Exploration of Bioactives from Rare and Endemic Species of Western Ghats for the Development of Potential Drug Lead against Infectious and Lifestyle Diseases

By

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Dedicated to my beloved Parents….

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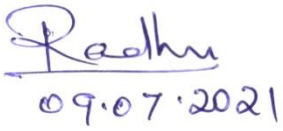

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Neethu Sivadas

TABLE OF CONTENTS

ABBREVIATIONS

ADME/T	: Adsorption, Distribution, Metabolism, Excretion/ Toxicity
AGEs	: Advanced Glycation End products
AMR	: Antimicrobial Resistance
API	: Ayurveda Pharmacopeia of India
ATCC	: American Type Culture Collection
brs	: Broad Singlet
BSA	: Bovine Serum Albumin
CC	: Column Chromatography
CC_{50}	: The 50 % Cytotoxic Concentration
CD	: Circular Dichroism
$CDCl_3$	: Deuterated Chloroform
CD_3COCD_3	: Deuterated Acetone
CD_3OD	: Deuterated Methanol
CFU	: Colony Forming Unit
CLSI	: Clinical & Laboratory Standards Institute
CNS	: Central Nervous System
cm	: Centi Meter
CoA	: Coenzyme A
COSY	: Correlation spectroscopy
°C	: Degree Celsius
d	: Doublet
2D	: Two Dimensional
DCM	: Dichloromethane
dd	: Doublet of doublets
DEPT	: Distortionless Enhancement by Polarization Transfer
DFT	: Density Functional Theory
DMSO	: Dimethyl Sulphoxide
DMEM	: Dulbecco's Modified Eagle Medium
ECD	: Electronic Circular Dichroism
ESKAP	: *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*
ESI	: Electrospray ionization

EtOAc : Ethyl acetate

FBS : Fetal Bovine Serum

FDA : Food and Drug Administration

FIC : Fractional Inhibitory Concentration

FT-IR : Fourier transform infrared

g : Gram

GC-MS : Gas Chromatography Mass Spectrometry

h : Hour

HCl : Hydrochloric Acid

HMBC : Heteronuclear Multiple Bond Correlation

HMQC : Heteronuclear Multiple Quantum Coherence

HPLC : High Performance Liquid Chromatography

HRMS : High-resolution mass spectrometry

IC_{50} : Concentration required for 50 % inhibition

IP : Intraperitoneally

IR : Infrared

IUCN : International Union for Conservation of Nature

J : Coupling Constant

kg : Killogram

m : Multiplet

M : Molar

M^+ : Molecular ion

MDR : Multidrug Resistance

mg : Milligram

MHA : Mueller-Hinton Agar

MHBII : Mueller-Hinton Cation Supplemented Broth II

MHz : Mega Hertz

MIC : Minimum Inhibitory Concentration

mL : Milliliter

mm : Millimeter

mM : Millimolar

MRSA : Methicillin-Resistant *Staphlococcus aureus*

MSSA : Methicillin-Sensitive *Staphlococcus aureus*

MTD	: Maximum Tolerable Dose
MTT	: (3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-2H-tetrazoliumbromide)
NK	: Nayopayam Kwatha
nm	: Nanometer
NMR	: Nuclear Magnetic Resonanace
NPs	: Natural Products
OD	: Optical Density
PAE	: Post Antibiotic Effect
PBS	: Phosphate-buffered saline
PDB	: Protein Data Bank
PMBN	: Polymyxin B Nonapeptide
ppm	: Parts Per Million
RPM	: Revolutions per minute
RT	: Room Temperature
s	: Singlet
SA	: *Staphlococcus aureus*
SD	: Standard Deviation
SI	: Selectivity Index
t	: Triplet
TCA	: Tricholroacetic Acid
TLC	: Thin Layer Chromatography
TMS	: Tetramethylsilane
TSB	: Tryptic Soy Broth
UV	:Ultraviolet
v/v	: Volume/Volume
VRE	: Vancomucin Resistant *Enterococci*
VRSA	: Vancomycin-Resistant *Staphlococcus aureus*
VSE	: Vancomucin Sensitive *Enterococci*
VSSA	: Vancomycin-Sensitive *Staphlococcus aureus*
WHO	: World Health Organisation
α	: Alpha
β	: Beta

