-cancer
12	12-Oleanen-3-yl acetate, (3α)-	Triterpene	Anti-inflammatory, antimicrobial.(Dutta et al., 2021)

**Bioactivity source: Dr.Duke's Phytochemical and Ethnobotanical Databases.

Table 5.9: Nature and biological activity of compounds detected in the root methanolic extract

Peak No.	Compound name	Nature of compound	Bioactivity**
1	Dodecanoic acid	Saturated fatty acid	Antioxidant activity (Lalitharani, 2009)
2	Globulol	Sesquiterpene	Antimicrobial activity (https://pubchem.ncbi.nlm.nih.gov/compound/ 12304985)
3	2-Naphthalene methanol, decahydro-α,α,4a-trimethyl-8-methylene-, [2R-(2α,4aα,8aβ)]-	Sesquiterpene	Insecticidal activity
4	Tetradecanoic acid	Saturated fatty acid	Antioxidant activity, anticancer, hypocholesterolaemic, nematicide (Lalitharani, 2009; Santhosh et al., 2014)
5	Hexadecanoic acid, methyl ester	Palmitic acid Ester	Antioxidant, hypocholesterolenic, antiandrogenic, flavor, nematicide (Lalitha et al., 2014)
6	6-(p-Tolyl)-2-methyl-2-heptenol	Sesquiterpene	Not reported
7	n-Hexadecanoic acid	Palmitic acid	Antioxidant activity, nematicide, hypocholesterolaemic, antiandrogenic, Hemolytic (Lalitharani, 2009; Santhosh et al., 2014)
8	cis-10-Heptadecenoic acid	Unsaturated fatty acids	Anti-inflammatory, antimicrobial (Dr. Duke's Phytochemical and Ethnobotanical Database)
9	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	Fatty acid ester	Anti-cancer (Yu et al., 2005)
10	C9-Octadecenoic acid (Z)-, methyl ester	Fatty acid ester	Antioxidant, antimicrobial (Dr. Duke's Phytochemical and Ethnobotanical Database)
11	cis-13-Octadecenoic acid	Fatty acid	Acidifier, acidulant (Dr. Duke's Phytochemical and Ethnobotanical Database)
12	Octadecanoic acid	Saturated fatty acid	Antimicrobial activity (Rahuman, 2000)
13	Urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)-	Triterpene	Anti-inflammatory, antioxidant, antimicrobial, anticancer, and hepatoprotective (Oh et al., 2021)
14	Lup-20(29)-en-3-ol, acetate, (3β)-	Triterpene	Antiproliferative activity(Boryczka et al., 2013)
15	Squalene	Triterpene	Antitumor, Antimicrobial, anti-inflammatory, antioxidant, cancer preventive, pesticide, immunostimulant, chemo preventive (Santhosh et al., 2014; Huang et al., 2009; Cardino et al., 2015)

16	dl-α-Tocopherol	Fat soluble vitamin	Analgesic, antidiabetic, antitumor, anti-inflammatory, antioxidant, antidermatitic, antiaging, anticancer, antileukemic, hypocholesterolaemic (Rajalakshmi & Mohan,2016)
17	Campesterol	Phytosterols	Anti-inflammatory, antidiabetic, and anticancer activities (https://pubchem.ncbi.nlm.nih.gov/compound/Campesterol)
18	Stigmasterol	Triterpene	Anti-inflammatory, antidiabetic, and anticancer activities(Miras-Moreno et al., 2016)
19	γ-Sitosterol	Pentacyclic triterpenoids	Anti-inflammatory effect (Dr. Duke's Phytochemical and Ethnobotanical Database)
20	β-Amyrin	Triterpene	Antibacterial and antifungal (Kwun et al., 2021)
21	Lup-20(29)-en-3-one	Triterpene	Antiproliferative activity(Boryczka et al., 2013)
22	4,22-Stigmastadiene-3-one	Steroid	Anti-inflammatory, antioxidant, anticancer, or antiviral activities(Miras-Moreno et al., 2016)
23	Lupeol	Triterpene	Anti-inflammatory, anti-virus, anti-diabetes, anti-cancer(Xu et al., 2018)
24	12-Oleanen-3-yl acetate, (3α)-	Triterpene	Anti-inflammatory, antimicrobial.(Dutta et al., 2021)

**Bioactivity source: Dr.Duke's Phytochemical and Ethnobotanical Databases.

The spectral properties and structural details of major bioactive compounds detected in the GC-MS analysis of methanolic extract of different components in *K. caryophyllata* are shown in the figures 5.6 (leaf component), 5.7 (stem component and 5.8 (root component).

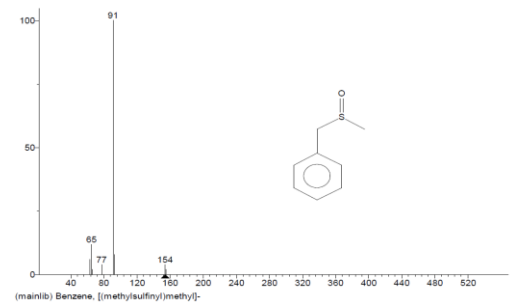

Fig. 5.6.a: Benzene, [(methylsulfinyl)methyl]- with RT value 6.12

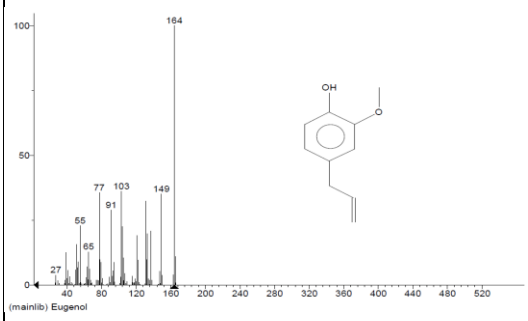

Fig. 5.6.b: Eugenol with RT value 24.110

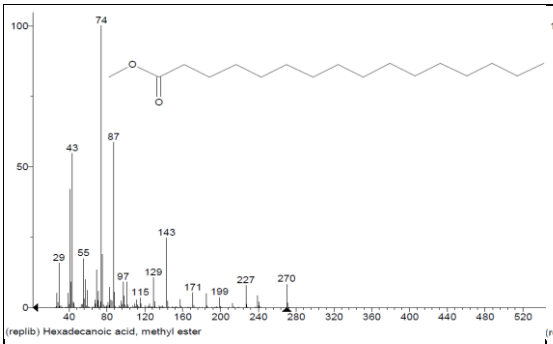

Fig. 5.6. c: Hexadecanoic acid, methyl ester with RT value 37.093

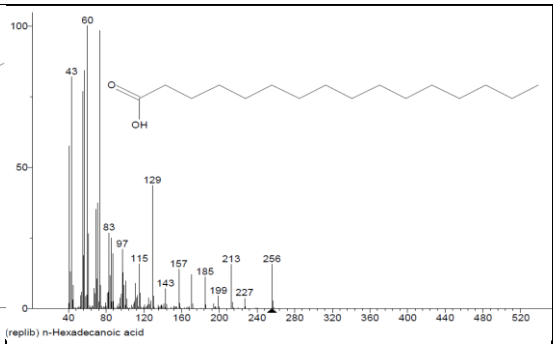

Fig. 5.6.d: n-Hexadecanoic acid with RT value 37.989

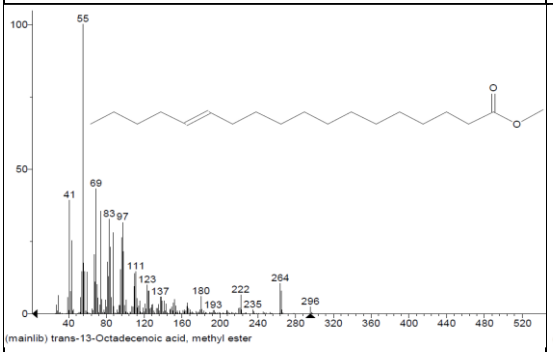

Fig. 5.6.e: trans-13-Octadecenoic acid, methyl ester with RT value 40.406

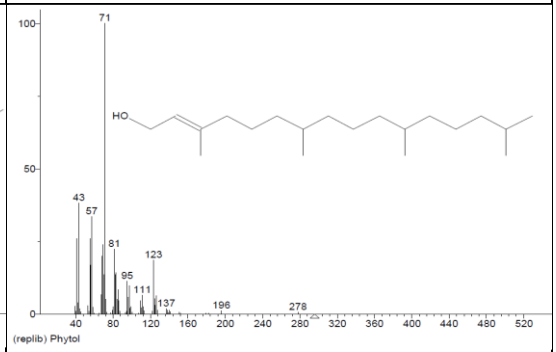

Fig. 5.6.f: Phytol with RT value 40.606

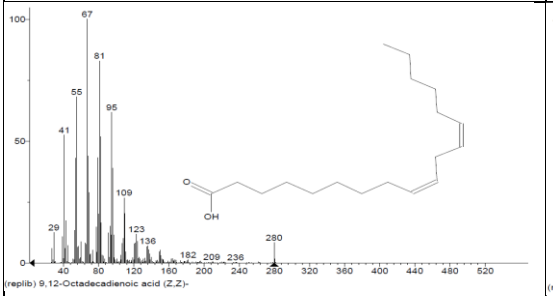

Fig. 5.6.g: 9,12-Octadecadienoic acid (Z,Z)- with RT value 41.145

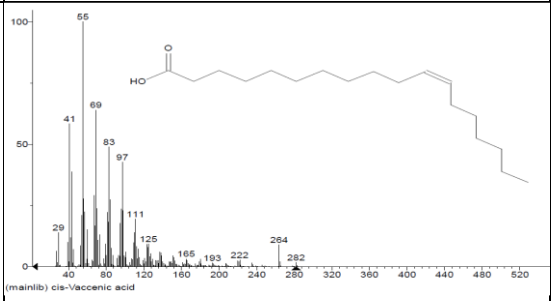

Fig. 5.6.h: cis-Vaccenic acid with RT value 41.284

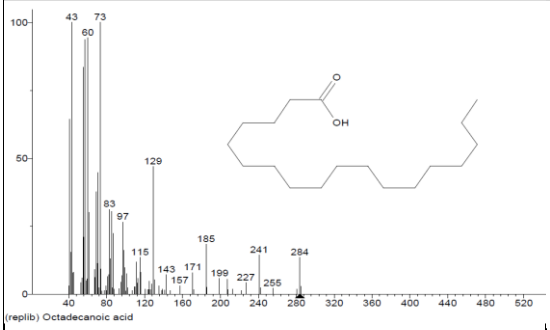

Fig. 5.6.i: Octadecanoic acid with RT value 41.690

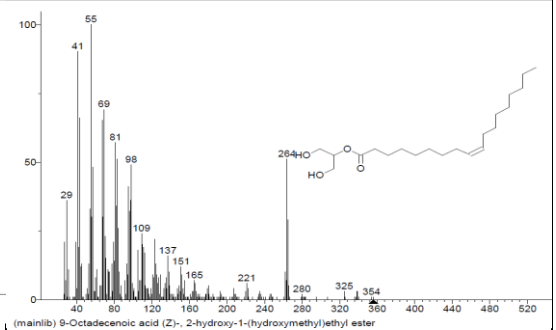

Fig. 5.6.j: 9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester with RT value 46.775

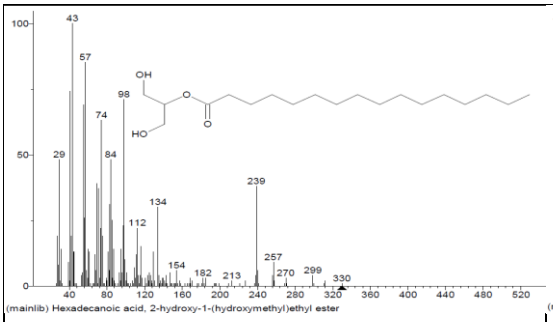

Fig. 5.6.k: Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester with RT value 47.451

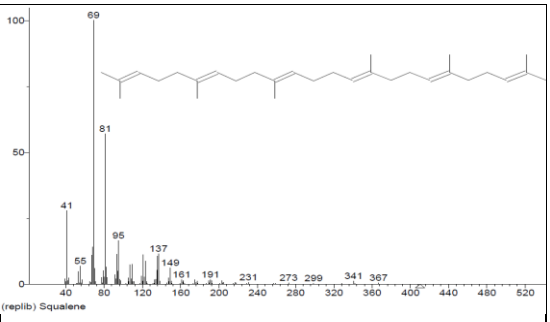

Fig. 5.6.l: Squalene with RT value 51.724

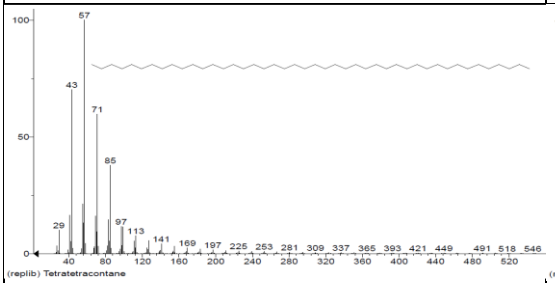

Fig. 5.6.m: Tetratetracontane with RT value 56.124

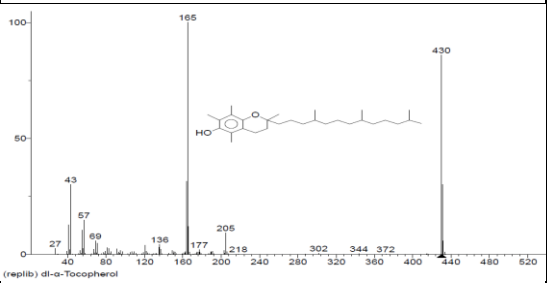

Fig. 5.6. n: dl-α-Tocopherol with RT value 56.649

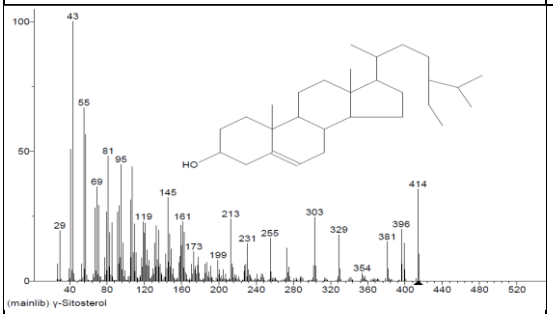

Fig. 5.6. o: γ-Sitosterol with RT value 60.761

Figure 5.6: Spectral properties and structure of major bioactive compounds detected in the GC-MS analysis of *K. caryophyllata* methanolic leaf extract

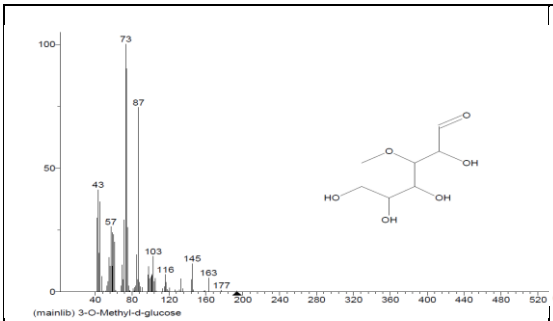

Fig. 5.7.a: 3-0-methyl glucose with RT value 31.63

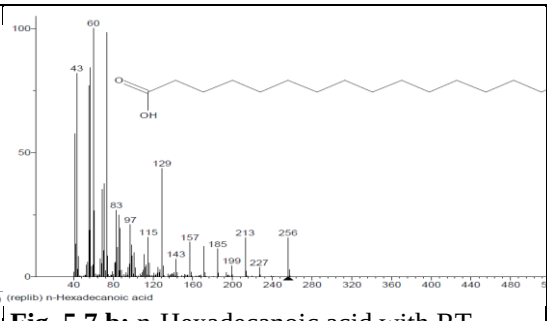

Fig. 5.7.b: n-Hexadecanoic acid with RT value 37.21

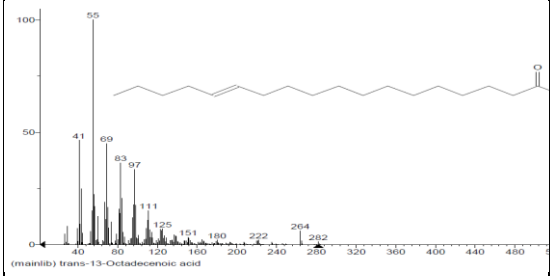

Fig. 5.7.c: Trans-13Octadecenoic acid with RT value 40.47

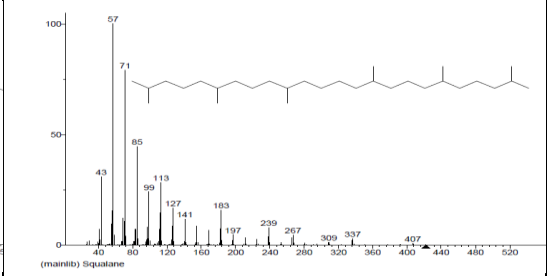

Fig. 5.7.d: squalene with RT value 48.62

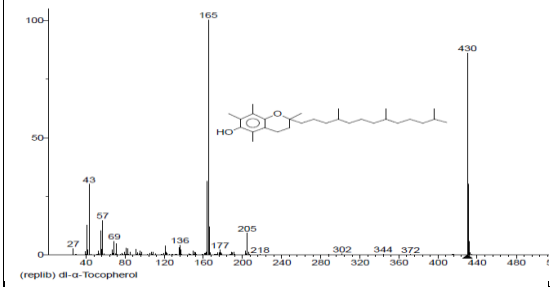

Fig. 5.7.e: dl-α tocopherol with RT value 55.42

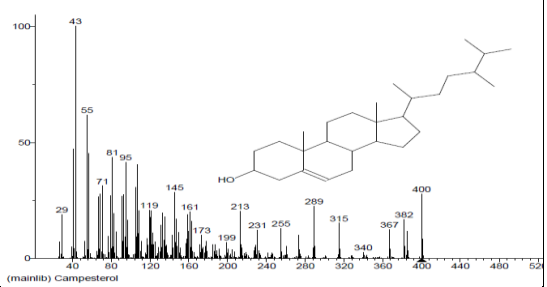

Fig. 5.7.f: campestrol with RT value 57.11

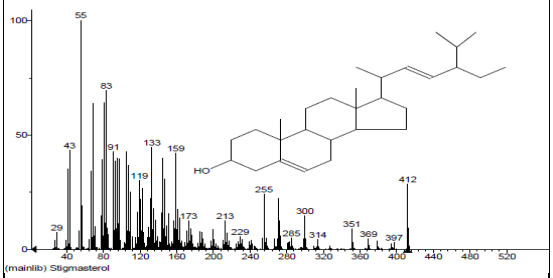

Fig. 5.7.g: stigmasterol with RT value 57.64

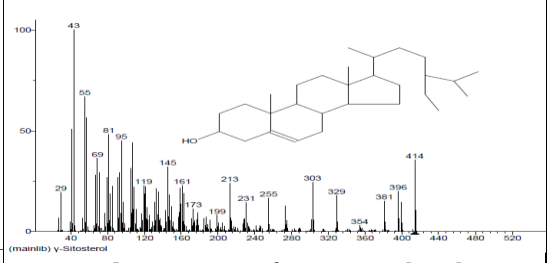

Fig. 5.7.h: Structure of γ-Sitosterol with RT value 59.01

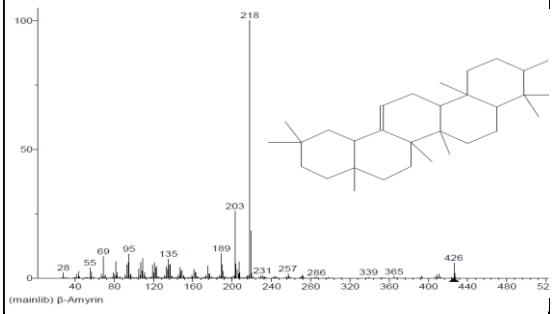

Fig. 5.7.i: β-Amyrin with RT value 59.84

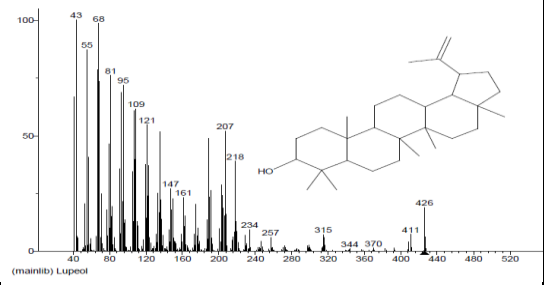

Fig. 5.7.j: Lup-20(29)-en-3-onewith RT value 60.29

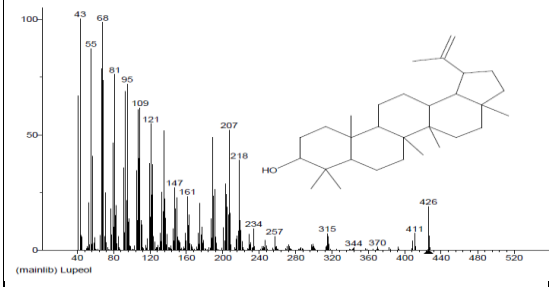

Fig. 5.7.k: Lupeol with RT value 61.05

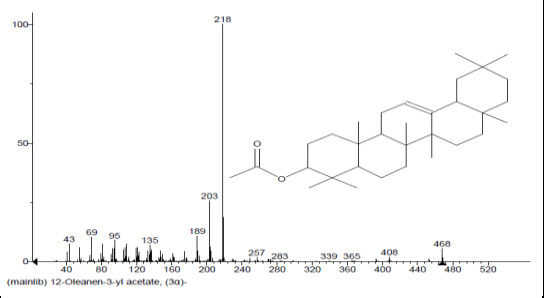

Fig. 5.7.l: 12-Oleanen-3-yl acetate, (3α)-with RT value 62.25

Figure 5.7: Spectral properties and structure of major bioactive compounds detected in the GC-MS analysis of *K. caryophyllata* methanolic stem extract

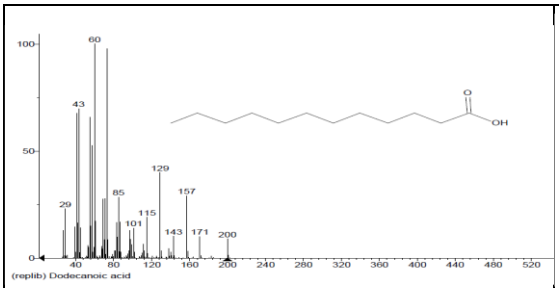

Fig. 5.8.a: Dodecanoic acid with RT value 28.95

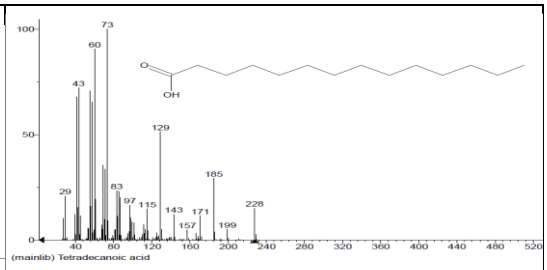

Fig. 5.8.b: Tetradecanoic acid with RT value 33.25

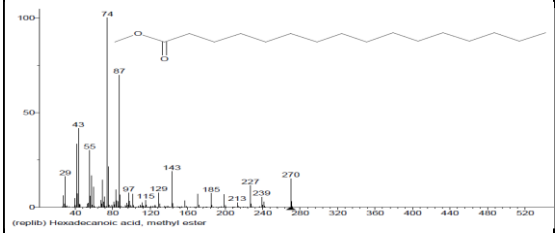

Fig. 5.8.c: Hexadecanoic acid,methyl ester with RT value 36.41

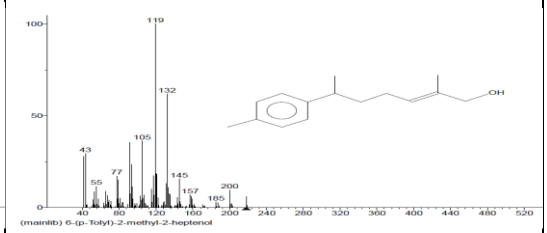

Fig. 5.8.d: Hexadecanoic acid, methyl ester with RT value 36.88

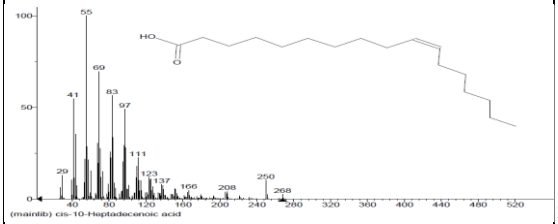

Fig. 5.8.e: n-Hexadecanoic acid with RT valu e 38.74

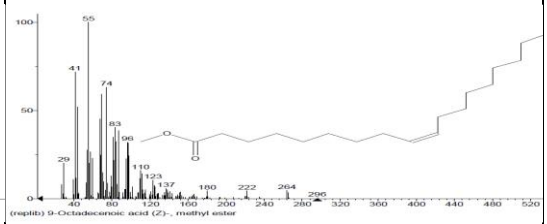

Fig. 5.8.f: C9-Octadecenoic acid (Z)-, methyl ester with RT value 39.69

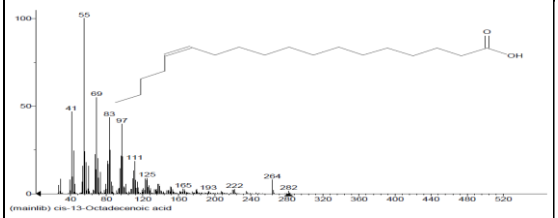

Fig. 5.8.g: Cis-13 Octadecenoic acid with RT value 40.66

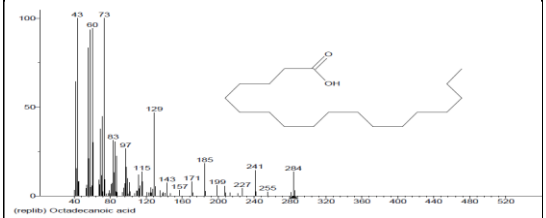

Fig. 5.8.h: Octadecenoic acid with RT value 40.99

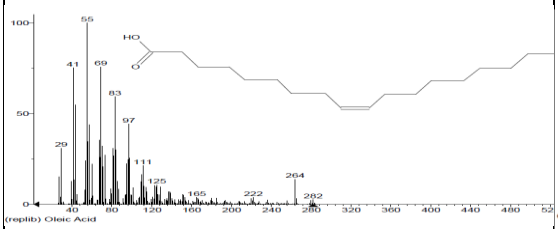

Fig. 5.8.i: Oleic acid with RT value 41.48

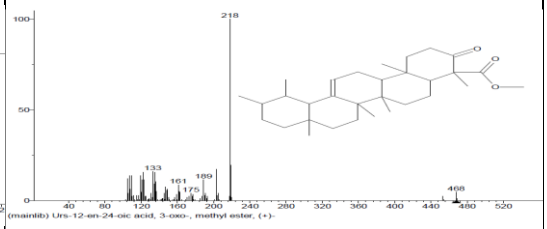

Fig. 5.8.j: Urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)- with RT value 42.24

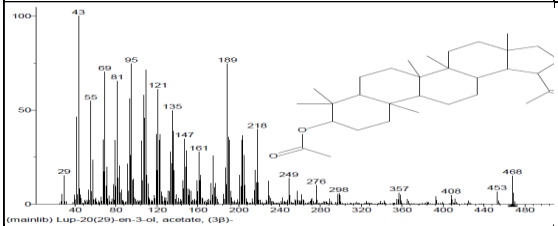

Fig. 5.8.k: Lup-20(29)-en-3-ol, acetate, (3β)- with RT value 43.98

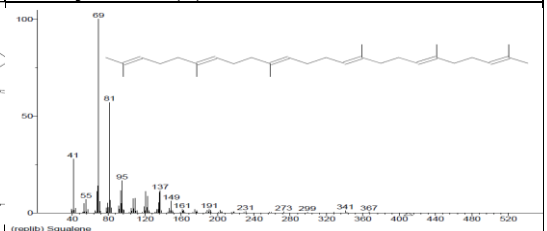

Fig. 5.8.l: Squalene with RT value 31.63

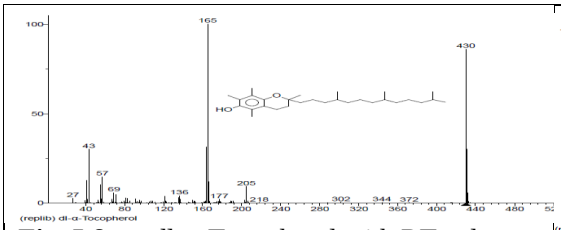

Fig. 5.8.m: dl-α-Tocopherol with RT value 50.96

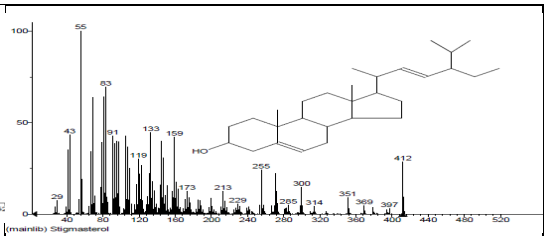

Fig. 5.8.n: stigmasterol with RT value 55.9

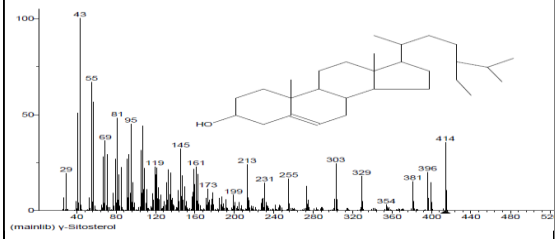

Fig. 5.8.o: γ-sitosterol with RT value 59.04

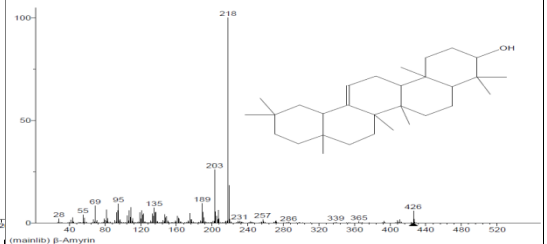

Fig. 5.8.p: β-Amyrin with RT value 59.89

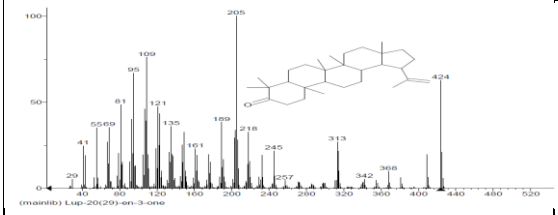

Fig. 5.8.q: Lup-(20(29)-en-3- one with RT value 60.33

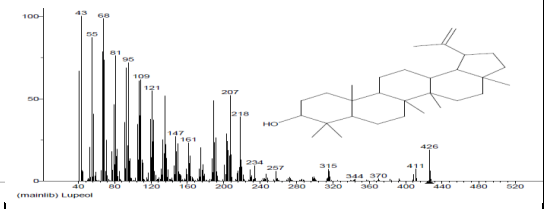

Fig. 5.8.r: Lupeol one with RT value 61.10

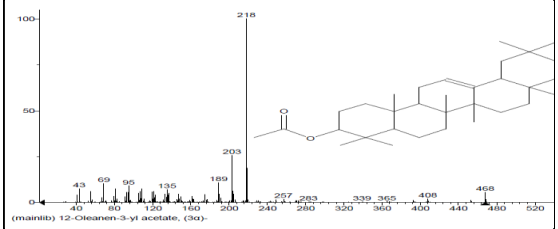

Fig. 5.8.s: 12-Oleanen-3-yl acetate, (3α)- with RT value 62.90

Figure 5.8: Spectral properties and structure of major bioactive compounds detected in the GC-MS analysis of *K. caryophyllata* methanolic root extract

HR LC-MS analysis of methanolic extracts of plant components

HR LC-MS analysis of methanolic extracts of leaf, stem and root in *K. caryophyllata* was performed preferably targeted for non-volatile compounds. Both positive and negative ionisation modes were used in HR LC-MS analysis to obtain a thorough profile of the compounds in the component extracts. These modes are based on how compounds ionise differently, offering unique but complementary data.

Methanolic leaf extracts

The HR LC-MS chromatogram profile obtained from the methanolic extracts of leaf identified 12 and 26 bioactive compounds with known medicinal properties

respectively in the positive and negative chromatogram (figure 5.9 & 5.10). The details of compounds identified such as name of compound, Retention Time, chemical formula, mass, mass accuracy and DB(decibel) difference are shown in table 5.10 (Positive chromatogram) and table 5. 11 (Negative chromatogram). The nature and bioactivity of compounds detected from both positve and negative chromatogram are depicted in the table 5.12. The details of spectral properties and structure of major bioactive compounds detected in the chromatograms are shown in figure 5.11.

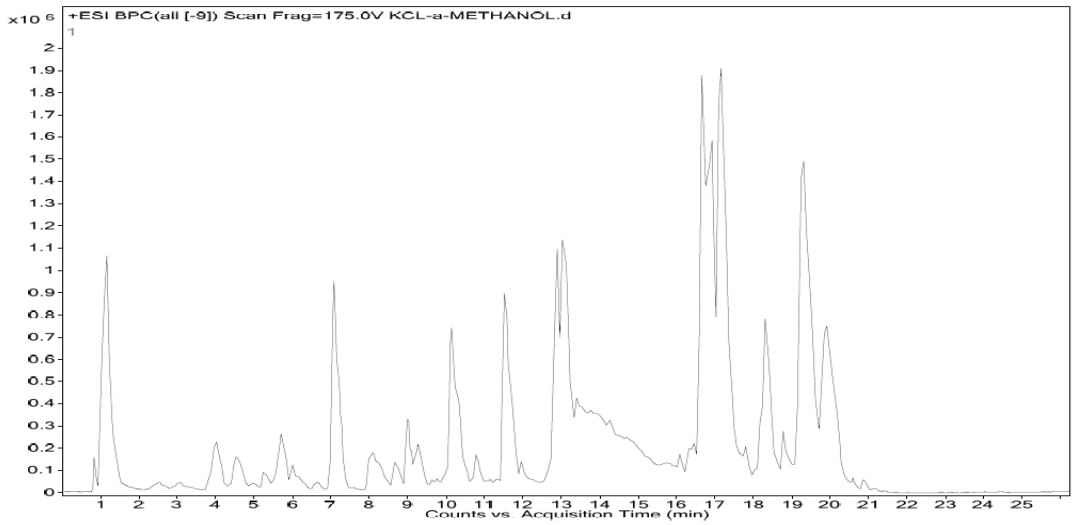

Figure 5. 9: Positive HR LC-MS chromatogram of methanolic leaf extracts of *K. caryophyllata*.

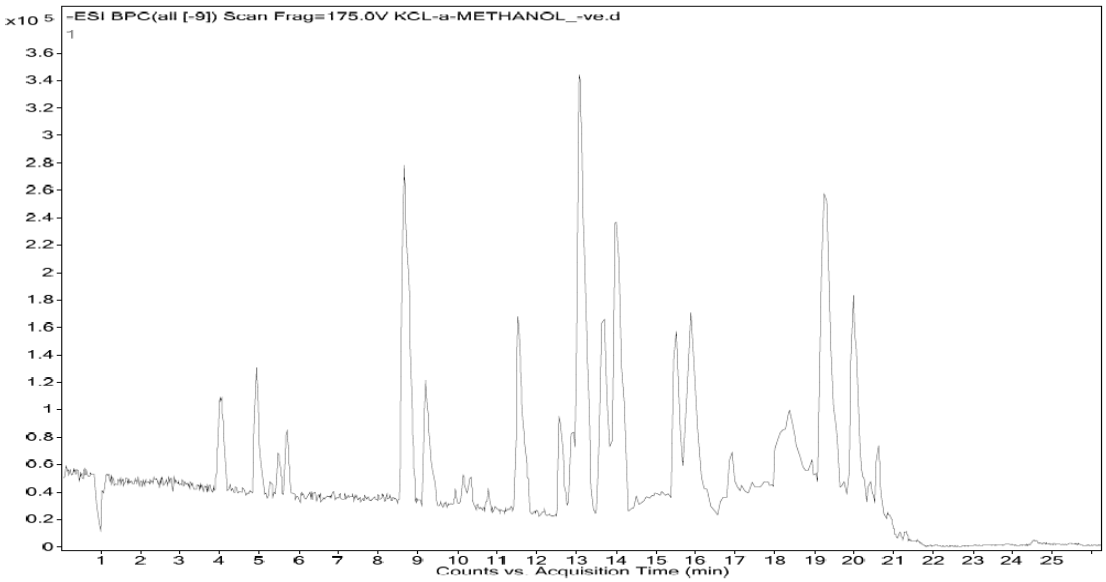

Figure 5. 10: Negative HR LC-MS chromatogram of methanolic leaf extracts of *K. caryophyllata*.

Table 5.10: Details of phytocompounds identified in the LC-MS analysis of methanolic leaf extract (Positive chromatogram)

Sl. No	Compound Name	Chemical Formula	Mass (m/z)	Retention Time (RT)	Mass Accuracy (ppm)	DB Difference (PPM)
1	3beta,6betaDihydroxy Nortropane	$C_7 H_{13} N O_2$	144.103	1.111	143.0961	-10.6
2	Umbelliferone	$C_9 H_6 O_3$	163.0405	3.801	162.0334	-10.24
3	Triprolidine	$C_{19} H_{22} N_2$	279.1876	4.824	278.1808	-8.86
4	(+)-Myrtenyl formate	$C_{11} H_{16} O_2$	181.124	9.341	180.1169	-10.25
5	Picrasin C	$C_{23} H_{34} O_7$	445.2151	11.51	422.2258	10.86
6	Panaquinquecol 1	$C_{18} H_{28} O_3$	293.2135	13.346	292.2062	-8.16
7	Deinoxanthin	$C_{40} H_{54} O_3$	583.4182	16.334	582.411	-6.4
8	Harderoporphyrin	$C_{35} H_{36} N_4 O_6$	609.2743	16.625	608.2673	-5.78
9	Kanokoside D	$C_{27} H_{44} O_{16}$	625.2694	16.847	624.2619	1.96
10	Stigmast-4-ene-3,6-dione	$C_{29} H_{46} O_2$	427.3598	17.582	426.3525	-6.35
11	Cucurbitachrome 1	$C_{40} H_{56} O_4$	601.4271	18.414	600.4205	-4.37
12	3-cis-Hydroxy-b,e, Caroten-3'-one	$C_{40} H_{54} O$	551.4281	19.579	550.4209	-6.28

Table 5.11: Details of phytocompounds identified in the LC-MS analysis of methanolic leaf extract (Negative chromatogram)

Sl. No	Compound Name	Chemical Formula	Mass (m/z)	Retention Time (RT)	Mass Accuracy (ppm)	DB Diff (ppm)
1	Flazine	$C_{17} H_{12} N_2 O4$	353.078	3.904	308.0801	-1.11
2	3,5-Dihydroxyphenyl 1-O-(6-O-galloyl-beta-D-glucopyranoside)	$C_{19} H_{20} O_{12}$	439.0898	4.905	440.0954	-0.06
3	Physalin K	$C_{28} H_{30} O_{12}$	617.188	5.301	558.171	4.9
4	Theasinensin C	$C_{30} H_{26} O_{14}$	609.134	5.507	610.1409	-14.16
5	Fukinolic acid	$C_{20} H_{18} O_{11}$	433.068	6.076	434.0752	-6.94
6	(-)-Quebrachamine	$C_{19} H_{26} N_2$	327.211	8.633	282.2131	-12.48
7	Methanophenazine	$C_{37} H_{50} N_2 O$	328.214	9.407	538.402	-18.03
8	Convalloside	$C_{35} H_{52} O_{15}$	711.3277	12.643	712.3326	-2.84
9	9-HOTE	$C_{18} H_{30} O_3$	293.207	12.917	294.215	15.36
10	Melleolide D	$C_{24} H_{31} Cl O_8$	541.184	13.074	482.1698	1.92

11	Jamaicamide C	C_{27} H_{39} Cl N_2 O_4	549.277	13.707	490.2625	-5.51
12	13-Deoxytedanolide	C_{32} $H_{50}O_{10}$	653.355	13.994	594.3385	3.27
13	26-Glucosyl-1,3,11,22-tetrahydroxyergosta-5,24-dien-26-oate	C_{34} H_{54} O_{11}	683.365	14.261	638.364	4.04
14	Lyciumoside IX	C_{35} $H_{56}O_{15}$	715.359	14.443	716.364	-2.93
15	8-C-Ascorbylepigallocatechin 3-gallate	C_{28} H_{24} O_{17}	691.118	15.072	632.1018	-0.75
16	Vobtusine	C_{43} H_{50} N_4O_6	717.375	15.56	718.3802	-10.02
17	Calotropin	C_{29} H_{40} O_9	577.263	15.805	532.264	6.02
18	Prodelphinidin A2 3'-gallate	C_{37} H_{28} O_{18}	819.1404	15.914	760.1244	4.15
19	Grossamide	C_{36} H_{36} N_2O_8	623.2441	16.945	624.2515	-6.87
20	Magnesium protoporphyrin monomethyl ester	C_{35} H_{34} MgN_4 O_4	643.228	17.143	598.2278	25.44
21	Squalamine	C_{34} H_{65} N_3O_5S	672.462	17.746	627.4664	-3.09
22	Corotoxigenin-3-O-alpha-L-rhamnopyranoside	C_{29} H_{42} O_9	579.279	18.488	534.2807	4.04
23	Synaptolepis factor K1	C_{36} H_{54} O_8	613.3824	18.655	614.3876	-9.93
24	33-Deoxy-33-hydroperoxyfurohyperforin	C_{35} H_{52} O_6	627.463	19.185	568.382	
25	Alloxanthin	C_{40} H_{52} O_2	6.9.388	19.847	564.3881	-9.93
26	Saikosaponin BK1	C_{48} H_{78} O_{17}	971.544	19.976	926.5432	-20.87

Table 5.12: Nature and biological activity of bioactive compounds detected in the HR LC-MS analysis of leaf methanolic extract of *K. caryophyllata*

Sl. No	Compound Name	Nature of compounds	Bioactivity
1	3beta,6beta Dihydroxynortropane	Alkaloids	Anti-inflammatory, and anti- allergy (https://pubchem.ncbi.nlm.nih.gov/compound/3beta_6beta-Dihydroxynortropane)
2	Umbelliferone	Phenolics	Antioxidant, anti-inflammatory, anti-hyperglycemic (Mazimba, 2017)
3	Triprolidine	Phenolics	Anti-inflammatory, antihistamine (Shim et al., 2022)
4	(+)-Myrtenyl formate	Bicyclic monoterpenoids	Antimicrobial, *anticancer*, anti-inflammatory and antioxidant (Awadh Ali et al., 2017)
5	Picrasin C	Triterpene	Antiplasmodial, anticancer (Chakraborty, 2013)
6	Panaquinquecol 1	Long-chain fatty alcohol	Cytotoxic and anticancerous (Christensen, 2020)
7	Deinoxanthin	Carotenoid	Antioxidant (Ji. HF, 2010)
8	Harderoporphyrin	Porphyrins	No data available
9	Kanokoside D	Terpene glycoside	Anti-obesity effect (Yuki et al., 2015)
10	Stigmast-4-ene-3,6-dione	Triterpenes	Anti-inflammatory and anti-allergic properties (Abubakar & Majinda, 2016)
11	Cucurbitachrome 1	Carotenoids	Antioxidant (Guerriero et al., 2018)
12	3-cis-Hydroxy-b,e, Caroten-3'-one	Terpene-isoprenoid	Potential in alleviating age-related diseases in humans (https://pubchem.ncbi.nlm.nih.gov/compound/53477759.)
13	Flazine	Alkaloids	Antidiabetic, Anti-inflammatory (Kim et al., 2016)
14	3,5-Dihydroxyphenyl 1-O-(6-O-galloyl-beta-D-glucopyranoside)	Phenolic glycosides	Anticancer, antidiabetic (Hassan et al., 2021)
15	Physalin K	Steroids	Anticancer, anti-inflammatory, antiparasitic, and antimicrobial (Meira et al., 2022)
16	Theasinensin C	Phenolics	Anti-inflammatory, anti-oxidant, antimicrobial (Liu et al., 2021)
17	Fukinolic acid	Terpene	Anti-inflammatory, antiviral, cytotoxic (Gavin et al., 2013).
18	(-)-Quebrachamine	Indole alkaloids	Anti-inflammatory (Zhao et al., 2023)
19	Methanophenazine	Terpene-Polyisoprenoid	Antifungal (Jo et al., 1998)

20	Convalloside	Cardiac glycosides	Cardiotonic (Demir et al., 2022)
21	9-HOTE	Sterol	Anti-inflammatory, Anti aggregatory (Meljon et al., 2012)
22	Melleolide D	Sesquiterpenoid	Cytotoxic, Antifungal (Dörfer et al., 2019)
23	Jamaicamide C	Lipopeptide	Neuro toxin (Dörfer et al., 2019)
24	13-Deoxytedanolide	Steroid	Antitumor (Smith et al., 2003)
25	26-Glucosyl-1,3,11,22-tetrahydroxyergosta-5,24-dien-26-oate	Terpene	Antimicrobial, antiinsecticide (Dawe, 2014)
26	Lyciumoside IX	Diterpene glycosides	Anti-inflammatory, antidiabetic (Yao et al., 2011)
27	8-C-Ascorbylepigallocatechin 3-gallate	Polyphenols	Prevent alchiemer's disease (AD) (Kanaoka et al.,1964)
28	Vobtusine	Indole Alkaloid	Cytotoxicic (https://pubchem.ncbi.nlm.nih.gov/ compound/Vobtusine).
29	Calotropin	Terpene-Cardiac glycosides	Anticancer (Koch et al., 2020)
30	Prodelphinidin A2 3'-gallate	Phenolics	Antiviral, Cytotoxicity (Cheng et al., 2002)
31	Grossamide	Phenolics	Anti-neuroinflammatory (Mostafa et al., 2018)
32	Magnesium protoporphyrin monomethyl ester	Ester	Anticancer drug (De Britto et al., 2001)
33	Squalamine	Terpene-Squalene Derivative	Antiangiogenic agent in Cancer, Antimicrobial (Limbocker et al., 2021)
34	Corotoxigenin-3-O-alpha-L-rhamnopyranoside	Terpene-Cardiac glycosides	Unknown
35	Synaptolepis factor K1	Diterpene	Antidiabetic, Antiaging effect (Pereira et al., 2019)
36	33-Deoxy-33-hydroperoxyfurohyperforin	Monoterpenoids	Antibacterial, Apoptotic properties, Antitumor, Antifungal (Lee et al., 2006)
37	Alloxanthin	Diterpenoid	Anti-inflammatory (Konishi et al., 2008)
38	Saikosaponin BK1	Diterpene	Anti-viral, anti-tumor, anti-inflammatory, anticonvulsant, anti-nephritis and hepatoprotective activity (Sulaiman et al., 2022)