γ	: Gama
δ	: Delta
ν_{max}	: Absorption maxima
μg	: Microgram
μL	: Microliter
μM	: Micromolar

PREFACE

Nature, the treasure trove of novel bioactive, chemically diverse secondary metabolites endlessly contributed towards the design and development of medications against life threatening diseases. The indigenous knowledge about medicinally important species and the highly enriched floristic diversity paved the foundation to modern drug discovery approaches. Encouraged with the excellent therapeutic potential of naturally derived compounds in clinics there is recent advancement of researches that emphasized on the development of new drug leads from secondary metabolites from plant species with the aid of sophisticated and innovative techniques. In recent years, natural products and their analogues pioneered as foremost therapeutic agents over synthetically derived drugs and were approved by FDA in clinics. The challenges associated with the treatment infectious and lifestyle diseases including drug resistance necessitates new alternatives to address the issues. In this scenario our efforts targeted the exploration of the hidden biodiversity of Southern part region of Western Ghats (Agasthyamala Biosphere Reserve). Our study focused on the identification of bioactive secondary metabolites from rare, endemic, medicinally important species of this region.

We were interested to investigate the less/unexplored wild nutmeg species of Agasthyamala Biosphere Reserve in terms of phytochemical and pharmacological aspects along with the comparative study of wild nutmeg species with the true nutmeg. Initially we have focused on the literature study of the renowned flowering plant family myristicaceae and the main features about the species of this family were outlined in Chapter **1**.

Chapter **2** describes in detail the bioprospecting of two unexplored rare and endemic species of wild nutmegs. Part A focused on the phytochemical analysis of the aerial parts of the species *Myristica beddomei subsp. spherocarpa* which is one of the sub species of *Myristica beddomei* along with the evaluation of isolates for their antiproliferative potential. Chemoprofiling of *Myristica trobogarii*, the newly identified species after 150 years from Western Ghats along with its biological screening was described in Part B of Chapter 2.

Chapter **3** focuses on the isolation of bioactives from the wild nutmeg *Myristica malabarica* and the true nutmeg *Myristica frgrans* and the application of the active constituents against the management of T2DM. Isolation of malabaricones the major bioactive constituent of the species and their in vitro antidiabetic potential was discussed

in part A while part B outlines the isolation and *in vitro* antidiabetic activity of lignans and neolignans from *Myristica fragrans*. The findings also help to a compare of the species in terms of their phytoconstituents.

Infectious diseases are emerged as an unmet threat due to inherent drug resistance of the diseases causing pathogens which in turn demanded the need of new antibacterial agents. Herein in Chapter **4** we focused the exploration of antibacterial potential of malabaricones. A detailed *in vitro* and *in vivo* antibacterial study of the compounds against MDR *S. aures* along with the synergistic combination study of compound with conventional antibiotics was also established.

First part of Chapter **5** deals with the phytochemical investigation of traditionally important medicinal plant *Hyptis capitata.* The leaf is well employed for skin diseases in traditional medicine. So we have also evaluated the antibacterial potential of the phytochemicals from the leaves of the species. The study also revealed that ecological and environmental factors play a crucial role in the development of phytoconstituents.

Scientific validation and standardization of herbal based formulations are crucial to determine the purity, efficacy and to gain acceptance in modern medicine. Most of the Ayurvedic formulations available today have no standardization protocols and are not validated based on the ingredient herbs. WHO encouraged the use of plant based formulations to cure ailments. They have introduced guidelines and protocols to determine the purity, safety and efficacy. In this regard we have attempted to develop standardization protocol for the Ayurvedic polyherbal formulation *Nayopayam Kwatha*. Second part of the chapter 5 describes the detailed study of standardization of the Nayopayam Kwatha formulation based on the marker constituents present in the individual herbs of the formulation.

Natural Products in Drug Discovery – An Overview

1A.1. Introduction

Nature, the versatile source of chemically diverse and unique bioactive molecules is always beneficial to humankind. Natural products (NPs) can be defined as "a chemical compound or substance produced by living organisms" and are characterized with enormous structural diversity and complexity. Owing to this structural diversity and unique pharmacological or biological activities they have been utilized in both traditional and modern medicine for treating various ailments. NPs offer several significant features when compared to synthetic molecules. Chemical novelty, diversity, specificity, binding efficiency, and ability to interact with biological targets associated with natural products cannot be achieved from structures derived synthetically. NPs and their related structural analogues always offer potential bioactive scaffolds for drug design and development [1].

1A.2. History of Natural Products in Drug Discovery

NPs have long back history of being used as active therapeutic agents. Plants were regarded as the promising avenue of such therapeutically active compounds. In ancient times, plants and its extracts were used to cure almost all ailments. The earliest documented records describing the use of plant extracts and oils was in 2600 BC in Mesopotamia followed by the Egyptian pharmaceutical record Ebers Papyrus in 1500 BC and the Chinese documented records including Materia Medica dated from 1100 BC, Shennong Herbal dated from ~100 BC and the Tang Herbal dated from 659 AD along with Greek documents. Charaka samhitha and Sushruta samhitha were the documented Indian Ayurvedic scripts highlighted the importance of plant based medications [2].

Identification of the analgesics morphine from opium poppy (*Papaver somniferum*) in 1804 was the major breakthrough in natural product based drug discovery. Throughout the century several purified bioactive natural products were extracted from numerous plant species include antimalarial drug quinine from the bark of *Cinchona officinalis* and cocaine from cocoa species. Another significant contribution was the pain reliever salicin from the bark of willow tree by Johannes Buchner in 1838. Subsequently it was derivatised by the more active compound acetylsalicylic acid (aspirin) [3]. Several traditional medicinal lead compounds (Fig. **1A.1**) led to the development of some well-known drugs such as khellin to chromolyn (bronchodilator),

galegine to metformin (antidiabetic drug) and papaverine to verapamil (antihypertensive drug) [4].

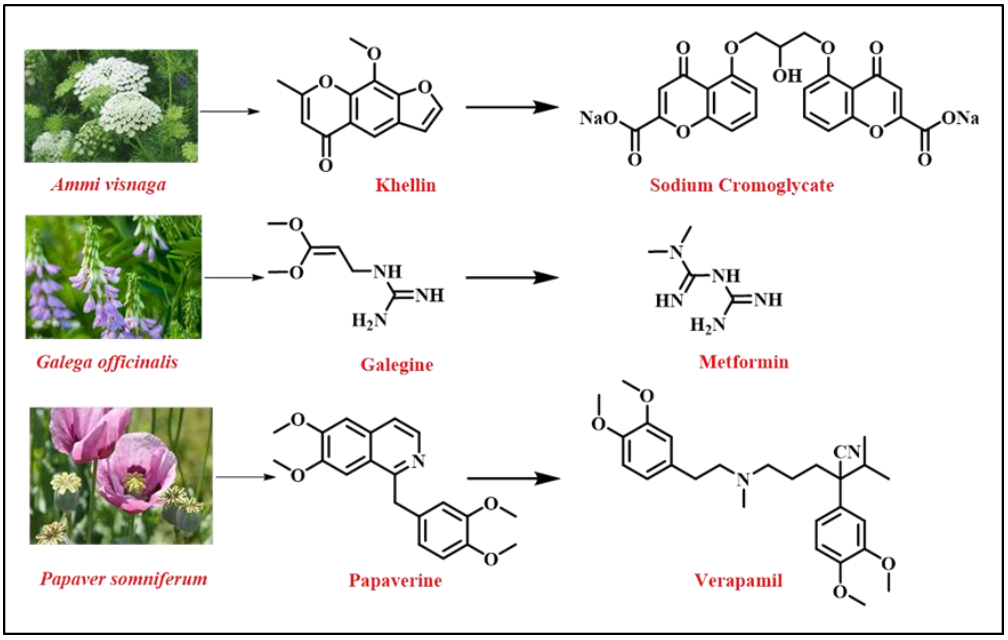

Figure 1A.1: Drugs based on traditional medicinal lead compounds

Identification of valuable therapeutic agents from natural sources continued in 20th century also. The discovery of penicillin (β-lactam antibiotic) in 1928 paved the way for the development of distinct class of antibiotics from microorganisms includes cephalosporins, tetracyclines, aminoglycosides, rifamycins, chloramphenicol and lipopeptides. During that time the therapeutic use of extracts and partly purified natural products were significantly replaced by the extra pure isolated compounds. The first antiviral (vidarabine) and anticancer drug (cytarbine) approved in clinical use was based on two nucleosides isolated from Caribbean marine sponges in 1950. Later in 1970 paclitaxel the anticancer drug isolated from *Taxomyces* species and is still used as an effective chemotherapeutic agent. Around 40% of the new drugs introduced in clinical use were either natural products or derivatives thereof [5].