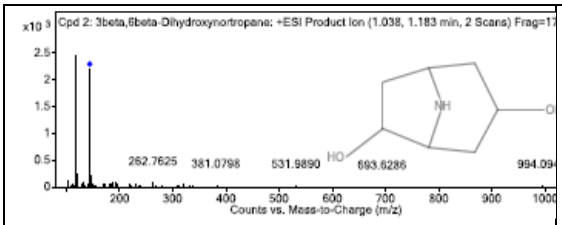

Fig.5.11.a:3beta, 6beta, Dihydroxy nortropane with RT value 1.11

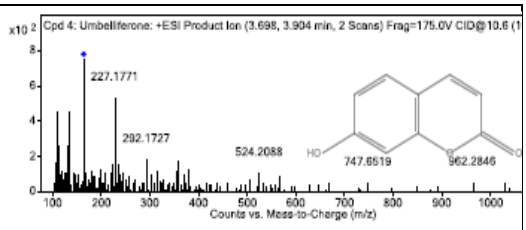

Fig. 5.11.b: umbelliferone with RT value 3.801

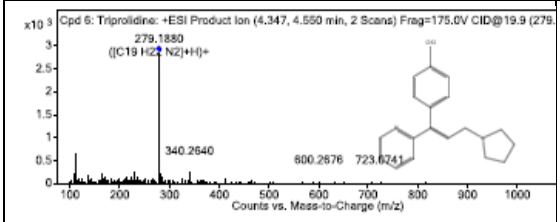

Fig. 5.11.c: Triprolidine with RT value 4.824

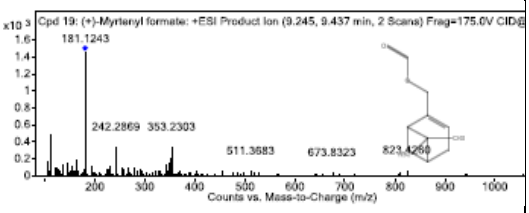

Fig. 5.11.d: (+)-Myrtenyl formate with RT value 9.341

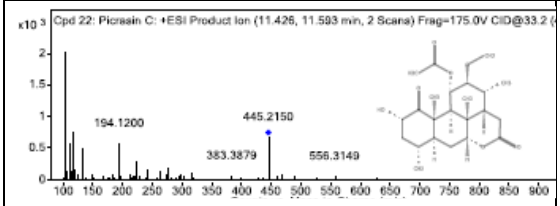

Fig. 5.11.e: Picracin C with RT value 11.51

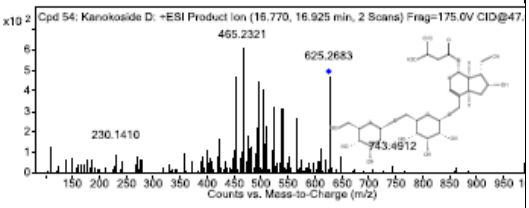

Fig. 5.11.f: Kanokoside D with RT value 17.172

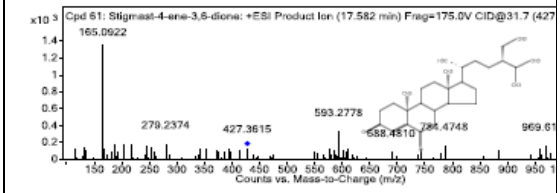

Fig. 5.11.g: Stigmast-4-ene-3,6-dione with RT value 17.401

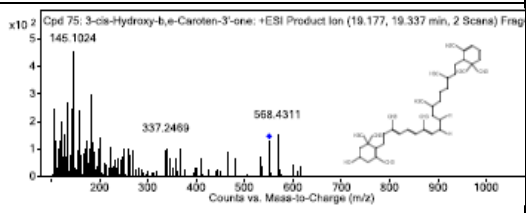

Fig. 5.11.h: 3-cis-Hydroxy-b,e, Caroten-3'-one with RT value 19.579

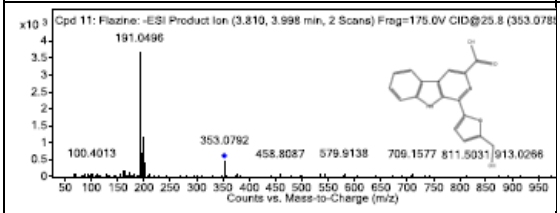

Fig. 5.11.i: Flazine with RT value 3.904

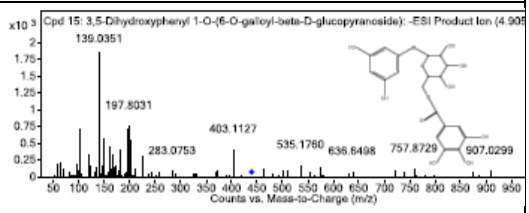

Fig. 5.11.j: 3,5-Dihydroxyphenyl 1-O-(6-O-galloyl-beta-D-glucopyranoside) with RT value 4.905

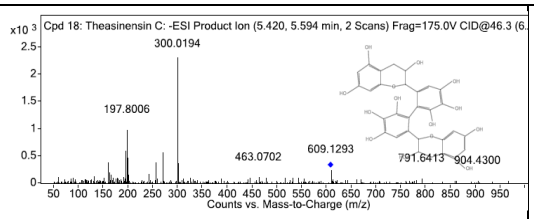

Fig. 5.11.k: Theasinensin C with RT value 5.507

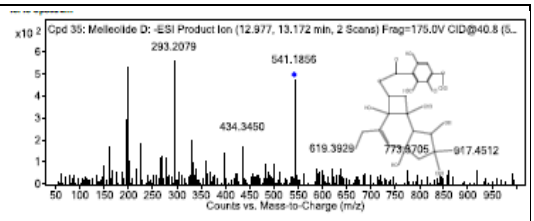

Fig. 5.11.l: Melleolide D with RT value 13.074

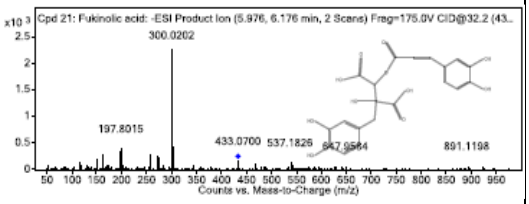

Fig. 5.11.m: Fukinolic acid with RT value 6.076

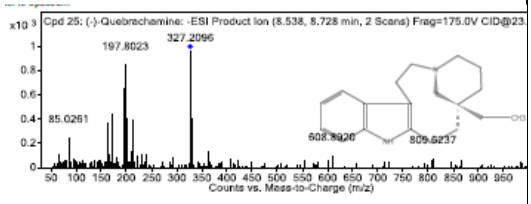

Fig. 5.11.n: (-)-Quebrachamine with RT value 8.633

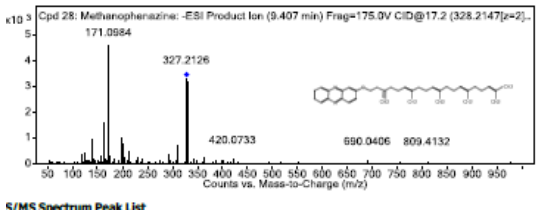

Fig. 5.11.o: Methanophenazine with RT value 9.407

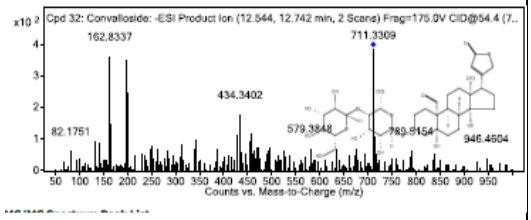

Fig. 5.11.p: Convalloside with RT value 12.643

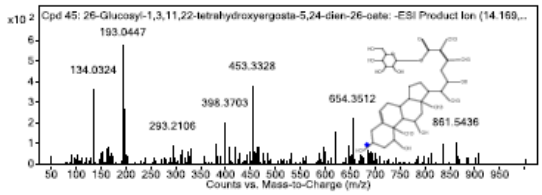

Fig. 5.11.q: 26-Glucosyl-1,3,11,22-tetrahydroxyergosta-5,24-dien-26-oate with RT value 14

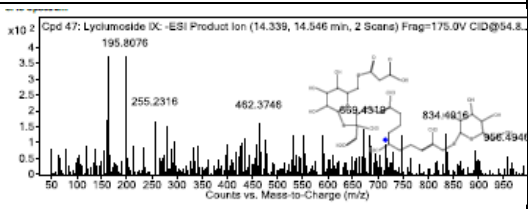

Fig. 5.11.r: Lyciumoside IX with RT value 14.443

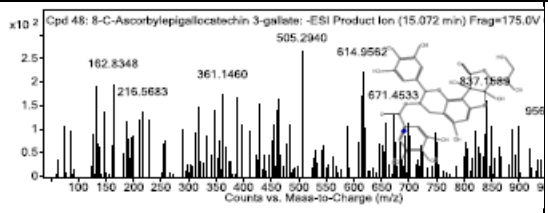

Fig. 5.11.s: (-)-Quebrachamine with RT value 8.633

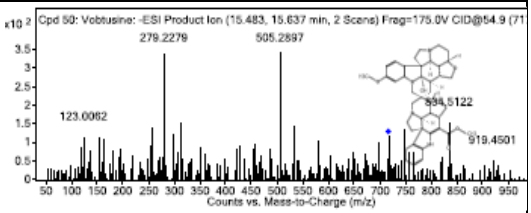

Fig. 5.11.t: Vobtusine with RT value 15.56

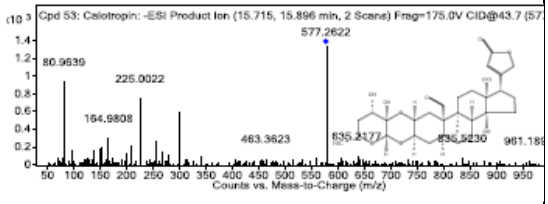

Fig. 5.11.u: Calotropin with RT value 15.805

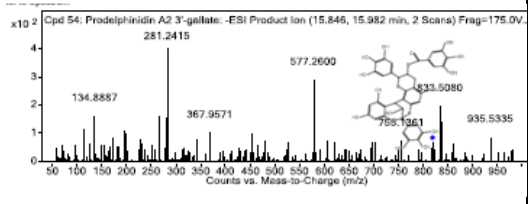

Fig. 5.11.v: Prodelphinidin A2 3'-gallate with RT value 15.914

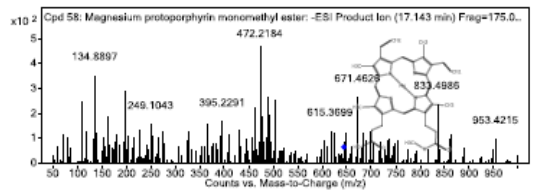

Fig. 5.11.w: Grossamide with RT value 17.143

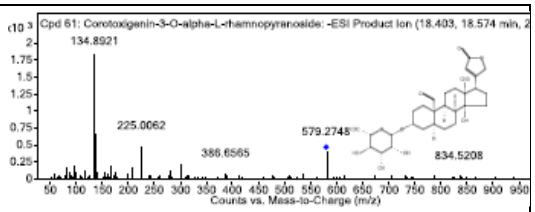

Fig. 5.11.x: Corotoxigenin-3-O-alpha-L-rhamnopyranoside with RT value 18.488

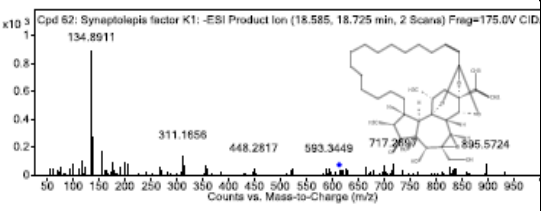

Fig. 5.11.y: Synaptolepsis factor K1 with RT value 18.

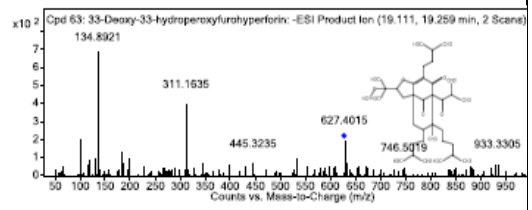

Fig. 5.11.z: 33-Deoxy-33 hydroperoxyfurohyperforin with RT value 19.185

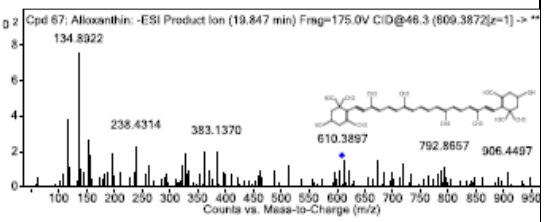

Fig. 5.11.a1: Alloxanthin with RT value 19.847

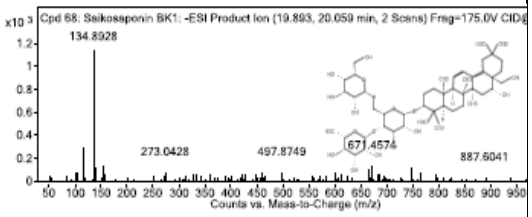

Fig. 5.11.b1: Saikosaponin BK1 with RT value 19.976

Figure. 5.11: Spectral properties and structure of major bioactive compounds detected in the LC-MS analysis of methanolic leaf extract of *K. caryophyllata*

Methanolic stem extracts

The HR LC-MS chromatogram profile obtained from the stem methanolic extracts identified 12 and 20 compounds with known medicinal properties respectively in the positive and negative chromatogram (figure 5.12 & 5.13). The details of compounds identified such as name of compound, retention time, chemical formula, mass, mass accuracy and DB difference are shown in table 5.13 (Positive chromatogram) and table 5.14 (Negative chromatogram). The nature and bioactivity of compounds detected from both positve and negative chromatogram are depicted in the table 5.15. The details of spectral properties and structure of major bioactive compounds detected in the chromatograms are shown in the figure 5.14.

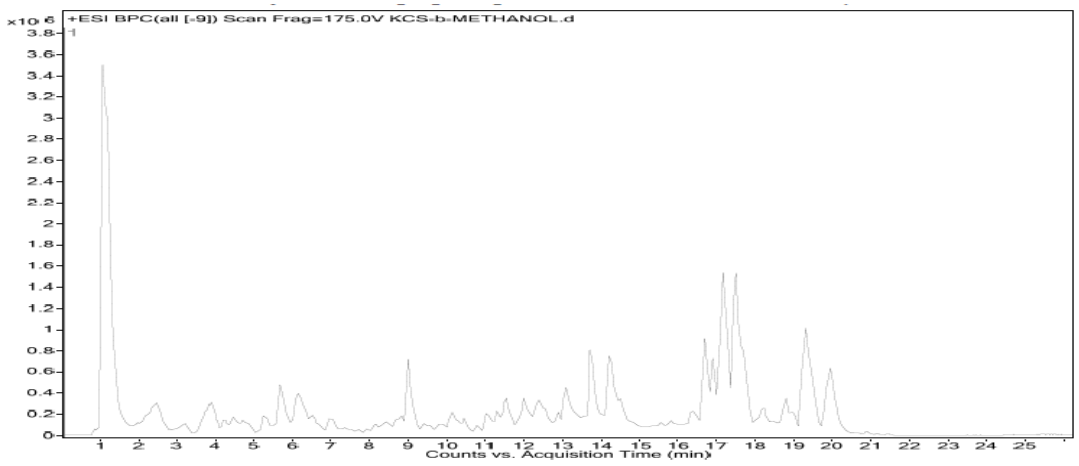

Figure 5. 12: Positive HR LC-MS chromatogram of methanolic stem extracts of *K. caryophyllata*.

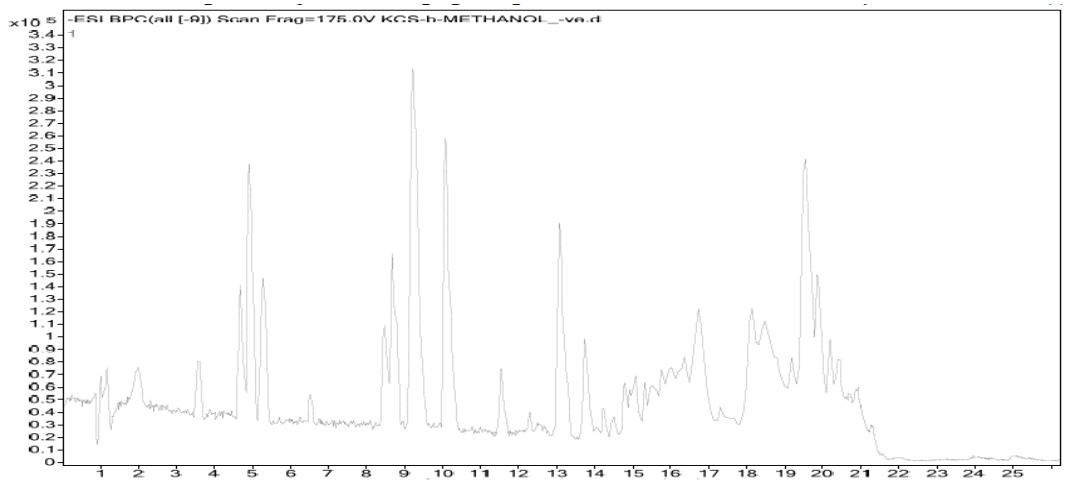

Figure 5. 13: Negative HR LC-MS chromatogram of methanolic stem extracts of *K. caryophyllata*.

Table 5.13: Details of phytocompounds identified in the LC-MS analysis of methanolic stem extract (Positive chromatogram)

Sl. No	Compound Name	Chemical Formula	Mass (m/z)	Retention Time (RT)	Mass Accuracy (ppm)	DB DIFF (PPM)
1	3beta,6beta-Dihydroxynortropane	$C_7 H_{13} N O_2$	144.1033	1.118	143.0961	-10.26
2	Lophophorine	$C_{13} H_{17} N O_3$	236.1301	1.508	235.1229	-8.72
3	Solanocapsine	$C_{27} H_{46} N_2 O_2$	453.346	4.579	430.3569	-2.14
4	Aschantin	$C_{22} H_{24} O_7$	401.162	5.521	400.1547	-6.15
5	Kurchessine	$C_{25} H_{44} N_2$	395.341	6.098	372.3516	-3.1
6	Quinidine	$C_{20} H_{24} N_2 O_2$	325.193	7.14	324.186	-6.8
7	Picrasin C	$C_{23} H_{34} O_7$	445.216	11.536	422.2259	10.78
8	Kanokoside D	$C_{27} H_{44} O_{16}$	625.2687	17.165	624.2614	2.45
9	Pheophorbide a	$C_{35} H_{36} N_4 O_5$	593.279	17.891	592.2719	-4.85
10	3beta-3-Hydroxy-18-lupen-21-one	$C_{30} H_{48} O_2$	441.374	19.146	440.3673	-6.13
11	Lappaconitine	$C_{32} H_{44} N_2 O_8$	607.295	18.82	584.3053	7.69
12	3-cis-Hydroxy-b,e-Caroten-3'-one	$C_{40} H_{54} O$	551.427	19.312	550.4201	-4.83

Table 5.14: Details of phytocompounds identified in the LC-MS analysis of methanolic stem extract (Negative chromatogram)

Sl. No	Compound Name	Chemical Formula	Mass (m/z)	Retention Time (RT)	Mass Accuracy (ppm)	DB DIFF (PPM)
1	Azelaic acid	$C_9 H_{16} O_4$	187.093	1.71	188.1006	23.48
2	9-Hydroxy-4-methoxypsoralen 9-glucoside	$C_{18} H_{18} O_{10}$	393.098	4.672	394.0942	-10.77
3	Physalin K	$C_{28} H_{30} O_{12}$	557.171	5.301	558.1759	-3.37
4	Melleolide D	$C_{24} H_{31} Cl O_8$	541.184	13.078	482.1707	0.03
5	Beta-Obscurine	$C_{17} H_{24} N_2 O$	331.201	13.758	272.1848	14.92
6	Disinomenine	$C_{38} H_{44} N_2 O_8$	715.327	14.935	656.3132	-5.19
7	Maclurin 3-C-(2"-p-hydroxybenzoyl-6"-galloyl-glucoside)	$C_{33} H_{28} O_{17}$	755.1308	15.327	696.1149	25.43
8	8-C-Ascorbylepigallocatechin 3-gallate	$C_{28} H_{24} O_{17}$	691.118	15.404	632.1044	-4.79
9	Prodelphinidin A2 3'-gallate	$C_{37} H_{28} O_{18}$	819.142	15.873	760.1266	1.32
10	Polyporusterone G	$C_{28} H_{44} O_5$	459.307	16.127	460.3143	9.93
11	(17alpha,23S)-Epoxy-28,29-dihydroxy-27-norlanost-8-ene-3,24-dione	$C_{29} H_{44} O_5$	472.318	16.54	472.3179	2.02
12	(6beta,8betaOH)-6,8-Dihydroxy-7(11)-eremophilen-12,8-olide	$C_{15} H_{22} O_4$	265.145	16.674	266.1515	1.49
14	Somniferine	$C_{36} H_{36} N_2 O_7$	607.250	17.248	608.2575	-8.55
15	(+)-Lyoniresinol 9-glucoside	$C_{28} H_{38} O_{13}$	627.232	17.494	582.2319	-1.19
16	Callystatin A	$C_{29} H_{44} O_4$	455.312	17.494	456.3187	11.62
17	2S-hydroxy-octadecanoic acid	$C_{18} H_{36} O_3$	299.256	17.494	300.2631	11.26
18	(2S)-2-hydroxyphytanic acid	$C_{20} H_{40} O_3$	327.287	19.488	328.2948	10.02
19	3,7-Dihydroxy-25-methoxycucurbita-5,23-dien-19-al	$C_{31} H_{50} O_4$	485.3665	19.796	486.3665	9.02
20	8-Hydroxyhyperforin 8,1-hemiacetal	$C_{35} H_{52} O_5$	611.403	20.254	552.3894	-14.38

Table 5.15: Nature and biological activity of bioactive compounds detected in the HR LC-MS analysis of stem methanolic extract of *K. caryophyllata*

Sl. No	Compound Name	Nature of compounds	Bioactivity
1	3beta,6beta-Dihydroxynortropane	Tropane alkaloid	Anti-inflammatory, and anti- allergy (https://pubchem.ncbi.nlm.nih.gov/compound/3beta_6beta-Dihydroxynortropane)
2	Lophophorine	Alkaloids	Antibacterial (Ibarra-Laclette et al., 2015)
3	Solanocapsine	Steroid alkaloid	Inhibitors of acetylcholinesterase (Pereira et al., 2019)
4	Aschantin	Alkaloid	Antiplasmodial, antagonistic, antagonistic, and chemopreventive .(Dr.Duke's phytochemical and Ethnobotanical Database)
5	Kurchessine	Aza-steroid alkaloid	Anti-inflammatory (https://pubchem.ncbi.nlm.nih.gov/compound/54612858)
6	Quinidine	Alkaloid	Adrenergic antagonist, an antimalarial, an anti-arrhythmia drug, (https://pubchem.ncbi.nlm.nih.gov/compound/441074)
7	Picrasin C	Triterpene	Antiplasmodial, anticancer (Cachet et al., 2012)
8	Kanokoside D	Terpene	Anti-obesity effect(Yuki et al., 2015)
9	Pheophorbide a	Chlorophyll derivative	Anti- proliferative (Saide et al., 2020)
10	3beta-3-Hydroxy-18-lupen-21-one	Triterpene	Anti-inflammatory (https://pubchem.ncbi.nlm.nih.gov/compound/3beta-3-Hydroxy-18-lupen-21-one)
11	Lappaconitine	Alkaloid	Analgesic, antipyretic (Chen et al., 2019)
12	3-cis-Hydroxy-b,e-Caroten-3'-one	Isoprenoid	Anticancerous (https://pubchem.ncbi.nlm.nih.gov/compound/3-cis-Hydroxy-b_e-Caroten-3_-one)
13	Azelaic acid	Dicarboxylic acid	Anti-inflammatory, antibacterial, keratolytic, comedolytic, and anti-oxidant activity (https://pubchem.ncbi.nlm.nih.gov/compound/Azelaic-acid)
14	9-Hydroxy-4-methoxypsoralen 9-glucoside	Coumarin glycosides.	17-beta-hydroxysteroid dehydrogenase-Inhibitor, Aryl-Hydrocarbon-Hydroxylase-Inhibitor.(Dr.Duke's phytochemical and Ethnobotanical Database)
15	Physalin K	Steroid	Anti-inflammatory activity (https://pubchem.ncbi.nlm.nih.gov/compound/Physalin-A#section=Chemical-Gene-Co-Occurrences-in-Literature)
16	Melleolide D	Terpene-Sesquiterpene	Antimicrobial activity and cytotoxic properties (https://pubchem.ncbi.nlm.nih.gov/compound/Melleolide-D)
17	Beta-Obscurine	Alkaloid	Acetylcholine esterase activity (Fu et al., 2016)
18	Sinomenine	Alkaloid	Analgesic, sedative, and anti-inflammatory effects,(Jiang et al., 2020)
19	Maclurin 3-C-(2''-p-hydroxybenzoyl-6''-galloyl-glucoside)	Phenolic glycosides	Antioxidant, anti-inflammatory, antimicrobial, anticancer (Liu et al., 2021)

20	8-C-Ascorbylepigallocatechin 3-gallate	Polyphenolics	Prevent Alzheimer's disease (Kanaoka et al.,1964)
21	Prodelphinidin A2 3'-gallate	Phenolics-Flavonoids	Antiviral(Cheng et al., 2002)
22	Polyporusterone G	Triterpene	Anticancer, Antidote(Dr.Duke's phytochemical and Ethnobotanical Database)
23	(17alpha,23S)-Epoxy-28,29-dihydroxy-27-norlanost-8-ene-3,24-dione	Triterpene	Analgesic, Anticancer(Dr.Duke's phytochemical and Ethnobotanical Database)
24	(6beta,8betaOH)-6,8-Dihydroxy-7(11)-eremophilen-12,8-olide	Terpene	Antiamyloid-Beta AntiTGF-beta, Beta-Galactosidase-Inhibitor Beta-Glucuronidase-Inhibitor.(Dr.Duke's phytochemical and Ethnobotanical Database)
25	Somniferine	Alkaloid	Antioxidant(Kumar & Kumar, 2021)
26	(+)-Lyoniresinol 9-glucoside	Glycoside-lignan	Antioxidant(Rahim et al., 2022)
27	Callystatin A	Diterpenoid	Cytotoxic (https://pubchem.ncbi.nlm.nih.gov/compound/Callystatin-A)
28	2S-hydroxy-octadecanoic acid	Fatty acid	Analgesic-Synergist, ANS-Stimulant, Anticancerc.(Dr.Duke's phytochemical and Ethnobotanical Database)
29	(2S)-2-hydroxyphytanic acid	Fatty acid	Anticarcinomic , AntiCrohn's. Antidiabetic.(Dr.Duke's phytochemical and Ethnobotanical Database)
30	3,7-Dihydroxy-25-methoxycucurbita-5,23-dien-19-al	Terpenoid	Allelopathic (Chen et al.,2017)
31	8-Hydroxyhyperforin 8,1-hemiacetal	Monoterpenoid	Antidepressant (Lee et al., 2006)

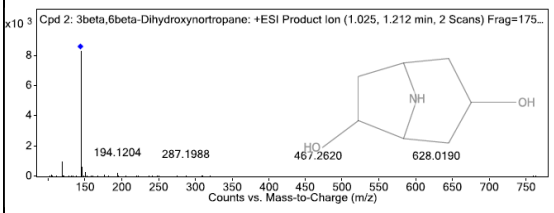

Fig. 5.14.a: 3beta,6beta-Dihydroxynortropane with RT value 1.118

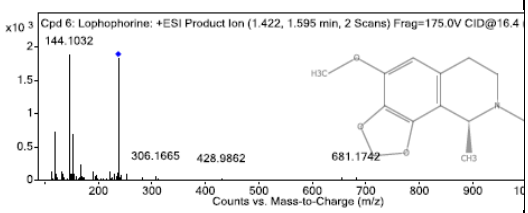

Fig. 5.14.b: Lophophorine with RT value 1.508

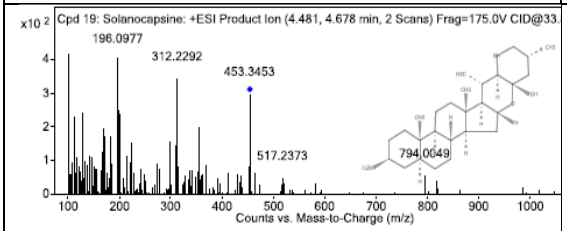

Fig. 5.14.c: Solanocapsine with RT value 19.185

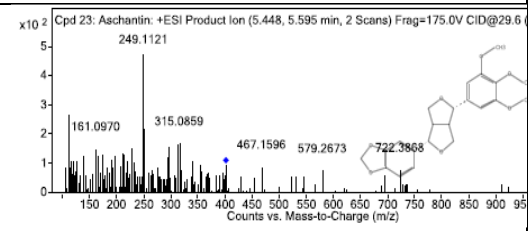

Fig. 5.14.d: Aschanthin with RT value 5.521

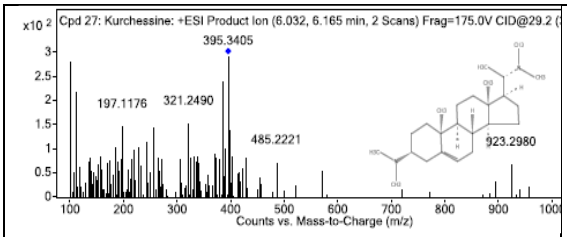

Fig. 5.14.e: Kurchessine with RT value 4.57

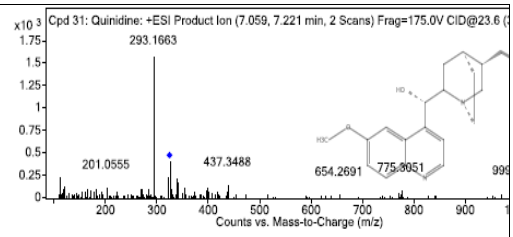

Fig. 5.14.f: Quinidine with RT value 7.14

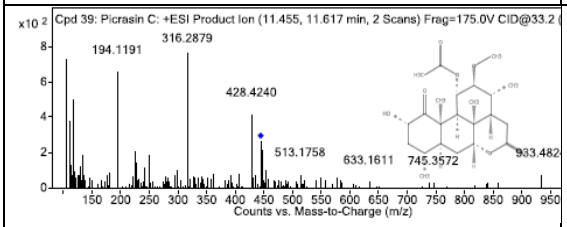

Fig. 5.14.g: Picrasin with RT value 19.185

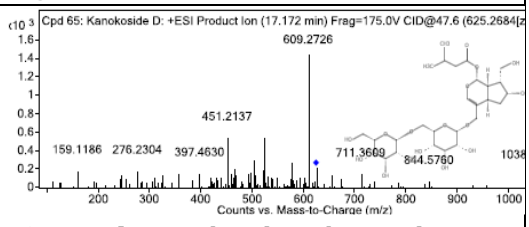

Fig. 5.14.h: Kanokoside with RT value 19.185

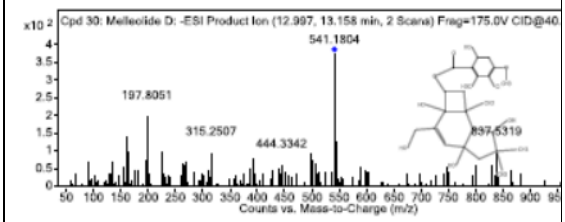

Fig. 5.14.i: Melleolide D with RT value 13.078

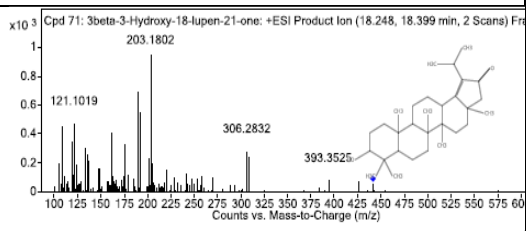

Fig. 5.14.j: 3beta 3-Hydroxy-18-lupen-21-one with RT value 19.185

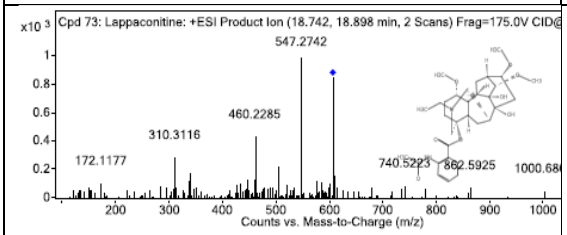

Fig. 5.14.k: Lappaconitine with RT value 19.185

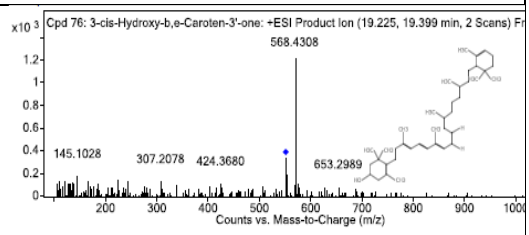

Fig. 5.14.l: 3-cis-Hydroxy-b,e-Caroten-3'-one with RT value 19.185

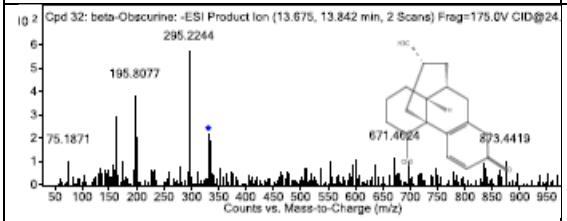

Fig. 5.14.m: Beta-Obscurine with RT value 13.758 present in *Kamettia caryophyllata*

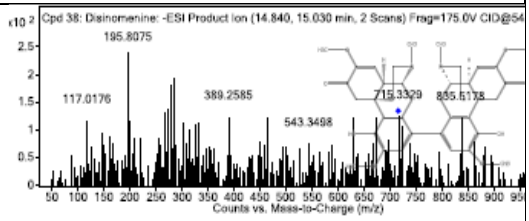

Fig. 5.14.n: Disinomenine with RT value 14.935

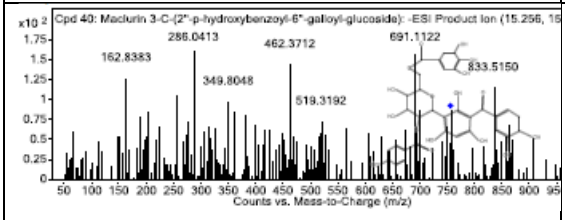

Fig. 5.14.o: Maclurin 3-C-(2"-p-hydroxybenzoyl-6"-galloyl-glucoside) with RT value 15.327

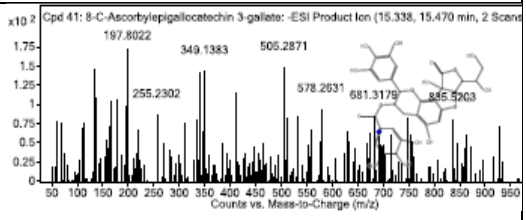

Fig.5.14.p: 8-C-Ascorbylepigallocatechin 3-gallate with RT value 15.404

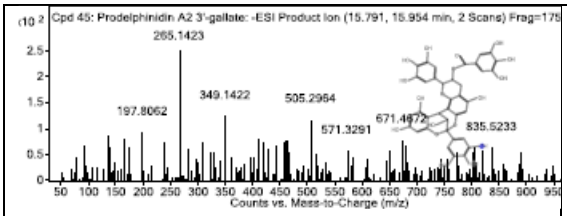

Fig. 5.14.q: Prodelphinidin A2 3'-gallate with RT value 15.873

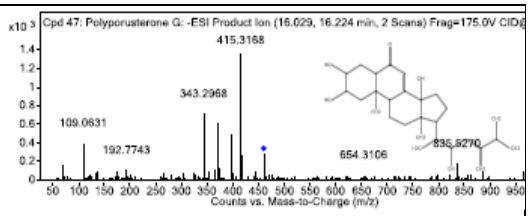

Fig. 5.14.r: Polyporusterone G with RT value 16.127

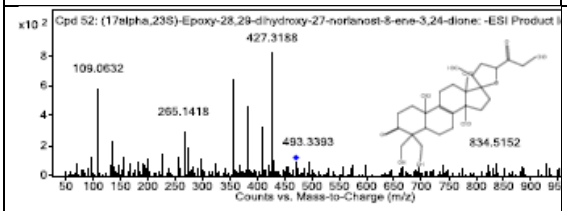

Fig. 5.14.s: (17alpha,23S)-Epoxy-28,29-dihydroxy-27-norlanost-8-ene-3,24-dione with RT value 16.54

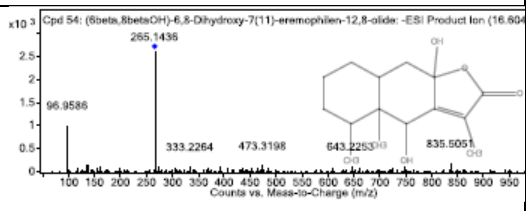

Fig.5.14.t: (6beta,8betaOH)-6,8-Dihydroxy-7(11)-eremophilen-12,8-olide with RT value 16.674

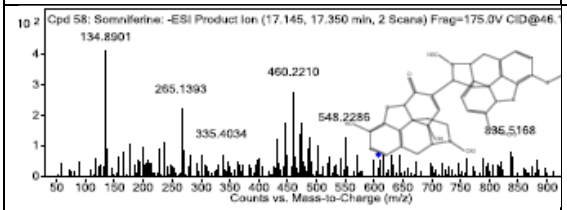

Fig. 5.14.u: Somniferine with RT value 17.360

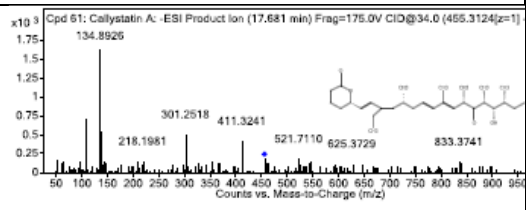

Fig. 5.14.v: Callystatin A with RT value 17.494

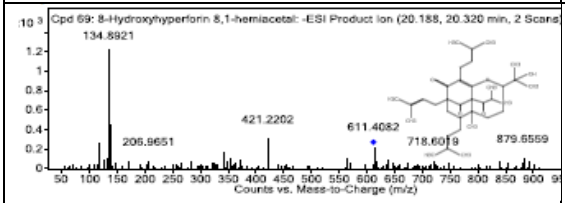

Fig. 5.14.x: 8-Hydroxyhyperforin 8,1-hemiacetal with RT value 20.254

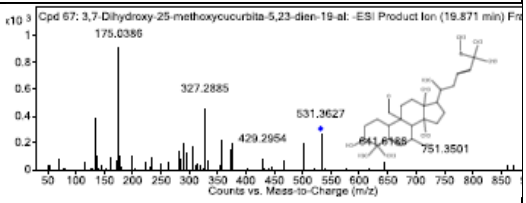

Fig.5.14.y: 3,7-Dihydroxy-25-methoxycucurbita-5,23-dien-19-al with RT value 19.796

Figure. 5.14: Spectral properties and structure of major bioactive compounds detected in the LC-MS analysis of methanolic stem extract of *K. caryophyllata*

Methanolic root extracts

The HR LC-MS chromatogram profile obtained from the root methanolic extracts identified 16 and 14 compounds with known medicinal properties respectively in the positive and negative chromatogram (figure 5.15 & 5.16). The details of compounds identified such as name of compound, retention time, chemical formula, mass, mass accuracy and DB difference are shown in table 5.16 (Positive chromatogram) and table 5.17 (Negative chromatogram). The nature and bioactivity of compounds detected from both positve and negative chromatogram are depicted in the table 5.18.

The details of spectral properties and structure of major bioactive compounds detected in the chromatograms are shown in the figure 5.17.

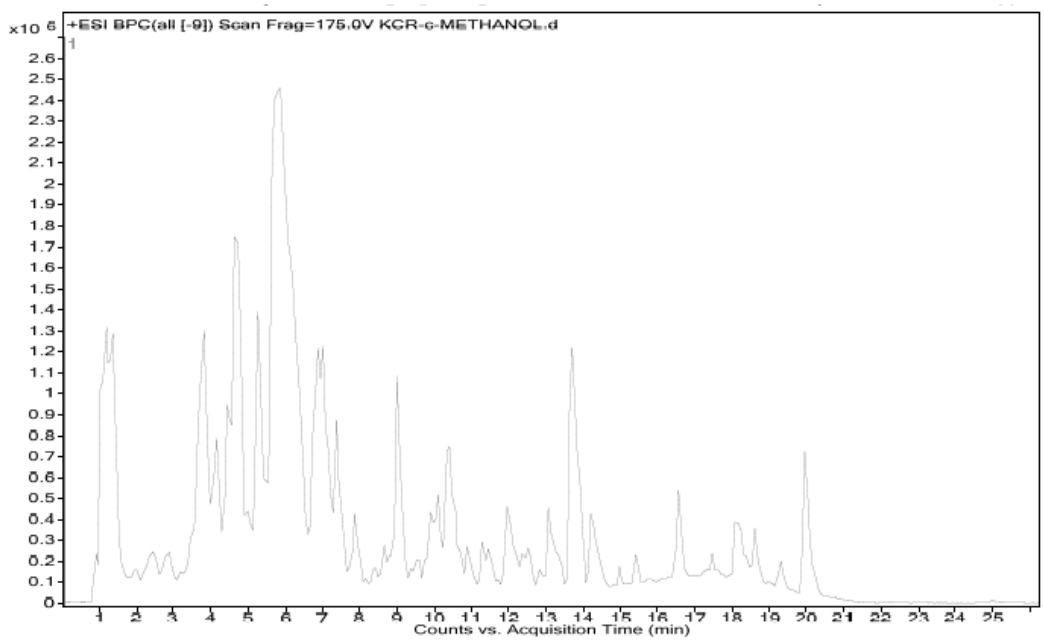

Figure 5. 15: Positive HR LC-MS chromatogram of methanolic root extracts of *K. caryophyllata*.

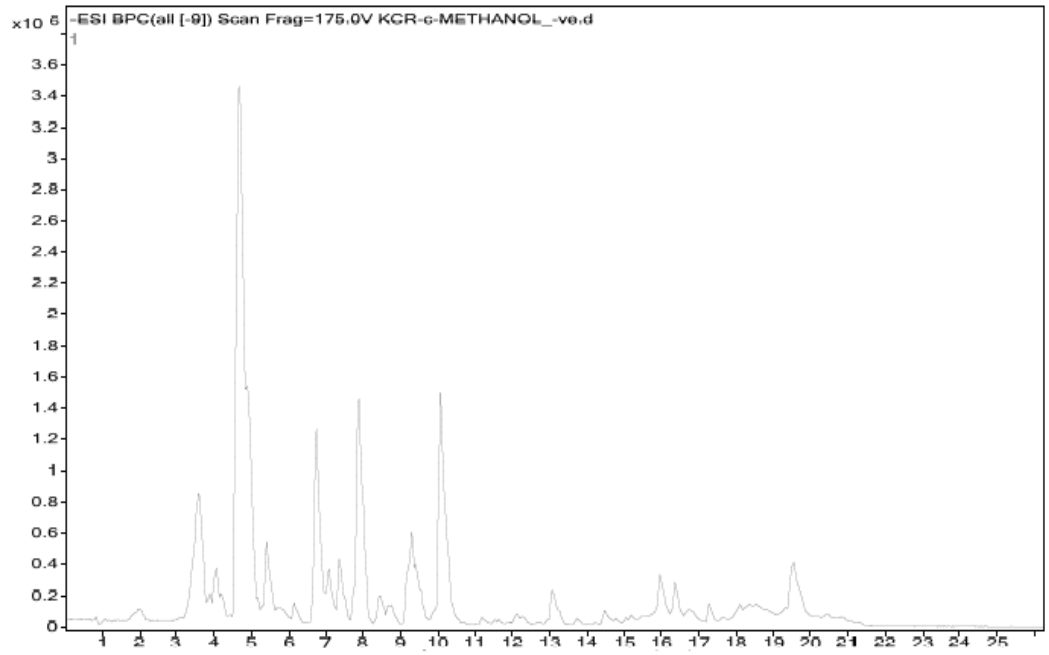

Figure 5. 16: Negative HR LC-MS chromatogram of methanolic root extracts of *K. caryophyllata*.