1A.3. Natural Product Drug Leads against Infectious and Lifestyle Diseases

Infectious and life style diseases are regarded as the looming threats to human lives and are the leading cause of increased rate of morbidity and mortality. Infectious diseases (contagious diseases, transmissible diseases or communicable diseases) are characterized by various ailments resulting from pathogenic microorganisms (bacteria, viruses, fungi or parasites) that infect the host organism and can be spread, directly or indirectly from one organism to another. While the lifestyle diseases (non-communicable

diseases) are originated from the sedentary lifestyle and unhealthy food habits which in turn results a group of chronic diseases including type 2 diabetes, cancer, cardiovascular diseases etc. The therapeutic area of diabetes, cancer and infectious diseases were extremely benefited from naturally derived active therapeutic agents and has long back history.

1A.3.1. Infectious Diseases

Discovery and development of natural products derived drugs laid the foundation to compete the diseases causing pathogens. Microorganisms played a significant role in the discovery of antibiotics rather than any other natural sources. Diverse array of bioactive drugs and drug leads were identified and employed against infectious diseases, amongst 69 % of all antibacterial agents were originated from natural products.

Actinomycetes and *Streptomyces* were the major group of bacteria contributed bioactive metabolites to antibiotic drug development. The antibiotic classes include peptides/glycopeptides, angucyclinone, tetracycline, macrolides, anthraquinones, polyenes, beta-lactum, anthracyclines etc. were derived from *Actinomycetes*. While *Streptomycetes* derived antibacterial agents are aminoglycosides (streptomycin, neomycin, and kanamycin), erythromycin, tetracycline, chloramphenicol vancomycin and thienamycin. *Streptomyces* were also the source of active antifungals such as macrolide polyene, nystatin, amphotericin B and natamycin. Nystatin, one of the first effective polyene antifungal agent played significant role as topical antifungal agent against oral, gastro-intestinal and genital candidosis. Amphotericin B is also a traditional polyene antifungal agent against life-threatening fungal infections caused by *Aspergillus* species [6]. Natural product based antifungal compounds in phase III clinical trials are ibrexafungerp and rezafungin (echinocandin skeleton).

Plant derived natural products including several alkaloids, organosulphur compounds, phenolic compounds, terpenes, coumarins etc. possessed potent antibacterial activity and were employed in clinical use either directly or co-administrated with existing antibiotics [7]. Artemisinin and its analogues were proved to be the effective antimalarial agents in clinical use. Alkaloids, terpenoids, flavonoids, various glycosides, lignans and proteins constituted important antiviral agents from plants against viruses like herpes virus, human immunodeficiency virus (HIV), influenza and hepatitis virus. Amongst flavonoids were well studied and exploited for their potential against several viral diseases such as influenza, dengue and Chikungunya [8-9]. Most of the FDA approved antiviral drugs used today are based on a natural product parent.

Natural products in their pure form and its derivatives constituted around 48 % of antibacterial agents approved by FDA up to 2019. Plazomicin, a semisynthetic aminoglycoside bactericidal antibiotic drug was approved by FDA in 2018 for targeting the infections with gram-negative aerobic bacteria in complicated urinary tract infections. Rifamycin (Aemcolo) is a bactericidal minimally absorbed antibiotic approved by FDA in November 2018 for the treatment of the non-invasive strains of *Escherichia coli* causing travelers' diarrhea. Lefamulin is a derivative of the original fungal natural product pleuromutilin. Sarecycline and omadacycline are the tetracycline derived antibiotics for bacterial skin and skin structure infections [10].

1A.3.2. Cancer

Cancer, the group of heterogeneous diseases is characterized as the second leading cause of morbidity and mortality worldwide. The area of cancer chemotherapy was greatly benefited from the discovery of natural product derived drugs. Diverse class of bioactive compounds identified from plants, microbes, marines and animal sources contributed potent anticancer drugs and drug leads. Plant derived compounds have long back history in the treatment of cancer. Most effective plant derived anticancer drug class in clinical use was the vinca alkaloids, taxanes, podophyllotoxins and camptothecins.

Vinca alkaloids (bis-indole alkaloids) such as vinblastine and vincristine isolated from *Catharanthus roseus* were the first plant derived drugs entered in clinical use. The effective anticancer semisynthetic analogues of vinca alkaloids are vinorelbine and vindesine. They induce apoptotic cell death by disrupting the microtubules in cancer cells. Improved formulation of vincristine *ie* the vincristine sulfate liposome injection was approved by FDA against relapsed Philadelphia chromosome-negative acute lymphoblast leukemia. The class of diterpene compounds isolated from *Taxus* genus is called taxanes. Paclitaxel (Taxol®), a taxane was isolated from *Taxus brevifolia* and its semisynthetic analogues include docetaxel (Taxotere®) and cabazitaxel (Jevtana®) are the second-generation semisynthetic taxane derivatives approved in 2010 by FDA for the treatment of metastatic hormone-refractory prostate cancer. Paclitaxel is used as an effective anticancer agent against several cancer types including non-small cell lung cancer, breast & ovarian cancer whereas docetaxel was found to be effective against breast cancer. The clinically-approved new albumin-bound formulation of paclitaxel is Abraxane® which offers significant advantages. Epipodophyllotoxin, isolated from the *Podophyllum* genus and its semisynthetic derivatives teniposide and etopside were also important chemotherapeutic agents employed in clinics. Camptothecins are another class of

chemotherapeutic agents isolated from *Camptotheca acuminate*. Clinically active semisynthetic analogues derived from camptothecin include topotecan, irinotecan (CPT 11) and belotecan. Topotecan and irinotecan were FDA approved drugs for the treatment of various types of cancer including ovarian, breast, lung and colon cancers [11-14].

Marine derived natural products were also approved for the treatment of various cancer types on account of their cytotoxicity and anticancer potential. Ecteinascidin 743, a complex alkaloid from the marine tunicate, *Ecteinascidia turbinate* was the first anticancer FDA-approved drug in 2015 for the treatment of relapsed ovarian cancer in conjunction with liposomal doxorubicin. Halichondrin B (polyether) and bryostatin (macrolide) were further examples of marine-derived anticancer agents isolated from sponges and bryozoan. Eribulin mesylate was approved by FDA in 2010 and is marketed under the name of Halaven which is a synthetic derivative of the polyketide halichondrin B. Brentuximab vedotin (Adcetris), an antibody drug conjugate was approved by FDA in 2011. It is composed of a tumor specific antibody and the pentapeptide monomethyl auristatin E, which is a derivative of dolastatin 10 and is used for the treatment of adult patients with primary cutaneous anaplastic large cell lymphoma. Lubrinectidin (Zepzelca), a derivative of ecteinascidin (trabectedin) from the tunicate, *Ecteinascidia turbinata* is the very recently FDA approved drug (2020) for the treatment of metastatic small cell lung cancer. Several other anticancer agents from marine sources are under last stages of clinical trials (Phase III/II) for anticancer therapy [15].

Microorganisms provided the foremost chemotherapeutic agents such as antitumor antibiotics which are extensively employed in clinical use. Members of anthracycline such as actinomycin, ansamycin, bleomycin, mitomycin, enediynes, staurosporines etc. constitutes major anticancer agents and were isolated from various *Streptomyces* species. Several clinically useful agents include, daunomycin related agents (doxorubicin, daunomycin, epirubicin), glycopeptides (bleomycins A2, blenoxane®), mitosanes (mitomycin C), enediynes (Mylotarg®), epothilones and its synthetic derivatives. Azaepothilone (16-azaepothilone B) was FDA approved drug against metastatic breast cancer in 2007. Carfilzomib (Kyprolis™), a synthetic analogue of epoxomicin was another FDA approved anticancer drug in 2012 against relapsed and refractory multiple myeloma [16].