Table 5.16: Details of phytocompounds identified in the LC-MS analysis of methanolic root extract (Positive chromatogram)

Sl. No	Compound Name	Chemical Formula	Mass (m/z)	Retention Time (RT)	Mass Accuracy (ppm)	DB Diff (ppm)
1	3beta,6beta-Dihydroxynortropane	$C_7 H_{13} N O_2$	144.104	1.131	143.0963	-11.65
2	Lophophorine	$C_{13} H_{17} N O_3$	235.123	1.436	235.1231	-8.27
3	Ruspolinone	$C_{14} H_{19} N O_3$	250.146	2.45	249.1386	-8.52
4	Cyclobuxine D	$C_{25} H_{42} N_2 O$	409.320	3.446	386.3311	-3.81
5	3-Hydroxyquinine	$C_{20} H_{24} N_2 O_3$	341.189	3.546	340.1814	-7.81
6	2',6'-Dimethoxy-4'-hydroxy acetophenone	$C_{10} H_{12} O_4$	197.083	4.632	196.0753	-8.49
7	Nb-Feruloyltryptamine	$C_{20} H_{20} N_2 O_3$	359.137	4.7	336.1473	0.15
8	Echitovenine	$C_{23} H_{28} N_2 O_4$	397.214	5.36	396.2074	-6.3
9	Capsaicin	$C_{18} H_{27} N O_3$	306.209	5.484	305.2015	-7.92
10	16-Methoxytabersonine	$C_{22} H_{26} N_2 O_3$	367.204	5.571	366.1966	-6.11
11	Kurchessine	$C_{25} H_{44} N_2$	395.341	5.718	372.3515	-2.77
12	Tabersonine	$C_{21} H_{24} N_2 O_2$	337.178	6.153	336.186	-6.69
13	Quinidine	$C_{20} H_{24} N_2 O_2$	325.194	7.046	324.1864	-7.97
14	Akuammidine	$C_{21} H_{24} N_2 O_3$	353.189	7.174	352.1816	-8.11
15	Brucine	$C_{23} H_{26} N_2 O_4$	395.199	7.178	394.1922	-7.44
16	Farnesyl pyrophosphate	$C_{15} H_{28} O_7 P_2$	383.136	7.903	382.1289	5.57

Table 5.17: Details of phytocompounds identified in the LC-MS analysis of methanolic root extract (Positive chromatogram)

Sl. No	Compound Name	Chemical Formula	Mass (m/z)	Retention Time (RT)	Mass Accuracy (ppm)	DB Diff (ppm)
1	Azelaic acid	$C_9 H_{16} O_4$	187.094	1.729	188.1009	21.07
2	Atovaquone	$C_{22} H_{19} Cl O_3$	411.102	3.401	366.103	-4.06
3	9-Hydroxy-4-methoxypsoralen 9-glucoside	$C_{18} H_{18} O_{10}$	393.092	4.592	394.0971	-18.01
4	Secoxyloganin	$C_{17} H_{24} O_{11}$	403.121	5.004	404.1285	8.35
5	7-Hydroxy-3-methoxy-1-primeverosyloxy xanthone	$C_{25} H_{28} O_{14}$	597.154	5.182	552.1542	-11.72
6	1-O-E-Cinnamoyl-(6-arabinosylglucose)	$C_{20} H_{26} O_{11}$	441.139	5.198	442.1458	3.9
7	Schidigeragenin C	$C_{27} H_{42} O_4$	429.297	5.992	430.3015	15.84
8	Polygonolide	$C_{12} H_{12} O_4$	265.072	6.753	220.0736	-0.16
9	Melleolide D	$C_{24} H_{31} Cl O_8$	541.185	13.096	482.1717	-1.9
10	L-Oleandrosyl-oleandolide	$C_{27} H_{46} O_{10}$	529.297	14.492	530.3051	7.55
11	Maclurin 3-C-(2''-p-hydroxy benzoyl-6''-galloyl-glucoside)	$C_{33} H_{28} O_{17}$	755.131	15.744	696.1155	24.61
12	(17alpha,23S)-Epoxy-28,29-dihydroxy-27-norlanost-8-ene-3,24-dione	$C_{29} H_{44} O_5$	471.3078	16.377	472.3151	7.99
13	(6beta,8betaOH)-6,8-Dihydroxy-7(11)-eremophilen-12,8-olide	$C_{15} H_{22} O_4$	265.1449	16.667	266.152	-0.75
14	beta-Boswellic acid acetate	$C_{32} H_{50} O_4$	497.3601	18.202	498.3672	7.42

Table 5.18: Nature and biological activity of bioactive compounds detected in the HR LC-MS analysis of root methanolic extract of *K. caryophyllata*

Sl. No	Compound Name	Nature of compounds	Bioactivity
1	3beta,6beta-Dihydroxynortropane	Tropane alkaloid	Anti-inflammatory, and anti- allergy (https://pubchem.ncbi.nlm.nih.gov/compound/3beta_6beta-Dihydroxynortropane)
2	Lophophorine	Isoquinolines (alkaloids)	Antibacterial properties (Ibarra-Laclette et al., 2011)
3	Ruspolinone	Pyrrolidine alkaloid	Organocatalyst (Eze et al., 2019)
4	Cyclobuxine D	Steroid Alkaloid	Anticancer, Antidote, Antidote , Antileukotriene-D4 (Dr.Duke's phytochemical and Ethnobotanical Database)
5	3-Hydroxyquinine	Cinchona alkaloid	Antibacterial activity (Rattanachak et al., 2022)
6	2',6'-Dimethoxy-4'-hydroxyacetophenone	Phenolics	Antibacterial (https://pubchem.ncbi.nlm.nih.gov/compound/2_6_-Dimethoxy-4_-hydroxyacetophenone)
7	Nb-Feruloyltryptamine	Cinnamic acids	Cytotoxic (https://pubchem.ncbi.nlm.nih.gov/substance/446189862)
8	Echitovenine	Monoterpenoid	Anticancer (Mohammed et al., 2021)
9	Capsaicin	Alkaloid	Analgesic, anti-inflammatory, antioxidant, antimicrobial, anticancerous (Phairong et al., 2020)
10	16-Methoxytabersonine	Alkaloid(CAtheranthus)	Cytotoxic, Anticancerous (Zhao et al., 2023)
11	Kurchessine	Aza-steroid alkaloid	Anti-inflammatory (https://pubchem.ncbi.nlm.nih.gov/compound/54612858)
12	Tabersonine	indole alkaloids	Anticancer
13	Quinidine	Alkaloid	Adrenergic antagonist, antimalarial, anti-arrhythmia drug, (https://pubchem.ncbi.nlm.nih.gov/compound/441074)
14	Akuammidine	Alkaloid	Anti-inflammatory and anti-asthmatic properties. (https://pubchem.ncbi.nlm.nih.gov/compound/Akuammidine)
15	Brucine	Alkaloid	Toxic
16	Farnesyl pyrophosphate	Sesquiterpenoid	Anti-parasitic reagents (Sun & Mckenna, 2011)

17	Azelaic acid	Dicarboxylic acid	Anti-inflammatory, antibacterial, and antioxidant activity (https://pubchem.ncbi.nlm.nih.gov/compound/Azelaic-acid)
18	Atovaquone	Naphthoquinone	Antimalarial, an antifungal agent (https://pubchem.ncbi.nlm.nih.gov/compound/Atovaquone)
19	9-Hydroxy-4-methoxypsoralen 9-glucoside	Coumarin glycosides.	17-beta-hydroxysteroid dehydrogenase-Inhibitor, Aryl-Hydrocarbon-Hydroxylase-Inhibitor.(Dr.Duke's phytochemical and Ethnobotanical Database)
20	Secoxyloganin	Secoiridoid glycoside	Antioxidant and anti-allergic properties (https://pubchem.ncbi.nlm.nih.gov/compound/Secoxyloganin)
21	7-Hydroxy-3-methoxy-1-primeverosyloxyxanthone	Glycoside	Antioxidant, anti-inflammatory, antimicrobial, anticancer, and neuroprotective effects (https://pubchem.ncbi.nlm.nih.gov/compound/7-Hydroxy-3-methoxy-1-primeverosyloxyxanthone)
22	1-O-E-Cinnamoyl-(6-arabinosyl glucose)	Glycoside	Antioxidant, anti-inflammatory, and antimicrobial effects (Bereksi et al., 2018)
23	Schidigeragenin C	Triterpenoid	Antioxidative, antidiabetic, hepatoprotective
24	Polygonolide	Phenolics	Anti-inflammatory, antifungal(Doss & Parivuguna, 2015)
25	Melleolide D	Sesquiterpene	Antimicrobial activity and cytotoxic properties (https://pubchem.ncbi.nlm.nih.gov/compound/Melleolide-D)
26	L-Oleandrosyl-oleandolide	Terpene-Cardiac glycoside	Potent cardiac effect(Salim et al., 2020)
27	Maclurin 3-C-(2''-p-hydroxy benzoyl-6''-galloyl-glucoside)	Phenolic glycosides	Antioxidant, anti-inflammatory, antimicrobial, anticancer
29	(17alpha,23S)-Epoxy-28,29-dihydroxy-27-norlanost-8-ene-3,24-dione	Triterpenoids	Analgesic, TNF-alpha-Inhibitor.(Dr.Duke's phytochemical and Ethnobotanical Database)
30	(6beta,8betaOH)-6,8-Dihydroxy-7(11)-eremophilen-12,8-olide	Terpenoids	Antibacterial, antioxidant and anticancer (Ruan et al., 2020)
31	beta-Boswellic acid acetate	Triterpenoid	Anti-inflammatory, anti-arthritic, anti-cancer, and anti-microbial (Dr.Duke's Phytochemical and Ethnobotanical Database)

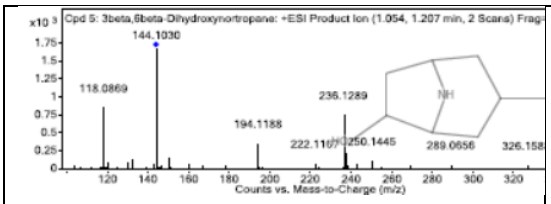

Fig.5.17.a:3beta,6beta-Dihydroxynortropane with RT value 1.131

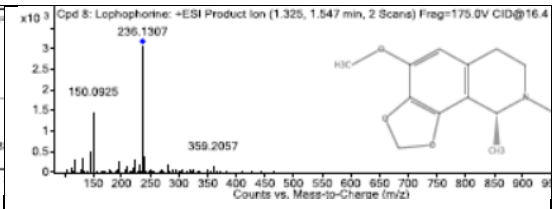

Fig. 5.17.b: Lophophorine with RT value 1.436

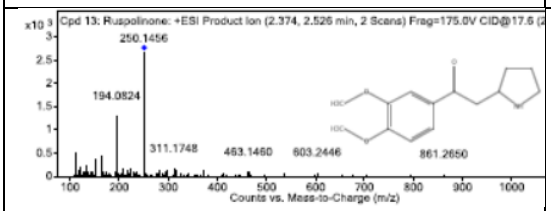

Fig. 5.17.c: Ruspolinone with RT value 2.45

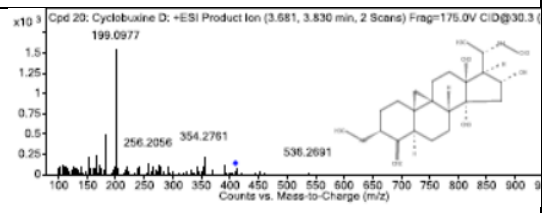

Fig. 5.17.d: Cyclobuxine D with RT value 3.446

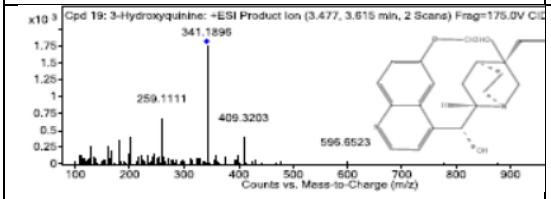

Fig. 5.17.e: 3-Hydroxyquinine with RT value 3.546

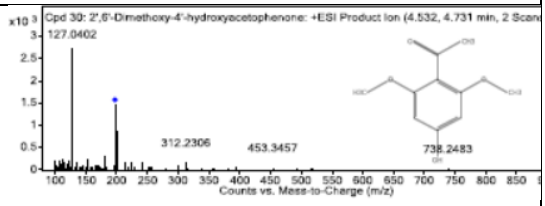

Fig. 5.17.f: 2',6'-Dimethoxy-4'-hydroxy acetophenone with RT value 4.632

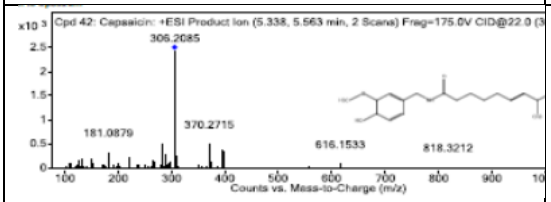

Fig. 5.17.g: Capsaicin with RT value 5.484 present

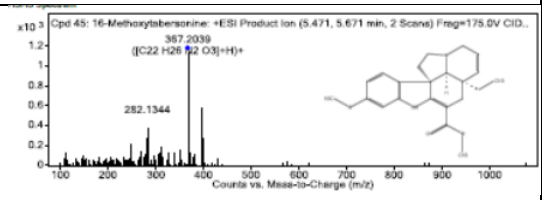

Fig.5.17.h: Structure of 16-Methoxytabersonine with RT value 5.571

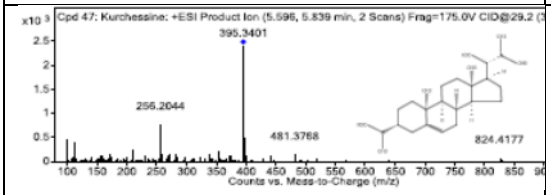

Fig. 5.17.i: Kurchessine with RT value 5.718

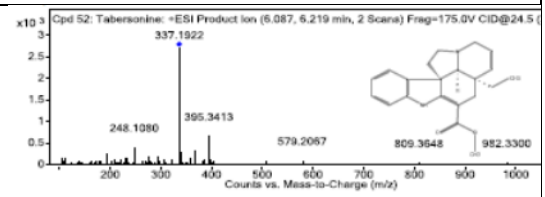

Fig. 5.17.j: Tabersonine with RT value 6.153

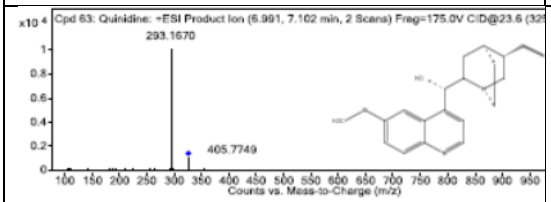

Fig. 5.17.k: Quinidine with RT value 7.046

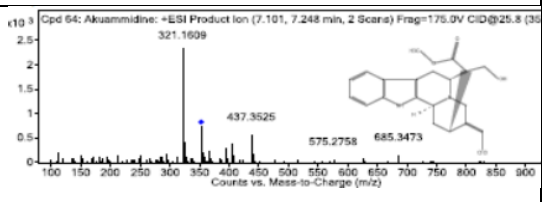

Fig. 5.17.l: Akuammidine with RT value 7.174

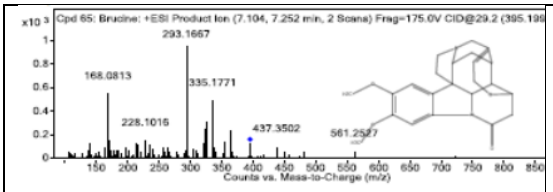

Fig. 5.17.m: Brucine with RT value 7.178

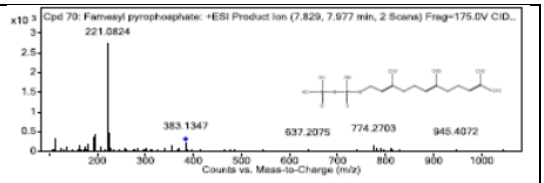

Fig. 5.17.n: Farnesyl pyrophosphate with RT value 7.903

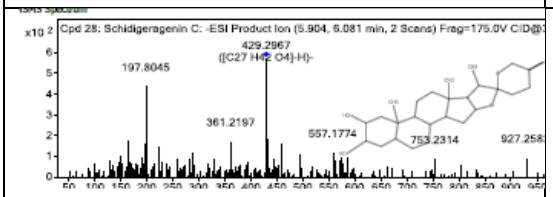

Fig. 5.17.o: 1 Schidigeragenin C with RT value 5.992

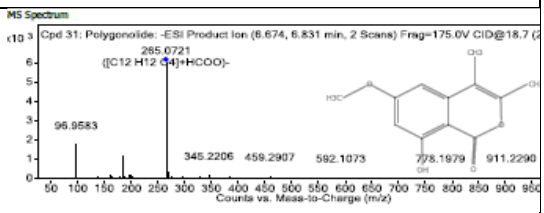

Fig. 5.17.p: Polygonolide with RT value 6.753

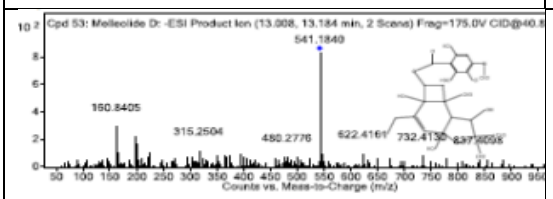

Fig. 5.17.q: Melleolide D with RT value 13.096

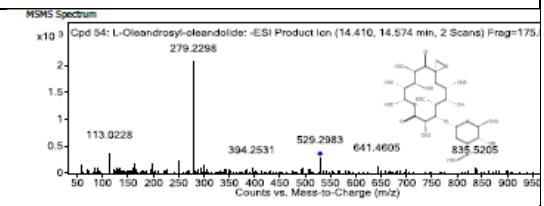

Fig. 5.17.r: L-Oleandrosyl-oleandolide with RT value 14.492

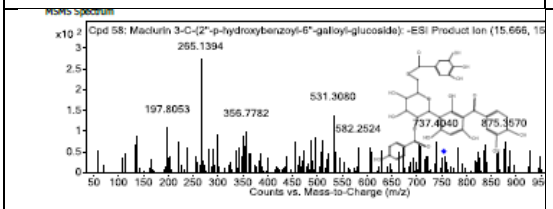

Fig. 5.17.s: Maclurin 3-C-(2"-p-hydroxy benzoyl-6"-galloyl-glucoside)with RT value 15.744

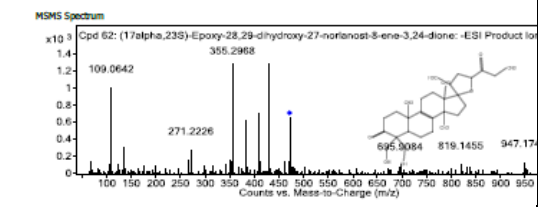

Fig. 5.17.t: (17alpha,23S)-Epoxy-28,29-dihydroxy-27-norlanost-8-ene-3,24-dione with RT value 16.377

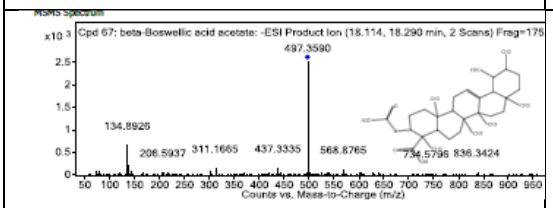

Fig. 5.17.u: beta-Boswellic acid acetate with RT value 18.202

Figure. 5.17: Spectral properties and structure of major bioactive compounds detected in the LC-MS analysis of methanolic root extract of *K. caryophyllata*

Quantitative analysis of secondary metabolites

In order to analyse the dominancy of major phytochemical groups detected in the study and for further in-depth analysis to identify various specific bioactive compounds present and for isolation and characterisation of selected compounds,

quantification of phytochemical groups was carried out in the selected methanolic extract of leaf, stem and root.

Estimation of total alkaloid content

The results of total alkaloid estimation in methanolic leaf, stem and root extract of *K. caryophyllata* obtained is shown in the figure 5.19. Total alkaloid content was estimated as mg atropine equivalent per gram of extract. The regression analysis established a robust linear relationship (y = 0.0121x + 0.0075) with a high correlation coefficient (R^2 = 0.9914), enabling the conversion of absorbance values to milligram atropine equivalents per gram of extract (figure 5.18). The alkaloid concentration was highest in leaf extracts (246.69±7.204mg AE/g) when compared to stem (133.47±5.785mg AE/g) and root extracts (100.96±9.399 mg AE/g)(figure 5.19).

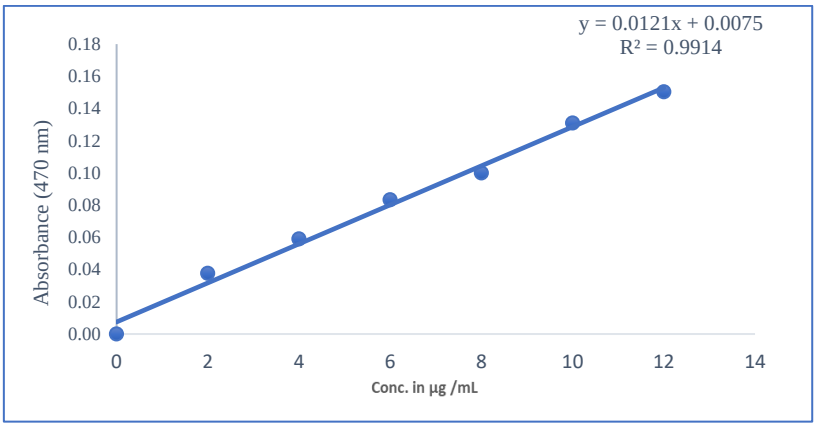

Figure 5.18: Calibration curve of atropine

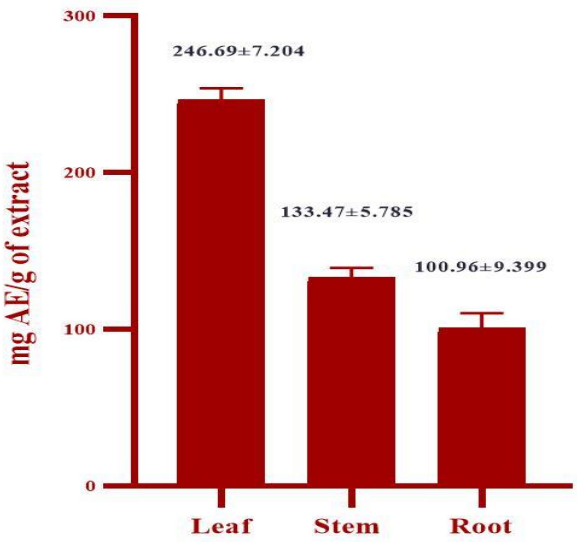

Figure 5.19: Total alkaloid content in methanolic extracts of *K. caryophyllata*

The statistical analysis of total alkaloid content using Duncan's multiple range test reveals that the variation in the content recorded among different plant components were significant (table 5.19). The alkaloid content in the leaf component differed significantly higher over the root and stem components while the lowest content of root significantly differed over leaf and stem contents.

Table 5.19: One-Way ANOVA and Duncan analysis of alkaloid content in the stem, root and leaf components of *K. caryophyllata*

One-Way ANOVA:

F value	P value	η2p (Effect size)
$F_{(2,6)}$= 303.213	P<0.001	η^2_p(Effect size)=0.99

Dunacn's Multiple Range test:

Subset for alpha=0.05			
Plant parts	**1**	**2**	**3**
Root	100.96		
Stem		133.47	
Leaf			246.69
Sig	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Estimation of total phenolic content

The determination of phenolic content among the plant parts of *Kamettia caryophyllata* revealed distinct differences. The amount of phenolic content present in the extract was calculated in terms of milligram gallic acid equivalents/gram of extract using the linear regression equation (figure 5.20). The leaf extracts exhibited the highest phenolic concentration (132.27±6.23 mg GAE/g) and this is immediately followed by stem extract (25.23±1.683 mg GAE/g) and the lowest concentration of 4.04±1.573 mg GAE/g recorded in the root extract (figure 5.21). The phenolic concentration of leaf extracts differed significantly higher over the concentration of other two component extracts. The phenolic concentration recorded by the root extract was significantly lower when compared to leaf and stem extracts (table 5.20).

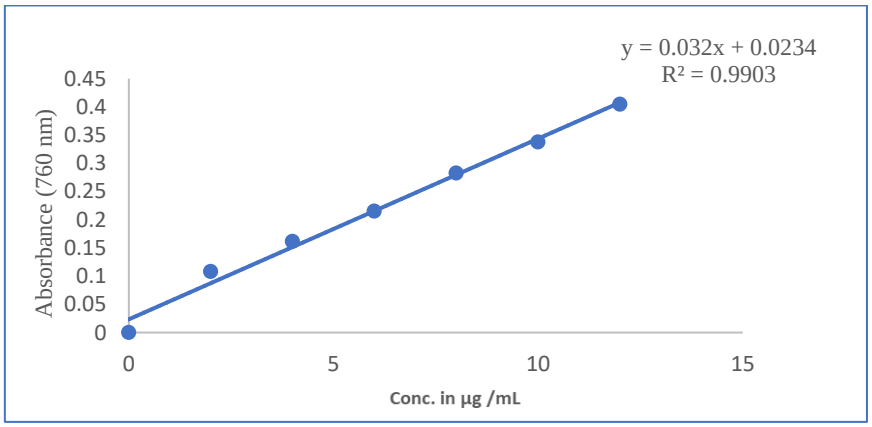

Figure 5.20: Calibration curve of Gallic acid

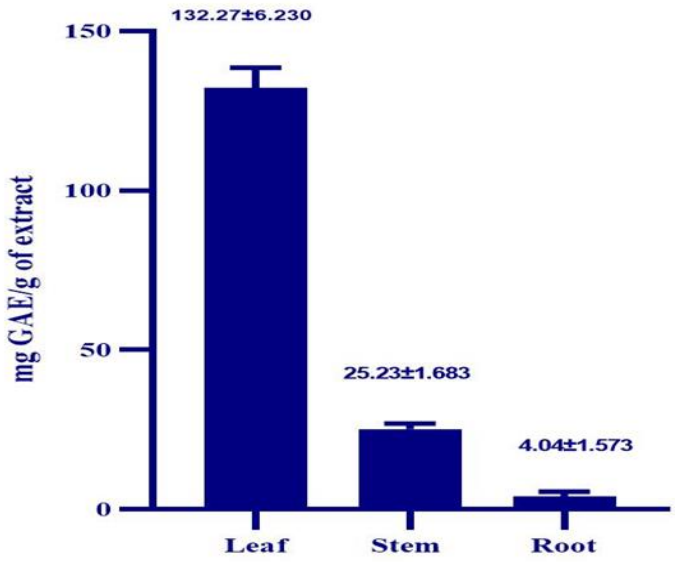

Figure 5.21: Total Phenolics content in methanolic extract of *K. caryophyllata*

Table 5.20: One-Way ANOVA and Duncan analysis of Phenolic Content among Leaf, Stem, and Root of *Kamettia caryophyllata*

One-Way ANOVA:

F value	P value	η2p (Effect size)
F(2,6) = 963.835	P<0.001	η^2_p(Effect size)=0.99

Duncan's Multiple Range Test:

Subset for alpha=0.05			
Plant parts	**1**	**2**	**3**
Root	4.04		
Stem		25.23	
Leaf			132.27
Sig.	1.00	1.00	1.00

Means for groups in homogeneous subsets are displayed.

Estimation of total flavonoid content

The linear regression equation from the calibration curve of quercetin was y=0.0181x-0.0076, while the correlation coefficient was 0.9864 (figure 5.22). Flavonoid levels were found to vary across the plant parts, with stem extracts demonstrating the highest concentration of 157.18±5.237mg QE/g, followed immediately by leaf extracts of 134.22±5.589mg QE/g which was followed by root extracts with a value of 72.89±6.475mg QE/g (figure 5.23). Duncan's multiple range test reveals, the total flavonoid content recorded in the stem component differed significantly higher over the values of leaf and root components and similarly, the total flavonoid content recorded in the root component was significantly lower over the values of stem and leaf components (table 5.21).

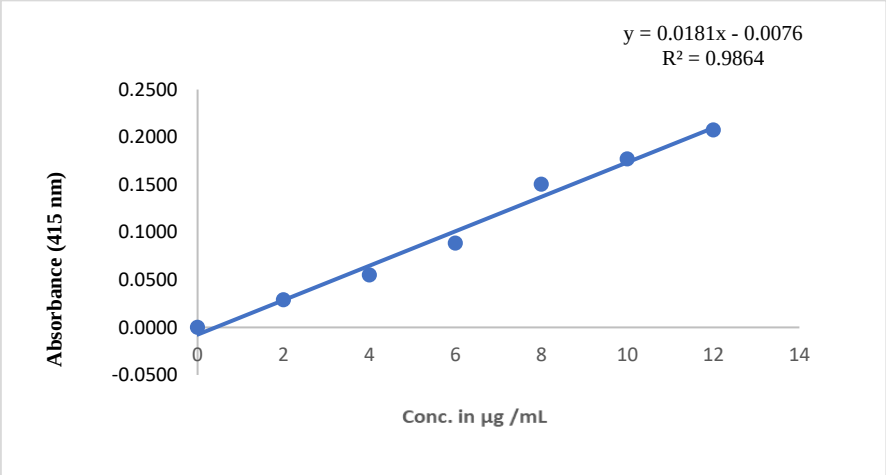

Figure 5.22: Calibration curve of Quercetin

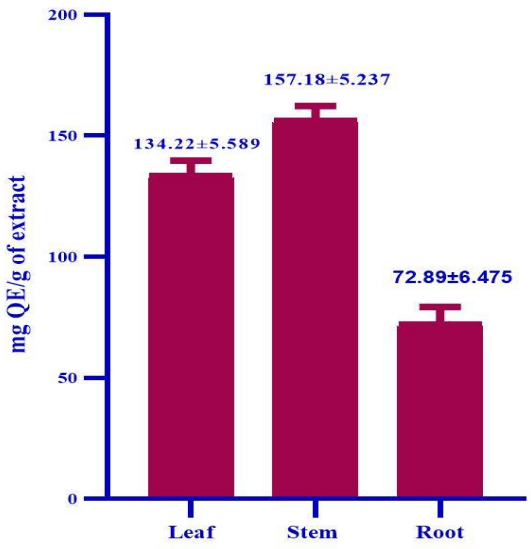

Figure 5:23: Total flavonoid content in the methanolic extracts of *K. caryophyllata*

Table 5.21: One-Way ANOVA and Duncan analysis of total flavonoid content in the leaf, stem, and root components of *K.caryophyllata*

One-Way ANOVA:

F value	P value	η2p (Effect size)
$F_{(2,6)}$= 169.889	P<0.001	η^2_p(Effect size)=0.983

Duncan's Multiple Range Test:

Subset for alpha=0.05			
Plant parts	**1**	**2**	**3**
Root	72.89		
Leaf		134.22	
Stem			157.18
Sig	1.00	1.00	1.00

Means for groups in homogeneous subsets are displayed.

Estimation of total tannin content

The amount of tannin present in the extract in terms of milligram tannic acid equivalents/gram of extract was calculated using the linear regression equation obtained from the calibration curve of tannic acid (figure 5.24). Regarding content of tannin levels, leaf extracts contained the most substantial amount (86.03±6.79mg TAE/g) when compared with stem extracts which recorded a lower concentration of 17.63±1.257 mg TAE/g and the root extracts which recorded the least concentration of 2.88±0.823mg TAE/g (figure 5.25). The statistical analysis revealed significant differences in tannin content among component parts. The difference in the tannin content of leaf component over the content of root and stem was significantly higher and similarly, the lowest content recorded in the root component differed significantly over the values of leaf and stem (table 5.22).

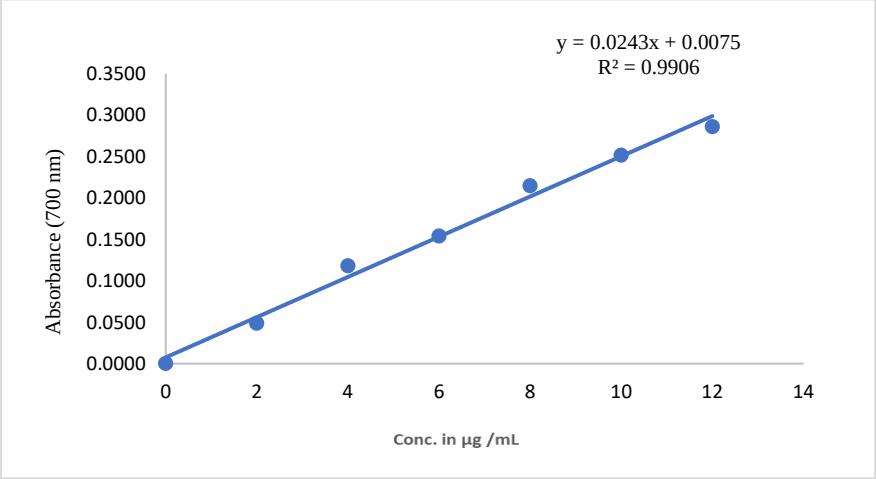

Figure 5.24: Calibration curve of Tannic acid

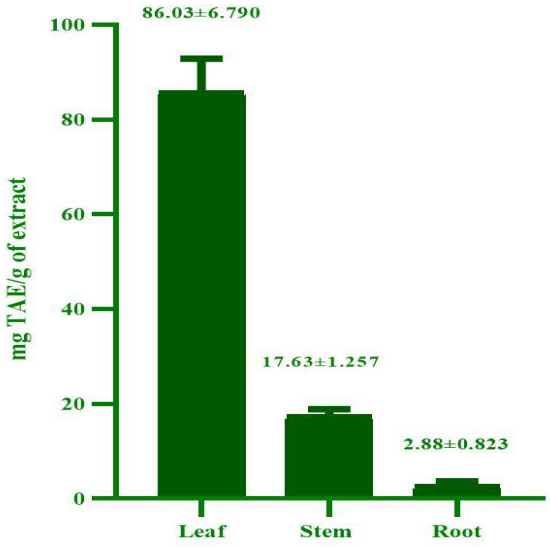

Figure 5.25: Total tannin content in methanolic extracts of *K. caryophyllata*

Table 5.22: One-Way ANOVA and Duncan analysis of tannin content in the leaf, stem, and root components of *K. caryophyllata*

One-Way ANOVA:

F value	P value	η2p (Effect size)
F(2,6) = 366.308	P<0.001	η^2_p(Effect size)=0.99

Duncan's Multiple Range Test:

Subset for alpha=0.05			
Plant parts	**1**	**2**	**3**
Root	2.88		
Stem		17.63	
Leaf			86.03
Sig	1.00	1.00	1.00

Means for groups in homogeneous subsets are displayed

Estimation of total terpene content

The quantification of terpene concentrations in different component parts of *K. caryophyllata* used a calibration curve based on linalool, allowing the calculation of terpene content in milligrams of linalool equivalents per gram of extract (figure 5.26). Leaf extracts exhibited the highest terpene content (257.9±6.324 mg LE/g), followed immediately by stem (130.641±4.622 mg LE/g) and then root (22.87±1.881 mg LE/g extracts (figure 5.27).

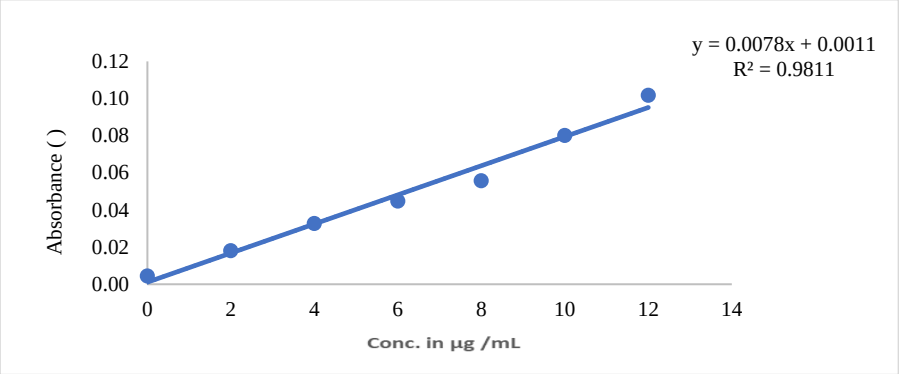

Figure 5.26: Calibration curve of Linalool

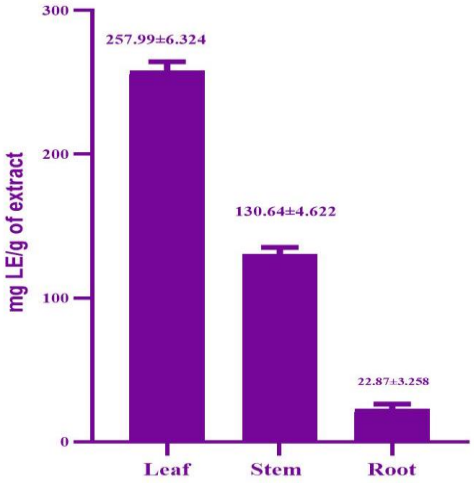

Figure 5.27: Total terpene content in methanolic extract of *K. caryophyllata*

The terpene content recorded in the leaf extracts differed significantly higher over the concentration of other two component extracts. The lowest content recorded by the root extract was significantly lower when compared to the terpene content of leaf and stem extracts (table 5.23).

Table 5.23: One-Way ANOVA and Duncan analysis of terpene content in the leaf, stem, and root components of *K.caryophyllata*

One-Way ANOVA:

F value	P value	η2p (Effect size)
$F_{(2,6)}$= 1732.279	P<0.001	η^2_p(Effect size)=0.998

Duncan's Multiple Range Test:

Subset for alpha=0.05			
Plant parts	**1**	**2**	**3**
Root	22.87		
Stem		130.64	
Leaf			257.99
Sig.	1.00	1.00	1.00

Means for groups in homogeneous subsets are displayed

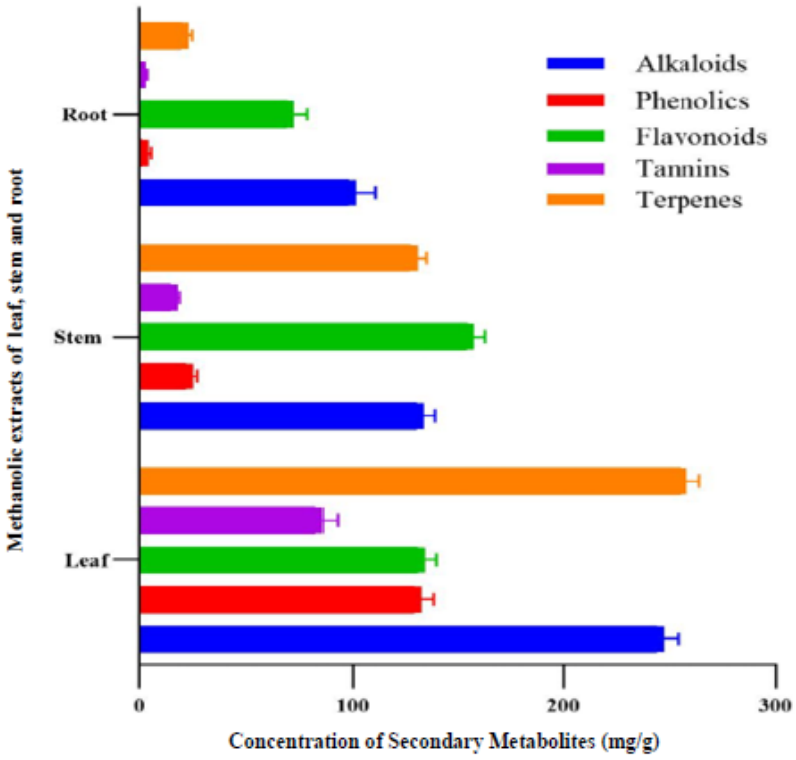

Figure 5.28: Concentration of secondary metabolites in leaf, stem and root methanolic extract of *K. caryophyllata* (mg/g)

Plants are recognized for their capability to produce over 50,000 secondary metabolites that hold medicinal and therapeutic significance (Patil et al., 2023). The family Apocyanaceae, to which *K. caryophyllata* belongs, is known for its diverse bioactive compounds, which give medicinal properties to the plants (Fadahunsi et al., 2021). Jayaraj et al. (2019) in their study reported, the phytochemical analysis of *Vinca rosea* plant extracts revealed the presence of alkaloids, xanthoproteins, terpenoids, tannins, aromatic acids, flavonoids, amino acids, saponins and phenolic compounds with potent therapeutic effects. The detection of bioactive groups like alkaloids, terpenoids phenolics, flavonoids, glycosides, tannins, saponins, proteins and carbohydrates in the preliminary qualitative phytochemical screening of *K. caryophyllata* is a clear indication of its pharmacological significance and its possible scopes for the utilisation in the development of drugs and treatment of many human ailments. Another important inference derived from the observation of the study and related literatures is that, many phytochemical groups detected in the plant are sharing common therapeutic or medicinal properties. This includes, the antioxidant property shared by bioactive groups like phenolics, alkaloids, flavonoids, glycosides and

tannins, as these phytoconstituents are already reported to have strong radical scavenging property (Ndukwe & Ikpeama, 2013). These chemical groups are also known to be potentially toxic to the growth and development of wide range of pathogenic microorganisms and in this respect, they are also sharing common antimicrobial property (Okwu & Josiah, 2006). Similarly, the chemical groups terpenoids, flavonoids, glycosides, alkaloids and tannins are further reported to have anticancer, anti-inflammatory and anti-allergic activity (Prieto et al., 1999; Priyanga et al., 2014). It is well known that synthetic drugs are mostly exerting their medicinal action based on a single xenobiotic compound while therapeutic action of herbal medicines is generally based on synergistic or additive action of multiple compounds acting at single or multiple target sites associated with a physiological process. This kind of combined effect of natural medicine is not only effective in eliminating wide range of ailments as well as pathogenic organisms, but also reduce the chances of pathogenic organisms developing resistance (Parekh, 2007).

The variation in the profiles of phytochemical groups as well as specific bioactive compounds obtained from different component parts of *K. caryophyllata* in different solvent extracts, underscore the importance of using multiple extraction solvents and different plant components in phytochemical studies. This is useful to get a comprehensive information picture regarding the diversity of phytoconstituents, their distribution within the plant body and solvents suitable for extraction of different phytoconstituents. The broad range of phytochemical groups detected in the methanol extracts of leaf, stem and root in *K. caryophyllata* compared to chloroform and petroleum ether extracts lead to focus on these specific extracts for further in-depth exploration and characterization in subsequent research endeavours (Masota et al., 2023). This strategic selection of methanol extracts from different plant parts sets the stage for more targeted investigations to elucidate the individual bioactive properties and potential synergistic effects in the plant, further highlighting the need for isolation and exploration of individual compounds (Nascimento et al., 2000).

Several specific bioactive compounds detected and identified in the present study through GC-MS and HR LC-MS analysis are reported to have interesting biological activities. The results of the HR LC-MS screening in the methanolic leaf extract of *K. caryophyllata* revealed, out of the 38 bioactive compounds detected in the methanolic

leaf extract of *K. caryophyllata*, 18 compounds were identified as terpenoids; 7 compounds as phenolics and 4 compounds as alkaloids, while the results of the GC-MS screening of the same revealed out of the 31 bioactive compounds, 4 compounds identified as terpenoids. Similarly, HR LC-MS screening of methanolic stem extract showed out of the 31 bioactive compounds detected, 11 compounds were identified as terpenoids; 3 compounds as phenolics and 10 compounds as alkaloids, while with respect to the GC-MS screening of the same revealed out of the total 12 compounds detected, 7 compounds identified as terpenoids. However, the screening of methanolic root extract revealed out of the total 31 compounds detected in the HR LC-MS screening, 8 compounds identified as terpenoids; 3 compounds as phenolics and 10 compounds as alkaloids and 12 compounds as alkaloids, while GC-MS screening of the same revealed out of the total 24 compounds detected, 12 compounds identified as terpenoids.

Overall, 69 bioactive compounds were detected in HR LC-MS and GC-MS screening of methanolic leaf extract of *K. caryophyllata*, out of which 24 compounds were found representing the phytochemical group terpenoids, while 7 compounds were representing phenolics, whereas only 4 compounds were representing alkaloids (table 5.7 & 5.12). This constitutes around 34.78%, 10.14% and 5.8% of the total compounds, respectively for the groups terpenoids, phenolics and alkaloids. With respect to the methanolic stem extract, out of the total 43 compounds detected (table 5.8 & 5.15), 18 compounds (41.86%) were representing terpenoids, while 3 compounds (6.98%) were representing phenolics, whereas 10 compounds (23.26%) were representing alkaloids. With respect to the methanolic root extract, out of the total 55 compounds detected (table 5.9 & 5.18), 20 compounds (36.36%) were representing terpenoids, while 3 compounds (5.45%) were representing phenolics, whereas 10 compounds (18.18%) were representing alkaloids. Based on these observations, the study inferred that the phytochemical group terpenoids stand as the major dominating group with highest number of bioactive compounds compared to other groups like alkaloids and phenolics. While comparing different component parts of the plant, the leaf component recorded the highest number of terpenoid compounds with diverse biological activities over stem and root components.

The GC-MS screening revealed the occurrence of several compounds in *K. caryophyllata* which were previously reported as having anti-inflammatory activities. This includes 8 specific bioactive compounds such as 3,7,11,15-Tetramethyl-2-hexadecen-1-ol; Trans-13-Octadecenoic acid, methyl ester; Phytol; 9,12-Octadecadienoic acid(Z,Z)-; Cis-Vaccenic acid; Squalene; dl-α-Tocopherol and γ-Sitosterol in the leaf component, 8 compounds like Trans-13-Octadecenoic acid; dl-α-Tocopherol; Campesterol; Stigmasterol; Squalene; γ-Sitosterol; Lupeol and 12-Oleanen-3-yl acetate, (3α)- in the stem component and 10 compounds such as cis-10-Heptadecenoic acid; Urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)-; Squalene; dl-α-Tocopherol; Campesterol; Stigmasterol; γ-Sitosterol; 4,22-Stigmastadiene-3-one; Lupeol and 12-Oleanen-3-yl acetate, (3α)- in the root component (Lalitha *et al*., 2014; Krishnamoorthy & Subramaniam, 2014; Santhosh *et al*., 2014; Jones, 2002; Haider *et al*., 2016; Rajalakshmi & Mohan,2016; Miras-Moreno et al., 2016; Huang et al., 2009; Cardino et al., 2015; Dutta et al., 2021). Similarly, the HR LC-MS screening revealed 15 compounds in the leaf component such as 3beta,6beta Dihydroxynortropane; Umbelliferone; Triprolidine; (+)-Myrtenyl formate; Stigmast-4-ene-3,6- dione; Flazine; Physalin K; Theasinensin C; Fukinolic acid; (-)-Quebrachamine; 9-HOTE; Lyciumoside IX; Grossamide; Alloxanthin and Saikosaponin BK1 and likewise, 7 compounds in the stem component such as 3beta,6beta-Dihydroxynortropane; Kurchessine; 3beta-3-Hydroxy-18-lupen-21-one; Azelaic acid; Physalin K; Sinomenine; Maclurin 3-C-(2"-p-hydroxybenzoyl-6"-galloyl-glucoside and 11 compounds in the root component such as 3beta,6beta-Dihydroxynortropane; Capsaicin; Kurchessine; Akuammidine; Azelaic acid; 7-Hydroxy-3-methoxy-1-primeverosyloxyxanthone; 1-O-E-Cinnamoyl-(6-arabinosyl glucose); Polygonolide; Maclurin 3-C-(2"-p-hydroxy benzoyl-6"-galloyl-glucoside); beta-Boswellic acid acetate were also reported to have anti-inflamatory activities (Mazimba, 2017; Shim et al., 2022; Awadh et al., 2017; Abubakar & Majinda, 2016; Kim et al., 2016; Meira et al., 2022; Liu et al., 2021; Gavin et al., 2013; Zhao et al., 2023; Meljon et al., 2012; Yao et al., 2011; Mostafa et al., 2018; Konishi et al., 2008; Sulaiman et al., 2022; Jiang et al., 2020; https://pubchem.ncbi.nlm.nih.gov; Phairong et al., 2020; Bereksi et al., 2018; Doss & Parivuguna, 2015). This observations in the study strongly indicates the plant possess anti-inflammatory property.