Biologically active compounds including peptides, proteins and enzymes with potential anticancer activity have been isolated from various animal sources especially from the venom of snakes, ants, bees, spider etc. Some examples include Viridistatin 2

(*Crotalus viridis*), Tzabcanin (*Crotalus tzabcan*), Chlorotoxin (*Leiurus quinquestriatus*), Vidatox 30 CH (*Rhopalurus junceus*), L-amino acid oxidase (*Ophiophagus hannah*) etc. Chlorotoxin (Phase III), Cilengtide (Phase II & III) and BAY86-7548 (Phase II & III) are in the clinical trial for cancer cell proliferation and metathesis [17].

1A.3.3. Diabetes

Diabetes mellitus, a group of chronic metabolic disorders is characterized by persistent hyperglycemia (elevated levels of blood glucose) arises as a consequence of relative or absolute deficiency of insulin production and secretion, or both. Diabetes are generally referred as type 1 (insulin dependent) characterized by absolute insulin deficiency associated with pancreatic *β*-cells destruction and type 2 (non-insulin dependent) originates from insulin resistance and impaired insulin secretion. Type 2 diabetes mellitus (T2DM) is the greatest health crises contributes around 90% of the diabetic population worldwide and its prevalence is projected to increase by >50% globally by 2045. Diabetic complications such as retinopathy, nephropathy, neuropathy and cardiovascular diseases (major risk factor) are the reason for increased rate of mortality. Environmental factors (Sedentary life style, unhealthy food habits etc.) and genetic factors create pathophysiological troubles which will ultimately leads to impaired glucose homeostasis in T2DM. Currently, ten classes of drugs are approved by the US Food and Drug Administration for the treatment of T2DM [18-20].

Natural products have pivotal role in contributing novel therapeutic agents for the prevention of T2DM with increased efficacy and lower side effects. Multiple regulating mechanism and signaling pathways associated with natural derived compounds have made them an appropriate candidate for the drug development against T2DM. Natural products including flavonoids, polyphenols, alkaloids, saponins, quinones etc exhibited potent antidiabetic activity and have the ability to ameliorate the status of T2DM. The first medicinal plant described with a clear antidiabetic effect was *Galega officinalis*. Metformin was the first line medication for the treatment T2DM which is a semisynthetic derivative of galegine (a guanidine). Pycnogenol water extracts from pine bark showed antidiabetic activity due to its digestive enzyme inhibition potential against α-glucosidase. Several antidiabetic drugs were derived from microorganisms also. Acarbose (Glucobay) from *Actinoplanes* is the most widely used digestive enzyme inhibitor acting on α-glucosidase, α-amylase, sucrose and maltase for efficient management of T2DM. Miglitol, originally obtained from various *Bacillus* and *Streptomyces* strains was the second-generation α-glucosidase inhibitor structurally similar to glucose. Voglibose is the

synthetic derivative of valiolamine, which is isolated from a fermentation broth of *Streptomyces hydroscopicus subsp. limoneus* is also an effecive α-glucosidase inhibitor. Extenatide (Byetta), triproamylin acetate (Normylin), liraglutide (Victoza) were the FDA approved antidiabetic drugs derived from natural products. Recently approved natural derived antidiabetic drugs against T2DM are Glucagon-like peptide-1 receptors, Lixisenatide (Lyxumia) in 2013 and Semaglutide (Ozempic) in 2017 which is alike extenatide [16, 21-23].

1A.4. Classification of Natural Products

Natural products always retain diversities in their structure, function and biosynthesis and are broadly classified into primary and secondary metabolites. Metabolites are regarded as the intermediates/products of various biochemical reactions involved in the living system. Primary metabolites include starch, cellulose, nucleic acids, chlorophyll pigment etc. are essential for the growth and development and are directly involved in major biological functions of the living organisms. Kossel in 1891 introduced the term "secondary metabolites" and is defined as a heterogeneous group of natural metabolic products functioning as defense or signaling molecules in ecological interactions, symbiosis, metal transport etc. which are not essentially required for the survival. Secondary metabolites often replaced by the term natural products due to its diversity in structure and broad range of bioactive potential. Medicinal properties of these secondary metabolites were impressive and were extensively utilized in traditional medicine and can act as potential drug candidates. Secondary metabolites are classified according to their vast diversity in source, structure, function and biosynthesis. A brief description of the classification is given below.