The antioxidant property is another major therapeutic property that is generally focussed on with plant chemical constituents. The GC-MS screening test conducted in the study revealed the presence of several bioactive compounds in *K. caryophyllata*, which were previously reported for antioxidant activity. This includes occurrence of eleven (11) bioactive compounds like Eugenol; Dodecanoic acid, methyl ester; Dodecanoic acid; Methyl tetradecanoate; Tetradecanoic acid; Hexadecanoic acid, methyl ester; n-Hexadecenoic acid; Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester; Tetratetracontane; Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester and dl-α-Tocopherol in the leaf component extract and similarly, detection of two compounds n-Hexadecanoic acid and dl-α-Tocopherol in the stem component extract and eight compounds such as Dodecanoic acid; Tetradecanoic acid; Hexadecanoic acid, methyl ester; n-Hexadecanoic acid; C9-Octadecenoic acid (Z)-, methyl ester; Urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)-; dl-α-Tocopherol and 4,22-Stigmastadiene-3-one in the root component extract (Gupta *et al*., 2002; Ravikumar *et al*., 2012; Lalitha *et al*., 2014; Lalitharani, 2009; Markkas & Madhuramozhi, 2015; Gnanavel & Mary Saral, 2013; Rajalakshmi & Mohan,2016; Sivakumar & Gayathri, 2015; Huang et al., 2009; Cardino et al., 2015; Oh et al., 2021; Miras-Moreno et al., 2016). Likewise, the HR LC-MS screening revealed occurrence of 5 compounds such as Umbelliferone; (+)-Myrtenyl formate; Deinoxanthin; Cucurbitachrome 1 and Theasinensin C in the leaf component and similarly, 4 compounds Azelaic acid; Maclurin 3-C-(2"-p-hydroxybenzoyl-6"-galloyl-glucoside) and Somniferine; (+)-Lyoniresinol 9-glucoside in the stem component and occurrence of 7 compounds such as Capsaicin; Azelaic acid; Secoxyloganin; 7-Hydroxy-3-methoxy-1-primeverosyloxyxanthone; 1-O-E-Cinnamoyl-(6-arabinosyl glucose); Schidigeragenin C; Maclurin 3-C-(2"-p-hydroxy benzoyl-6"-galloyl-glucoside) and (6beta,8betaOH)-6,8-Dihydroxy-7(11)-eremophilen-12,8-olide also reported to have antioxidant activity (Mazimba, 2017; Awadh Ali et al., 2017; Ji. HF, 2010; Guerriero et al., 2018; Liu et al., 2021; Kumar & Kumar, 2021; Rahim et al., 2022; https://pubchem.ncbi.nlm.nih.gov; Phairong et al., 2020; Bereksi et al., 2018; Ruan et al., 2020). The occurrence of this much number of bioactive compounds having antioxidant activity revealed through GC-MS and LC-MS screening, indicates that plant *K. caryophyllata* possess considerable level of antioxidant property.

There are many specific phytocompounds detected in *K. caryophyllata* reported to have antimicrobial property. The GC-MS screening detected 10 bioactive compounds in this respect such as 3,7,11,15-Tetramethyl-2-hexadecen-1-ol; 9-Hexadecenoic acid, methyl ester, (Z)-; Phytol; Cis-Vaccenic acid; Benzene, [(methylsulfinyl)methyl]-; 2-Hydroxy-gamma-butyrolactone; Pentadecanoic acid, methyl ester; Squalene; Octadecanoic acid, methyl ester and Octadecanoic acid in the leaf extract, while 4 compounds detected such as β-Amyrin; Lupeol; Squalene and 12-Oleanen-3-yl acetate, (3α)- in the stem extract and 9 specific compounds such as Globulol; cis-10-Heptadecenoic acid; C9-Octadecenoic acid (Z)-, methyl ester; Octadecanoic acid; Urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)-; Squalene; β-Amyrin; Lupeol and 12-Oleanen-3-yl acetate, (3α)- in the root extract and reveals antimicrobial property of the plant (Lalitha et al., 2014; Santhosh et al., 2014; Hirotani et al., 1991; Moorthy & Boominathan, 2011; Belakhdar et al., 2015; Rahuman, 2000; Kwun et al., 2021; Huang et al., 2009; Cardino et al., 2015; Dutta et al., 2021; Oh et al., 2021; Xu et al., 2018). In addition to these, the HR LC-MS screening also detected 10 compounds with antimicrobial property such as (+)-Myrtenyl formate; Physalin K; Theasinensin C; Fukinolic acid; Methanophenazine; Melleolide D; 26-Glucosyl-1,3,11,22-tetrahydroxyergosta-5,24-dien-26-oate; Prodelphinidin A2 3'-gallate; Squalamine and 33-Deoxy-33-hydroperoxyfurohyperforin in the leaf component extract and similarly, 5 compounds in the stem extract (table 5.8 & 5.15) and 13 compounds in the root component extract (table 5.9 & 5. 18) (Awadh et al., 2017; Meira et al., 2022; Liu et al., 2021; Gavin et al., 2013; Jo et al., 1998; Dörfer et al., 2019; Dawe, 2014; Cheng et al., 2002; (Limbocker et al., 2021; Lee et al., 2006; Sulaiman et al., 2022; Ibarra-Laclette et al., 2015; https://pubchem.ncbi.nlm.nih.gov; Rattanachak et al., 2022; Phairong et al., 2020; Bereksi et al., 2018; Doss & Parivuguna, 2015; Ruan et al., 2020). These observations are further indicating that the plant possess antimicrobial property

The quantitative determination of secondary metabolites in the methanolic extract of leaf, stem and root of *K. caryophyllata* revealed significant variation in the content of major phytochemical groups alkaloids, phenolics and terpenoids among these plant parts(figure 5.28). The highest concentration of these phytochemical groups was recorded in the leaf component and among this, the group terpenoids recorded comparatively highest concentration in the leaf component than in the stem and root.

This observation in the study was supported and well substantiated by the inference obtained from GC-MS and HR LC-MS screening, where the highest number of specific bioactive compounds with chemical nature belongs to terpenes recorded in the methanolic leaf extract. Further, many unknown peaks were observed in HR LC-MS and GC-MS chromatograms of the leaf, stem, and root extracts, highlighting the complex nature of *K. caryophyllata*. These unidentified peaks suggest the existence of numerous compounds that have not yet been characterised, indicating a rich and diverse phytochemical composition (EI Sayed et al., 2020). A diverse category of compounds was most abundant in the leaf extract, followed by the stem and root extracts. This diversity may account for superior therapeutic properties of the leaf component compared to those of stem and root. Additionally, the synergistic and additive actions of these diverse compounds likely enhance the overall medicinal properties of the leaf extract compared to stem and root extracts.

CONCLUSION

The production of herbal formulations and isolation of therapeutic potential compounds have been the major research areas for a long time. This chapter has validated the phytochemical constituents in different solvent extracts of leaf, stem, and root components of *K. caryophyllata,* which have not been much reported previously. The preliminary screening done for phytochemical groups like alkaloids, phenolics, flavonoids, terpenoids etc. in leaf, stem and root component extracts of *Kamettia caryophyllata* revealed most abundant and diverse types can be extracted from the leaf component specifically in methanolic extract, compared to all other solvent extracts including stem and root. The detection and identification of specific bioactive compounds in different component extracts performed using HR LC-MS and GC-MS analysis provided insight into the phytochemical profile and revealed variation in composition, nature and bioactivity of specific chemical compounds across different plant parts. The highest number of bioactive compounds with more diverse activity has been recorded in the leaf component followed immediately by root and then stem component. The quantitative estimation of various secondary metabolites in methanolic extract of different component parts indicates majority of the secondary metabolites including terpenoids, alkaloids and phenolics have highest concentration in the leaf component compared to stem and root. Among various

phytochemical groups quantified in the leaf component, the group terpenoids was found dominating over other groups. Overall, the study concluded that the leaf component extract and particularly the methanolic extract of *K. caryophyllata* have comparatively more diverse and higher concentration of various phytochemical groups and corresponding higher number of specific bioactive compounds with interesting biological activities like anti-inflammatory, antioxidant and antimicrobial activities, suggesting their superior pharmacological potential. The study suggests isolation and characterization of specific bioactive compounds of interest and to conduct necessary experiments on their biological activities for confirmation and safety.

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Chapter 6

Bioactivity Studies of *Kamettia caryophyllata*

CONTENTS

INTRODUCTION

Medicinal plants have been used traditionally for centuries across different cultures and are recognized for their therapeutic applications. The medicinal plants contain a wide range of phytochemicals, which have a wide range of physiological effects in humans and animals (Rabizadeh et al., 2022). Secondary metabolites are chemical molecules produced by plants that remain unknown in their direct roles in growth and development, but instead they are found to exist as compounds of defense providing protection for plants from various biotic and abiotic stresses (Asadi Samani et. al., 2016; Kinghorn, 1994; Demain & Fang, 2000). Understanding the use of plants by traditional healers in treating various ailments as well as the knowledge about the role of phytochemicals in defense and protection mechanism of plants have provided valuable insights into their potential applications in treating human ailments. (Ahmad & Ahmad, 2019). This has stimulated the interest of researchers and scientists to search for phytochemicals in plants that possess pharmacological properties and therapeutic potential(Wink, 2010). Now a days, the screening of phytochemicals and their bioactivity evaluation studies is one of the basic objectives in the field of therapeutic industry.

In vitro bioassays are the first step in the preclinical screening process for herbal

remedies, allowing researchers to make informed decisions about which plants or compounds are worth investigating more closely. These studies are crucial in analysing and evaluating several fundamental properties like antioxidant, antimicrobial, cytotoxic and anti-inflammatory activities. More often, plants contain phytochemicals with antioxidant properties to manage oxidative stress due to free radicles generated under various stress conditions. Examining the antioxidant activity in plant extracts enables us to figure out its capacity to defend against hazardous free radicals causing oxidative stress linked to many health problems in humans (Sujatha & Sekar, 2019). Similarly, there are several phytocompounds that exhibit antimicrobial properties including antibacterial, antifungal and antiviral activities. In order to develop new antimicrobials, it is essential to evaluate the plant extracts' ability to fight against different pathogenic organisms through antimicrobial activity assessment (Khan et al., 2013). Certain studies claim that cytotoxicity assessment helps to find out therapies that mainly target and damage potentially harmful cells while sparing healthy ones by providing only minor damage as possible (Nguyen et al., 2017). Researchers explore the anti-inflammatory characteristics of plant extracts to learn about their potential for reducing inflammation, a prevalent characteristic in numerous illnesses and disorders. Understanding these characteristics is essential to create therapies for various inflammatory diseases in humans (Miras-Moreno et al., 2016).

A lot of bioactivity studies have already been carried out to reveal scientific evidences to therapeutic efficacy of medicinal plants, which have been traditionally used by ancient healers for centuries. For instance, the phytochemical catechins present in *Camellia sinensis* is scientifically proved as strong antioxidant in humans (Siddiqui et al., 2004). Carson et al. (2006) reported strong potent antimicrobial activity of tea tree oil extracted from the plant *Melaleuca altemifolia* against wide range of pathogenic organisms. Hamidpour et al. (2013) reported anti-inflammatory property of phytocompound boswellic acids extracted from *Boswellia serrata*. Likewise, Linde et al. (2008) has scientifically demonstrated the effectiveness of *Hypericum perforatum* plant extract to treat depression. Such studies highlight the importance of scientific evidences in validating traditional uses and expanding the therapeutic applications of medicinal plants. Scientific studies will help the researchers and scientists to

identify and isolate the specific bioactive principles in medicinal plants, which may be useful in enhancing the therapeutic efficacy either by their direct application in required concentrations or by utilizing them for developing new combinations and formulations of drugs for treating various specific diseases and disorders.

The World Health Organization's (WHO) Traditional Medicine Strategy (2014-2023) emphasises evaluating traditional medicine. Bioactivity screening approaches are recommended for randomly collected common or untested plants. This approach involves a broad screening and further investigation of the most promising activity using a specific bioassay, which helps to ensure the quality, safety, appropriate usage and efficacy of traditional medicines, along with other specified objectives. The present chapter intend to investigate and standardise the therapeutic potential of leaf, stem, and root methanolic extracts of *Kamettia caryophyllata* by evaluating its bioactivity properties like antioxidant, antimicrobial, cytotoxic and anti-inflammatory activities using a variety of bioassays.

REVIEW OF LITERATURE

Medicinal plants, all over the world, have been found to possess crucial bioactive chemicals that can aid in the prevention of many ailments such as cancer, diabetes,heart disease etc. (Prasathkumar et al., 2021). The main benefit of employing medications made from plants is that they are typically safer than synthetic alternatives, have powerful therapeutic effects and are less expensive. Different plant species manufacture secondary metabolites in different ways. These secondary metabolites are molecules that do not appear to be required for plant growth but on the other hand, have been shown by research to have significant functions in plants, such as protection against ultraviolet radiation exposure, resistance to illnesses caused by viruses, fungus, bacteria, and phytopathogens, and keeping herbivores at bay (Guerriero et al., 2018). These secondary metabolites, which belong to three major families known as polyphenols, terpenes and alkaloids, are the most interesting in therapeutics.

Plants are an essential source for discovering novel medical compounds for drug development, and secondary metabolites are sources of medicines from plants. The

biologically active phytochemicals interact with biological targets and modulate physiological processes, making them valuable for healthcare applications. Secondary metabolites are generally regarded as the compounds of defense and signalling molecules of an organism against environmental stresses and predators and involved in various molecular interactions like symbiosis, competition, and metal ions transport (Kinghorn, 1994; Demain & Fang, 2000). Secondary metabolites are involved in improving health as they can act as antibiotics, anabolises, immunomodulators, and growth promoters. Some act as nutraceuticals, pesticides, insecticides, and pheromones and display established health-promoting effects and significant roles as disease eradicators (Thirumurugan et al., 2018). Medicinal plants often contain multiple bioactive compounds that can work synergistically, enhancing their therapeutic effects. This phenomenon is commonly observed in traditional herbal remedies. For example, the combination of curcumin and piperine from black pepper (*Piper nigrum*) has been shown to increase the bioavailability and effectiveness of curcumin (Shoba et al., 1998).

According to previous research reports, crude extracts and isolated chemicals of Apocynaceae family members have various pharmacologically advantageous qualities, such as antioxidant, antimicrobial, cytotoxic, and anti-inflammatory activities. (Bhadane et al., 2018). Apocynaceae plants, including *Tabernaemontana divaricata, Rauvolfia serpentina, Carissa carandas, Catharanthus roseus, Nerium oleander* and *Plumeria alba* exhibit a wide range of pharmacological effects including antimicrobial, antioxidant, anti-inflammatory, and anticancer qualities and are potentially useful to treat various human and animal health issues (Islam & Lucky, 2019). The existing literature about Apocyanaceae member *Kamettia caryophyllata*'s bioactive potential is evidently lacking. The whole plant has been traditionally used for lichen and leprosy, weakening, tearing, arthritic pains, itching and scabies, spasms and epilepsy, cachexia, and anodyne poison (Manilal & Remesh, 2010).

Medicinal plants are often rich in antioxidants, which aid in neutralising toxic free radicals and reduce oxidative stress in the body. Green tea *(Camellia sinensis)* contains catechins with strong antioxidant properties (Siddiqui et al., 2004). Free radical species are characterised by one or more unpaired electrons, formed continuously in the living body and removed by nonenzymatic and enzymatic

reactions. When these mechanisms fail to defend against oxidative stress, they can damage proteins, carbohydrates, lipids, and DNA, which cause different kinds of diseases. The antioxidant effects of plants contribute to their protective role against various chronic diseases, including cardiovascular disorders and neurodegenerative diseases. Phytochemicals such as flavonoids, phenolic compounds, and carotenoids act as potent anti- oxidants, preventing cell damage caused by free radicals. Research studies have demonstrated the role of phytochemical antioxidants in preventing oxidative damage and reducing the risk of chronic diseases (Bhatt et al.,2013; Surh, 2003). Many plants under the Apocyanaceae family exhibit antioxidant properties and are used for therapeutic purposes (Halliwell, 1996). Teugwa et al. (2013) observed that the Apocyanaceae plant *Picralima nitida* leaves have antioxidant properties and could be used in herbal medicine.

The DPPH assay is a quick, easy and affordable approach for determining antioxidant properties of herbal extracts. It is a spectrophotometric technique that measures the change in DPPH concentration after it reacts with an antioxidant. Every evaluation of antioxidants as well as phytochemical capabilities of plant extraction should include an anti-free-radical activity analysis using DPPH (Halliwell, 2011; Ye et al., 2015). Antioxidant ability should not be tested with a single method; at least three different in vitro antioxidant methods must be performed together to determine antioxidant activity. A pure-only method does not reflect antioxidant activity. Therefore, the methods to be used in analysis for research purposes should be carefully selected and applied (Munteanu & Apetrei, 2021). Hydrogen peroxide (H_2O_2) scavenging activity of antioxidants present in plant extracts has been determined widely by measuring decrement of H_2O_2 in an incubation system containing H_2O_2 and the scavenger using the classical UV-method at 230 nm (Keser et al., 2012; Ahmad et al., 2014; Ruch et al., 1989). This is a simple and rapid colorimetric assay to determine H_2O_2 scavenging activity of plant extracts based on the reaction system where H_2O_2 rapidly reacts with phenol and 4-aminoantipyrine in the presence of horseradish peroxidase (HRP) to produce a pink coloured quinoneimine dye (Modrovich, 1983). H_2O_2 scavengers will eventually result in decreased production of this particular chromophore. This method is applied to standard antioxidants ascorbic acid, gallic acid and tannic acid in addition to selected plant extracts to determine their hydrogen peroxide scavenging abilities. The phosphomolybdenum assay is another method used to measure the total

antioxidant capacity of plant extracts and their ability to scavenge free radicals. This assay is based on the reduction of phosphomolybdate ion in the presence of an antioxidant resulting in the formation of a green phosphate/MoV complex which is measured spectrophotometrically at 695 nm.

Microbial infections are considered as a significant public health problem worldwide. Many countries in Africa and other parts of the world have continued to encourage screening programs of plants used in traditional medicine to authenticate their antimicrobial activities and possible inclusion in primary health care (Bereksi et al., 2018). Medicinal plants with antimicrobial properties make them useful in combating infectious diseases. Many plant compounds exhibit anti-bacterial, anti-viral, anti-fungal, and anti-parasitic activities. For example, tea tree oil derived from *Melaleuca altemifolia* has shown significant anti-microbial effects against a wide range of microorganisms (Carson et al., 2006). Garlic *(Allium sativum)* exhibits potent antimicrobial activity against both Gram-positive and Gram- negative bacteria (Naganawa et al., 1996). The antimicrobial properties of medicinal plants provide potential therapeutic options in the management of infectious diseases.

Bacterial infection can also occur due to multi-drug resistance which leads to mortality and morbidity (Kumar et al., 2009). For that reason, antibiotic resistance has become a global concern. The increase in the multi-drug resistance of bacteria threatens the therapeutic efficacy of several drugs. Therefore, new antibacterial drugs are needed to treat various diseases with low toxicity and less price. For that purpose, secondary metabolites from plants are currently considered for developing new drugs because they are rich in natural compounds. Using different solvent systems, numerous researchers have studied plants' antibacterial activities of leaves, flowers, stems, roots, and fruits (Jain et al., 2019).

According to Chusri et al., in 2014, the Apocynaceae family members like *Allamanda cathartica, Cerbera manghas, Thevetia peruviana Adenium obesum, Catharanthes roseus, Holarrhena antidysenterica, Nerium oleander, Plumeria obtusa, Plumeria rubra, Wrightia pubescens, Alstonia scholaris, Cerbera odollam, Rouwolfia serpentina* are well-known for its wide range of bioactive constituents that possess antimicrobial qualities. Grujic et al., in 2014, investigated the antimicrobial activity

of ethanol and diethyl ether extracts of *Vinca major, V. minor,* and *V. roseus* using the microdilution method; the MIC values were found to vary from 19.53-2500 µg/ml. The inhibitory effect of *Thevetia peruviana* extract against *Klebsiella schum* was evaluated using the agar well diffusion method (Raghavendra & Mahadevan, 2011). According to Parastoo et al. (2012), the Agar well diffusion method is frequently employed to assess the antibacterial activity of plants or microbial extracts. A novel acylated triterpene was identified from the alcoholic leaf extract of *Rauvolfia vomitoria*, and it has shown antifungal properties against the drug-resistant fungus *Candida albicans*, with a minimum inhibitory concentration (MIC) of 64 µg/ml (Fannang et al., 2011).

Since the mid-1900s, cytotoxic studies have attracted significant attention because of their ability to reveal possible antitumor and anticarcinogenic properties which have historically served as a foundation for the creation of revolutionary anticancer medications. As reported by Fiskesjo in 1985, the *Allium cepa* assay is widely used to investigate cytotoxicity in plant species. The *Allium* test correlates with other test methods, such as the MIT-217 cell test that is used in vivo mice, rats, or human subjects, as demonstrated by Fiskesjo and Levan in 1993. This association points to the *Allium* assay's potential to replace laboratory animals in toxicological research is noted by Abu and Mba in 2011. One potential explanation for the impact of plant extract on the mitotic index is that it breaks down DNA synthesis or inhibits spindle formation. When a plant extract is applied, the mitotic index decreases, suggesting that the phytochemicals interfere with the regular sequence of mitosis and may even stop cell division (Abidal & Thoppil, 2003). Aydin and Mammadov, in 2019, state that a positive relationship exists between the prevalence of chromosomal abnormalities and the extract's concentration.

The cytological effects of medicinal plant extracts were demonstrated by numerous researchers. *Azadirecta indica, Morinda lucida, Cymbopogon citratus, Mangifera indica,* and *Carica papaya* were the five medicinal plants whose aqueous extracts were used to test the cytotoxic and genotoxic properties using the *Allium cepa* assay (Akinboro & Bakare, 2007). All of the examined extracts have been shown to trigger mitotic spindle disruption in *A. cepa* and to have mitodepressive effects on the division of cells. Shehab and Adam, in 1983, evaluated the cytological effect of *Anastatica*

hierochuntica water extract on *A. cepa* and observed the presence of various aberrations such as de-spiralation in anaphase and prophase, chromosome bridge, disturbed metaphase and anaphase, and lagging chromosomes. Cytotoxic effect of *Parthenium hysterophorus* on cells of *A. cepa* root tips showed significant aberrations like nuclear vacuolation, polyploidy, clumped metaphase, ball metaphase, chromosome fragmentation and laggards (Seema &Chauhan, 2002). Trypan blue exclusion tests determine the number of live and dead cells based on the principle that intact plasma membranes in live cells exclude specific dyes, whereas dead cells do not(Ribeiro et al., 2020).

Inflammation is a natural defense mechanism of the body. However, chronic inflammation can contribute to the development of several diseases like arthritis, asthma, and inflammatory bowel disease. The phytochemicals possess anti-inflammatory property, inhibiting the production of pro-inflammatory molecules and modulating signaling pathways involved in inflammation and these compounds have shown potential in managing inflammatory conditions and reducing the risk of associated diseases (Gupta et al., 2012; Wang et al., 2013).

Medicinal plants with anti-inflammatory properties offer potential therapeutic benefits. For instance, the anti-inflammatory effects of curcumin from turmeric *(Curcuma longa)* have been extensively studied (Gupta et al., 2012). *Boswellia serrata*, commonly known as frankincense, possesses anti-inflammatory properties attributed to its active compounds such as boswellic acids (Hamidpour et al., 2013). These plant-based anti-inflammatory agents hold promise in the management of inflammatory conditions. The methanolic root extract of *Ricinus communis* (Euphorbiaceae) was investigated in Wistar albino rats for its anti-inflammatory. The study finds that in both acute and chronic inflammatory models in rats, the methanolic *Ricinus communis* root extract possesses considerable anti-inflammatory action (Ilavarasan et al., 2006). The study showed that the apocyanaceae member, *Hancornia speciosa* fruit extract has anti-inflammatory properties in animal models, and it suggested that the rutin and chlorogenic acid content in the extract may be the cause of these properties (Torres-Rêgo et al., 2016). The findings of Castro et al. (2014) supported the traditional use

of *Cryptostegia grandiflora* leaves for their anti-inflammatory efficacy. Ofori and Borquaye (2019) studied the anti-inflammatory potential of the *Strophanthus gratus* (Apocynaceae) ethanol extract and fractions were evaluated using a carrageenan-induced paw edema model in 7-day-old chicks. It was shown that 129.7 ± 10.5 mg/kg of crude extract was needed to mitigate 50% of the generated inflammation. However, the use of medicinal plants for various treatments faces challenges in ensuring quality control, preventing adulteration and contamination, promoting sustainable sourcing and conservation, protecting intellectual property rights, establishing standardized dosage guidelines, addressing limited scientific evidence, and implementing robust regulatory frameworks. These challenges require measures such as quality control, testing, sustainable practices, legal frameworks, research collaboration, and harmonized regulations to ensure the safe and effective utilization of medicinal plants. (Canter et al., 2005; Kala et al., 2006; Mamedov, 2012).

MATERIALS AND METHODS

Plant sample extraction

Kamettia caryophyllata leaves, stems, and roots were carefully cleaned under tap water, air-dried in the shade, and ground into a powder. 1000-gram of powdered plant material was put in a Soxhlet system with methanol as the extraction solvent. After filtering the resultant extract, the filtrate was evaporated in a rotary evaporator at a pressure lower than 40°C until a constant weight was reached. For additional examination, the dried extract was kept in a refrigerator at 4 degrees Celsius.

SECTION A

Antioxidant Activity Analysis

Phosphomolybdenum scavenging assay

Principle: The antioxidant in the plant extract can reduce the molybdenum (VI) present in the reagent to molybdenum (V) and develop a green phosphate/ molybdenum (V) complex at an acid pH. This complex demonstrates maximal absorbance at 695 nm, indicating antioxidant activity.

Reagents: 28 mM sodium phosphate, 0.6 M sulfuric acid, and 4 mM ammonium molybdate.

Protocol: The phosphomolybdenum technique, as outlined by Prieto et al. (1999), was used to determine the total antioxidant capacity of the *K. caryophyllata* methanol extract. The following reagent solution was mixed with 0.1 ml of *K. caryophyllata* extract: 28 mM sodium phosphate, 0.6 M sulfuric acid, and 4 mM ammonium molybdate. For ninety minutes, the reaction mixture-containing test tubes were incubated at 95ºC. After allowing the mixture to cool at average room temperature, the absorbance was observed at 695 nm using a UV-visible spectrophotometer (Systronic, 2203) against blank. Ascorbic acid was used as the standard, and a reagent and solvent without extract were used to make up the control solution. The percentage of scavenging activity at various test drug doses was calculated, and the IC50(half maximal Inhibitory Concentration) value of test drugs was compared with that of ascorbic acid, which was used as the standard.

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

Principle: The DPPH, a stable free radical, displays a deep violet hue in solution due to a broad absorption band centred at roughly 517 nm. When it reacts with an antioxidant, it reduces the violet colour to colourless or pale yellow. This feature allows one to watch the reaction visually and can be used to calculate the radical scavenging activity of the extract.

DPPH + AH → DPPH-H + A

Reagent: 0.1 mM DPPH solution is prepared in methanol

Protocol

With a few minor modifications, the Cheng et al. (2006) procedure was applied to assess the extract's ability to scavenge free radicals using DPPH. 1.5 ml of *K. caryophyllata* methanolic extracts (leaf, stem and root) at different concentrations was mixed with 0.5 ml of a 0.1 mM DPPH solution in methanol. The standard used was ascorbic acid (2–12 µg/ml) and the mixture was then incubated for 30 minutes, and the absorbance at 517 nm was measured. Methanol was utilised as a blank, and the control solution comprised of the reagent and the solvent without extract. Lower

absorbance indicated more significant DPPH free radical scavenging activity. The assay was calculated as indicated:

Scavenging activity = [(Abs. of control − Abs. of the sample)/ (Abs. of control] × 100

The absorbance of the sample denotes the sample being tested, and the control denotes the absorbance of the control sample.

Hydrogen Peroxide (H_2O_2) scavenging assay

Principle: Hydrogen peroxide is a weak oxidant that can detrimentally affect certain enzymes by oxidising their essential active groups. When hydrogen peroxide enters the body, it may generate hydroxyl radicals. The hypothesis behind the hydrogen peroxide scavenging assay is that H_2O_2 will react with the antioxidant present in the plant extract and convert Hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). Then, spectrophotometry is used to quantify the drop in H_2O_2 concentration.

Materials: Hydrogen peroxide solution (40 mM) prepared in phosphate buffer (pH 7.4)

Ascorbic acid standard (1mg/ml)

Protocol: The extract's capability to scavenge hydrogen peroxide (H_2O_2) was estimated according to the method (Ruch et al., 1989). 0.6 mL of 40 mM hydrogen peroxide was mixed with different concentrations (10–100 µg/mL) of extracts and incubated for 10 minutes. Ascorbic acid was used as a standard. The absorbance of each solution at 230nm was measured against a blank containing only phosphate buffer using a UV-visible spectrophotometer. Antioxidants present in the extract neutralise hydrogen peroxide, which reduces the absorbance.

The % of scavenging activity was calculated as follows:

Scavenging activity = [(Abs. of control − Abs. of the sample)/ (Abs. of control] × 100

Calculation of IC50

The half maximal inhibitory concentration (IC50) of extracts and standards from all the assays was computed from graphs by plotting extract concentration in abscissa vs. the percentage inhibition taken in the Y coordinate. A linear regression equation derived from the graph obtained the IC50 values for the extracts and ascorbic acid.

SECTION B

Antimicrobial Activity Assay

Agar Well Diffusion Method

Collection of test organisms and preparation of stock culture

Cultures of 5 pathogenic microorganisms were used for *invitro* analysis. All the microorganisms, namely *Candida albicans*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa* were collected from the Microbiology lab, Jubilee Mission Medical College & Research Institute, Thrissur, Kerala, India.

Materials

Muller-Hinton Agar medium-38 grams of MHA powder in 1 litre of distilled water.

Potato dextrose agar (PDA) medium: 39 gm of Commercial PDA Powder to 1 Litre of Distilled water. Boil while mixing to dissolve. Autoclaved for 15 min at 121°C.

Determination of antimicrobial activity

Antimicrobial activity tests followed the Agar Well Diffusion Method (Magaldi et al., 2004). DMSO (dimethyl sulfoxide) was used to dissolve the methanolic extract of leaf, stem and root of *K. caryophyllata* and filtered through a 0.2 µm nylon filter. For the assay, 200 µl culture suspension of the tested microorganisms (carrying 10^6 colony-forming units /ml of bacteria cells and 10^8 spores/ml of fungal strains) were evenly dispensed on Muller-Hinton Agar medium and Potato dextrose agar (PDA) medium, respectively. Wells were then created using a sterile borer (3 mm depth and 4 mm diameter), and each well was loaded with 100 µl of the sample extract. DMSO, without any extract, served as the negative control. Additionally, 100 µl of various

concentrations of plant solvent extracts (25 µg, 50 µg and100 µg) were added using a sterile syringe into the agar wells, allowing them to diffuse at room temperature for 24 hours at 37°C for bacteria and 72 hours at 30°C for fungal strains.

In the assessment of antimicrobial activity, the diameter of the growth inhibition zones, including the 4 mm diameter of the well, was measured in millimetres. Triplicates were maintained, and readings were taken in three different fixed directions, with the average values recorded. Chloramphenicol was used as the positive standard for bacteria, and Clotrimazole served as the positive standard for fungi.

SECTION C

In Vitro Cytotoxicity Assessment

Cytotoxicity assessment using Trypan Blue exclusion method

Drug preparation for in vitro studies

To make stock solutions, 10 mg of a methanolic extract of various *Kamettia caryophyllata* plant extracts were dissolved in 200 µl dimethyl sulphoxide (DMSO) and mixed with one ml of distilled water. Different dilutions of the methanolic extracts of the leaves, stems, and roots were prepared.

In vitro cell lines

Ehrlich Ascites Carcinoma (EAC) and Daltons Lymphoma Ascites (DLA) cells were utilised as cancer cell lines in mice. Control cells were derived from rat spleen. The research was carried out at the Amala Cancer Research Institute, Thrissur.

Propagation of tumour cell lines

The Daltons Lymphoma Ascites and Ehrlich Ascites Carcinoma cells were maintained in the intraperitoneal cavity of Swiss albino mice. At first, 1 x 10^6 cells (100 µl) were injected into the intraperitoneal cavity of mice. After 15 days, the cells were aspirated using a 1 ml syringe and phosphate-buffered saline. The cells were washed three times in Phosphate Buffered Saline (PBS) to remove impurities. The number of cells was counted using a haemocytometer and made up to 100 µl PBS containing 1 x 10^6 cells.

Then, the cells were injected into the intraperitoneal cavity of another mouse, and it continued at 15-day intervals.

Preparation of control cell lines

Healthy Swiss albino Mice were anaesthetised with carbon dioxide, and the tissue from the spleen was removed. After that, it was broken into a single-cell suspension in a Dulbecco's Modified Eagle Medium (DMEM) complete medium with antibiotics and filtered with mesh cloth. After three rounds of washing, the recovered cells were suspended in a predetermined volume of DMEM complete media containing antibiotics and then counted.

Procedure

DLA and EAC tumour cells were used in cytotoxic tests. Mice spleen cells were employed as a control. 100µl Viable cell suspensions (1 x 10^6 cells) were added to tubes holding methanolic extracts (leaf, stem, and root) of *K. caryophyllata* at different concentrations such as 1.25 µg/ml, 2.5 µg/ml, 5 µg/ml, 10 µg/ml, 25 µg/ml, and 50 µg/ml. Phosphate buffered saline (PBS) increased the volume to 1 ml. The only thing in the untreated control tube was cell suspension. Following a three-hour incubation period at 37°C, the cell suspension was combined with 100 µl of 1% Trypan Blue, allowed to stand for two to three minutes, and then loaded onto a haemocytometer (Babu et al., 2002) and observed through the microscope. Dead cells whose cell membrane is altered can imbibe the blue colour of the Trypan Blue dye. However, live cells with intact cell membranes remain unstained in the dye.

Dead cells take up the blue colour of trypan blue, and live cells do not take up the dye. The number of stained and unstained samples was counted separately, and the percentage of cytotoxicity was calculated after comparing it with the untreated control.

$$\% \text{ cytotoxicity} = \frac{No\ of\ dead\ cells}{No\ of\ live\ cells + No\ of\ dead\ cells} \times 100$$

SECTION D

Anti-inflammatory Studies

I. Invitro anti-inflammatory analysis

Trypsin inhibition assay

Principle: Proteinases cause tissue damage in the lysosomal granules of neutrophils during inflammatory reactions. Therefore, proteinase inhibitors in the drug or plant extracts block proteinase and provide a prominent level of protection from inflammation (Leela Prakash et al., 2011).

Materials: 0.06 mg trypsin dissolved in 1 ml of 20mM Tris-HCl Buffer, PH 7.4; 10-100 µg/ml from a stock concentration of 10mg/ml of plant extracts; 0.8% Azocasein prepared in 20 mM $NaHCO_3$, PH 8.1

Procedure: The trypsin inhibition assay method of Marchetti et al. in 1998, with slight modifications, was performed in the study. 20 µl of different concentrations of *K. caryophyllata* leaves, stem, and root extracts (10–100 µg/ml from a stock concentration of 10 mg/ml) were mixed with 0.5 ml of 0.06 mg trypsin in 1 ml of 20mM Tris-HCl Buffer, PH 7.4 with a final volume of 1 ml. 0.5 ml of 0.8% Azocasein in 20 mM NaHCO3, PH 8.1, was added to the reaction mixture, and the analysis mixture was incubated at 37^0C for 20 minutes after an initial 5 minutes of incubation. Trichloro acetic acid solution, one millilitre of 10%, was added to halt the process and subjected to centrifugation. After the 10-minute centrifugation at 3,000 rpm, the supernatant was collected and mixed with 1 millilitre of 1 molar NaOH. The absorbance was then recorded at 440 nm. Buffer alone is kept as a blank, and a reagent and solvent without extract were used to make up the control solution.

The percentage of proteinase inhibitory activity was calculated by following the equation

$$\% \text{ of Inhibition of proteinase} = \frac{\text{Control OD} - \text{Treated OD}}{\text{Control OD}} \times 100$$

Nitric oxide scavenging assay

Principle: At the physiological pH, Sodium nitroprusside spontaneously generates nitric oxide, which interacts with oxygen and generates nitrite ions, the amount of

which can be measured using the Griess reagent. Nitric oxide scavengers in the plant extracts can compete with oxygen and decrease the amount of nitrite ions produced. Nitric oxide acts as a pro-inflammatory mediator and can injure the tissue and cause inflammation (Mfotie Njoya et al., 2017).

Procedure: 20 µl of various concentrations of the *K.caryophyllata* methanol extracts mixed with 0.5 ml of 10 milli molar of sodium nitroprusside in phosphate buffer saline (PH 7.4) were incubated at room temperature for 150 minutes (Marcocci et al. 1994)). After incubation, 0.5 ml Griess reagent (1% sulfonamide and 0.1% naphthalene ethylenediamine dihydrochloride in 2.5% Orthophosphoric acid) was added to the reaction mixture. The absorbance was measured at 546 nm. The inhibition of nitric oxide generation was estimated by comparing the absorbance values of the control with those of the treatments. Phosphate buffer saline is kept as a blank, and a reagent and solvent without extract were used to make up the control solution.

$$\% \text{ of Inhibition of nitric oxide generation} = \frac{\text{Control OD} - \text{Treated OD}}{\text{Control OD}} \times 100$$

The in vitro anti-inflammatory investigation conducted in the leaf, stem and root methanolic extracts of *K. caryophyllata* showed promising outcomes in the leaf methanolic extract. Thus, more research was planned in the invivo model to evaluate anti-inflammatory activities in the leaf extract.

Calculation of IC50

The half maximal inhibitory concentration (IC 50) of extracts and standards from all the assays was computed from graphs by plotting extract concentration in abscissa vs. the percentage inhibition taken in the Y coordinate. A linear regression equation derived from the graph obtained the IC50 values for the extracts.

II. Invivo anti-inflammatory analysis

Study using leaf methanolic extract of *K. caryophyllata*

Prior to conducting the animal studies, permission was obtained from the Institutional Animal Ethical Committee (ACRC/IAEC/21(2)-P12). All the animals were handled according to the Institutional Animal Ethical Committee guidelines. The research was carried out at the Amala Cancer Research Institute, Thrissur.

Drug preparation for *in vivo* studies

The leaf methanol extract was used for the study. 2% propanol is used to make the drug's stock solution. 10 mg /ml solution has been prepared for further use.

Experimental animals

Swiss albino mice (25-30 gm) of either sex belonging to the 8 - 10 weeks age group were procured from the Small Animal Breeding Station, College of Veterinary and Animal Science Mannuthy, Thrissur. Female mice were nulliparous and non-pregnant. The animals were maintained under sterilized environmental conditions and 22 – 28^0C temperature, 60 - 70% relative humidity, 12 hours dark/light cycle, and fed with standard rat feed (Sai Feeds, India) and water *ad libitum*. Animals were acclimatized to the laboratory environment prior to 7 days before the experimentation and marked uniquely for identification.

Acute Toxicity study

The Organisation for Economic Cooperation and Development (OECD) guidelines were followed in evaluating the acute toxicity study of *K. caryophyllata* methanolic leaf extract. The study employed female Swiss albino mice weighing 25–30 grams. Two groups of three animals each were formed. Vehicle control (2 percent propanol) was provided to group 1. In contrast, group 2 received a dose of 2500 milligrams of drug per kilogram body weight of mice. An oral feeding cannula connected to a 1 ml syringe was used to administer the medication. Each animal's body weight was ascertained before dose administration, and the dose was computed based on that weight. Following the start of the dosage, each animal was monitored separately for the first thirty minutes, then every six hours at half-hour intervals and once daily for the next fourteen days. Throughout the study period, specific observations were made on mortality, toxicity indicators, body weight, and behavioural alterations. Every three days, food, water, and body weight were recorded. The animals were starved for a whole night and sacrificed, and a necropsy was done after 14 days.

In vivo Anti-inflammatory Assay

Male Swiss albino mice weighing between 25 to 30 g were split into five groups, each with six mice (figure 6.1). In order to identify each mouse in each group, during the advancement of experimental data recording, one mouse in each group was left unmarked, next marked in the head, next on the trunk, next on the tail, next on the trunk and tail and the last mouse marked on all parts head, trunk and tail. This marking helped in identifying individual mice within the group (figure 6. 2).

Carrageenan-Induced Paw Oedema in Swiss Albino Mice- Acute Inflammatory Model

The control group, known as Group I, was left untreated. Vehicle control (2% propylene glycol) was assigned to Group II. Group III received 10 mg/kg of the usual medication, diclofenac. The animals in test Groups IV and V were given 250 and 500 mg/kg body weight of methanolic extract of *K. caryophyllata* diluted in two different percentages of propylene glycol. The animals were given two doses of the plant extract for seven days—250 mg and 500 mg/kg body weight(figure 6.3). The animals in the control and test groups were fed orally using an oral gavage needle. Animals in Group III received diclofenac, a common anti-inflammatory medication. On the seventh day of the experiment, 0.02 ml of freshly prepared carrageenan (0.1% carrageenan in 0.9 % saline solution) was injected sub planarly into the left hind paw of mice in each group one hour after the drug administration. Using Vernier Callipers, paw thickness was measured(figure 6.3) one hour before and every hour after carrageenan injection(Boominathan et al., 2004).

The percentage of inhibition was calculated according to the following

$$\% \text{ of inhibition} = \frac{\text{Control } [(\text{PT}-\text{PO}) \text{ control} - (\text{PT}-\text{PO}) \text{ treated}]}{(\text{PT}-\text{PO}) \text{ control})} \times 100$$

(PT= Paw thickness at various time intervals and PO= Initial Paw thickness)

Formalin-Induced Paw Oedema in Swiss Albino mice- Chronic Inflammatory model

The formalin-induced paw oedema method determined anti-inflammatory activity in the chronic models (Chau, 1986). Group I was the control group kept as untreated. Group II was given vehicle control (2% propylene glycol). Group III was given standard drug diclofenac (10 mg/kg). Groups IV and V comprised test groups in which the animals were fed 250 mg and 500 mg/kg body weight of *K. caryophyllata* methanolic extract dissolved in two Percentage propylene glycol. The animals were treated with the plant extract in two doses, 250 mg and 500 mg/kg body weight, respectively, for seven days. The animals in the control and test groups were fed orally using an oral gavage needle. Animals were pre-treated for seven days with respective drugs or diclofenac and continued for the next seven consecutive days. On the eighth day, chronic inflammation was induced by a sub-plantar injection of 0.02ml of 2% freshly prepared formalin on all the animals' right hind paws. Paw thickness was measured with digital Vernier callipers before and after the formalin injection and every day for seven days.

The percentage of inhibition was calculated according to the following

$$\% \text{ of inhibition} = \frac{\text{Control } [(\text{PT}-\text{PO}) \text{ control} - (\text{PT}-\text{PO}) \text{ treated}]}{(\text{PT}-\text{PO}) \text{ control}} \times 100$$

(PT= Paw thickness at various time intervals and PO= Initial Paw thickness)

Figure 6.1: Weighing mouse for grouping

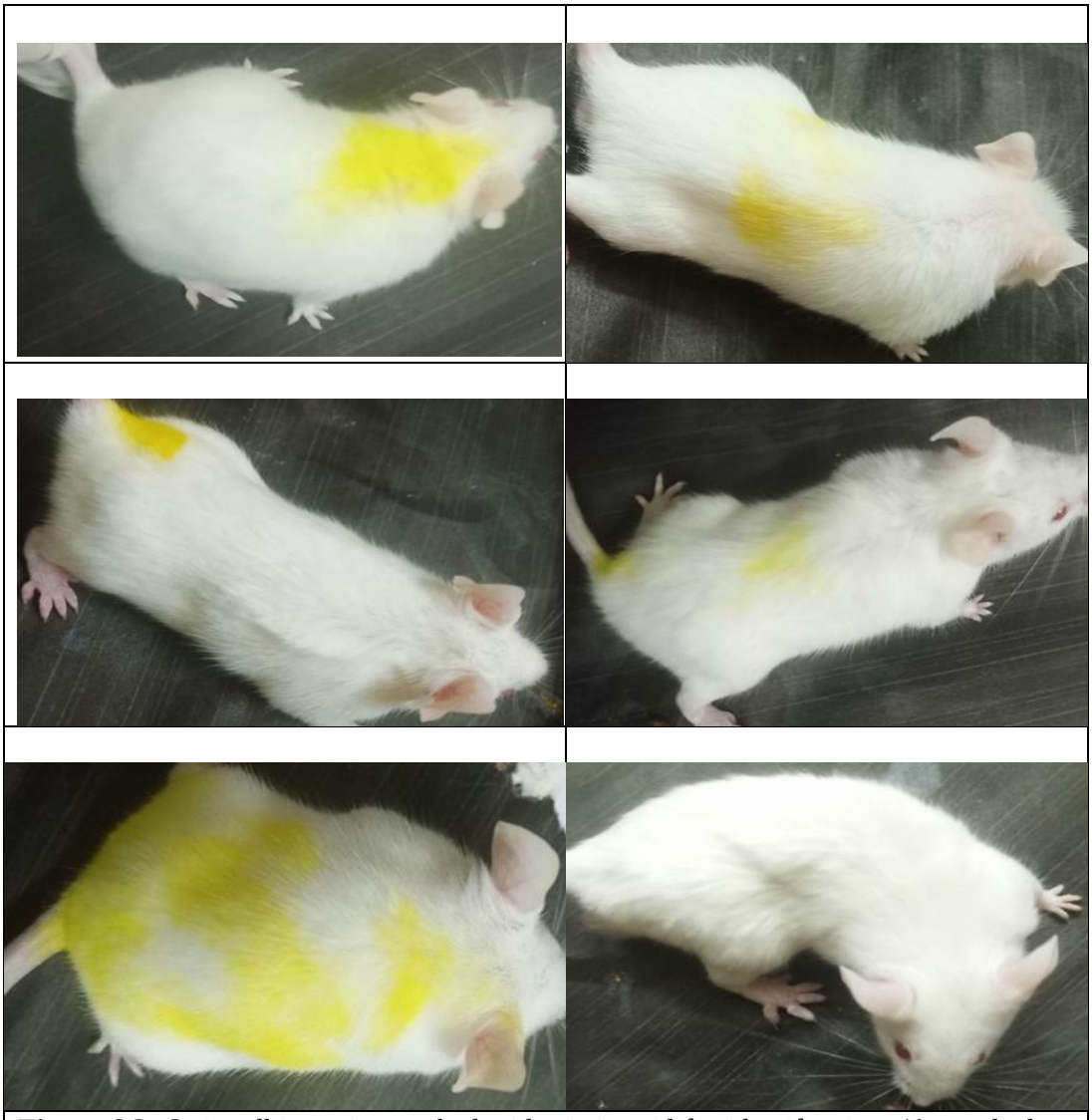


Figure 6.2: Swiss albino mice marked with picric acid for identification. (A. marked on the head, B. marked on trunk, C. marked on the tail; D. marked on the trunk and tail; E marked on the head, trunk and tail; F. colourless)

Figure 6. 3: A. Injecting inflammatory agents; B. measurement of hind paw using vernier callipers

Statistical analysis

The observations were reported as a mean± standard deviation. Statistical analysis was done using SPSS software and One-Way ANOVA with Duncan analysis was performed.

RESULTS AND DISCUSSIONS

SECTION A

Invitro antioxidant studies

Invitro antioxidant activity was performed in the leaf, stem, and root methanolic extracts of *Kamettia caryophyllata* using different antioxidant assays such as Phosphomolebdenum assay, DPPH assay, and Hydrogen peroxide assay. Ascorbic acid was used as the standard for all the tests.

Phosphomolybdenum assay

Phosphomolebdenum assay examined the total antioxidant activity of methanolic leaf, stem, and root component extracts of *K. caryophyllata* and the percentage inhibition and IC50 values of standard ascorbic acid and all the component extracts are depicted in table 6.1 and represented graphically in figure 6.4. Among the plant component extracts, the leaf extract displayed the highest antioxidant activity with an inhibition percentage of 69.98 and lowest IC50 value of 64.87µg/ml, followed immediately by the stem extract (inhibition percentage- 53.73% and IC50 value 94.89µg/ml) and the lowest activity (51.67%) with a highest IC50 value of 117.66µg/ml recorded by the root extract (table 6.1). The scavenging effect of component extracts and ascorbic acid was found concentration-dependent. This was in the range of 2-12µg/ml for ascorbic acid, while in the range of 10-100µg/ml for leaf and stem extracts, whereas it was in the range of 10-120µg/ml for root extract.

Statistical analysis using One-way ANOVA and Duncan's analysis clearly revealed significant difference in half-maximal inhibitory concentration (IC50) values between various component extracts of *K. caryophyllata* as well as with standard ascorbic acid (table 6.2). Among the component extracts, the difference in IC50 value of leaf extract was significantly lower over stem and root, while that of root extract was significantly

higher over leaf and stem extracts and revealed comparatively higher antioxidant potential of leaf component. However, when comparing with standard ascorbic acid, the IC50 value displayed by all the component extracts were significantly higher and hence comparatively lower antioxidant activity.

Table 6.1: Phosphomolybdenum assay- Percentage inhibition by methanolic extracts of leaf, stem, and root of *K. caryophyllata*

Sl. No	Concen. (µg)	% of Inhibition Ascorbic acid (std.)	Concen. (µg)	% of Inhibition (Mean± SD)		
				Leaf methanolic extract	Stem methanolic extract	Root methanolic extract
1	Control	0±0	Control	0±0	0±0	0±0
2	2	25.11±4.56	10	10.96±3.77	8.72±2.55	-
3	4	33.14±2.51	20	23.19±5.99	13.78±3.39	10.38±2.87
4	6	51.42±5.71	40	36.39±1.89	22.24±3.34	15.77±2.55
5	8	62.81±5.88	60	53.27±1.07	29.30±4.27	21.07±1.43
6	10	70.01±5.78	80	60.18±1.65	41.60±4.39	33.43±4.08
7	12	75.22±3.41	100	69.98±0.99	53.73±2.74	45.43±2.67
8	-	-	120	-	-	51.67±2.01
IC50		7.0361±0.46		64.87±0.88	94.89±8.0	117.66±7.46

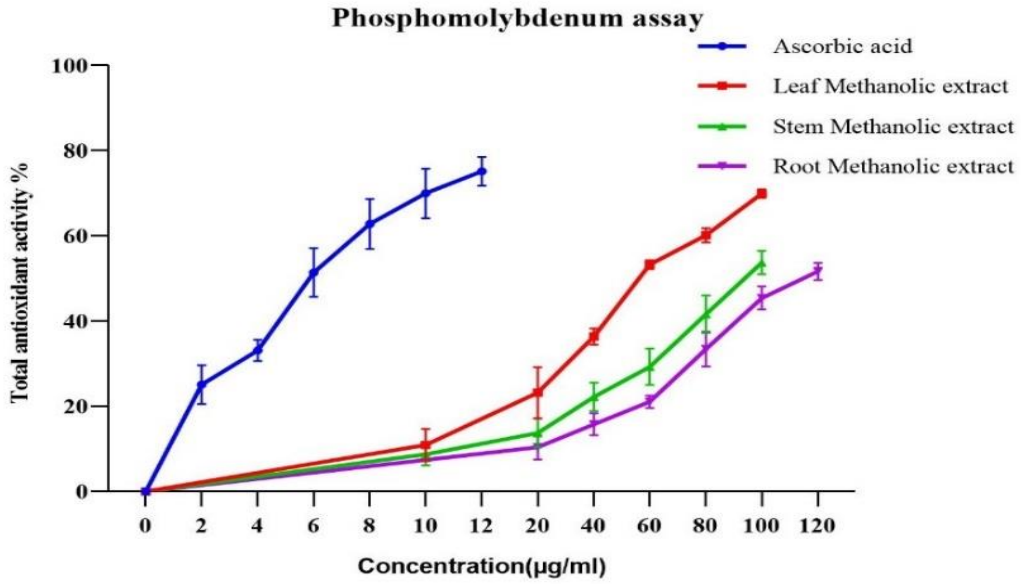


Figure 6.4: In vitro Phosphomolebdenum assay of leaf, stem, and root methanolic extract

Table 6.2: One-Way ANOVA and Duncan analysis of IC50 value obtained with leaf, stem, and root extract of *K. caryophyllata*

One-Way ANOVA:

F value	P value	η^2_p (Effect size)
$F_{(3,20)}$ = 455.117	P<0.001	η^2_p(Effect size)= 0.985

Duncan's Multiple Range Test:

	Subset for alpha=0.05			
Plant parts	**1**	**2**	**3**	**4**
Standard	7.0361			
Leaf		64.8665		
Stem			94.8911	
Root				117.6578
Sig.	1.00	1.00	1.00	1.00

Means for groups in homogeneous subsets are displayed

2,2-diphenyl-1-picrylhydrazyl DPPH Radical scavenging assay

The DPPH radical scavenging activity measured as percentage of inhibition exhibited by methanolic extract of leaf, stem and root of *K. caryophyllata* and the standard ascorbic acid is shown in table 6.3 & figure 6.5). Among the component extracts, the leaf extract recorded the highest percentage of inhibition (74.30%) with IC50 value of 7.97µg/ml., followed immediately by stem extract (65.88%) with IC50 value of 9.48µg/ml and both these extracts recorded their highest inhibition percentage at extract concentration of 12µg/ml. The lowest inhibition percentage exhibited by the root extract with IC50 value of 67.64µg/ml and their highest inhibition of 74.17% recorded only at extract concentration of 100µg/ml. However, when comparing with DPPH radical scavenging activity displayed by the standard ascorbic acid (90.96% with IC50 value 5.42µg/ml), the activity of all plant extracts was significantly lower (table 6.4). The half-maximal inhibitory concentration (IC50 value) of leaf extract differed significantly lower over the root extract, while the difference with the stem extract was not significant, whereas the IC50 value recorded by the root extract was significantly higher over the values of both leaf and stem extracts.

Table 6.3: Inhibition percentage due to DPPH radical scavenging activity by methanolic extracts of leaf, stem, and root of *K. caryophyllata*

Sl. No	Concen. (µg/ml)	% of inhibition (Mean ±SD)			Concen. (µg/ml)	% inhibition (Mean± SD)
		Ascorbic acid	Leaf methanolic extract	Stem methanolic extract		Root methanolic extract
1	Control	0 ±0	0 ±0	0±0	Control	0±0
2	2	21.82 ±0.39	6.46 ±0.27	7.59 ±1.75	10	12.66 ±3.01
3	4	46.16 ±2.21	18.60 ±0.23	15.39 ±1.16	20	21.19 ±0.53
4	6	73.71 ±4.25	41.72 ±0.62	24.09 ±3.97	40	37.27 ±3.39
5	8	78.02 ±4.29	51.41 ±0.40	39.24 ±3.32	60	50.46 ±4.10
6	10	88.51 ±3.28	63.22 ±2.94	52.83 ±5.45	80	60.92 ±6.07
7	12	90.96 ±4.12	74.30 ±1.25	65.88 ±0.47	100	74.17 ±2.49
IC50		5.42± 0.216	7.97 ±0.076	9.84 ±0.49		67.64 ±4.01

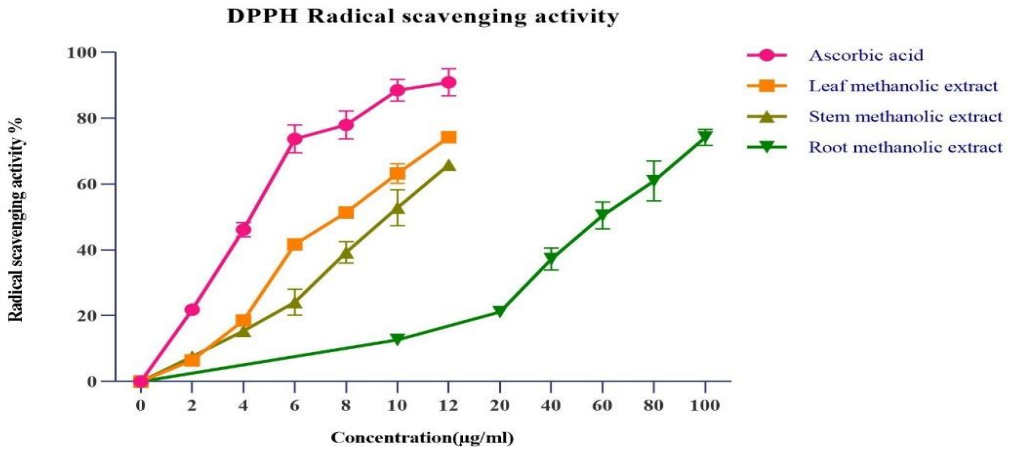


Figure 6.5: In vitro DPPH radical scavenging activity of leaf, stem and root methanolic extract of *K. caryophyllata*

Table 6.4: One-Way ANOVA and Duncan analysis of IC50 value of DPPH radical scavenging activity

One-Way ANOVA:

F value	P value	η^2_p (Effect size)
$F_{(3,20)}$ = 1316.729	P<0.001	η^2_p(Effect size)= 0.994

Duncan's Multiple Range Test:

Subset for alpha=0.05			
Plant parts	**1**	**2**	**3**
Standard	5.42		
Leaf		7.97	
Stem		9.84	
Root			67.64
Sig.	1.00	0.13	1.00

Means for groups in homogeneous subsets are displayed

Hydrogen peroxide scavenging assay

The table 6.5 and figure 6.6 shows the percentage of inhibition exhibited by the leaf, stem, and root methanolic extracts of *K. caryophyllata* and the standard ascorbic acid under hydrogen peroxide scavenging activity. The data clearly indicates strong antioxidant activity displayed by all the component extracts where, the highest inhibition percentage of 65.07 with lowest IC50 value of 68.66 µg/ml was recorded by leaf extract, which differed positively significant over the stem and root extracts but negatively significant over the standard ascorbic acid which in turn displyed an inhibition percentage of 82.39 with an IC50 value of 54.81 µg/ml. Following the leaf extract, the immediate highest inhibition of 53.91% and IC50 value of 87.7µg/ml was exhibited by the stem extract and the lowest inhibition percentage of 42.96 with highest IC50 value of 121.78 µg/ml was recorded by the root extract, which differed significantly lower over the stem and leaf extracts (table 6.6). The scavenging effect of all the extracts and ascorbic acid was found concentration-dependent in the range of 10-100µg/ml.

Table 6.5: Percentage inhibition due to H_2O_2 radical scavenging activity by methanolic extracts of leaf, stem, and root of *K. caryophyllata*

Sl. No	Conc. (µg)	% Inhibition of Ascorbic acid	% Inhibition of component extracts		
			Leaf Methanolic extract	Stem Methanolic extract	Root Methanolic extract
1	Control	0±0	0±0	0±0	0±0
2	10	12.42±0.50	8.99±0.57	4.89±0.38	5.67±0.54
3	20	21.03±0.69	14.76±1.46	9.29±0.08	7.44±0.944
4	40	39.34±0.98	34.14±1.07	25.15±0.97	14.47±1.03
5	60	61.26±0.95	49.21±0.84	37.58±0.71	21.62±0.86
6	80	76.62±1.01	61.04±1.42	46.38±0.32	33.76±0.41
7	100	82.39±1.38	65.07±0.96	53.91±0.56	42.96±1.80
IC50		54.81±0.64	68.66±0.48	87.71±0.84	121.78±3.09

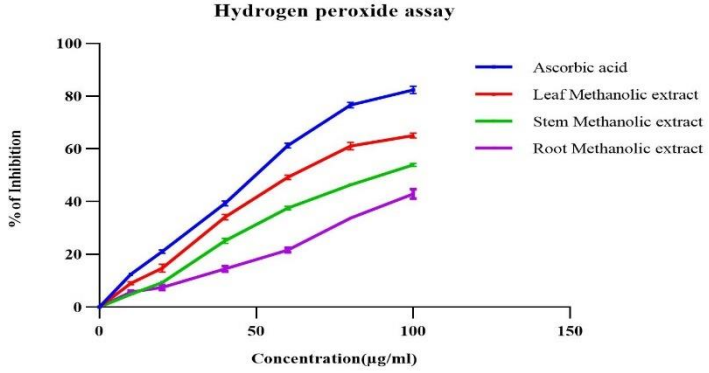


Figure 6.6: In vitro Hydrogen peroxide scavenging activity of leaf, stem, and root methanolic extract of *K. caryophyllata*

Table 6.6: One-Way ANOVA and Duncan analysis of Hydrogen peroxide scavenging activity in the leaf, stem, and root of *K. caryophyllata*

One-Way ANOVA:

F value	P value	η^2_p (Effect size)
$F_{(3,20)}$ = 1855.13	P<0.001	η^2_p(Effect size)= 0.996

Duncan's Multiple Range Test:

Extracts	Subset for alpha=0.05			
	1	2	3	4
Standard- Ascorbic acid	54.8100			
Leaf		68.6600		
Stem			87.7167	
Root				121.7767
Sig.	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed

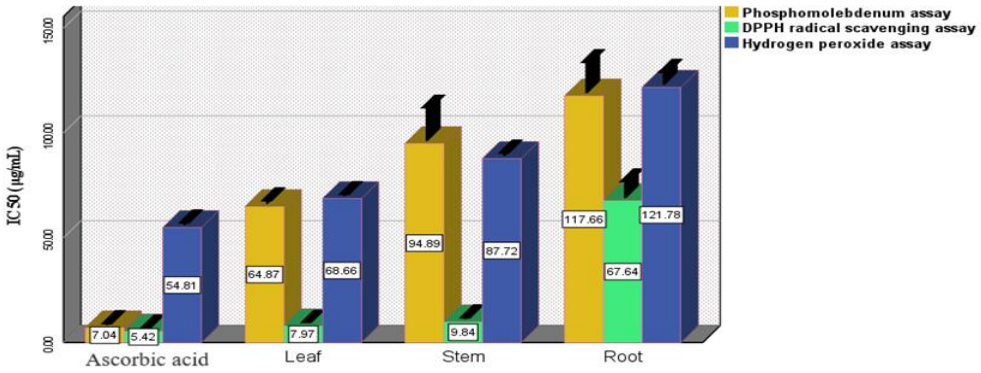


Figure 6.7: Comparison of antioxidant activity in terms of IC50 value of leaf, stem and root methanolic extracts and ascorbic acid in various radical scavenging assay

Antioxidants are essential compounds that protect the body from damage caused by free radical-induced oxidative stress. The continued such oxidative stress may cause chronic inflammation, which leads to most atrocious diseases such as cancer, cardiovascular, diabetes, pulmonary and neurological diseases (Lobo et al., 2010; Conner & Grisham. 1996). The natural antioxidants from plant resources including phenolic acids, flavonoids and carotenoids are renowned for their diverse array of biological activities, which encompass anti-inflammatory, antibacterial, antiviral, antiaging, and anticancer properties (Xu et al., 2017). The lower values of half-maximal inhibitory concentration (IC50) of compounds signify greater free radical scavenging activities of the compound and indicates greater oxidative stress reducing capacity. The lower IC50 values of different component extracts of *K. caryophyllata*, particularly the methanolic leaf extract, displayed in various radical scavenging assays

in the present study have given valuable insights into their potential antioxidant activities and associated health benefits(figure 6.7). As a pure synthetic compound, the standard ascorbic acid in the study showed superior activity over all plant component extracts analysed however, as natural and without any or less side effects, the activity exhibited by crude extracts is highly considerable and promising. Further, with the scope for isolation of specific bioactive component or components with additive and synergistic antioxidant activities in pure form from these crude extracts, there may be chances for significant enhancement in the antioxidant activities, which may be more or comparable with standard ascorbic acid activities.

Among different component extracts, the leaf extract showcased the most effective free radical scavenging activity, followed by stem extract and the least activity recorded by root extract in all the radical scavenging assays performed in the study. This clearly confirms higher potential of radical scavenging activity by the leaf extract over stem and root extracts. These differences may be due to variation in the bioactive molecules with antioxidant property in terms of diversity and concentration in different component parts of *K. caryophyllata* (Khan et al., 2012; Phuyal et al., 2020). The study further observed variation in the IC50 value of leaf, stem as well as root extracts against different ROS like DPPH, H_2O_2 and Phosphomolebdenum targeted by different radicle scavenging assay like DPPH assay, H_2O_2 assay and Phosphomolybdenum assay performed in the study. The varying reactivities of specific bioactive compounds having antioxidant activity in the component extracts towards different ROS species might be the reason for variations in the IC50 value. The study revealed the radicle scavenging effect of all the component extracts and ascorbic acid was concentration-dependent.

Therefore, the study confirms potential antioxidant activity of plant *K. caryophyllata* and reveals that the intensity of these activities was found varying according to the component part selected for extraction and dosage of extract used. The highest radicle scavenging activity inferred in the study was with leaf component extract, irrespective of ROS species that may be due to several interconnected factors. The leaves usually harbour higher concentrations of phenolic compounds and flavonoids, both can substantially contribute to the plant's overall antioxidant content (Ribeiro et al., 2020) and again, the creation of strong defense system such as carotenoids, chlorophyll and

tocopherols in the leaves may be boosting their antioxidant capacity (Biswal et al., 2012; Mesa & Munne-Bosch, 2023) against oxidation stress due to generation of ROS during photosynthesis (Gogoi & Basumatary, 2018). Further, the structural and functional roles of leaves and their higher metabolic activity can serve as adaptive mechanisms against environmental stressors, ultimately fostering a higher concentration of antioxidants.

SECTION B

Antimicrobial activity studies

The Agar Well Diffusion method was employed to assess antimicrobial activity in terms of zones of inhibition generated by different concentrations such as 25 µg/ml, 50 µg/ml and 100 µg/ml of leaf, stem and root methanolic extracts of *K. caryophyllata* against pathogenic microorganisms such as *Candida albicans, Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa*. Chloramphenicol was chosen as a positive control against pathogenic bacteria in this study, due to its broad-spectrum antimicrobial activity against gram-positive and gram-negative bacteria. Chloramphenicol targets the 50S ribosomal subunit of bacteria and inhibits protein synthesis by blocking peptidyl transferase, an enzyme crucial for peptide bond formation, and preventing aminoacyl-tRNA binding to the peptidyl transferase site (Maddison & David, 2017). Clotrimazole was used as standard against *Candida albicans*, as an antifungal medicine, it makes pores on the fungal membrane and causes damages (Sawyer et al., 2007).

The results revealed differences in the antimicrobial activity of different component extracts against the tested pathogens. The details of the zone of inhibition produced by different component extracts and control treatments against tested five pathogenic organisms are depicted in table 6.7. The methanolic leaf extracts exhibited comparatively better zones of inhibition against most of the tested pathogens and this was immediately followed by better performance exhibited by the stem extract and least performance by the root extract(figure 6.8). The data revealed that a direct correlation exist between the zone of inhibition produced and concentration of the extract used.

Table 6.7: Antimicrobial activity (as zone of inhibition (ZOI)) of leaf, stem and root methanolic extract of *K. caryophyllata* against different pathogenic microorganisms.

Pathogens	Concentration (µg/ml)	Zone of inhibition (ZOI) (mm)		
		Leaf	Stem	Root
Candida albicans	Positive control	17.83 ±.75	17.83 ±.75	17.83 ±.75
	Negative control	0	0	0
	25 µg/ml	8.5 ±0.55	0	0
	50 µg/ml	11.67 ±0.52	11.00 ±0.89	0
	100 µg/ml	13.58 ±0.49	14.33 ±0.52	0
Escherichia coli	Positive control	27.17 ±1.16	27.17 ±1.16	27.17 ±1.16
	Negative control	0	0	0
	25 µg/ml	0	0	0
	50 µg/ml	14.50 ±0.55	0	8.67 ±0.52
	100 µg/ml	17.50 ±0.55	11.33 ±0.82	10.17 ±0.75
Staphylococcus aureus	Positive control	13.83 ±0.98	13.83 ±0.98	13.83 ±0.98
	Negative control	0	0	0
	25 µg/ml	0	0	0
	50 µg/ml	12.50 ±0.54	0	0
	100 µg/ml	14.17 ±0.75	9.00 ±0.89	0
Klebsiella pneumonia	Positive control	28.00 ±0.89	28.00 ±0.89	28.00 ±0.89
	Negative control	0	0	0
	25 µg/ml	0	0	0
	50 µg/ml	18.67 ±0.52	0	14.67 ±0.55
	100 µg/ml	25.5 ±0.55	18.33 ±0.82	21.00 ±0.63
Pseudomonas aeruginosa	Positive control	29.33 ±0.82	29.33 ±0.82	29.33 ±0.82
	Negative control	0	0	0
	25 µg/ml	13.00 ±0.89	0	0
	50 µg/ml	15.33 ±0.82	13.50 ±0.55	0
	100 µg/ml	19.83 ±1.94	17.00 ±0.89	0

The leaf extract produced highest zone of inhibition over stem and root extracts at highest extract concentration of 100 µg/ml used against all the pathogenic organisms, with an exception in the case of *Candida albicans* and it was 13.58mm, 17.50mm, 14.17mm, 25.5mm and 19.83mm respectively against *Candida albicans, Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia* and *Pseudomonas aeruginosa*. When comparing the ZOI produced by the leaf extract with that of ZOI produced by the positive control, it was 76.16%, 64.41%, 102.46%, 91.07% and 67.61% of the ZOI

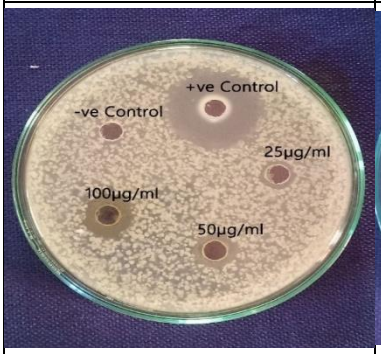
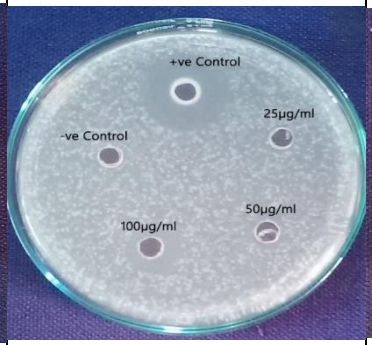
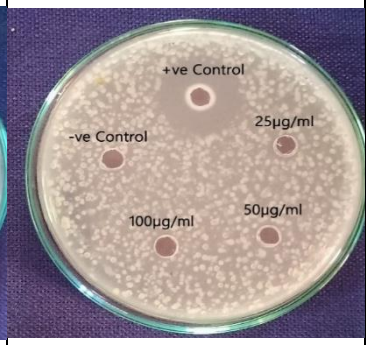
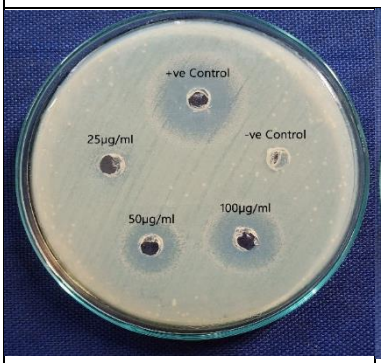
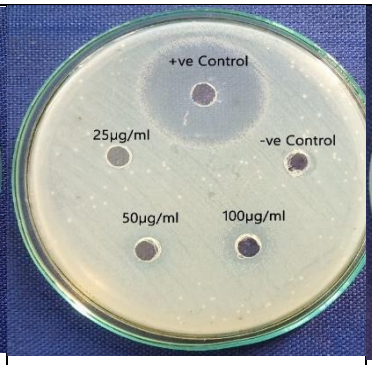
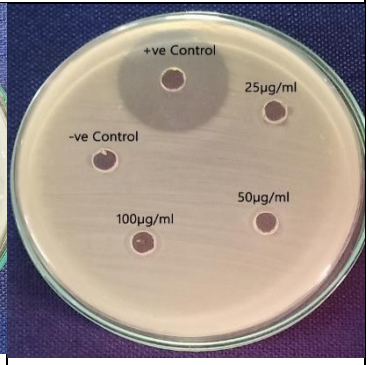
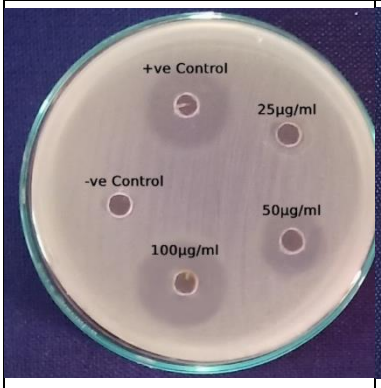
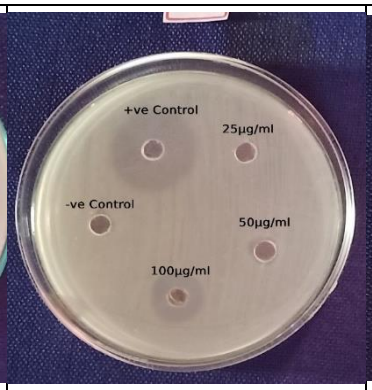
produced by the positive control respectively for *Candida albicans, Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia* and *Pseudomonas aeruginosa*. This confirms potential antimicrobial activity of methanolic leaf extrat against all the tested pathogenic organisms.

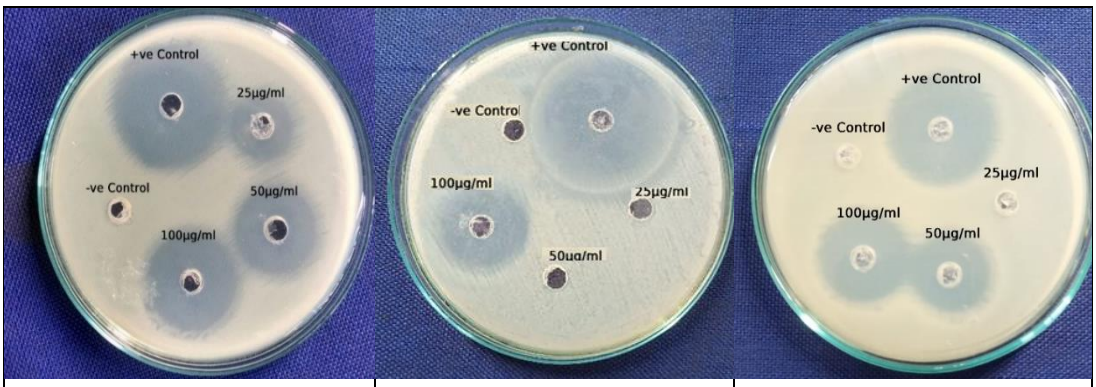
With respect to the stem extract, the highest ZOI produced at 100 µg/ml concentration was 14.33mm, 11.33mm, 9.00mm, 18.33mm and 17.00mm respectively against *Candida albicans, Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia* and *Pseudomonas aeruginosa* and it was about 80.37%, 41.70%, 65.08%, 65.46% and 57.96% of the ZOI produced by the positive control respectively. With an exception in the case of *Candida albicans*, the ZOI produced by stem extract was comparatively lower over the ZOI produced by the leaf extract against pathogens. With an exception of 5.23% increase in the ZOI produced against *Candida albicans*, the treatment of stem extract caused a reduction of 35.26%, 36.49%, 28.12% and 14.27% over the ZOI produced by the leaf extract against *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia* and *Pseudomonas aeruginosa* respectively.

The antimicrobial activity and the highest ZOI produced with respect to root extract at 100 µg/ml concentration was 0.00mm, 10.17mm, 0.00mm, 21.00mm and 0.00mm respectively against *Candida albicans, Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia* and *Pseudomonas aeruginosa*. The percentage of ZOI produced by root extract when compared to positive control was 0.00%, 37.43%, 0.00%, 75.00% and 0.00% respectively for *Candida albicans, Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia* and *Pseudomonas aeruginosa*. Only with an exception of 12.71% increase over the ZOI induced by the stem extract against *Klebsiella pneumonia*, the root extract caused reduction in the ZOI agaiinst all the pathogenic organism compared to that of leaf as well as stem extracts. The treatment using root extract caused a reduction of 100%, 41.89%, 100%, 17.65% and 100% respectively over the ZOI produced by the leaf extract against *Candida albicans, Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia* and *Pseudomonas aeruginosa*.

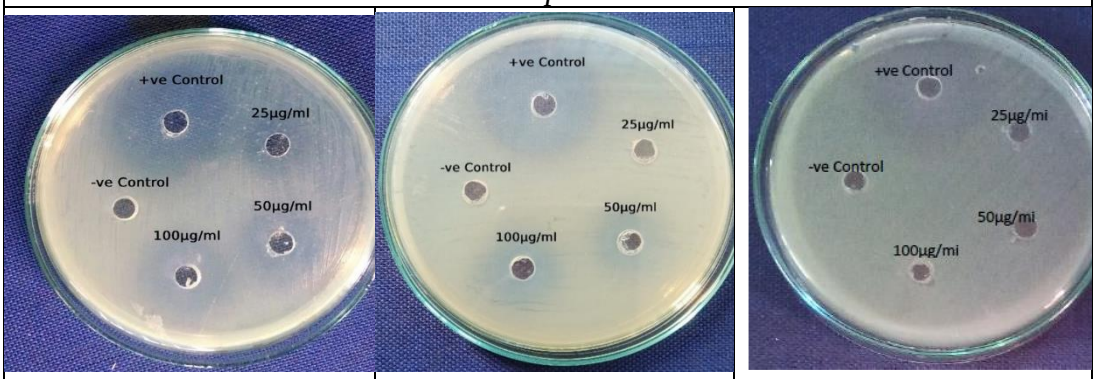
No antimicrobial activity or ZOI was detected at 25 µg/ml concentration against *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumonia* with respect to

leaf extract, while it was at 25 µg/ml & 50 µg/ml against *Escherichia coli, Staphylococcus aureus* & *Klebsiella pneumonia* and at 25 µg/ml concentration alone against *Candida albicans,Pseudomonas aeruginosa* with respect to stem extract. However, with respect to root extract all the tested concentrations against *Candida albicans, Staphylococcus aureus* & *Pseudomonas aeruginosa* and as well as at 25 µg/ml concentration alone against *Escherichia coli* & *Klebsiella pneumonia* displyed any ZOI or antimicrobial activity.

Leaf extract	Stem extract	Root extract
		
Candida albicans		
		
Escherichia coli		
		
Staphylococcus aureus		



Klebsiella pneumonia



Pseudomonas aeruginosa

Figure 6.8: Antimicrobial activity (ZOI) of leaf, stem, and root methanolic extract of *Kamettia caryophyllata* against tested pathogens

The statistical analysis revealed the differences in ZOI produced at highest concentration of 100µg/ml by various extracts and the control against different tested pathogens were significant (table 6.8 -6.12). With an exception against *Candida albicans*, the leaf component extract among different extracts recorded significantly highest ZOI against all pathogens, whereas in the case of former, the stem methanolic extract displyed significantly highest ZOI. Similarly, significantly lowest ZOI against tested pathogens was recorded by root methanolic extract, with an exception against *Klebsiella pneumonia,* where stem extract recorded significantly lowest ZOI (table 6.11).

Table 6.8: One-Way ANOVA and Duncan analysis of antimicrobial activity (ZOI) of leaf, stem and root methanolic extracts of *K. caryophyllata* against *Candida albicans*

One-Way ANOVA:

Pathogens	**F**	**P value**	**η^2_p (Effect size)**
Candida albicans	1374.61	<0.001	0.994

Duncan's multiple tests

Extracts	**Subset for alpha=0.05**			
	1	**2**	**3**	**4**
Root	0.00			
Leaf		13.58		
Stem			14.33	
Positive control				17.83
Sig.	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed

Table 6.9: One-Way ANOVA and Duncan analysis of antimicrobial activity (ZOI) of leaf, stem and root methanolic extracts of *K. caryophyllata* against *Eschertia coli*

One-Way ANOVA:

Pathogens	**F**	**P value**	**η^2_p (Effect size)**
Eschertia coli	500.90	<0.001	0.987

Duncan's multiple tests

Extracts	**Subset for alpha=0.05**			
	1	**2**	**3**	**4**
Root	10.167			
Stem		11.33		
Leaf			17.50	
Positive control				27.17
Sig.	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed

Table 6.10: One-Way ANOVA and Duncan analysis of antimicrobial activity (ZOI) of leaf, stem and root methanolic extracts of *K. caryophyllata* against *Staphylococcus aureus*

One-Way ANOVA:

Pathogens	F	P value	η^2_p (Effect size)
Staphylococcus aureus	448.47	<0.001	0.985

Duncan's multiple tests

Extracts	Subset for alpha=0.05		
	1	2	3
Root	0.00		
Stem		9.00	
Positive control			13.83
Leaf			14.17
Sig.	1.000	1.000	0.459

Means for groups in homogeneous subsets are displayed

Table 6.11: One-Way ANOVA and Duncan analysis of antimicrobial activity (ZOI) of leaf, stem and root methanolic extracts of *K. caryophyllata* against *Klebsiella pneumonia*

One-Way ANOVA:

Pathogens	**F**	**P value**	**η^2_p (Effect size)**
Klebsiella pneumonia	209.92	<0.001	0.997

Duncan's multiple tests

Extracts	**Subset for alpha=0.05**			
	1	**2**	**3**	**4**
Stem	18.33			
Root		21.00		
Leaf			25.5	
Positive control				28.00
Sig.	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed

Table 6.12: One-Way ANOVA and Duncan analysis of antimicrobial activity (ZOI) of leaf, stem and root methanolic extracts of *K. caryophyllata* against *Pseudomonas aeruginosa*

One-Way ANOVA:

Pathogens	**F**	**P value**	**η^2_p (Effect size)**
Pseudomonas aeruginosa	685.29	<0.001	0.990

Duncan's multiple tests

Extracts	**Subset for alpha=0.05**			
	1	**2**	**3**	**4**
Root	0.0			
Stem		17.00		
Leaf			19.83	
Positive control				29.33
Sig.	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed

The findings of the present study revealed significant antimicrobial property of different component extracts of *K. caryophyllata*, specifically methanolic leaf component extract against five selected pathogenic organisms and highlight the scope for potential applications in medical and pharmaceutical contexts. Diverse phytochemical groups and specific compounds identified in different components of *K. caryophyllata* in the study have been previously reported for their antibacterial activity, and they exert their effects through various mechanisms. The phytochemical group alkaloid and specific alkaloid compounds detected in component extracts have been reported to impact bacterial cell walls, causing structural irregularities, affecting cellular function and it may interfere with cell division and disrupt membrane integrity

(Yan et al., 2021). The group terpenes and their specific compounds detected in the study is reported to have antibacterial activity and this might be due to their disruptive properties to bacterial cell membranes by interacting with their lipid components and their interference with bacterial respiration (Nik et al., 2019). Similarly, the phytochemical group phenolics and many related specific compounds detected in *K. caryophyllata* have also been reported to have great antibacterial properties mainly due to their ability to disrupt bacterial cell membranes, leading to increased permeability and their interfere with bacterial enzymes and proteins (Muthu et al., 2021). Further, occurrence of flavonoids and tannin compounds in the plant particularly in the leaf component might have also contributed to the antimicrobial activity. Flavonoids can inhibit various enzymes that are essential for bacterial growth and survival and also inhibit bacterial DNA replication (Shamsudin et al., 2022). Tannins can chelate iron, which is vital for bacterial metabolism, thereby inhibit bacterial cell wall synthesis and disrupt the cell membrane (Bittner et al., 2021). Dal Piaz et al. (2018) reported that terpenes can block fungal growth by inhibiting membrane permeability. Kim et al. (2018) reported that the alkaloids have anti-fungal properties by reducing the biofilm formation and similarly, polyphenols and flavonoids present in plants exhibit a cooperative antifungal impact against *Candida albicans* (Rhimi et al., 2020).

The antimicrobial study clearly inferred that *K. caryophyllata* methanolic extracts possess concentration-dependent antimicrobial activity. The leaf extract consistently outperformed stem and root extracts against most of the tested pathogenic microorganisms, indicating its potential as a natural antimicrobial source. Leaves are often the site of active secondary metabolite production, including compounds of antimicrobial properties. Significantly higher concentration of diverse secondary metabolites with antimicrobial property in the leaf component compared to stem and root might be the reason which is leading to more vigorous antimicrobial activity. Further, it is worth noting that the antimicrobial activity caused by phytochemicals is a complex interplay of multiple factors; different phytochemicals sharing common antimicrobial property may be targeting a single or different aspects of bacterial or fungal physiology. Therefore, these synergistic and additive effects of these active chemicals should be taken into consideration for the antimicrobial activity.

SECTION C

Cytotoxicity Study Using Trypan Blue Exclusion Test

The Trypan Blue exclusion test is widely used to assess cell viability. Viable cells possess intact membranes unstained with Trypan Blue dye, but non-viable cells absorb Trypan Blue and exhibit a blue colour. This method is commonly employed to evaluate cytotoxicity of plant drugs. The details of results obtained from the cytotoxicity evaluation of methanolic leaf, stem and root extracts with a concentration ranging from 1.25µg/ml to 50 µg/ml for leaf and stem extracts and 50 µg/ml to 150 µg/ml for root extract on cancer cell lines Ehrlich Ascites Carcinoma (EAC) and Daltons Lymphoma Ascites (DLA) and on healthy spleen cells are depicted in the table 6.13, table 6.14 and table 6.15.

Table 6.13: Cytotoxicity % of methanolic leaf extracts of *K. caryophyllata* on cancer cell lines EAC and DLA and healthy spleen cells

Sl. No	Concentration of leaf extract (µg/ml)	Cytotoxicity% (Mean± SD		
		Spleen cell	DLA	EAC
1	Control	0.47±0.54	0.707±0.047	0.70±0.46
2	1.25	3.67±0.34	2.68±0.36	5.75±1.29
3	2.5	3.88±0.46	8.07±0.78	11.48±1.63
4	5	4.16±0.48	15.90±1.00	24.24±2.16
5	10	4.36±0.44	29.76±1.01	40.98±1.45
6	25	7.10±1.37	64.39±1.71	89.95±1.59
7	50	11.15±1.16	83.46±1.27	98.97±1.07

Table 6.14: Cytotoxicity % of methanolic stem extracts of *K. caryophyllata* on cancer cell lines EAC and DLA and healthy spleen cells

Sl. No	Concentration of stem extract (µg/ml)	Cytotoxicity% (Mean± SD		
		Spleen cell	DLA	EAC
1	Control	0.01±0.02	0.002±0.005	0.02±0.04
2	1.25	2.73±0.70	3.11±1.17	16.88±0.44
3	2.5	3.17±0.51	5.77±0.68	7.81±1.75
4	5	3.12±0.58	8.22±1.15	10.39±0.69
5	10	4.19±0.93	11.27±0.95	21.41±2.07
6	25	4.01±0.48	22.05±1.74	30.56±2.37
7	50	5.10±3.2	34.35±1.11	70.96±1.80

Table 6.15: Cytotoxicity % of methanolic root extracts of *K. caryophyllata* on cancer cell lines EAC and DLA and healthy spleen cells

Sl. No	Concentration of root extract (µg/ml)	Cytotoxicity% (Mean± SD		
		Spleen cell	DLA	EAC
1	Control	0.01±0.018	0.0025±0.004	0.02±0.038
2	50	2.7±0.60	4.07±0.44	8.00±0.41
3	70	3.17±0.80	6.55±0.34	13.42±0.48
4	90	3.13±0.58	7.65±1.15	10.39±0.69
5	110	4.19±0.49	11.27±0.46	17.45±0.36
6	130	4.01±0.39	15.67±0.45	24.35±0.31
7	150	3.81±0.67	18.02±0.53	27.47±0.49

The data indicates a direct correlation between cytotoxicity % and concentration of the extract used and it was more evident with respect to cytotoxicity response of different cell lines against leaf and stem extracts. Among different component extracts, the methanolic leaf extract induced highest reduction in the cell viability and recorded highest cytotoxicity of 98.97% for EAC cell line; 83.46% for DLA cell line and 11.15% for healthy spleen cells (figure 6.9), whereas the lowest viability reduction and cytotoxicity % recorded by the root extract and it was 8.00% for EAC cell line; 4.07% for DLA cell line and 2.7% for spleen cells at 50 µg/ml concentration and at a higher concentration of 150 µg/ml root extract, it was 27.47%, 18.02% and 3.81% respectively (figure 6.11). Most importantly, the study noted that even at the highest concentration of 150 µg/ml of different component extracts of *K. caryophyllata* tested, the cytotoxic effect on healthy spleen cells was very minimal or feeble in comparison with that of cancer cell lines EAC and DLA.

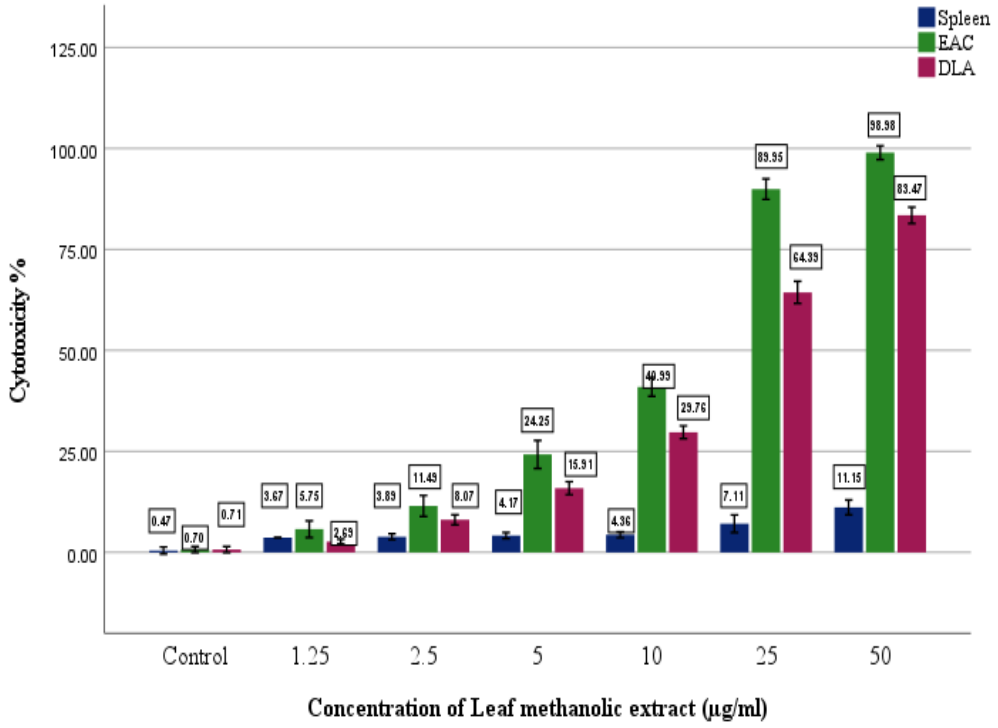


Figure 6.9: *In vitro* cytotoxicity of methanolic leaf extract of *K. caryophyllata* on spleen cells and cancer cell lines EAC and DLA

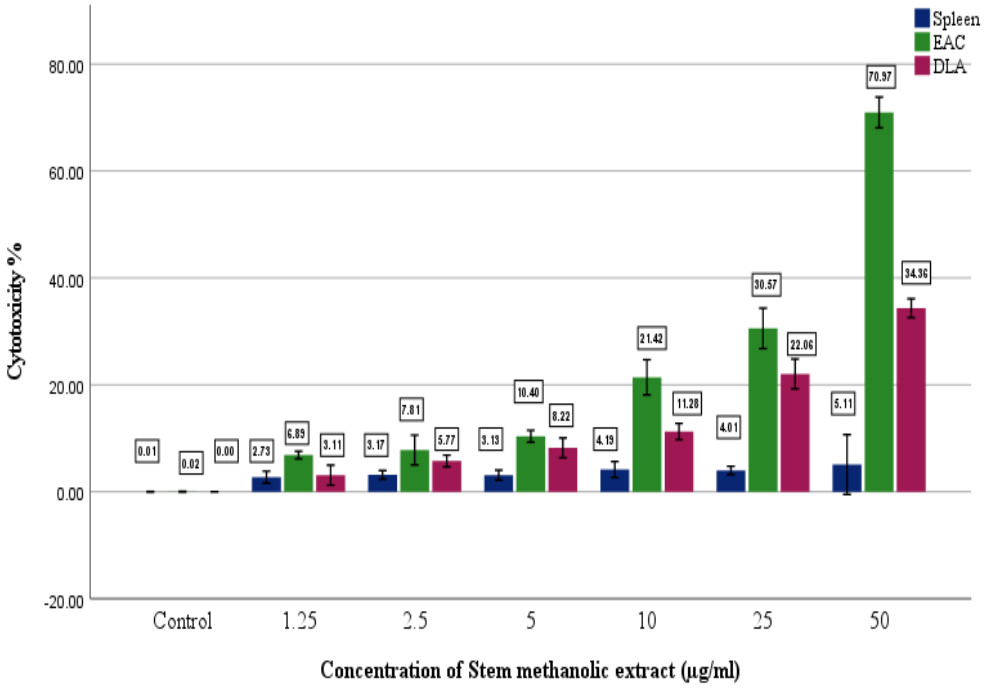


Figure 6.10: *In vitro* cytotoxicity of methanolic stem extract of *K. caryophyllata* on spleen cells and cancer cell lines EAC and DLA

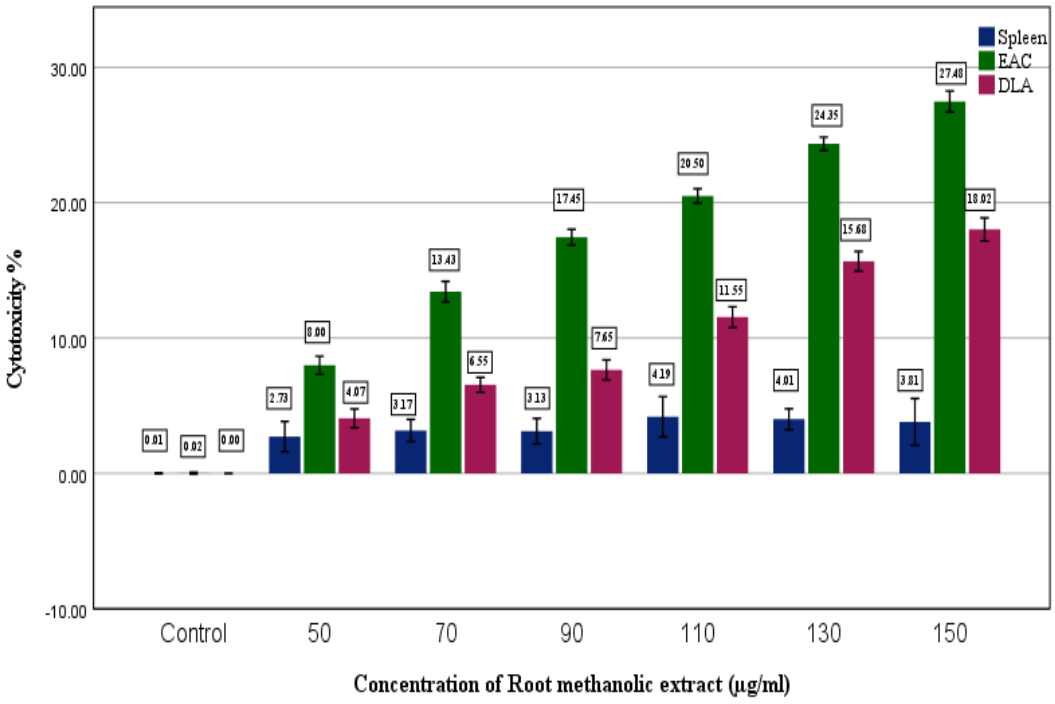


Figure 6.11: *In vitro* cytotoxicity of methanolic root extract of *K. caryophyllata* on spleen cells and cancer cell lines EAC and DLA

The analysis of half-maximal inhibitory concentration values (IC50 values) of different component extracts against EAC and DLA cancer cell lines revealed that the leaf methanolic extract recorded lowest IC50 value for causing 50% viability reduction of these cancer cell lines compared to stem and root methanolic extracts (table 6.12). This was 19.83µg/ml of leaf extract against EAC cell lines and 23.27µg/ml against DLA cell lines. In contrast, the root extract displayed weaker activity and hence recorded the highest IC50 value and this was 264.23µg/ml against EAC cell lines and 307.45µg/ml against DLA cell lines. The statistical analysis revealed differences in IC50 values among component extracts against different cell lines which differed significantly with each other as in table 6.17 & table 6.18.

Table 6.16: IC50 values of methanolic leaf, stem and root methanolic extracts of *K. caryophyllata* against cancer cell lines EAC and DLA

IC 50	Leaf (µg/ml)	Stem(µg/ml)	Root(µg/ml)
DLA cell lines	23.27 ± 4.72	71.34 ±2.67	307.45±1.75
EAC cell lines	19.83 ±2.09	35.43 ±0.97	264.23±0.82

Table 6.17: One-Way ANOVA and Duncan analysis of IC 50 of methanolic leaf, stem and root extracts of *K. caryophyllata* against DLA cancer cell lines

One-Way ANOVA:

F value	P value	η^2_p (Effect size)
$F_{(1,6)}$ = 8545.687	P<0.001	0.991

Duncan's Multiple Range Test:

Extract	Subset for alpha = 0.05		
	1	2	3
Leaf	23.27		
Stem		71.33	
Root			307.45
Sig.	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed

Table 6.18: One-Way ANOVA and Duncan analysis of IC 50 of methanolic leaf, stem and root extracts of *K. caryophyllata* against EAC cell lines

One-Way ANOVA:

F value	P value	η^2_p (Effect size)
$F_{(1,6)}$ = 37523.72	P < 0.001	0.99

Duncan's Multiple Range Test:

Extract	Subset for alpha = 0.05		
	1	2	3
Leaf	19.82		
Stem		35.42	
Root			264.23
Sig.	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed

Since 1980s, the in vitro screening approach for bioactive compounds of medicinal value has become efficient, which involves testing compounds across various concentrations against the target including cancer cell lines to assess their ability to inhibit cell growth or induce cell death (Menon et al., 2022). Further, by performing *in vitro* screening and evaluation of plant-based extracts and natural compounds used by traditional medicinal knowledge, there is greater scope for finding potent bioactive compounds with medicinal value (Almeida et al., 2014). The findings of the present study reveal remarkable cytotoxic potential of *K. caryophyllata* methanolic extracts and particularly the leaf extract against DLA and EAC cancer cell lines. The

significantly lower IC50 values displayed by methanolic leaf extract over the stem and root extracts offers valuable insights into its chemical composition and mechanisms underlying the observed cytotoxic activities. The screening for phytocompounds done previously in the present study revealed the occurrence of large number of specific bioactive compounds which were previously reported for anticancer activity in the methanolic leaf extracts. These compounds include Phytol; Squalene; dl-α-Tocopherol; Methyl tetradecanoate; Tetradecanoic acid; 9,12-Octadecadienoic acid (Z,Z)-, methyl ester; Trans-13-Octadecenoic acid, methyl ester; 9-Octadecenoic acid (Z)- 2-hydroxy-1-(hydroxymethyl) ethyl ester identified in the GC-MS analysis (Lalitha et al., 2014; Santhosh et al., 2014; Rajalakshmi & Mohan,2016; Ravikumar et al., 2012; Lalitharani, 2009; Yu et al., 2005; Krishnamoorthy & Subramaniam, 2014; Godswill et al.,2014) and similarly, compounds identified in the HR LC-MS screening such as Picrasin C; (+)-Myrtenyl formate; Panaquinquecol 1; 3,5-Dihydroxyphenyl 1-O-(6-O-galloyl-beta-D-glucopyranoside); Physalin K; Fukinolic acid; Melleolide D; 13-Deoxytedanolide; Vobtusine; Calotropin; Prodelphinidin A2 3'-gallate; Magnesium protoporphyrin monomethyl ester; Squalamine; 33-Deoxy-33-hydroperoxyfurohyperforin and Saikosaponin BK1 (Chakraborty, 2013; Awadh Ali et al., 2017; Christensen, 2020; Hassan et al., 2021; Meira et al., 2022; Gavin et al., 2013; Dörfer et al., 2019; Smith et al., 2003; Koch et al., 2020; Cheng et al., 2002; De Britto et al., 2001; Limbocker et al., 2021; Lee et al., 2006 and Sulaiman et al., 2022). The significant and robust cytotoxic activity of methanolic leaf extract inferred in the study can be attributed to the occurrence of these vast number of specific phytocompounds known for their anticancer properties (Zhao et al., 2019).

SECTION D

Invitro Anti-inflammatory Trypsin Inhibition Assay

Invitro anti-inflammatory Trypsin Inhibition assay performed on leaf, stem, and root methanolic extract of *Kamettia caryophyllata* with a concentration range from 10-100 µg/ml exhibited differences in response and inhibition percentage. The details of inhibition % is depicted in the table 6.19.

Table 6.19: Percentage inhibition trypsin activity and IC 50 values of component extracts

SL. NO	Concentration (µg/ml)	% of Inhibition (Mean± SD)		
		Leaf methanolic extract	Stem methanolic extract	Root methanolic extract
1	Blank/Control	0±0	0±0	0±0
2	10	4.24±1.79	12.61±0.30	0±0
3	20	13.57±0.54	16.69±0.29	0±0
4	40	23.59±4.77	19.90±1.75	0±0
5	60	33.71±3.26	23.94±0.54	0±0
6	80	43.05±0.64	28.55±0.69	0±0
7	100	49.37±0.74	30.98±0.42	0±0
IC50		94.73± .13	336.38±6.10	-

Absorbance values are expressed as mean ± SD, n=6

The data indicates, in the range of 10-100 µg/ml extract concentration, the root extract did not exhibit any inhibitory activity against trypsin while, the leaf and stem extracts displayed dose-dependent inhibition (figure 6.12). The leaf methanolic extract caused maximum level of inhibition, followed by stem extract. The linear regression equation obtained from the constructed plot of different concentrations of plant extracts were used to calculate their IC50 value for performing 50 percent inhibition of trypsin activity (table 6.19). The lowest IC50 value for 50% trypsin inhibition was recorded by the methanolic leaf extract (94.73µg/ml), which differed significantly over the very high IC50 value of 336.38µg/ml recorded for stem extract (table 6.20). This indicates comparatively high-level trypsin inhibition activity of methanolic leaf extract.

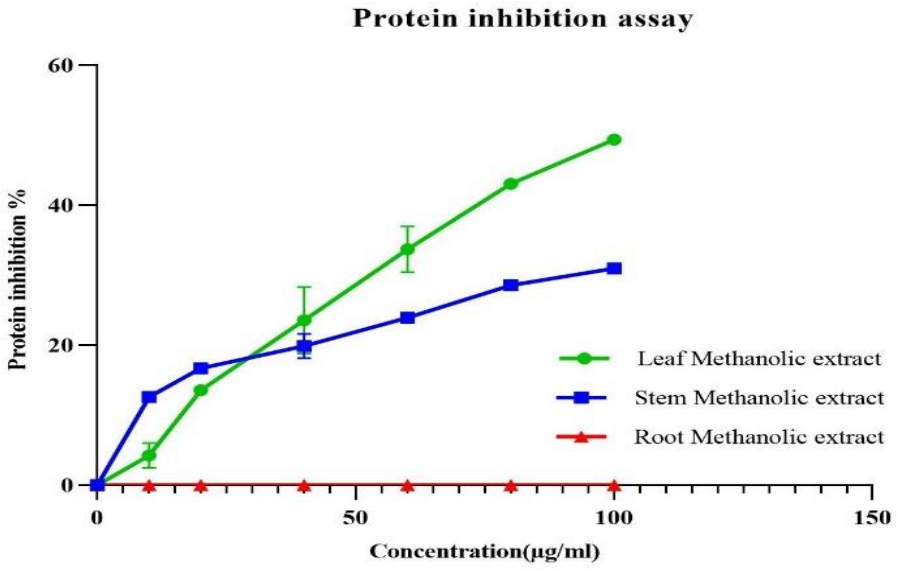


Figure 6.12: Trypsin inhibition activity of leaf, stem and root extracts of *K. caryophyllata*

Table 6.20: One-Way ANOVA and Duncan analysis of IC 50 values for Trypsin inhibition activity among Leaf, Stem, and Root of *K. caryophyllata*

One-Way ANOVA:

F value	P value	η^2_p (Effect size)
$F_{(2,9)}$ = 9710.116	P<0.001	η^2_p(Effect size)= 0.999

Duncan's Multiple Range Test:

Extract	Subset for alpha = 0.05	
	1	2
Leaf	94.7300	
Stem		336.3874
Sig.	1.000	1.000

Means for groups in homogeneous subsets are displayed

Nitric oxide inhibition assay

Invitro anti-inflammatory activity of leaf, stem, and root methanolic extracts using Nitric oxide inhibition assay shows, all the component extracts caused inhibition in the generation of nitric oxide in a dose dependant manner but with differences between extracts along the tested concentrations ranging from 10-100 µg/ml (table 6.21). Among the different extracts, leaf extract showed the highest inhibition activity, followed immediately by stem extract and the lowest activity by root extract. In all the three cases, a progressive increasing trend was observed in nitric oxide scavenging activity with increasing concentration of extracts (figure 6.13). The IC50 values calculated from the linear regression graph reveals that the lowest half-maximal inhibitory concentration values were recorded by the methanolic leaf extract (74.82µg/ml) which differed significantly lower over the IC50 values of root as well as stem extracts (table 6.22).

Table 6.21: Percentage inhibition of nitric oxide generation and IC50 values of component extracts of *K. caryophyllata*

Sl. No.	Concentration (µg)	% of Inhibition (Mean± SD)		
		Leaf Methanolic extract	Stem Methanolic extract	Root Methanolic extract
1	Control	0±0	0±0	0±0
2	10	6.41±0.638	4.79±0.74	6.593±1.06
3	20	12.67±4.31	9.72±1.9	13.553±1.00
4	40	31.03±2.73	18.2±1.44	23.441±1.44
5	60	56.02±8.92	29.00±1.61	26.582±0.96
6	80	59.16±5.13	43.34±2.70	32.487±0.90
7	100	61.32±2.08	51.48±2.39	43.348±0.6
IC50		74.82±4.67	96.93±2.86	178.66±2.05

Absorbance values are expressed as mean ± SD, n=6

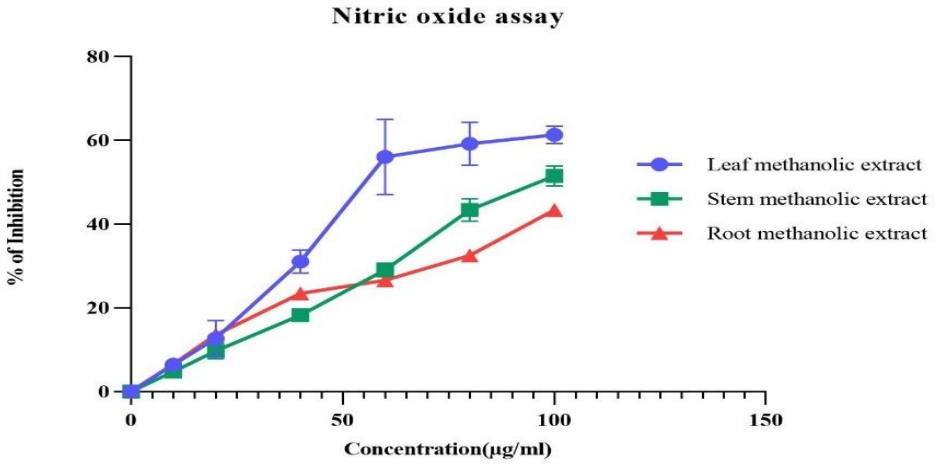


Figure 6.13: Nitric oxide inhibition activity of leaf, stem and root extracts of *K. caryophyllata*

Table 6.22: One-Way ANOVA and Duncan analysis of IC 50 values for Nitric oxide inhibition activity of leaf, stem, and root extract of *K. caryophyllata*

One-Way ANOVA:

F value	P value	η^2_p (Effect size)
$F_{(2,9)}$ = 1051.298	P<0.001	η^2_p(Effect size)= 0.995

Duncan's Multiple Range Test:

Extract	Subset for alpha = 0.05		
	1	2	3
Leaf	74.8229		
Stem		96.9320	
Root			178.6550
Sig.	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed

The results of both Trypsin as well as Nitric oxide inhibition assay revealed the methanolic leaf extract of *K. caryophyllata* has significantly lower half-maximal inhibitory concentration (IC50) values than stem and root extracts. Lower IC50 value indicates higher inhibitory activity for 50% reduction of inflammation causing agents trypsin and nitric oxide. Trypsin is a serine protease enzyme that induces inflammatory signals by activating proteinase-activated receptor-2 (Knecht et al., 2007; Meyer-Hoffert et al., 2004) while, nitric oxide (NO) is a proinflammatory signalling molecule, and excessive NO generation might cause inflammation under unusual circumstances (Sharma et al., 2007). Therefore, the inference of the present study highlights the effectiveness of methanolic leaf extract of *K. caryophyllata* to a greater extend in remediating inflammatory problems by inhibiting or scavenging inflammatory signalling molecule generated under stress conditions.

In vivo Anti-inflammatory Studies of *K. caryophyllata Leaf Extract*

Acute toxicity study

Acute toxicity studies were conducted to determine the maximum tolerance level for a single dose. No mortality or any signs of behavioural changes or toxicity were observed after oral administration of methanolic extract of *Kamettia caryophyllata* up to the dose level of 2500 mg/kg body weight in Swiss albino mice. 2% propylene glycol was administered in the control group. Even at the highest dose level of 2500mg/kg body weight, which is ten times the therapeutic effective dose (TED), all animals survived and were healthy indicating that the leaf methanolic extract of *K. caryophyllata* did not cause any toxicity in mice(table 6.23).

Table 6.23: Effect of methanolic leaf extract of *K. caryophyllata* on mortality of mice

Group	Drug	Dose	Dead/Total	% of death
Group 1	2% Propylene glycol	250µl/kg body weight	0/3	Nil
Group 2	Methanolic extract of *Kamettia caryophyllata dissolved in 2% Propylene glycol*	2500mg/kg body weight	0/3	Nil

Effects of leaf extract on behavioural characteristics of mice

The details of behavioural characteristics of mice monitored and recorded both prior to and following the application of methanolic extract from the leaves of *K. caryophyllata* is displayed in the table 6.24. Various behavioural factors were evaluated including motor activity, arching and rolling, tremors, convulsions, respiration, salivation, diarrhoea, sedation, skin colour, and eye lacrimation. None of the mice showed any behavioural changes during the first 24 hours as well as for the next fifteen days of the study.

Table 6.24: Effect of *K. caryophyllata* leaf extract on behavioural characteristics in mice

Behaviour signs of mice	Response	
	Group 1	Group 2
Motor activity	Normal	Normal
Arching and rolling	Nil	Nil
Tremor	Nil	Nil
Convulsions	Nil	Nil
Respirations	Normal	Normal
Salivation	Nil	Nil
Diarrhoea	Nil	Nil
Sedation	Nil	Nil
Skin colour	Normal	Normal
Eye Lacrimation	Nil	Nil

Effect of leaf extract on body weight of mice

In order to check and confirm any notable difference that occurred in the body weight of mice administered with leaf extract of *K. caryophyllata* at a dose of 2500mg/kg body weight, it was compared with a control group which did not receive leaf extract and the data were collected at an interval of 3 days up to 14th day and the details obtained is depicted in the table 6.25 & figure 6.14.

Table 6.25: Comparison of body weight between control and leaf extract administered groups

Days after treatment	Body weight of mice groups	
	Control mice	Leaf extract administered mice
1	30.23 ±2.84	29.50 ±0.72
4	30.63 ±2.58	29.53 ±0.55
7	30.70 ±2.59	29.93 ±0.61
11	30.27 ±3.14	29.73 ±0.80
14	30.04 ±2.40	29.81 ±0.61

The data indicates either the mice that received methanolic leaf extract or those not received leaf extract exhibited no considerable decrease or increase in body weight and did not find any significant difference in the body weight between the two groups of mice (table 6.26). This reveals that, the administration of leaf extracts up to a concentration of 2500 mg/kg body weight of mice did not cause any toxicity.

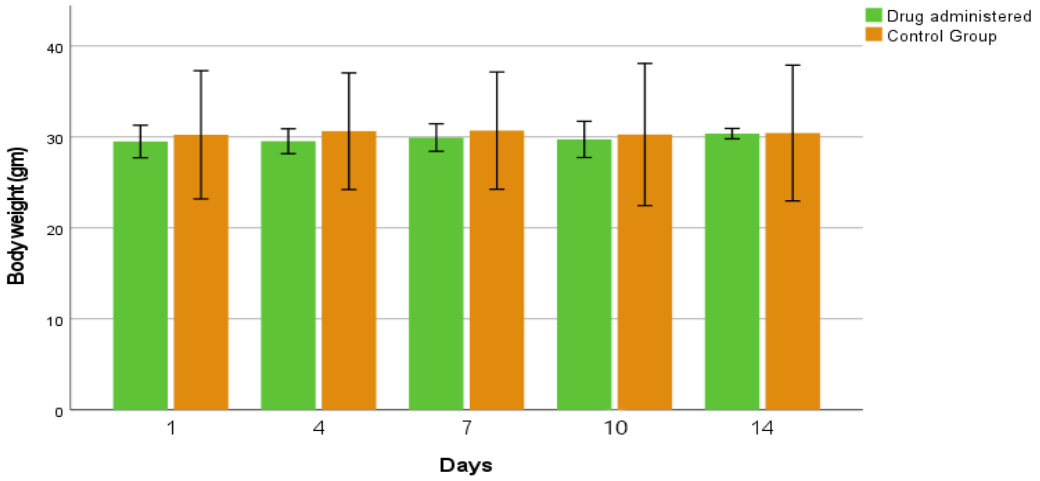


Figure 6.14: Effect of methanolic leaf extract *K. caryophyllata* on body weight of mice

Table 6.26: One-Way ANOVA on the effect of leaf extract on body weight in mice

F value	P value	η^2_p (Effect size)
$F_{(1,4)}$= 0.375	0.574	0.085

Effect of leaf extract on food consumption of mice

Both the control group as well as the leaf extract administered groups of mice consumed food as almost normal without much noticeable difference (table 6.27 & figure 6.15) and statistical analysis confirmed no significant difference between the groups (table 6.28).

Table 6.27: Comparison of food consumption between control and leaf extract administered groups

Days after treatment	Consumption of food in mice groups	
	Control mice	Leaf extract administered mice
1	14.33±1.52	13.63±0.78
4	14.07±2.10	13.70±1.47
7	13.73±1.10	15.00±1.0
11	14.17±0.76	14.833±0.29
14	14.16.5±1.28	15.17±1.25

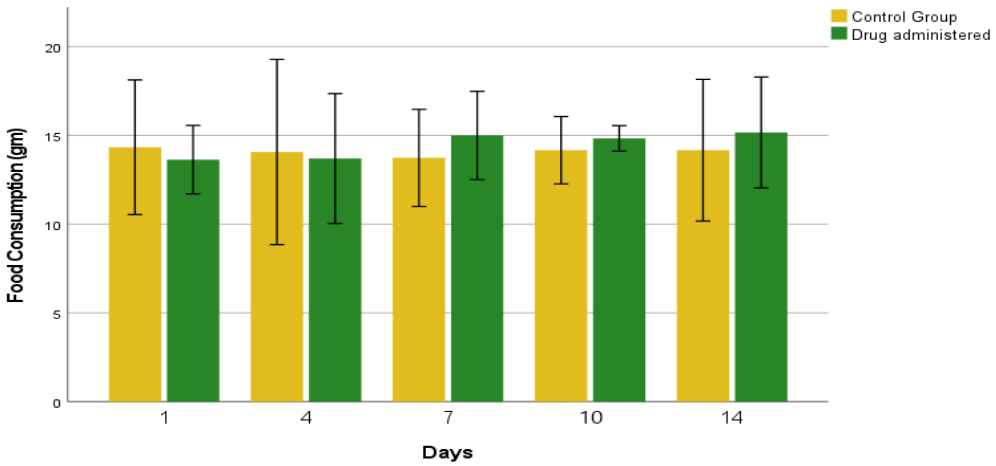


Figure 6.15: Effect of methanolic leaf extract of *K. caryophyllata* on food consumption

Table 6.28: One-Way ANOVA on the effect of leaf extract on food consumption in mice

F value	P value	η^2_p (Effect size)
$F_{(1,4)}$= 0.017	0.903	0.04

Effect of leaf extract on water consumption in mice

All the test animals in the control and leaf extract administered groups exhibit no notable difference in water consumption throughout the study (table 6.29 & figure 6.16). ANOVA results indicate no significant variations in water consumption between the groups (table 6.30).

Table 6.29: Comparison of water consumption between control and leaf extract administered groups

Days after treatment	Consumption of water in mice groups	
	Control mice	Leaf extract administered mice
1	17.57±0.78	16.4±1.44
4	16.03±1.0	15.07±0.90
7	14.83±1.19	14.40±0.53
11	15.2±1.65	15.63±1.24
14	15.87±1.35	15.39±1.09

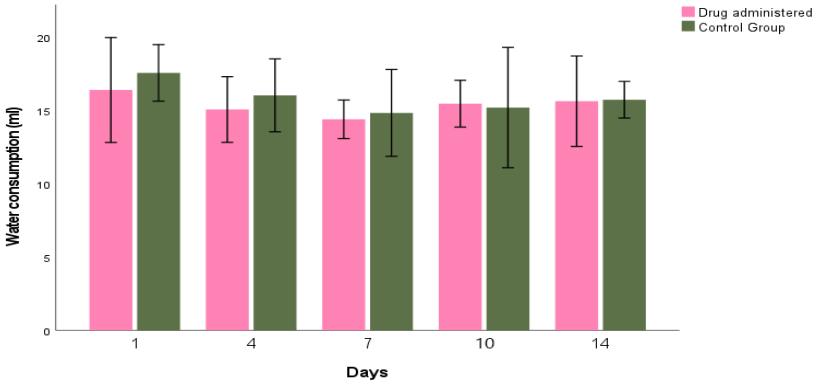


Figure 6.16: Effect of leaf extract of *K. caryophyllata* on water consumption in mice

Table 6.30: One-Way ANOVA on the effect of leaf extract on water consumption in mice

F value	P value	η^2_p (Effect size)
$F_{(1,4)}$= 0.720	0.44	0.15

Necropsy for checking abnormalities

Necropsy has been done to check whether any abnormalities occurred in internal organs because of plant leaf extract administration. No abnormalities observed in the plant drug administered group as well as in the control group (figure 6.17). Overall, the findings of the study suggest the methanolic extract of *K .caryophyllata* is safe for oral administration in mice up to a dose level of 2500 mg/kg body weight. These results support further investigation of methanolic leaf extract for therapeutic applications.

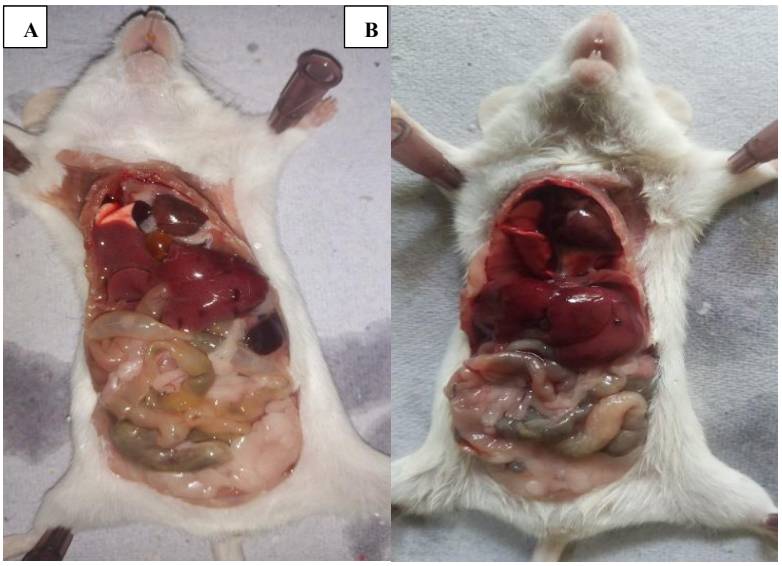


Figure 6.17: Necropsy images. (**A.** Group1- Vehicle control; **B.** Group2- Drug administered)

In vivo Anti-inflammatory studies

Carrageenan-Induced Paw Oedema in Swiss Albino Mice- Acute Inflammatory Model

The *in vivo* anti-inflammatory activity of the methanolic leaf extract of *K. caryophyllata* was validated through the Carrageenan-induced paw oedema model. The inflammation was induced by carrageenan only one hour after the plant drug was administered. Subplantar injection of carrageenan caused progressive inflammation of the left hind paw of mice and monitored hourly over 6 hours. The observations have been documented in table 6.31.

Table 6.31: The anti-inflammatory activity analysis of mice groups treated differentially in the Carrageenan-induced Paw Oedema model

Mice groups	Carrageenan induced Paw Oedema thickness at different intervals (in mm)						
	0 hour	1st Hour	2nd Hour	3rd Hour	4th Hour	5th Hour	6th Hour
Carrageenan alone (control)	0.00	0.99±0.24	1.58±0.22	1.44±0.35	1.41±0.41	1.22±0.31	1.22±0.09
2%Propylene Glycol (vehicle) + Carrageenan	0.00	1.58±0.28	1.64±0.29	1.65±0.38	1.50±0.40	1.32±0.46	1.07±0.17
Diclofenac (standard) + Carrageenan	0.00	0.79±0.24	1.21±0.31	1.24±0.12	0.90±0.27	0.72±0.06	0.56±0.14
Methanolic leaf extract 500mg/kg body wit + Carrageenan	0.00	1.38±0.25	1.19±0.17	1.02±0.13	0.99±0.04	0.72±0.14	0.46±0.11
Methanolic leaf extract 250mg/kg body wit + Carrageenan	0.00	1.49±0.22	1.43±0.25	1.24±0.16	1.16±0.36	0.94±0.38	0.45±0.11

The mice groups administered with high dose of 500mg/kg body weight as well as a low dose of 250mg/kg body weight of methanolic leaf extract after carrageenan injection exhibited a progressive decrease in the paw thickness throughout the monitoring period from 1st hour to 6th hour, though caused a comparatively higher inflammation and paw thickness in the 1st hour. However, the mice group administered

with 2% propylene glycol (vehicle) and similarly, those mice group that received diclofenac (standard) and those received carrageenan alone (control) exhibited a progressive increase in the paw thickness up to 3rd hour and there after exhibited a progressive decrease till the 6th hour as in other cases (figure 6.18). The data indicates, comparatively higher paw thickness due to inflammation was exhibited throughout the monitoring hours by mice group administered with 2% propylene glycol (vehicle) after carrageenan injection, with the largest paw thickness of 1.65mm recorded at 3rd hour. Similarly, with certain exception in the initial hours, comparatively lower paw thickness was exhibited by mice groups which received both high and low doses of methanolic leaf extracts. The reduction of inflammation induced lowest paw thickness of 0.45mm and 0.46mm at 6th hour respectively under the influence of leaf extract dosages of 250mg/kg body weight and 500mg/body weight which were found lower than the paw thickness of 0.56mm recorded under the influence of standard diclofenac(figure 6.19).

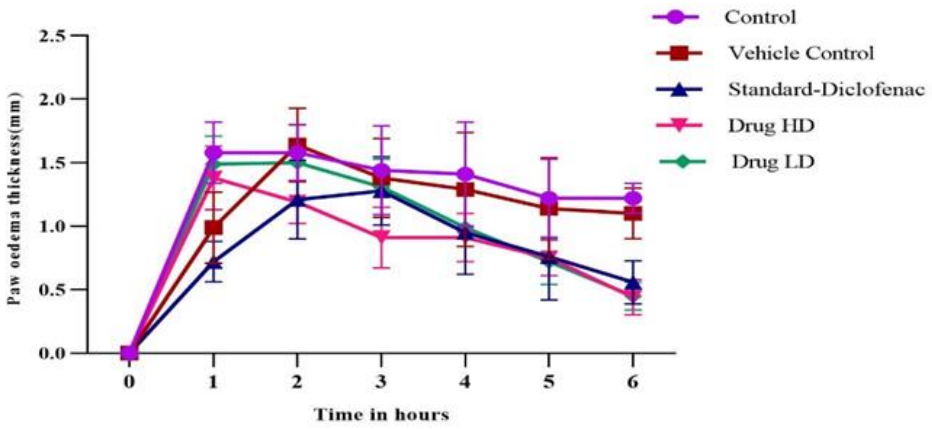
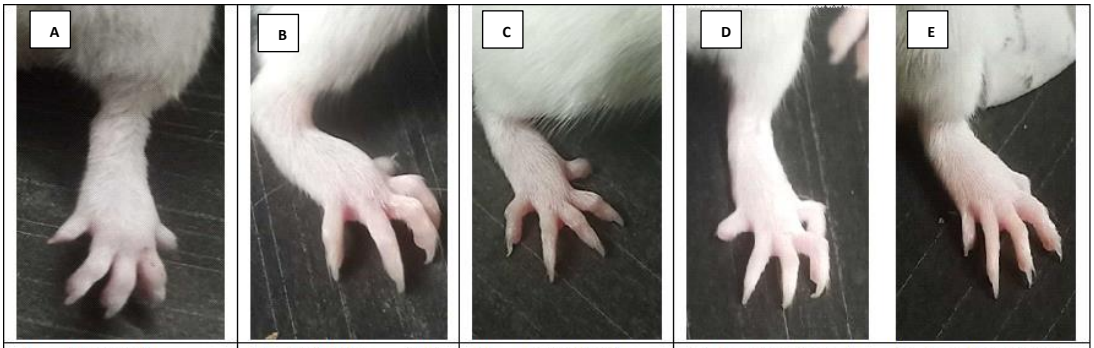


Figure 6.18: Comparative analysis of anti-inflammatory activity in treated mice groups



A. Control - Carrageenan	B. Vehicle - 2% propylene glycol	C. Standard – Diclofenac	D. Leaf extract - 500mg/body wit	E. Leaf extract - 250mg/body wit

Figure 6.19: Paw thickness mice groups in the carrageenan-induced Paw Oedema model

The statistical analysis of the data revealed, the reduced inflammation level and paw thickness induced under the remedial effect in mice groups which were administered with high dose (500mg/kg body weight) as well as low dose (250mg/kg body weight) of *K. caryophyllata* methanolic leaf extracts and the standard diclofenac differed significantly lower over the higher level inflammation and paw thickness induced by carrageenan alone as well as carrageenan & vehicle 2%propylene administered mice groups (table 6.32). However, though reduction in inflammation and paw thickness induced by leaf extracts was lower than that induced by standard diclofenac, the differences were not significant and similarly, the difference in paw thickness between the carrageenan alone and the carrageenan & 2% propylene administered mice groups were also not significant.

Table 6.32: One-Way ANOVA and Duncan analysis of final paw thickness between different mice groups in the Carrageenan-induced Paw Oedema model

One-Way ANOVA:

F value	P value	η^2_p (Effect size)
$F_{(4,25)} = 41.94$	P<0.001	0.870

Duncan's Multiple Range Test:

Type of mice groups	Subset for alpha = 0.05	
	1	2
Leaf extract -Low dose (250mg/body wt)	0.45	
Leaf extract -High dose (500mg/body wt)	0.46	
Standard – Diclofenac	0.56	
Vehicle - 2%Propylene Glycol		1.07
Control - Carrageenan		1.22
Sig.	0.207	0.080

Means for groups in homogeneous subsets are displayed

The percentage inhibition of Carrageenan induced paw oedema thickness in mice

Table 6.33 shows the percentage inhibition of carrageenan-induced inflammation as reduction in paw oedema thickness by different treated mice groups at final 6th hour of monitoring. In the study, it was noticed that starting from 1st hour to 6th hour of monitoring, both high as well as low doses of *K. caryophyllata* leaf extracts exhibited a progressive reduction in carrageenan-induced inflammation as reduction in paw

oedema thickness of mice (table 6.33). In the final 6th hour of monitoring, the high dose of (500mg/kg body weight) *K. caryophyllata* methanolic leaf extract recorded the highest inhibition of 63.36% and this was followed immediately by inhibition of 62.12% recorded by low dose of (250mg/kg body weight) leaf extract. In contrast, the standard drug diclofenac recorded an inhibition of 54.27%, which was 9.09% and 7.85% respectively lower than the inhibition recorded by high and low doses of leaf extract, but these differences where found in insignificant(table 6.34). The least percentage inhibition of 11.43% was recorded by the mice group treated with 2% propylene glycol (figure 6.20).

Table 6.33: The percentage inhibition of carrageenan-induced inflammation paw oedema thickness in treated mice groups

Mice groups	Percentage of inhibition at 6th hour
Control carrageenan alone	-
Vehicle -2% propylene glycol	11.43 ±14.22
Standard – Diclofenac	54.27 ±11.36
K. caryophyllata methanolic extract High dose of 500 mg/kg body wt.	63.36 ±13.58
K. caryophyllata methanolic Extract Low dose of 250 mg/kg body wt.	62.12 ±8.98

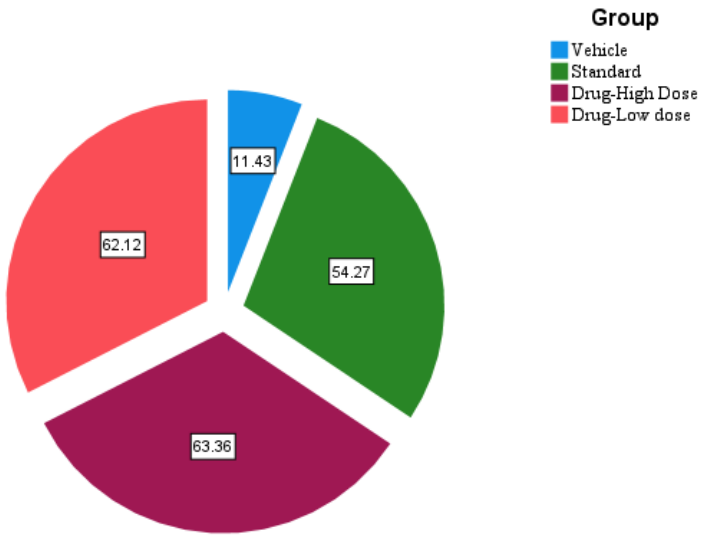


Figure 6.20: The percentage inhibition in the inflammation paw oedema thickness in treated mice groups

Table 6.34: One-Way ANOVA and Duncan analysis of percentage of inhibition in the carrageenan-induced inflammation paw oedema thickness in treated mice groups

One-Way ANOVA:

F value	P value	η^2_p (Effect size)
$F_{(2,9)} = 24.29$	P<0.001	0.786

Duncan's Multiple Range Test:

Extract	Subset for alpha = 0.05	
	1	2
Vehicle	11.43	
Standard		54.27
Drug- Low dose		62.12
Drug-High dose		63.36
Sig.	1.000	0.237

Means for groups in homogeneous subsets are displayed

The inference of the present study clearly highlights the effectiveness of methanolic leaf extracts as an anti-inflammatory agent. The finding also offers valuable insights into specific phytocompounds of leaf extract and their mechanisms behind the anti-inflammatory activities. The two-phase oedema reaction seen in the rat paw after injecting carrageenan under the skin consists of two separate stages (Posadas et al., 2004). The first stage begins about an hour after injection and is marked by the discharge of prostaglandins created by cyclooxygenase enzymes (COX), along with histamine, serotonin, and bradykinin. Following this, the postponed stage, which typically lasts around an hour, is linked to the invasion of neutrophils and the continuous production of prostaglandins (Bohlin et al., 2007). In addition, nitric oxide (NO) free radicals produced by neutrophils and inflammatory cytokines like interleukin-1 β (IL-1 β) and tumour necrosis factor (TNF-α) are released in this stage, adding to the acute inflammatory response (Gilligan et al., 1994). The carrageenan-induced anti-inflammatory model is widely recognised and utilised for evaluating the anti-inflammatory properties of natural and synthetic substances (Patil et al., 2019). As a phlogistic agent, carrageenan is non-antigenic and has no apparent systemic effects. (Boominathan et al., 2004). Its sulphated sugars trigger the inflammatory mediators and complement system, stimulating phospholipase A2, which starts the early stage of inflammation and is furthered by cytotoxic effects. (Osadebe & Okoye, 2003; Duwiejua et al., 2002). Postcapillary venules dilate due to the action of

carrageenan, causing inflammatory cells and fluid to exude and several proinflammatory mediators to be released. Anti-inflammatory substances can inhibit these inflammatory actions. The screening of phytochemical compounds previously performed in the methanolic leaf extract of *K. caryophyllata* identified a vast number of specific bioactive compounds of anti-inflammatory property (chapter-5). The significant anti-inflammatory activity of methanolic leaf extract revealed in this *in vivo* study can be attributed to the occurrence of these compounds known for their anti-inflammatory activities.

Formalin- -Induced Paw Oedema in Swiss Albino mice- Chronic Inflammatory Model

The formalin-induced paw oedema model is another well-established acute inflammatory model used to evaluate the anti-inflammatory potential of pharmacological agents. Here, the sub plantar injection of formalin produced a progressive swelling of the right hind paws of the animals and the thickness of paw oedema was observed in various treatment groups for six days. The reduction in the thickness of paw oedema is recorded in table 6.35. During the initial day, the formalin-treated control group showed a thickness of 1.48 mm, whereas the vehicle control group had a slightly greater thickness of 1.5 mm. The use of Diclofenac led to a thickness of 1.38 mm, reduced somewhat compared to the control groups. The paw oedema thickness of 1.62mm and 1.50mm recorded in the groups treated with *K. caryophyllata* methanolic leaf extract respectively for high and low doses was comparable to that of the control groups. Maximum paw thickness was observed on the first day of treatments except in the vehicle group. In the vehicle control group, inflammation slightly increased on the second day of 1.6 mm thickness. Minimum thickness was observed in the group that received the standard drug Diclofenac. The paw oedema thickness reduced in all the groups, but the volume of reduction was different in different groups (figure 6.21). Maximum reduction of inflammation was observed in the diclofenac-administered group on the sixth day (0.62mm). The high-dose and low-dose groups also showed a significant decrease, compared to the control group. The paw oedema thickness of the high-dose group significantly decreased to 0.65 mm, while that of low-dose group decreased to 0.76mm on the sixth day. This indicate the great potential of *K. caryophyllata* methanolic leaf extract, particularly at

higher dose to match the effectiveness of the standard medication using diclofenac for treating inflammation.

Table 6.35: Paw oedema thickness of mice groups in Formalin Induced Anti-inflammatory Model.

Mice groups	Paw oedema thickness different mice groups at different intervals (in mm)						
	0 day	1st day	2nd day	3rd day	4th day	5th day	6th day
Formalin alone (control)	0	1.48±0.22	1.43±0.20	1.43±0.37	1.19±0.25	1.06±0.15	1.02±0.14
2%Propylene Glycol (vehicle) + Formalin	0	1.5±0.32	1.6±0.033	1.28±0.48	1.07±0.18	1.02±0.20	0.98±0.14
Diclofenac (standard) + Formalin	0	1.38±0.09	1.32±0.11	1.23±0.16	1.08±0.39	0.93±0.26	0.62±0.11
Methanolic leaf extract 500mg/kg body wt + Formalin	0	1.62±0.07	1.30±0.09	1.09±0.13	0.82±0.13	0.81±0.08	0.65±0.06
Methanolic leaf extract 250mg/kg body wt + Formalin	0	1.50±0.16	1.30±0.22	1.36±0.39	1.02±0.30	0.85±0.18	0.76±0.08

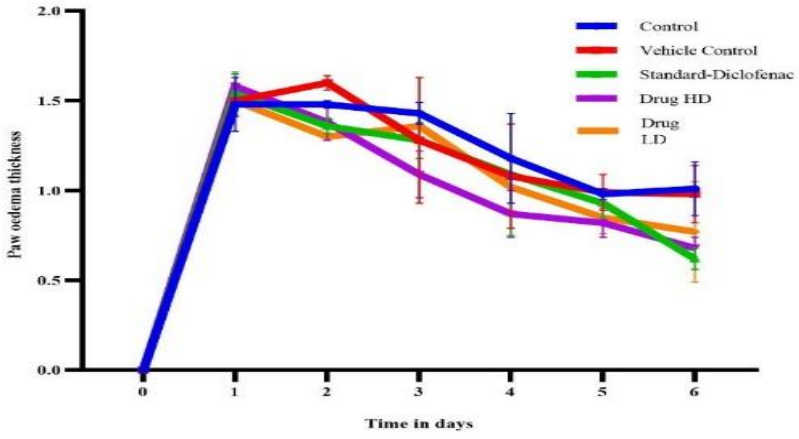


Figure 6.21: Comparison of Paw oedema thickness of different mice groups in Formalin Induced Anti-inflammatory model

The data obtained on the final 6th day was statistically analysed and revealed that the differences in paw oedema thickness among mice groups treated with standard formalin, high and low doses of methanolic leaf extracts were found not significant, while their differences over the values of thickness obtained in mice group treated with vehicle 2% propylene glycol as well as control mice group that received formalin

alone were significantly lower. The study also revealed that the differences in paw thickness between vehicle group and control group was insignificant (table 6.36).

Table 6.36: One-Way ANOVA and Duncan analysis of final day Paw Oedema thickness of mice in Formalin Induced Anti-inflammatory model

One-Way ANOVA:

F value	P value	η^2_p (Effect size)
$F_{(4,25)} = 16.229$	P<0.001	0.720

Duncan's Multiple Range Test:

Extract	Subset for alpha = 0.05	
	1	2
Standard	0.623	
Drug-High dose	0.647	
Drug- Low dose	0.763	
Vehicle		0.978
Control- Formalin		1.03
Sig.	0.053	0.453

Means for groups in homogeneous subsets are displayed

The percentage inhibition of formalin-induced paw oedema thickness

The percentage inhibition in the formalin-induced inflammation of paw oedema thickness exhibited by different treated mice groups on 6th day of monitoring is shown in the table 6.37. The mice group that received the standard diclofenac recorded the highest inhibition percentage (38.89%). This was immediately followed by an inhibition percentage of 36.06% recorded by mice group which received high dosage of *K. caryophyllata* leaf extracts (figure 6.22) and compared to standard, it was only 2.83% less inhibition and not differed significantly. Followed by high dosage, the next higher percentage of inhibition was recorded by low dosage of leaf extract and it was 25.16% inhibition, which differed significantly lower over the percentage of standard as well as high dosage treatments. The lowest inhibition value of 4.08% in the study was recorded by mice group which received 2% propylene glycol after formalin application, and it differed significantly lower over the percentage of all the treated groups (table 6.38 & figure 6.23).

Table 6.37: The percentage inhibition of formalin-induced inflammation paw oedema thickness in treated mice groups

Mice groups	Percentage of inhibition at 6th hour
Control formalin alone	-
Vehicle -2% propylene glycol	4.08 ±13.7
Standard – Diclofenac	38.89 ±7.16
K. caryophyllata methanolic extract High dose of 500 mg/kg body wt.	36.60 ±6.78
K. caryophyllata methanolic Extract Low dose of 250 mg/kg body wt.	25.16 ±8.17

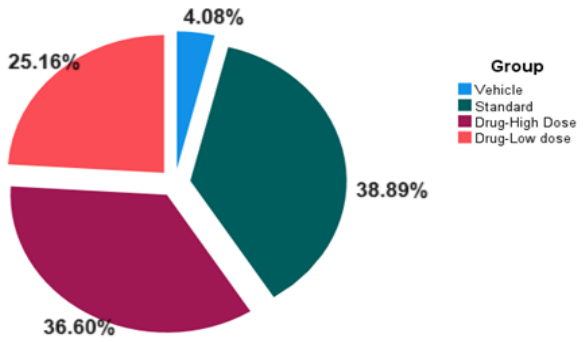


Figure 6.22: The comparison of percentage inhibition in the inflammation paw oedema thickness induced by formalin in treated mice groups

Table 6.38: One-Way ANOVA and Duncan analysis of percentage of inhibition in the ormalin-induced inflammation paw oedema thickness in treated mice groups

One-Way ANOVA:

F value	P value	η^2_p (Effect size)
$F_{(3,20)} = 24.29$	P<0.001	0.720

Duncan's Multiple Range Test:

Extract	Subset for alpha = 0.05		
	1	2	3
Vehicle	4.08		
Drug- Low dose		25.16	
Drug-High dose			36.60
Standard			38.89
Sig.	1.000	1.00	0.678

Means for groups in homogeneous subsets are displayed

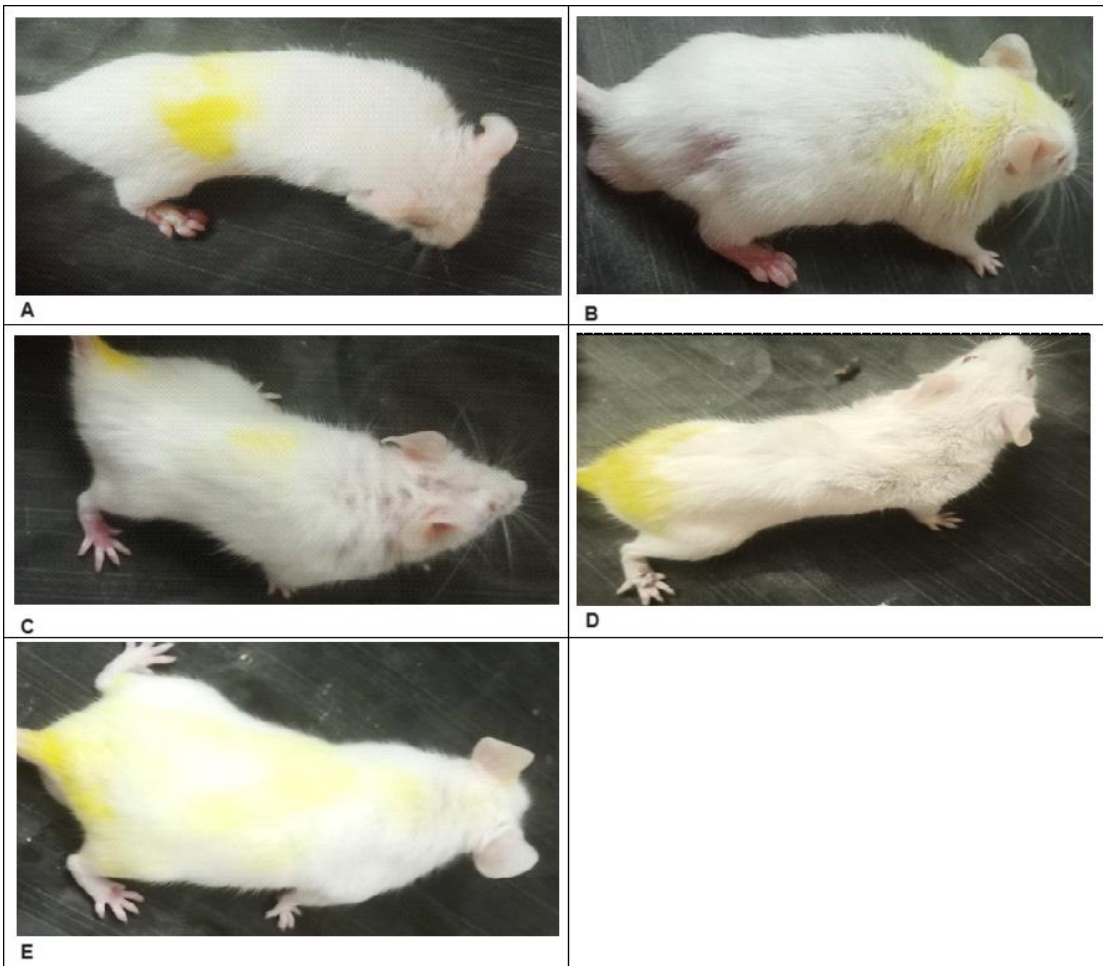


Figure 6.23: The paw oedema thickness of mice after the sixth day of treatment in Formalin Induced Anti-inflammatory model. (A. control; B. vehicle; C. standard -diclofenac; D. low dose of leaf methanolic extract. E. high dose of leaf methanolic extract)

The findings of formalin-induced inflammation paw oedema model study showed great potential of *K. caryophyllata* methanolic leaf extract particularly at high dose to match the effectiveness of the standard medicine in treating inflammation. This formalin-induced inflammation model is very similar to arthritis disease in humans and is regarded as one of the top experimental methods for testing the lasting of anti-inflammatory benefits of various drugs (Patil et al., 2019). As in the case of carrageenan-induced anti-inflammatory model, the formalin-induced inflammation displays a two-phase pattern with separate neurogenic and inflammatory stages. In the initial neurogenic stage, bradykinin and substance P are pivotal in mediating pain and inflammation. Later in the inflammatory phase, histamine, serotonin (5-HT), prostaglandins, and bradykinin play a role in maintaining the inflammatory reaction (Sofidiya et al., 2014). Nitric oxide and prostaglandins were released more from the

spinal cord during formalin-induced inflammation, as reported by Malmberg and Yaksh in 1992. This phenomenon is linked to hypersensitivity to pain.

Medications like corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs) mainly target the peripheral nervous system and specifically inhibit the second phase of inflammation. These findings highlight the complexity of the inflammatory response and the diverse mechanisms by which medications can exert their anti-inflammatory effects. Formalin-induced inflammation can aid in developing targeted therapeutic strategies for managing inflammatory conditions, including arthritis in humans (Lee-Kubli & Calcutt, 2014). The traditional use of *K. caryophyllata* for arthritic pains is documented in the Hortus Malabaricus, Volume IX (Manilai & Remesh, 2010). The bioactive compounds present in the *K.caryophyllata* may have the ability to reduce inflammation associated with arthritis.

The primary aim of this study was to compare the anti-inflammatory effects of leaf methanolic extract of *K. caryophyllata* with the standard anti-inflammatory drug diclofenac. Diclofenac, a well-known nonsteroidal anti-inflammatory drug (NSAID) was used as the benchmark in the present *invivo* studies to compare the anti-inflammatory effect of leaf methanolic extract of *K. caryophyllata*. Diclofenac is frequently used as a first-line treatment for both acute and chronic pain and inflammation resulting from a range of arthritis conditions. According to the National Center for Biotechnology Information (2024), non-steroidal anti-inflammatory medication Diclofenac, derived from phenylacetic acid, can inhibit the enzymes cyclooxygenase (COX)-1 and -2 that produce prostaglandins (PGs), which have a role in pain and inflammatory signalling (Crofford et al., 2016; DuBois et al., 1998).

In both *in vivo* models conducted in the present study, the results indicated that the selected high and low doses of *K. caryophyllata* leaf methanolic extracts, particularly high dose of 500mg/kg body weight of mice were as effective as standard medicine Diclofenac in reducing the inflammation thickness of paw oedema. The statistical analysis, including ANOVA and post hoc test-Duncan's analysis confirmed significant reduction in paw oedema thickness in the treatment groups compared to the control group. Therefore, the findings of this study confirmed the potential of *K. caryophyllata* leaf methanolic extract as a natural anti-inflammatory agent, as it could be attributed to its bioactive compounds present in the extract, which possess anti-

inflammatory properties by inhibiting inflammatory mediators and pathways. Phytochemical analysis conducted on the extracts of *K. caryophyllata* highlights the abundance of terpenoids, alkaloids, phenolics and flavonoids in the leaf methanolic extract.

Prior research has shown that different terpenoids have anti-inflammatory properties that can influence different signalling pathways related to inflammation to reduce the inflammatory reaction caused by carrageenan and other similar agents (Lu et al., 2014). Terpenoids can inhibit TNFα-induced ICAM-1 and nitric oxide synthase (iNOS) expression to reduce NO production in human cells (Ribeiro et al., 2020). Various terpenoid bioactive compounds of anti-inflammatory property detected in the study through HR LC-MS and GC-MS screening such as Squalene; γ-Sitosterol; Phytol; Stigmast-4-ene-3,6- dione; (+)-Myrtenyl formate; Fukinolic acid; Squalamine; Lyciumoside IX; Alloxanthin; Saikosaponin BK1 etc. might have contributed to this anti-inflammatory activity (Huang et al., 2009; Cardino et al., 2015; Awadh Ali et al., 2017; Lalitha et al., 2014; Santhosh et al., 2014; Abubakar & Majinda, 2016; Gavin et al., 2013; Yao et al., 2011; Konishi et al., 2008; Sulaiman et al., 2022).

Like terpenoids, there are a number of alkaloid compounds, as previously reported, with anti-inflammatory properties that may affect inflammatory pathways and reduce inflammation. The alkaloid inhibits lymphocyte proliferation induced by antigen and mitogen, natural killer cell cytotoxicity, mast cell histamine release, IL-1 secretion by human monocytes, and PAF's effect on platelets (Seow et al., 1989). The different alkaloid compounds of anti-inflammatory property identified in the LC-MS and GC-MS analysis of leaf extract like Flazine; 3beta,6beta Dihydroxynortropane; (-)-Quebrachamine etc. may also have contributed to the observed anti-inflammatory potential in the study (Kim et al., 2016; Zhao et al., 2023).

Similarly, phenolic compounds are known for their antioxidant as well as anti-inflammatory properties. The present study also detected the occurrence of several specific phenolic compounds having anti-inflammatory property in the leaf component of *K. caryophyllata* and major compounds includes Grossamide; Umbelliferone; Triprolidine; Theasinensin C etc. (Mazimba, 2017; Shim et al., 2022; Liu et al., 2021; Mostafa et al., 2018). Their occurrence in methanolic leaf extract

implies its capacity to eliminate free radicals and decrease oxidative stress, ultimately inhibiting inflammation and tissue damage (Wink, 2015). The synergistic and additive anti-inflammatory action of these diverse phytochemicals including terpenes, phenolics and alkaloids in *K. caryophyllata* leaf extract might have contributed to the overall effectiveness in reducing or inhibiting inflammation in both the models performed and indicate its promise as a treatment for inflammatory conditions, by potentially affecting inflammatory pathways or inflammatory mediators or other agents of inflammation. The findings further highlight, the inflammation-reducing effects of *K. caryophyllata* and its potential as a promising option for future research in creating anti-inflammatory drugs. More investigation is needed to clarify the molecular targets and signalling pathways of bioactive compounds exhibiting their anti-inflammatory properties.

CONCLUSION

The study of bioactivity properties of *Kamettia caryophyllata* such as antioxidant, antibacterial, cytotoxic and anti-inflammatory, conducted using methanolic extracts of different components revealed wide range of pharmacological effects and its potential as a promising candidate for future research. The antioxidant studies using phosphomolybdenum, DPPH, and hydrogen peroxide scavenging assays showed notable differences in IC50 values among various components and revealed significant and higher antioxidant property exhibited by the leaf extract with lowest IC50 values. This could be due to the occurrence of more diverse and higher number of antioxidant compounds offering greater free radical scavenging activities, compared to stem and root components. The study further revealed that the radicle scavenging effect of all the component extracts was concentration-dependent and the inference of the study have given valuable insights into their chemical composition and potential antioxidant activities and associated health benefits.

The antimicrobial activity studies against different tested pathogenic microorganisms displayed a dose-dependent action reaching peak antimicrobial effectiveness at higher levels. The leaf extract showed the strongest antimicrobial activity against all tested pathogens, with *Pseudomonas aeruginosa* being the most sensitive. The antimicrobial activity may be due to phytochemicals present in the component extract causing disruption of cell wall, inhibiting enzymes essential for bacterial growth, interfering

with DNA replication, disrupting cell membranes and chelating essential nutrients like iron required for growth.

The cytotoxicity study of methanolic extracts from different components of *K. caryophyllata* using Trypan Blue exclusion method revealed that the leaf methanolic extract has exhibited greater cytotoxic effects against DLA and EAC cancer cell lines than stem and root extracts. The IC50 values were significantly lower for leaf extracts, indicating their superior efficacy in inhibiting cancer cell growth and suggests that the cytotoxic compounds responsible for the anticancer effects are more abundant in the leaf component. The findings of the study are significant in cancer research, where novel therapeutic options are urgently needed.

The *in-vitro* anti-inflammatory assays revealed that the leaf methanolic extract of *K. caryophyllata* has more potent anti-inflammatory activity than the stem and root extracts. Followed by this, the study conducted *in-vivo* anti-inflammatory analysis, including Carrageenan-induced and Formalin-induced inflammation models in order to further ensure its anti-inflammatory therapeutic properties and revealed that the leaf methanolic extract outperformed all the bioactivity studies compared to stem and root extract. The anti-inflammatory effects of *K. caryophyllata* extract can be attributed to its bioactive compounds, including alkaloids, phenolics, flavonoids and terpenoids. These compounds possess anti-inflammatory properties by inhibiting inflammatory mediators and pathways, reducing oxidative stress and modulating cytokine expression and enzyme activity associated with inflammation. The collaborative effect of these active compounds may account for the notable anti-inflammatory effectiveness observed in the study. The observations suggest that *K. caryophyllata* methanolic extract holds promise as a potential treatment for inflammatory conditions. Further research is warranted to elucidate the precise molecular mechanisms and signalling pathways involved in its anti-inflammatory effects, paving the way for developing novel therapeutic strategies.

The occurrence of more diverse and higher number of phytocompounds with specific bioactivity has been identified in the methanolic leaf extract over stem and root extracts through various phytochemical screening procedures like HR LC-MS and GC-MS in the study. The synergistic and additive action of these diverse phytochemicals including terpenes, phenolics and alkaloids in *K. caryophyllata* leaf

extract might have contributed to the overall effectiveness. The overall findings of the bioactivity studies highlight the promising role of *K. caryophyllata* as a valuable source of natural antioxidant, antimicrobial, anticancerous and anti-inflammatory compounds with potential therapeutic applications. Further research into the specific bioactive compounds and their mechanisms of action is essential for harnessing the full therapeutic potential of *K. caryophyllata* in the prevention and management of various diseases. Additionally, clinical studies are warranted to validate the efficacy and safety of *K. caryophyllata* extracts for human use, paving the way for developing evidence-based therapeutic interventions.

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Chapter 7

Isolation, purification and characterisation of squalene from leaf methanolic extract of *Kamettia caryophyllata*

CONTENTS

INTRODUCTION

Drug development requires the extraction and separation of bioactive components from plants in order to create more reliable and potent treatments. Terpenes and terpenoids are the major bioactive phytocompounds of essential oils and their pharmacological properties have extensively been analysed. These bioactive substances, which are made up of many isoprene units, make up the greatest group of organic substances found in different plants. Monoterpenes, sesquiterpenes, diterpenes, triterpenes, and tetraterpenes are different classes of terpenoid compounds, reported to play key roles in pharmacological industry and human and animal health (Perveen, 2018). According to Zhao et al. (2023), they have a variety of pharmacological properties such as anti-inflammatory (Silva et al., 2015), antioxidant (Falleh et al., 2020; Mimica-Duke et al., 2003), antimicrobial (Falleh et al., 2020; Burt, 2004), anticancer (Dordevic et al., 2007), immunomodulatory (Mediratta et al., 2002), antibacterial, antifungal, antiviral and neuroprotective qualities.

Squalene, also known as 2, 6, 10, 15, 19, 23-hexamethyl-2, 6, 10, 14, 18, 22-tetracosahexaene, is a naturally occurring triterpene that is gaining considerable interest for its diverse biological properties and possible uses in different pharmacological sectors of interest (Ghimire et al., 2015). Its anti-inflammatory,

antioxidant, antimicrobial and anti-cancer capabilities make it a sought-after ingredient within the pharmaceutical, nutraceutical, cosmetic, and food industries (Cardeno et al., 2015; Govind Rao & Achaya, 1968; Huang et al., 2009). In the past, squalene mainly came from the livers of sharks and whales, which were highly valued for their believed healing powers, especially in Japanese society (Rosales-Garcia et al., 2017). The significant amount of squalene found in the liver oils of marine animals was associated with protective benefits against bacterial and fungal infections and related inflammations, particularly in cases like Eczema and dry skin (Ulrikh & Smolovskaya, 2022). Many plant sources such as wheat germ, grape seed oil, olive oil, soybean oil, peanuts, corn, and amaranth contain squalene, with olive oil being the main commercial source and amaranth having the highest squalene content (Huang et al., 2009).

In order to harness the power of a specific phytocompound, it is necessary to isolate that particular compound from plant and this involves its extraction, purification, identification and structuration. This process is essential for acquiring a refined and characterised version of the compound from the plant. In the present study, terpenoids were found in higher quantities in the methanolic leaf extract of *Kamettia caryophyllata*. The GC-MS screening of methanolic extracts from the leaf, stem, and root revealed the occurrence of different bioactive compounds, with squalene being the specific compound consistently found in all the component extracts. Moreover, the study detected a compound squalamine in the HR LC-MS screening of methanolic leaf extract, which is a conjugated form of squalene. The consistent occurrence of the compound squalene in all the component extracts in the study and considering their known potent and diverse pharmacological effects such as anti-inflammatory, antioxidant, antimicrobial, anti-cancerous, anti-hyperglycemic properties and further, the knowledge regarding the use of *K. caryophyllata* plant in traditional system of medicine for treating arthritis, scabies, itches etc. and associated pain and inflammation as documented in the Hortus Malabaricus (Manilal & Remesh, 2010), prompted the present investigation to isolate and characterise squalene from the methanolic leaf extract of unexplored *K. caryophyllata* for broadening the scope of its potential benefits.

REVIEW OF LITERATURE

Plants used in traditional system of medicine may contain a vast number of phytochemical substances that may be suitable for treating various chronic and infectious diseases and their separation either as pure phytocompounds or as standardized plant extracts, provide enormous opportunities and scope for new drug leads due to the unmatched occurrence of chemical diversity (Cos et al., 2006). Because of enhancing interests on phytochemicals diversity in the screening programs, seeking bioactive compounds of therapeutic value from medicinal plants has considerably increased globally. The initial and major steps to isolate and employ phytochemical compounds of biological interests from medicinal plants are extraction of phytochemicals, screening of compounds, isolation, characterization, structurisation and toxicological as well as clinical evaluation of bioactive compounds. The analysis of these bioactive compounds in plant extracts involve the applications of usual preliminary screening assays for phytochemical detection, chromatographic techniques like High performance Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC) and also non-chromatographic techniques like FTIR - Fourier Transform Infra-Red technique (Sasidharan et al., 2011).

Terpenes, the most abundant and varied secondary metabolites in plants and animals, include larger classes such as sterols and squalene found in animals (Masyita et al., 2022). Terpenoids play a crucial role in numerous physiological processes in plants. Prakash et al. (2017), pointed out the importance of terpenoids in self-defence and protection, regulating membrane fluidity, respiration, photosynthesis and growth in plants. Squalene, a highly unsaturated triterpene, has a molecular weight of just over 400 (Nistor et al., 2022). It was Japanese researcher Dr.Mitsumaru Tsujimoto in 1906 who discovered squalene by isolating the unsaponifiable part of shark liver oil and identifying the presence of a highly unsaturated hydrocarbon. The long-term sustainability of these oceanic animals and the presence of substances like cholesterol in the liver oils of marine creatures have limited their widespread use in beauty products. Consequently, there is a growing interest in exploring alternative plant-based sources for squalene (Okada & Matsumoto, 2004).

Deiana et al. (2001) reported that squalene has been found in multiple plant sources, such as rice bran, amaranth, wheat germ, carrots, alfalfa, and lettuce. Olive oil,

soybean oil, grape seed oil, and corn also contain squalene and olive oil is the primary commercial source, while *Amaranthus sp*. is also notably abundant in squalene, with approximately 5.1-7.7% extracted and purified from its seeds (Huang et al., 2009).

Squalene, a triterpenoid, has attracted considerable interest due to its wide range of beneficial properties in pharmaceuticals, cosmetics, and nutraceuticals. Farvin et al. (2006) emphasised squalene's ability to lower lipid levels, act as an antioxidant, and stabilise cell membranes. Subsequent research has highlighted anti-inflammatory, antioxidant, antimicrobial, and anti-cancerous properties (Cho et al., 2009: Latief et al., 2020). Additionally, squalene is used as an adjuvant in vaccines to boost immune responses and improve efficacy (Fox, 2008).

Research into the extraction, isolation and determination of squalene has explored various techniques. Traditional methods often involve mechanical pressing or chemical extraction using nonpolar solvents such as hexane and ether. Popa et al. (2015) noted that solvent-based methods can achieve yields of over 98%. Czaplicki et al. (2012) investigated squalene content in amaranth oil using extraction methods such as supercritical fluid extraction, cold pressing, and chloroform/methanol extraction. Reddy et al. (2021), studied the presence of squalene in the ethanolic extract of *Clerodendron serratum* through GC-MS analysis. Squalene was eluted at a retention time of 17.5 minutes, which matches the retention time of the standard squalene peak, thereby confirming its presence in the extract. Additionally, Nenadis and Tsimidou (2002) proposed a fractional crystallisation method for the rapid and cost-effective determination of squalene in virgin olive oil. A study by Jose et al. (2023) demonstrated the significant anti-inflammatory and antioxidant effects of squalene isolated from methanolic extract of *Simarouba glauca,* in an experimental animal model.

Various analytical techniques, including saponification, silica gel column chromatography, Thin-layer chromatography (TLC), High-performance liquid chromatography (HPLC), Fourier-transform infrared spectroscopy (FTIR), Nuclear Magnetic Resonance spectroscopy (NMR) and Gas chromatography-Mass spectrometry (GC-MS) can be employed to extract, purify, and characterise compounds. Saponification facilitates the hydrolysis of lipids and helps to achieve the yield of terpene and sterols, which aids in squalene extraction from plant materials.

He et al. (2002) developed a method to isolate and purify squalene from amaranth grain, which showed a significant increase from 4.2% to 43.3% in squalene content after saponification and subsequent column chromatography. Wu et al. (2022) developed a method for the determination of squalene, phytosterols, and tocopherols in edible oil, utilising solid phase extraction, saponification, and gas chromatography-mass spectrometry (GC-MS) for precise and efficient analysis of these compounds. Atiku et al. (2021), isolated squalene from *Ficus sycomorus* leaf extract by flash column chromatography accompanied by TLC, GCMS, and NMR. Patel et al. (2020) reported that column chromatography separates plant constituents based on their differential affinity towards the stationary phase. According to Borisove et al. (2021), Thin Layer Chromatography is a fast and cost-effective analysis to identify plant compounds. Renukadevi (2023) detected squalene from *Ganoderma lucidum* by employing the TLC method, and 10 % sulphuric acid was used as a spraying agent to visualise squalene.

High-performance liquid chromatography is a powerful method for both qualitative and quantitative analysis of phytocompounds and it enables accurate measurement and sensitivity in intricate mixtures. Analytical approaches for determining squalene involve HPLC methods, such as HPLC-RID, which separates squalene from triglycerides effectively by using a mobile phase consisting of a 1:1 ratio of acetone and acetonitrile (Popa et al., 2015). In another study, squalene was identified in the seed oil of *Euonymus europaeus L.* using hexane extraction followed by high-performance liquid chromatography with UV detection (HPLC UV), where squalene was observed to have a retention time of 9 minutes with a mobile phase of acetonitrile:2-propanol in the ratio 90:10 (Vrubel et al., 2019).

FTIR helps to detect functional groups and assess purity, while GC-MS offers precise identification and quantification using mass-to-charge ratios. FTIR analyses the characterisation of compounds by detecting characteristic absorption bands related to its structure, aiding in its identification and purity assessment. Latief et al. (2020) confirmed the presence of squalene by FTIR analysis from the methanolic extract of *Abroma augusta.* GC-MS analysis identified the presence of squalene and its molecular weight from the GC-MS spectrum of the isolated fraction. GC-MS separates and detects individual compounds within a mixture based on their mass-to-

charge ratio and retention times for providing highly accurate identification of the compound and allowing for its quantification in complex matrices (Reddy et al., 2021).

MATERIALS AND METHODS

Isolation of unsaponifiable from leaf methanolic extract of *Kamettia caryophyllata*

Ten grams of a methanolic leaf extract from *Kamettia caryophyllata* were precisely measured and put into a flask, and then 100 ml of alcoholic potassium hydroxide solution was added. The combination was softly heated with a reflux condenser for approximately one hour until the saponification was done. Following that, the condenser was washed with approximately 10 millilitres of ethyl alcohol. After cooling, the mixture was moved to a separating funnel, making sure it was fully transferred by rinsing the flask with 95% ethyl alcohol and adding 80 millilitres of distilled water for dilution. The extractable material was obtained by vigorously shaking the mixture with 50 millilitres of petroleum ether and allowing it to settle until distinct layers formed. The bottom layer was moved with soap solution, and the procedure of extracting with ether was done six more times again, using 50 ml of petroleum ether each time. When an emulsion was present, a small quantity of ethyl alcohol or alcoholic potassium hydroxide was included accordingly. The combined ether extracts were washed three times with 25 ml of aqueous alcohol, shaking and removing the alcohol-water layer afterwards. The ether layer was washed with 20 millilitres of water multiple times until the wash water did not change colour when a few drops of 1% phenolphthalein indicator solution were added (figure 7.1). Efforts were made to prevent the ether layers from being removed during the washing process. The petroleum ether layer was then dried with anhydrous sodium sulphate and filtered, and the solvent was removed with a rotary evaporator (figure 7.2). The unreactive (unsaponifiable) portion was collected for further analysis (He et al., 2002).

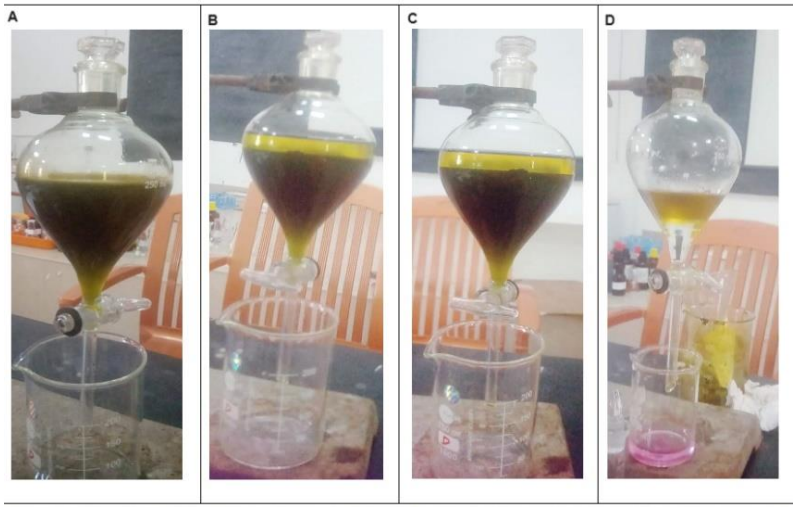

Figure 7.1: Steps of isolation of unsaponifiable leaf extract of *K. caryophyllata*. (A. mixture added in separating funnel; B-C- A distinct layer formed after adding petroleum ether; C. washing with water and checking pH with phenolphthalein indicator

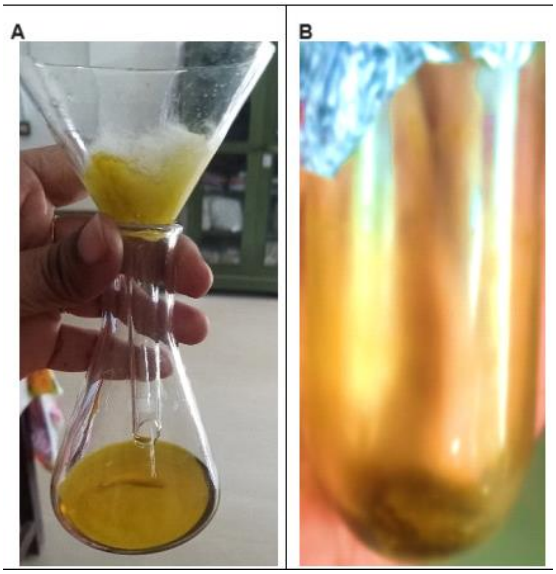

Figure 7.2: A. Filtered unsaponifiable in ether layer; B unsaponifiable dried using rotary evaporator

Column Chromatography

The unsaponifiable fraction was further purified for squalene using column chromatography on a silica gel column (24 g, 100–200 mesh, Himedia). Silica gel was heated for 30 minutes in a preheated oven. The column was filled with a slurry of silica gel in petroleum ether. A solution of 0.34 grams of the unsaponifiable fraction in 5 ml of petroleum ether was prepared and loaded onto the column. Elution was performed by washing the column with 1% diethyl ether in petroleum ether at a flow rate of approximately 1.0 ml/min. Fractions were collected in test tubes with appropriate labelling during the elution process (He et al., 2002).

Identification of components from the isolated fraction

Thin layer chromatography

Thin-layer chromatography (TLC) was employed as the detection method, confirming the presence of squalene in the fractions by analysing their relative mobility values (Rf). A pre-coated silica gel 60F254 (0.25 mm, Merck, Germany) TLC plate was used for the study. The mobile phase was optimised using different percentages of petroleum ether and ethyl acetate. Both the purified samples and standard squalene were allowed to run in a chromatographic chamber saturated with a volatile solvent mixture of the mobile phase. The solvent mixture of petroleum ether and ethyl acetate was adjusted to a ratio of 8:2. The spots on the plates were visualised using 10% sulfuric acid as the spraying reagent and baking the TLC plate in a hot air oven for 5–10 minutes at 70°C. The Rf value for each spot on the TLC plate was noted (Patel et al., 2020; Renukadevi, 2023).

The retention factor (Rf) was calculated using the following formula

$$\mathrm{RF} = \frac{The\ distance\ travelled\ by\ the\ component}{The\ distance\ travelled\ by\ the\ solvent}$$

High-Performance Liquid Chromatography (HPLC)

The standard solution was prepared by dissolving 1 µg of squalene in 1 ml of HPLC-grade acetone (Merck, Germany), followed by filtration through a membrane filter with a pore size of 0.2 to 0.4µm. The isolated squalene fraction was also prepared at a concentration of 1µg/ml in HPLC-grade acetone and filtered using a membrane filter. The solvent system used consisted of HPLC-grade acetone and 98.9% pure 2-propanol (Merck, Germany).

Squalene identification was carried out using an Agilent 1260 semi-preparative HPLC system equipped with a quaternary pump, diode array detector, and fraction collector. Chromatographic separation was performed on an Eclipse Plus C18 column (4.6mm ×250mm, 5µm) at ambient temperature. The mobile phase consisted of acetone and 2-propanol in a 95:5 ratio, with a flow rate of 1.0ml/min and an injection volume of 20µl. Isocratic elution was employed. Optical detection was achieved using a UV-VIS-DAD detector, continuously monitoring at 214nm (Vrubel et al., 2019). The presence of squalene in the samples was confirmed by comparing their retention time

with that of the squalene standard, thus verifying its identity in the analysed samples using the HPLC system.

FTIR Analysis to Identify the functional groups

For FTIR analysis, 0.5 mg of the isolate was directly analysed using an FTIR spectroscope (Perkin Elmer Pte Ltd) equipped with an ATR (Attenuated Total Reflectance) attachment featuring a diamond crystal. The instrument operated with a scan range from 4000cm^{-1} to 400cm^{-1}, a resolution of 0.2cm^{-1}. The FTIR-ATR method was applied to identify and characterise the functional groups present in the isolate based on their unique infrared absorption patterns.

GC-MS analysis for confirming squalene

The presence of squalene in the purified fraction was further confirmed through GC-MS analysis, which validated the isolation and identification of squalene. The analysis with GC-MS was performed by utilising an Agilent 7890A gas chromatograph connected to a 5975C mass spectrometer equipped with a triple-axis detector. The diluted sample was filtered using a 0.2µm Nylon syringe filter and then transferred into vials for analysis. A DB-5MS column (30m x 0.250mm, 0.25µm film thickness) was used for chromatographic separation. A 2µl isolated fraction was injected in spitless mode, with helium (99.9995% purity) as the carrier gas at a flow rate of 1ml/min. The electron impact (EI) mode with an ionisation energy of 70eV was used for detection, and the injector temperature was maintained at 280°C. The oven temperature was programmed to start at 40°C and held for 5 minutes, followed by three ramps: the first at 10°C/min to 100°C, the second at 7°C/min to 150°C, and the third at 5°C/min to a final temperature of 280°C, where it was held for 5 minutes. The entire GC process ran for a total duration of 75 minutes. Compound identification was based on a comparison with the NIST-08 mass spectral database.

RESULTS AND DISCUSSIONS

Plants store many chemical compounds with various biological activities. Identifying these compounds become critical with respect to understanding their exact mechanism of biological activities. GC-MS analysis of methanolic leaf extract of *Kamettia caryophyllata* initially identified and confirmed the occurrence of squalene compound

which is well known for its diverse potential health benefits. The isolation of squalene was done using the saponification method, where saponifiable elements, such as fatty acids got separated from unsaponified fractions that included terpenes in which squalene compounds were present (Popa Bebeanu et al., 2015). The unsaponified fractions were collected, dried, and weighed. About 0.347 g of unsaponifiable fractions were obtained after saponification of 10gm of *K. caryophyllata* leaf methanolic extract. The Salkowski reaction test provided additional verification and verified the occurrence of terpene compound (Khadabadi et al., 2013). It was subjected to Thin Layer Chromatography along with standard squalene and confirmed the presence of three compounds, including squalene (figure 7.3A). Moreover, the band obtained in TLC changed to reddish brown when 10% H2SO4 was sprayed and heated, indicating that the substance was terpenoid squalene.

Unsaponifiable fraction was further purified by column chromatography on silica gel (100-200 mesh) using a solvent system of 2% diethyl ether in petroleum ether. Twenty-five fractions were collected, and fractions 15-25 showed a single compound on TLC using Petroleum ether: ethyl acetate (8:2) as a solvent system. TLC analysis with standard squalene confirmed the presence of squalene in specific fractions, indicated by an Rf value of 0.75 on silica gel plates. They were pooled together and washed with acetone and concentrated to yield pure squalene compound (figure 7.3B). The compound isolated from the leaf extract was pale yellow in colour and liquid in nature.

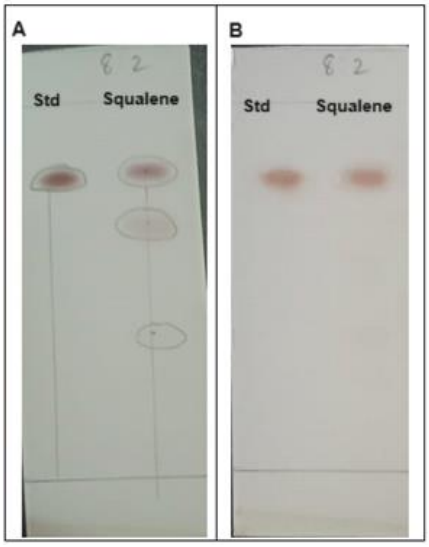

Figure 7.3: TLC Images (A. unsaponifiable fraction with standard squalene; B. isolated squalene with standard squalene)

High-performance liquid chromatography (HPLC) was employed to identify squalene in the unsaponifiable fraction of *K. caryophyllata* leaf extract and confirmed occurrence of squalene by comparing the retention time of its peak in the HPLC chromatogram of the standard with the corresponding peak in the chromatogram of the unsaponifiable fraction (figures 7.5 & 7.6). To ensure that no peaks originated from the solvent, HPLC-grade acetone, used for sample preparation, was also run and no significant peaks were detected (figure 7.4). Squalene was identified with a retention time of 3.956 minutes in the standard and 3.946 minutes in the unsaponifiable fraction. In addition to squalene, four other prominent peaks were observed in the unsaponifiable fraction. After column chromatography, the squalene fraction was collected, analysed using thin-layer chromatography (TLC), and subsequently loaded into the HPLC. The chromatogram showed a prominent peak at a retention time of 3.947 minutes, confirmed the presence of concentrated squalene (figure 7.7). The fraction corresponding to this peak at 3.947 minutes was collected with HPLC fraction collector and subjected to further analysis by FTIR and GC-MS.

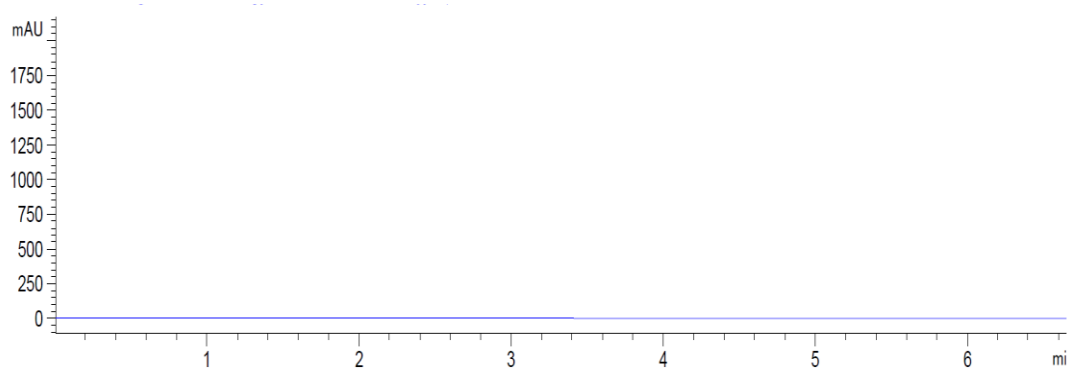

Figure 7.4: A. HPLC chromatogram of blank (Acetone)

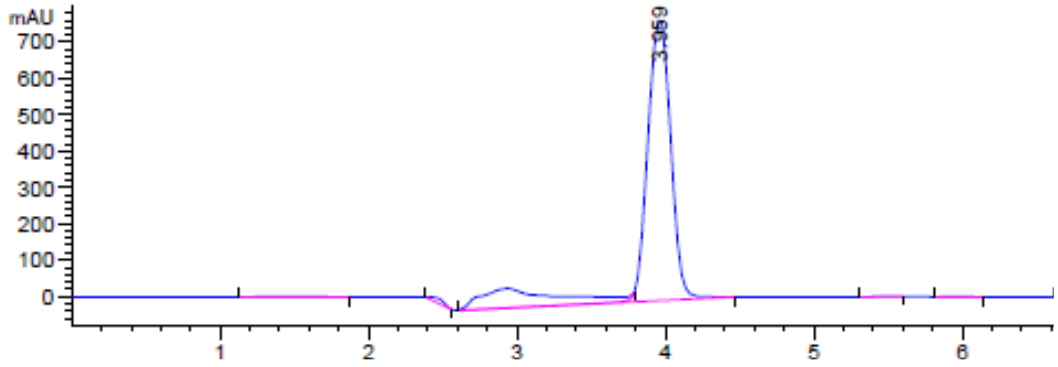

Figure 7.5: HPLC chromatogram of 1microgram per ml of standard squalene

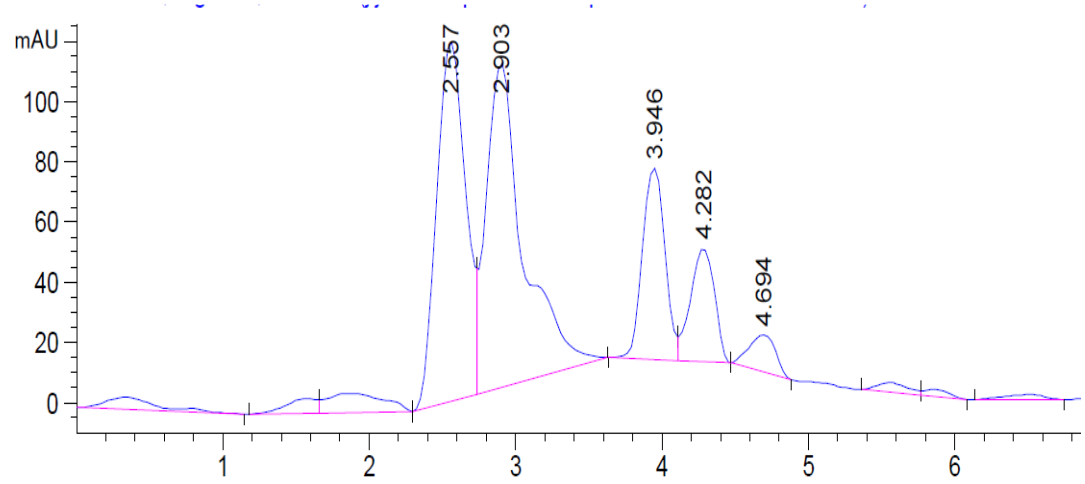

Figure 7.6: HPLC chromatogram of unsaponifiable leaf methanolic extract

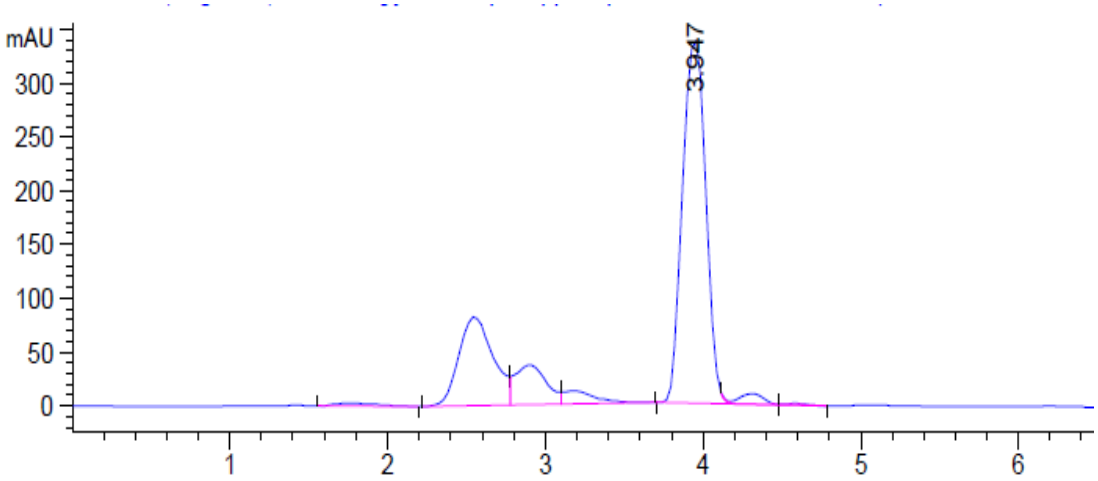

Figure 7.7: HPLC chromatogram of the isolated compound from leaf methanolic extract of *K. caryophyllata*

The fraction corresponding to the peak at retention time of 3.947 minutes obtained in the HPLC was collected for FTIR analysis. To identify functional groups present in the isolated compound, the Fourier transform infrared (FTIR) spectra was prepared and presented in figure 7.8. The FTIR spectra showed some specific peaks to identify the functional group and that includes the absorbance between 3000 - 2850 cm^{-1} representing C-H stretching from alkanes, the absorbance between 1640 - 1600 cm^{-1} representing C=C stretching from alkenes and the absorbance between 995 - 885 cm^{-1} representing C-H bending of alkene. The NIST Chemistry Webbook explains that the infrared spectrum of squalene displays unique absorption peaks which support the spectrum of isolated compound. The peak at 3500-3000 cm^{-1} may be due to impurities or solvent traces, as squalene does not contain hydroxyl groups.

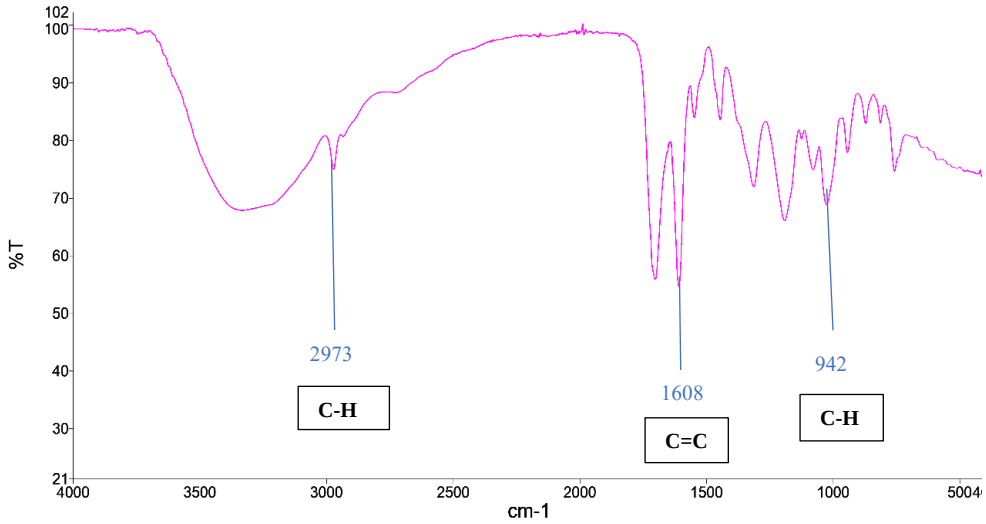

Figure 7.8: FTIR spectrum of the isolated compound from leaf methanolic extract of *K. caryophyllata*

The characterisation of squalene was done with a GC-MS analysis to determine the molecular weight of the compounds. The chromatogram which is shown in figure 6.9, displayed a single prominent peak with a retention time of 69.365 minutes, indicating the successful isolation of squalene from other compounds (figure 7.9). The 100% peak area further validated the purity and precise identification of the compound. The structure and mass spectrum of squalene were obtained from the NIST library, further confirming the identity of the compound through its characteristic fragmentation pattern (figure 7.10). The characterised isolate displayed a fragmentation pattern of molecular ions valued at 410.0 MW in the MS data, which was identical to the MS data of squalene compounds extracted from *Abroma augusta* methanolic extract (Latief et al., 2020).

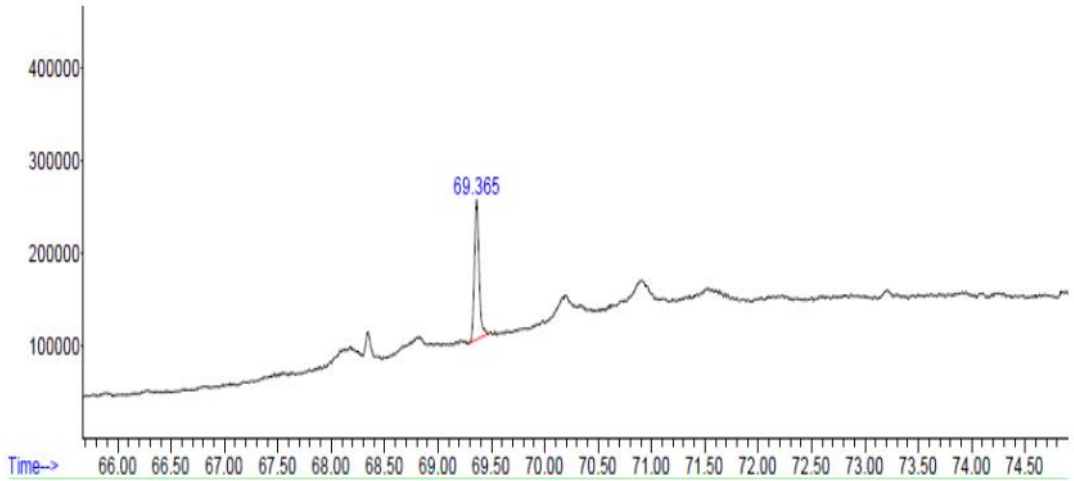

Figure 7.9: GC-MS chromatogram of the isolated compound from leaf methanolic extract of *K. caryophyllata*

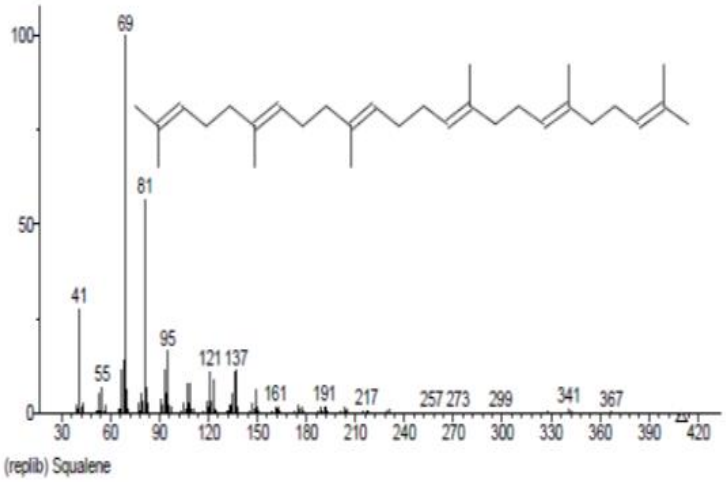

Figure 7.10: Structure of the isolated compound from leaf methanolic extract of *K. caryophyllata*

The methanolic extract from *K. caryophyllata* leaves showed a variety of important phytochemical groups like terpenes, alkaloids, phenolics etc. emphasising the plant's notable pharmacognostic possibilities. Out of these, terpenes were found in higher amounts in the leaves than other components of the plant. The phytochemical analysis of methanolic extracts from leaf, stem, and root using GC-MS screening showed the existence of various bioactive compounds, of which the specific bioactive compound squalene was consistently reported in all the component extracts and this squalene holds a substantial pharmacological significance with diverse biological activities such as anti-inflammatory, antioxidant, antimicrobial, anti-cancerous, anti-hyperglycemic properties etc. Furthermore, squalamine, a conjugated compound of squalene, was also detected in the leaf extract through LC-MS analysis.

Sanchez-Quesada et al. (2018) reported that squalene can reduce inflammation under various stress conditions by focusing on key pro-inflammatory mediators like TLR4, iNOS, COX-2, and MPO. Research done by Jose et al. (2023) demonstrated that squalene isolated from *Simarouba glauca* has a suppressing effect on the synthesis of inflammatory mediators like MPO, COX-2, NO, PGE2, TNF-α, and IL-6 in the paw tissue of mice, and it may be a promising therapeutic option for different inflammatory conditions. In a study by Latief et al. (2020), the anti-inflammatory effects of squalene isolated from methanol extract of *Abroma augusta* in rat models with chronic inflammation are found efficacious. Ulrikh and Somolovskaya in 2022 reported that squalene boosts the production of anti-inflammatory cytokines in pro-inflammatory

macrophages and aids in tissue remodelling by attracting eosinophils and neutrophils for phagocytosis.

According to Cardeno et al. (2015) squalene exhibits antioxidant and anticancer properties besides its anti-inflammatory property. Govind and Achaya, (1968) noted its antioxidant capabilities, and suggest its utility as a therapeutic agent. Huang et al. (2009) stated that squalene is less prone to peroxidation and appears to function as a suppressor of singlet oxygen in the skin. This function assists in shielding human skin from lipid peroxidation due to UV exposure and other oxidative damaging sources. Gunes (2013) corroborated that squalene serves as a potent antioxidant in the skin, guarding against lipid peroxidation from UV radiation and other forms of oxidative agents.

Ulrikh and Somolovskaya (2022) conducted antimicrobial studies on the squalene component of amaranth oil, confirming its antibacterial properties, which could reduce the number of microbes in wounds or injured tissues of mice. It is reported that shark liver oil, which is rich in squalene, can provide protection against bacterial and fungal infections, particularly in individuals with atopic dermatitis and xerosis-related skin lesions (Gunes, 2013). Nazemi et al. (2022) demonstrated that the compound squalene isolated from *Stichopus hermanni* is very effective as antimicrobial agent against pathogenic organisms *Bacillus cereus, Staphylococcus aureus* and *Candida albicans*. Further, Ibrahim and Mohamed (2021) explored the relationship between the antioxidant and anti-inflammatory properties of squalene and found it to be highly beneficial for cardiovascular health, while it was Widyawati et al. (2018) who verified that squalene exhibits anti-hyperglycemic properties and provides pancreatic protection in STZ-induced diabetic rats. It was based on these promising pharmacological significances that, a systematic examination was undertaken for separation, identification, structuration and confirmation of squalene from *K. caryophyllata*, so as to provide scientific evidence in support of their ethnomedicinal use. The present investigation has isolated and confirmed the presence of squalene as a major contributing factor for effective treatments in traditional medicinal system. But the scope is open for the study of several other diverse bioactive compounds detected and identified in the preliminary screening studies for better outcome.

CONCLUSION

The study of separating and identifying the lipid-soluble bioactive substance squalene from *Kamettia caryophyllata* leaf extract has produced significant outcome and impact. Squalene was isolated and purified with the help of saponification and column chromatography, yielding pure squalene fractions. Numerous analytical methods were used, such as TLC, HPLC, FTIR, and GC-MS, all of them offered satisfactory confirmation on the existence, purity, and structural properties of squalene. For confirming the existence of specific functional groups, FTIR spectroscopy has been performed. GC-MS revealed mass spectra and structure of squalene. The fact that squalene from *K. caryophyllata* was successfully isolated and characterised highlights the plant species' potential for medical applications. The results establish a strong basis for possible uses in pharmaceutical and nutraceutical fields because squalene is known to have strong diverse biological activities. The present investigation not only establishes the existence and purity of squalene in *Kamettia caryophyllata* but also it has provided scientific evidence and confirmation to the ethenomedicinal uses and their effectiveness in the traditional system of medicine for treating diseases like arthritics, scabies, itches etc. as documented in the Hortus Malabaricus (Manilal & Remesh, 2010). Further, this offers a solid foundation for upcoming studies in the biomedical, pharmaceutical, and biotechnological fields, possibly assisting in the creation of cutting-edge treatments derived from natural sources.

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Chapter 8

General Discussion

Worldwide, people use medicinal plants for the treatment of various diseases from ancient times and in the present days, considering their sustainability in efficacy, safety and the ability to reduce the chances of developing resistance by pathogenic organisms compared to synthetic drugs (Parekh, 2007), the demand of these natural resources have multiplied several times. Further, herbal medicine gained widespread global recognition when the World Health Organization (WHO) advocated for the use of traditional herbal remedies, particularly in developing nations, to address healthcare gaps not covered by modern synthetic medicine. Today, approximately 75–80% of the global population, predominantly in developing regions, relies on traditional medicine.

In plant-based drug development, a critical step is the collection and analysis of relevant research data, including traditional and ethnomedical knowledge. Ensuring the standardisation and quality control of medicinal plants is also an essential process. The future progress of herbal medicine analysis depends largely on accurate identification, standardisation, and quality assurance through reliable methodologies (Ravishankar & V.J, 2007). Due to similar names in medicinal plants, and factors such as limited knowledge of authentic sources, visual similarities, and careless collection methods, medicinal plants are often mistakenly substituted or adulterated (Farnsworth & Loub, 1980). It is necessary to assess and standardize the plants used in the formulations by using array of pharmacognostic and phytochemical evaluation tools, such as macroscopy, microscopy, powder microscopy, histochemical, physicochemical & phytochemical screening and evaluation followed by bioactivity studies and isolation, identification and structuration of specific bioactive compounds of crude medicinal plants (Kokate, 2001).

Pharmacognostic studies play an important role in verifying and authenticating medicinal plants by assessing their physical, chemical, and anatomical characteristics,

which ensures the consistency and purity of plant products, thereby preventing misidentification and adulteration (Chanda, 2014). Moreover, these studies help to establish reference standards, which are vital for creating pharmacopeial monographs and ensuring that herbal products comply with regulatory requirements (Alamgir, 2017).

The presence of phytochemical compounds, such as alkaloids, phenolics, and terpenes, is responsible for their therapeutic properties (Trease & Evans, 2009). This knowledge is vital for drug discovery as it helps identify compounds with significant pharmacological activities, such as antimicrobial, antioxidant, anti-inflammatory, or anticancer effects (Dalir & Safarnejad, 2017). These studies provide scientific evidence to support their medicinal claims and open new avenues for drug development by isolating and optimising active compounds from medicinal plants.

Plant bioactivity can be evaluated through in vitro and in vivo studies (Khan et al., 2013; Patil et al., 2019). In vitro experiments offer initial knowledge of bioactivity, while in vivo studies confirm the physiological relevance of the bioactive compounds (Dorfer et al., 2019). Plant-derived bioactive compounds are important for drug development when they exhibit promising biological activities and effects. Now a days, many modern pharmaceuticals have been derived from these naturally occurring bioactive substances. By correlating pharmacognostic and phytochemical data with bioactivity, the traditional knowledge of plant-based treatments can be validated. This can lead to the discovery of novel therapeutic agents or inspire the development of new and different plant-derived compounds for pharmaceutical applications.

In this context, the current study has been conducted on *Kamettia caryophyllata*, a plant referenced in Volume IX of *Hortus Malabaricus*. This plant has been used in Indian traditional system of medicine to treat conditions such as to alleviate arthritic pain, treat skin conditions like itches, scabies, lichen and leprosy, and manage cachexia, spasms and epilepsy and for its anodyne properties (Manilal, 1984; Mohan, 2005; Manilal & Remesh, 2010).

The World Health Organisation has observed certain degradations in the quality and efficacy of plant-based traditional medicine due to adulteration or misidentification of medicinal plants and has put forth specific guidelines for documentation and

monograph preparation of medicinal plant species to avoid such issues (Agarwal & Goyal, 2021; WHO, 2013). In the present study, morphological analysis as well as molecular level analysis were carried out, aimed to overcome challenges related to reliable identification and authentication of *K. caryophyllata*. The major morphological features were noted includes absence of modified climbing structures, reddish stem with abundant lenticels, reddish leaf midrib, presence of white latex, and follicle fruits with winged seeds. It expects that this kind of prominent morphological features will be useful as primary step towards establishment of identity. It was Hebert et al. (2003), who proposed the DNA barcoding analysis with rbcL and matK genes as a simple, economical and faster method with precision and accuracy with respect to the identification and authentication of raw plant material in the preparation of traditional medicine, to ensure the level of quality by finding out adulteration, misidentification and substitution (Sinha et al., 2012). By integrating both morphological and molecular approaches, the plant was successfully authenticated. Evolutionary analysis provided valuable knowledge into the close relationship between *K. caryophyllata* and the closely related species *K. chandeei*. The successful deposition of the rbcL and matK gene sequences in GenBank, the National Centre for Biotechnology Information, with accession numbers MH201080.1 and MH681795.1, will be useful for upcoming research. Researchers can utilise this genetic information for additional research on the identification, authentication, and evolutionary relationships of *K. caryophyllata* and its related species. Having these sequences in a global database ensures they can be accessed for comparisons, studies on evolutionary relationships, and plant identification, contributing to the progress of botanical and pharmaceutical research.

It is essential to conduct a thorough pharmacognostic assessment of *K. caryophyllata*, describing its macroscopic, organoleptic, microscopic and fluorescence properties, to establish its authenticity and purity prior to carrying out any tests. The smooth texture and olive-green colour and bitterness of the leaf powder, the rough texture of stem and root powder may influence processing methods and solubility as a herbal drug (Preuwenberg et al., 2010; Sims & Gamon, 2002; Vermerris et al., 2010) and these features can be considered to verify the authenticity and to ensure quality of products in herbal sectors that utilise this plant (Akbar et al., 2014; Chanda, 2014; Kavitha et al., 2014). Further, the leaf characteristics noted such as anomocytic stomata, stomatal

index (10.22), vein islet number (07), vein termination (05), and palisade ratio (1:6) etc. can also be considered as fundamental identifying features of *K. caryophyllata* in the herbal sector in future (Garga & Das, 2017; Kannabiran & Ramassam, 1988; Amponsah et al., 2014; Kavitha et al., 2014).

One of the simple and less expensive method to establish proper identification of raw plant materials is microscopic analysis by taking simple free hand anatomical sections (Choundhury et al., 2015; Dabur et al., 2007; Prakash et al., 2013). The prominent features noted were hypodermal stomata, oil globules and secretary cavities in leaf component and in contrast, presence of lenticels, 4-5 layers of phloem fibres prior to endodermis, hollow pith with scattered stone cells in the stem component, while prominent features like loosely arranged beaded cells in the cortex with lacunar regions and crystal cells and starch grains in the phloem region of root component, all of which can be used to authenticate and identify *K. caryophyllata* in the herbal sector (Singh et al., 2010; Kumar et al., 2012;).

The powder microscopy is another easy, simple as well as less expensive method used in pharmacognostic studies to ensures the purity of the herbal drug and detects any adulterants present in the sample by comparing with similar physical characteristics (Dutta & Chaurasia, 2023).The powder microscopic observations of *K. caryophyllata* done in the study revealed the occurrence of several specific features such as starch grains, calcium oxalate crystals and various cell structures to different components, that can be used as a reference for determining the plant's identity, assessing its quality, and gaining insights into potential therapeutic properties (Florence et al., 2015; Kumar et al., 2012; Zalke et al., 2013).

The identification of the medicinal plant as well as for the qualitative assessment of crude drug by the fluorescence analysis approach can be considered as an essential step for the pharmacognostical evaluation, and this is based on the anticipation that the fluorescence behavioural pattern exhibited by a particular plant powder may be unique to that plant in some respect. The fluorescence analysis carried out in the present study observed the development of orange fluorescence colour when the leaf powder was treated with acetic acid as well as with 1N NaOH in methanol and exposed to UV-light of 366nm wavelength. However, in the stem powder, in addition to a similar observation as in the case of leaf powder above, a yellow fluorescence

was also developed when treated with 25% ammonia and exposed to UV-light of 366nm. Whereas, the root powder developed green fluorescence with 50% KOH and white fluorescence with acetic acid, both under UV-light of 366nm, and a yellow fluorescence with 25% ammonia and exposed to UV254nm wavelength. Besides various fluorescence colour developments, a number of non-fluorescence colours were also found to develop when powders were treated with various chemical reagents and this may be in accordance with the nature and type of constituents present in the plant materials (Preetham et al., 2015). The fluorescence as well as the non-fluorescence colour behavioural pattern exhibited by *K. caryophyllata* against the selected chemical reagents in the study can be used for identification as well as qualitative assessment of the crude plant drug in the future.

Further, the study of physicochemical parameters such as water-soluble and alcohol-soluble extractive values, total ash content, water-soluble ash, acid-insoluble ash, moisture content, and pH can effectively ensure the purity and consistency of *K. caryophyllata* in herbal formulations, helping to prevent adulteration. The variations in moisture content or loss on drying among the leaves, stems, and roots reveal their physiological differences, which may impact the stability of herbal products made from these parts (Nguyen et al., 2017; Siraj et al., 2020). The study also revealed differences in total ash, water-soluble ash, and acid-insoluble ash content among the leaf, stem and root, reflecting their diverse inorganic compositions. These investigations are important for determining the mineral and inorganic matter distribution within the plant, which can play an essential role in evaluating the quality of herbal drugs (Alam & Us Saqib, 2015; Chandel et al., 2011; Chidambaram & Aruna, 2013). Leaves had higher total ash and water-soluble ash content, suggesting a higher content of inorganic substances like carbonate and phosphate salts compared to stems and roots. This indicates that the leaves may be more suitable for therapeutic uses where these inorganic compounds are desirable (Tang et al., 2018). The acid-insoluble ash content recorded in the study was minimal across all plant components, with a comparatively higher value observed in the root powder than in the leaf and stem powders, likely caused by impurities from sand or soil during the preparation process. The variation in the pH level in different components of a plant may be due to differences in the concentration of organic acidic/basic substances, which can influence the stability and efficacy of bioactive compound extractions. In the present

study, the leaf component exhibited the lowest pH value, while the stem component was only slightly acidic, and the root component with pH value in between and this information is useful for medicinal applications, as pH plays a significant role in formulations and therapeutic use (Muyumba et al., 2021; Shahriar et al., 2012).

The leaf component of *K. caryophyllata* showed the highest extractive yield than stem and root, both for water-soluble (12.32%) and alcohol-soluble (9.67%), indicating a higher concentration of phytoconstituents and this data is very relevant for selecting plant component for medicinal or commercial purposes to get higher yield of bioactive compounds. The higher water-soluble extractive values compared to alcohol-soluble indicates more compounds are water-soluble, which could influence the choice of extraction methods in herbal medicine preparation (Regupathi & Chitra, 2015; Thomas et al., 2008).

Histochemical methods are important for analysing the qualitative determination of various cellular components found in cells (Yang et al., 2018; Krishnan et al., 2003). The various histochemical detection and localisation tests of primary metabolites like carbohydrates, starch, proteins and lipids and secondary metabolites like phenolics, alkaloids, tannins, lignin etc. were carried out in the study to locate their spatial distribution in the plant body. The phenolics were observed as dark stains in the cortical region of all the plant parts, whereas localisation of alkaloids in the study showed primarily located in the cortical cells, xylem and phloem cells of the leaf, stem and root and in the pith region of the stem. The reddish-brown colouration seen in the cortex and xylem confirmed the presence of tannins while lignin was found deposited in xylem cells in the leaf, stem and root, in the parenchymatous cells and palisade mesophyll cells of leaves, and in the cortex cells of the stem. The presence of these primary and secondary metabolites in specific localisation in the plant parts using various histochemical tests can be used while preparing the monograph for identifying the plant and also to support their therapeutic significance (Hutzler et al., 1998; Badria & Aboelmaaty, 2019; EI Babili et al., 2021).

All these findings of pharmacognostic parameters along with morphological and molecular characteristics and physicochemical properties, may collectively serve as reference standards to identify adulteration in crude drugs, ensuring that products available in the market meet the required quality standards. Further, this can provide

practical guidance for traditional medicine practitioners in selecting the appropriate plant parts for therapeutic use, supporting the safe and effective use of *K. caryophyllata* in herbal medicine. The pharmacognostic standardisation of *K. caryophyllata* done in this study may represent the first step towards preparing a monograph for the plant. No other studies have been reported on this species, and was lacking pharmacopoeia standards for proper identification and authentication. The findings of this research provide a foundation for future standardisation efforts and the development of a comprehensive monograph for *K. caryophyllata*.

The therapeutic potential of plant species primarily resides in their diverse secondary metabolites with distinct biological activities, which can serve as valuable and safe alternative to artificial drugs in human and animals (Krishnaiah, 2007; Naseem et al., 2014). The most important strategy and decisive step in the selection of such a plant for pharmacological interest is by collecting the traditional knowledge if any available regarding the use of natural plant resources in folk medicine, followed by phytochemical screening to identify different phytochemical groups and specific bioactive compounds with known or promising medicinal values. The results of qualitative phytochemical screening done in the study indicated variation in the phytochemical profile of leaf, stem and root and revealed maximum diversity of chemical groups in the leaf component compared to stem and root components and this can be attributed to the difference in distribution of chemical constituents in different component parts. The inference of the study further revealed differences in the diversity of phytochemical groups extracted from different plant components into different extraction solvents, and this could be the result of differential affinity or solubility properties of different phytochemical groups. The polar solvent methanol was found most effective for *K, caryophyllata* to extract a wide range of bioactive phytochemicals including alkaloids, phenolics, tannins, flavonoids, terpenes, glycosides and saponins compared to chloroform and petroleum ether (Kumar, 2017). The occurrence of these phytochemical groups in *K. caryophyllata* plant was also further confirmed qualitatively in the study through various histochemical localisation tests. The inference of the study indicates the methanolic extract of *K. caryophyllata* may have diverse pharmacological properties such as antioxidant, anti-inflammatory, antimicrobial and cytotoxic effects (Dharman & Anilkumar, 2018; Ray et al., 2018). The study further noted that different phytochemical groups detected in the plant are

sharing common bioactive properties. The bioactive groups like phenolics, alkaloids, flavonoids, glycosides and tannins are sharing antioxidant and antimicrobial properties, as these chemical groups are reported to have strong radical scavenging activity and potential toxic nature to the growth and development of wide range of pathogens (Ndukwe & Ikpeama, 2013; Okwu & Josiah, 2006). Similarly, terpenoids, alkaloids and tannins are further reported to share anti-inflammatory, anti-allergic and anticancer properties (Prieto et al., 1999; Priyanga et al., 2014).

The study collected a comprehensive information on phytochemical composition including both volatile as well as non-volatile compounds in the methanolic leaf, stem and root component extracts of *K. caryophyllata*. The phytochemical screening using HR LC-MS and GC-MS techniques detected and identified a large number of specific chemical compounds of volatile and non-volatile nature. On comparison of different component extracts, the methanolic leaf extract has the highest number of compounds which are reported to have interesting diverse biological activities compared to stem and root extracts. Overall, 69 specific compounds were detected and identified in the methanolic leaf extract, out of which 34.78% of compounds were found representing the phytochemical group terpenoids, while 10.14% represented phenolics, whereas only 5.8% of compounds represented alkaloids. This was immediately followed by 55 compounds identified in the root methanolic extract, where 36.36% of compounds were representing terpenoids, 5.45% representing phenolics and 18.18% were representing alkaloids and the lowest count of 43 compounds detected with respect to methanolic stem extract where, 41.86% were representing terpenoids, 6.98% representing phenolics and 23.26% were representing alkaloids. This observation clearly inferred that the phytochemical group terpenoids stand as the major dominating group in all the components with the highest number of terpenoid compounds occurring in the leaf component over stem and root.

Another important inference of phytochemical screening study is, there are several specific bioactive compounds detected in *K. caryophyllata* which are sharing common therapeutic or medicinal properties. The study identified about 23 compounds in the leaf component (table 5.7 &5.12); 15 compounds in the stem (table 5.8 & 5.15) and 21 compounds in the root (table 5.9 &5.18) which were previously reported for anti-inflammatory activity (Prieto et al., 1999; Priyanga et al., 2014). Similarly,

compounds detected in *K. caryophyllata* which were previously reported for antioxidant property (Ndukwe & Ikpeama, 2013) includes 16 compounds identified in the leaf component (table 5.7 & 5.12); 6 in the stem component (table 5.8 & 5.15) and 15 in the root component (table 5.9 &5.18). Antimicrobial activity is another important therapeutic property shared by several compounds detected in the plant (Okwu & Josiah, 2006) and it includes 20 compounds from the leaf (table 5.7 &5.12), 9 compounds from the stem (table 5.8 & 5.15) and 22 compounds from the root component (table 5.9 &5.18). Similarly, 24 compounds were detected in the leaf component (table 5.7 &5.12); 15 compounds in the stem (table 5.8 & 5.15) and 20 compounds were detected in the root component (table 5.9 &5.18) of *K. caryophyllata* were sharing cytotoxic and anti-cancerous properties (Prieto et al., 1999; Zhao et al., 2016). It is well known that synthetic drugs are mostly exerting their medicinal action based on a single xenobiotic compound while therapeutic action of herbal medicines is generally based on synergistic or additive action of multiple compounds acting at single or multiple target sites associated with a physiological process. This kind of combined effect of natural medicine is not only effective in eliminating wide range of ailments as well as pathogenic organisms, but also reduce the chances of developing resistance by pathogenic organisms (Parekh, 2007). Based on these observations and validations on total number and diversity of specific bioactive compounds and further considering the inference obtained previously from quantitative estimation of diverse phtochemical groups in different component extracts, the study strongly revealed that the leaf methanolic extract of *K. caryophyllata* possess significantly higher chemical composition and diversity and has more potent and diverse biological activity such as anti-inflammatory, antioxidant, antimicrobial and cytotoxic & anticancer properties over the stem and root extracts.

The bioactivity studies highlight the importance of scientific evidences in validating traditional uses and expanding the therapeutic applications of medicinal plants. Scientific studies will help the researchers and scientists to identify and isolate the specific bioactive principles in medicinal plants, which may be useful in enhancing the therapeutic efficacy either by their direct application in required concentrations or by utilizing them for developing new combinations and formulations of drugs for treating various specific diseases and disorders**.** In vitro bioassays are the first step in the preclinical screening process for herbal remedies,

allowing researchers to make informed decisions about which plants or compounds are worth investigating more closely. These studies are crucial in analysing and evaluating several fundamental properties like antioxidant, antimicrobial, cytotoxic and anti-inflammatory activities.

The Phosphomolebdenum assay, DPPH assay, and Hydrogen peroxide assay were carried out to evaluate *in vitro* antioxidant activity of methanolic component extracts of *K. caryophyllata* and the leaf extract exhibited lowest half-maximal inhibitory concentration (IC50) values over stem and root extracts. The lower IC50 values signify greater free radical scavenging activities of the leaf extract and indicates greater oxidative stress reducing capacity compared to stem and root extracts. The higher antioxidant activity can be attributed to higher biodiversity and concentration of specific bioactive molecules with antioxidant property in the methanolic leaf extract (Khan et al., 2013; Phuyal et al., 2020). The leaves usually harbour higher concentrations of phenolic compounds and flavonoids, both can substantially contribute to the plant's overall antioxidant content (Ribeiro et al., 2020). Though the pure synthetic standard ascorbic acid showed superior activity over all plant component extracts analysed in the study, considering them as natural and without any or less side effects, the activity exhibited by crude extracts and particularly the leaf extract, is highly considerable and this has given valuable insights into their potential antioxidant activities and associated health benefits. Further, with the scope for isolation of specific bioactive compound (s) with additive and synergistic antioxidant activities in pure form from these crude extracts, there may be chances for significant enhancement in the antioxidant activities, which may be more or comparable with standard ascorbic acid activities.

The Agar Well Diffusion method employed to assess the antimicrobial activity of methanolic leaf, stem and root extracts revealed differences in antimicrobial activity against tested pathogenic microorganisms. The size in the zone of inhibition induced by different component extracts at different tested concentrations indicates a concentration-dependent antimicrobial activity and higher activity at higher concentrations. However, the results clearly revealed the leaf extract consistently outperformed stem and root extracts against most of the tested pathogenic microorganisms, indicating its higher potential as a natural antimicrobial source.

Significantly higher concentration of diverse secondary metabolites having antimicrobial property revealed through quantitative estimation and the occurrence of enormous number of diverse bioactive compounds of antimicrobial activity revealed through GC-MS and HR LC-MS screening in the leaf component compared to stem and root might be the reason leading to more vigorous antimicrobial activity. The diverse phytochemicals like alkaloids, phenolics and terpenoids and their specific compounds sharing common antimicrobial property is higher, their synergistic and additive effects should be taken into consideration while targeting a single or different aspects of microbial physiology, as its impacts on microbial cell wall and cell membrane is highly disruptive causing structural irregularities, affecting cellular function, interfere with cell division, increased permeability, interfere with enzymes and proteins (Yan et al., 2021; Nik et al., 2019; Mutha et al., 2021).

The cytotoxic study of different methanolic component extracts of *K. caryophyllata* on DLA and EAC cancer cell lines using Trypan Blue exclusion method revealed, the leaf methanolic extract has greater cytotoxic effects. Significantly lower IC50 values of leaf extracts compared to stem and root extracts, indicate their superior efficacy in inhibiting cancer cell growth. The quantitative determination of secondary metabolites carried out in the chapter 5 revealed, the quantity of secondary metabolites having cytotoxic and anticancer property is highest in the leaf methanolic extract and similarly, the GC-MS and HR LC-MS screening identified 20 bioactive compounds from leaf extract, 15 from stem extract and 17 bioactive compounds from root extract that possess cytotoxic and anticancer properties. These observations done in the study are well substantiating and supporting the lower IC50 values of leaf extracts for inhibiting cancer cell growth compared to stem and root extracts. Significantly lower IC50 values displayed by methanolic leaf extract offer valuable insights into its chemical composition and mechanisms underlying the observed cytotoxic activities.

The *in vitro* anti-inflammatory assays conducted in the study revealed the leaf methanolic extract of *K. caryophyllata* has the highest anti-inflammatory activity compared to the stem and root extracts. The phytochemical screening using HR LC-MS and GC-MS of different component extracts revealed the leaf methnolic extract of *K. caryophyllata* has the highest number of 23 specific bioactive compounds with anti-inflammatory properties and this strongly substantiated the finding of both

Trypsin Inhibition as well as Nitric oxide inhibition *in vitro* anti-inflammatory assays. The study firmly indicates that the plant *K. caryophyllata* possesses anti-inflammatory properties with the highest activity recorded in the leaf component, followed by stem and least activity in the root component and this was further supported by the qualitative detection as well as the quantitative estimation of major phytochemical groups like terpenoids, alkaloids and phenolics in the study.

K. caryophyllata is traditionally used to treat arthritis, scabies, itches as mentioned in Hortus Malabaricus (Manilal, 1984; Manilal & Remesh, 2010). Arthritis is primarily characterised by inflammation in the joints (Li et al., 2014; Sharma et al., 2007), while scabies is a parasitic skin infection that leads to intense itching and inflammation in the skin (Arlian & Morgan, 2017), and it is known that anti-inflammatory drugs can reduce the symptoms of these conditions. In this context, the anti-inflammatory effect of methanolic leaf extract of *K. caryophyllata* was conducted using *in vivo* anti-inflammatory animal models such as Carrageenan-induced and Formalin-induced inflammation models in Swiss albino mice. Since *K. caryophyllata* is an experimentally unexplored plant, an acute toxicity study was conducted in Swiss albino mice to determine a safe dose for *in vivo* testing. The study confirmed a dosage up to 2500mg methanolic extract/kg body weight of mouse was safe for use. Based on this, $1/5^{th}$ (500mg) and $1/10^{th}$ (250mg) of this dose were selected for the anti-inflammatory study**.**

In the carrageenan-induced inflammation model, *K. caryophyllata* methanolic leaf extract exhibited remarkably higher efficacy in reducing hind paw oedema thickness of mice induced with inflammation, indicating its potential as an anti-inflammatory agent. Both low dose (250mg/kg body weight) and high dose (500mg/kg body weight) of leaf extract exhibited greater anti-inflammatory activity than the standard diclofenac. This finding offers valuable insights into specific bioactive compounds having anti-inflammatory activity in the leaf extract and their mechanisms behind the anti-inflammatory activity. The reduction in paw oedema thickness of inflammation induced by *K. caryophyllata* leaf extract might be due to their ability to inhibit inflammatory actions, possibly through modulation of the cyclooxygenase pathway or due to their inhibitory effect on pro-inflammatory and inflammatory mediators or other inflammatory agents' activity triggered by sulphated sugars of carrageenan

(Konishi et al., 2008; Posadas et al., 2004; Ricciotti & Fitzgerald, 2011). Similarly, the formalin-induced inflammation model which resembles arthritis in humans (Mohan et al., 2019; Sofidiya et al., 2014) also revealed promising potential anti-inflammatory effects of methanolic leaf extract, particularly at higher dose of 500mg/body weight, which was something comparable to the activity of standard diclofenac. Comparatively higher concentration of phytochemical groups like alkaloids, phenolics and terpenoids together with the occurrence of more diverse and abundant number of specific bioactive compounds sharing common anti-inflammatory property in the leaf component of *K. caryophyllata* might have contributed synergistically to the anti-inflammatory effects, by inhibiting inflammatory mediators and pathways (Miras-Moreno et al., 2016; Wang et al., 2014).

Phytochemical profiling of the leaf extract with GC-MS and LC-MS studies revealed that terpenes are most abundant in the leaves. GC-MS analysis of leaf, stem, and root extracts identified squalene as a triterpene consistently found in all the component extracts. Also, the LC-MS analysis detected squalamine, a conjugated form of squalene specifically found in leaf extracts. Squalene is a compound known for substantial pharmacological significance with diverse biological activities such as anti-inflammatory, antioxidant, antimicrobial, anti-cancerous and anti-hyperglycemic properties (Cardeno et al., 2015; Huang et al., 2009; Rajeswari et al., 2012). These promising pharmacological significances have prompted the present study for further investigation into the isolation, identification and structuration of squalene from the methanolic leaf extract of *K. caryophyllata***,** so as to provide scientific evidence in support of their ethnomedicinal use. Different analytical techniques including TLC, Column chromatography, HPLC, FTIR, and GC-MS were employed and satisfactorily validated and confirmed the presence, purity, and structural properties of squalene. The FTIR spectroscopy has been performed for confirming the existence of specific functional groups like C-H stretching from alkanes, C=C stretching from alkenes and C-H bending of alkene and the GC-MS used was to reveal the mass spectra and structure of squalene. The successful isolation and characterisation of squalene having strong diverse biological activities from methanolic leaf extracts highlights the scope for strong potential uses of *Kamettia caryophyllata* in pharmaceutical fields for medical applications. The present investigation not only demonstrated the pharmacological significance of methanolic leaf extracts of *K. caryophyllata* and

confirmed the occurrence and purity of bioactive compound squalene with diverse pharmacological activities, including anti-inflammatory, antioxidant, antimicrobial and cytotoxic effects but also it has provided scientific evidence and confirmation to the ethenomedicinal uses and their effectiveness in the traditional system of medicine for treating diseases like arthritics, scabies, itches etc. as documented in the Hortus Malabaricus (Manilal & Remesh, 2010). The methanolic leaf extract consistently showed superior bioactivity across the studies, making it a promising candidate for further research into its therapeutic potential. However, major clinical trials are necessary for further validation of safety and effectiveness of methanolic leaf extract and specific bioactive compounds like squalene isolated with respect to human consumption for treatment.

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Chapter 9

Summary and Conclusion

Precise identification, standardisation and quality control measures are essential for the safety, purity and efficacy of herbal medicines. The objective of the present study was to standardise the raw plant drug *Kamettia caryophyllata* (Roxb.) Nicolson & Suresh, a climber belonging to the family Apocyanaceae, known to have uses in the Indian traditional system of medicine for treating arthritics, itches, scabies, leprosy, spasm, epilepsy etc. as documented in Hortus Malabaricus. To the best of knowledge, no detailed pharmacognostic and scientific chemical analysis has been previously reported on this plant.

The present study attempts to reveal the quality, purity, identity, and bioactive principles of pharmacological values of *K. caryophyllata* by conducting pharmacognostic, physicochemical, phytochemical and bioactivity analysis in the different components via leaf, root and stem. Further, the study aims to validate the scientific evidences in support of traditional medicinal use of the plant via isolation, purification and structuration of specific bioactive compound of interest.

As a primary step towards the establishment of identity of *K. caryophyllata*, it was successfully authenticated by integrating both morphological and molecular level approaches. The DNA barcoding by utilising rbcL and matK gene sequences provided valuable knowledge into the close relationship between *K. caryophyllata* and the related species *K. chandeei*. Further, for preparing valuable reference markers, various pharmacognostic features of the plant were utilised. This includes macroscopic and organoleptic characteristics of the plant components, the information on spatial distribution of phytochemical groups, fluorescence colour behavioural pattern exhibited based on differences in chemical composition, as well as prominent microscopic features noted in the anatomical sections and in the powder microscopy.

The analysis of the physicochemical parameters revealed differences in total ash, water-soluble ash, acid-insoluble ash content and in extractive yield of both water-

soluble and alcohol-soluble among the plant components, reflecting the variations in phytochemical compositions. Leaves had higher total ash, water-soluble ash content, and extractive yield suggesting a higher content of phytoconstituents, indicating it to be more suitable for therapeutic uses than other components. All the findings of pharmacognostic parameters, morphological and molecular characteristics and physicochemical properties, collectively contributed to the preparation of monograph of the plant and may serve as a reference standard to identify adulteration in crude drugs. Further, this can provide practical guidance for traditional medicine practitioners in selecting the appropriate plant part for effective use of *K. caryophyllata* in herbal medicine.

The qualitative phytochemical screening using various reaction tests revealed the significant occurrence of a wide range of phytochemical groups including alkaloids, terpenes, phenolics, tannins, flavonoids, glycosides, saponins, carbohydrates, and proteins in the methanolic extracts of leaf, stem and root components, compared to chloroform and petroleum ether extracts. The phytochemical screening using GC-MS and HR LC-MS for specific bioactive compounds revealed a higher number and diverse bioactive compounds in the methanolic leaf, stem and root extracts. Overall, the screening for phytochemicals concluded that the leaf component extract and particularly the leaf methanolic extract of *K. caryophyllata* have comparatively more diverse and higher concentration of various phytochemical groups and corresponding higher numbers of specific bioactive compounds with interesting biological properties like anti-inflammatory, antioxidant and antimicrobial activities, suggesting their superior pharmacological potential. The quantitative estimation of various secondary metabolites in methanolic extract of different component parts indicates majority of the secondary metabolites including terpenoids, alkaloids and phenolics have highest concentration in the leaf component compared to stem and root. Among various phytochemical groups quantified in the leaf component, the group terpenoids was found dominating over other groups.

The study of bioactivity properties of *K.caryophyllata* such as antioxidant, antimicrobial, cytotoxic and anti-inflammatory, conducted using methanolic extracts of different plant components revealed a wide range of pharmacological effects and its potential as a promising candidate for future research. The antioxidant studies using phosphomolybdenum, DPPH, and hydrogen peroxide scavenging assays showed the

highest antioxidant properties in the methanolic leaf extract. The antimicrobial activity study using Agar well diffusion method against different tested pathogenic microorganisms showed that the methanolic leaf extract was very effective compared to other plant components. Similarly, the cytotoxicity study using Trypan Blue exclusion method revealed that the leaf methanolic extract has exhibited greater effect against DLA and EAC cancer cell lines than stem and root extracts. The *in-vitro* anti-inflammatory study using Trypsine inhibition assay and Nitric oxide inhibition assay revealed that the leaf methanolic extract has more potent anti-inflammatory activity than the stem and root extracts. Followed by this, the study conducted *in-vivo* anti-inflammatory analysis, including Carrageenan-induced and Formalin-induced inflammation models, revealed a similar trend.

In all the bioactivity studies, the significant outperformance of the methanolic leaf extract over stem and root is attributed to the synergistic and additive properties of an enormous number of diverse bioactive compounds with multiple targeted activities. Another inference noted in the study is the dose-dependent activity of the methanolic extract in the selected concentration range.

The study identified that the phytochemical group, terpenoids, were found in all component extracts with dominance in the leaf. The phytochemical screening using GC-MS and HR LC-MS techniques revealed the consistent occurrence of a terpenoid compound, squalene, in all the component extracts, which has diverse pharmacological properties such as anti-inflammatory, antioxidant, antimicrobial and anti-cancerous. Through the isolation of this potential bioactive compound, squalene, scientific evidence and confirmation of the effectiveness of the use of *K. caryophyllata* in traditional medicine has been achieved.

The overall findings of the study conclude the promising role of *K. caryophyllata* as a valuable source of natural medicine in the prevention and management of various diseases. Further, this study offers a solid foundation for upcoming research in the medical field, possibly contributing to pioneering treatments using natural sources. Additionally, clinical studies are warranted to validate the efficacy and safety of *Kamettia caryophyllata* extracts for human use, paving the way for developing evidence-based therapeutic interventions.

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Chapter 10
Recommendations

Based on the pharmacognostic evaluation, bioactivity analysis and isolation of bio active compound squalene conducted in the present study together with the documentation in Hortus Malabaricus, *Kamettia caryophyllata* is recommended as a strong candidate for future therapeutic research.

The following further recommendations are proposed in this context:

1. Additional Subacute Toxicity Studies

Conducting additional subacute toxicity studies is crucial to assess the effects of *K. caryophyllata* on the internal organs of animal models. These studies will provide insights into the safety and potential side effects of the plant's extracts, informing dosage guidelines for future research and applications. Clinical trials are vital before experimenting in human models.

2. Isolation and Identification of bioactive Compounds

The activity of squalene isolated from *K. caryophyllta* has to be compared with other available forms of squalene to check for variations, if any. The isolation and structural elucidation of more promising bioactive compounds identified in this study through screening techniques is recommended for further validation of the plant.

3. Molecular target and signalling pathway

Both high and low doses of the leaf methanolic extract of *K. caryophyllata* in the study significantly reduced hind paw swelling in carrageenan and formalin anti-inflammatory animal models; additional research is needed to clarify the molecular targets and signalling pathways utilised by the bioactive compounds to exhibit their anti-inflammatory properties.

4. Propagation Methods

Kamettia caryophyllata being endemic to the Southern Western Ghats, it is essential to establish effective propagation methods. This will ensure the conservation and availability of the plant for future studies and applications.

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Certificate

This is to certify that the Angiosperm material vide Collection Number 9315 is *Kamettia caryophyllata* (Roxb.) Nicolson& Suresh, belongs to the family Apocyanceae, collected by Jiji. P. G, Research scholar, Department of botany, Sree Narayana College, Nattika, Thrissur, Kerala.

The Material has been deposited at SNMH! (International Herbarium accredited by New York Botanical Garden) With Accession No 2002 and 2003.

Maliankara

22-09-2017

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Dr. Sunil C N

Dr. C.N. Sunil
Asso. Professor & Research Guide
P.G. & Research Dept. of Botany
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GenBank

Kamettia caryophyllata ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast

GenBank: MH201080.1

FASTA Graphics

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GenBank

Kamettia caryophyllata maturase K (matK) gene, partial cds; chloroplast

GenBank: MH681795.1

FASTA Graphics

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Phone: 0487 2307968

Institutional Animal Ethics Committee (IAEC)

(Reg. No. 149/PO/Rc/S/1999/CPCSEA)

Amala Cancer Research Centre Society

AMALANAGAR - 680 555, THRISSUR, KERALA, INDIA

Ref :

Date: 02.12.2021

Certificate

This is to certify that the project proposal no. ACRC/IAEC/21(2)–P12 entitled 'Pharmacognostic analysis and Bioactivity studies in *Kamettia caryophyllata* (Roxb.) Nicolson& Suresh' submitted by Dr. Achuthan C R (on behalf of Ms. Jiji P G) has been approved by the IAEC of Amala Cancer Research Centre in its meeting held on 02.12.2021 and 66 Swiss albino mice have been sanctioned under this proposal.

Authorized by	Name	Signature	Date
Chairman:	Dr. T D Babu	.....	02.12.2021
Member Secretary:	Dr. Achuthan C R	.....	02.12.2021
Main Nominee of CPCSEA:	Dr. C B Devanand	.....	02.12.2021

PUBLICATIONS & PRESENTATIONS

A. PUBLICATIONS

Jiji, P. G., & Subin, M. P. (2023). Phytochemical profiling and bioactivity of methanolic stem extract of *Kamettia caryophyllata* (Roxb.) Nicolson & Suresh: A comprehensive study. *Journal of the Botanical Society of Bengal*, *77*(2), 56–68.

Jiji, P. G., & Subin, M. P. (2017). Qualitative phytochemical screening and GC-MS analysis in the leaf methanolic extracts of *Kamettia caryophyllata* (Roxb.) Nicolson & Suresh. *Paripex – Indian Journal of Research*, *6*(4),471-479.

B. PRESENTATIONS

Jiji, P. G., & Subin, M. P. (2020). Preliminary phytochemical analysis and antimicrobial activity studies of methanolic root extract of *Kamettia caryophyllata* (Roxb.) Nicolson & Suresh. In *Proceedings of the International Conference on Climate Change: Adaptation and Mitigation* (158–172). ISBN: 978-81-944730-0-8. St. Thomas College (Autonomous), Thrissur, Kerala.

Jiji, P. G., & Subin, M. P. (2020). Preliminary phytochemical analysis and antimicrobial activity studies of methanolic stem extract of *Kamettia caryophyllata* (Roxb.) Nicolson & Suresh. In *International Conference on Homeopathy & Phytomedicine.* (Abstract No. 79), 79-80. Department of Biochemistry, St. Thomas College, Pala, Kerala, in association with National Ayush Mission.

Jiji, P. G., & Subin, M. P. (2018). Preliminary phytochemical analysis in the petroleum ether extracts of leaf component in *Kamettia caryophyllata* (Roxb.) Nicolson & Suresh. In *Proceedings of the International Conference on Phytomedicine, Medicinal Plants: International Journal of Phytomedicine and Related Industries, Special issue*(1), 105–106. (NAAS: 5.12).Bharathiar University, Coimbatore, Tamil Nadu, India.