1A.4.1. Source of Origin

Plants, microorganisms, marine organisms and animal world are the major source of secondary metabolites. From the ancient time itself they have been explored for their bioactive potential.

1A.4.1.1. Plant Derived Secondary Metabolites

Plants constitute around 80% of the bioactive secondary metabolites and are well documented for their medicinal uses for years. Plants represent rich source of simple to complex lead molecules including flavonoids, alkaloids, terpenoids, tannins etc. To date, 35,000- 70,000 plants have been screened for their pharmacological use and they have largely contributed to herbal medications [24]. Plant based traditional medicine still satisfies the primary health care needs of around 80% of the population (WHO News

2002). The ease of availability and its enormous bioactive potential make them always a better choice of interest. Several plant derived molecules played a prominent role in the treatment of various diseases in their natural forms. Alkaloids, steroidal glycosides and terpenoids constitute the major class of compounds used as unmodified drugs derived from several plant species. The significant examples of plant derived natural compounds were discussed below.

Artemisinin, a sesquiterpene lactone, an active constituent of *Artemisia annua* has gained great attention owing to its unique mechanism of action against malaria. Digitoxin, the cardiac glycoside was isolated from *Digitalis purpurea*. Different types of alkaloids from the plants were found to have high therapeutic potential. Amaryllidaceae type alkaloid galanthamine from *Galanthus woronowii* and other species of this genus was widely used for the treatment of Alzheimer's disease. Tropane type alkaloids [atropine, (-)-hyoscyamine] from the species of *solanaceae* family were used in several medications. Opium alkaloids codeine and morphine were the major bioactive constituents of opium resin, obtained from the immature fruits of *Papaver somniferum* and were the pharmacologically important drugs as analgesic agents with considerable use today. Paclitaxel, a nitrogen containing diterpenoid isolated from the bark of *Taxus brevifolia* was a potent anticancer agent derived from plants. Quinidine and quinine are quinoline alkaloids obtained from *Cinchona* species possessed antiarrhythmic and antimalarial activity. Anticancer vinca alkaloids including vincristine and vinblastine were isolated from *Catharanthus roseus* and camptothecin and its analogs were initially discovered from *Camptotheca acuminate* [25-26].

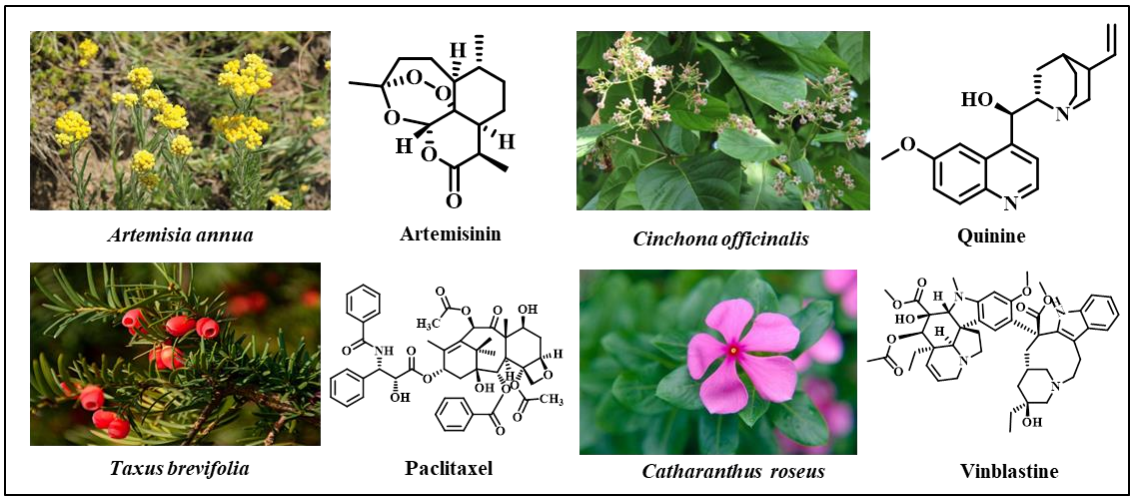

Figure 1A.2: Important plant derived drugs

Apart from the pure plant derived natural products, semisynthetic and synthetic derivatives of plant secondary metabolites were also employed in therapeutics. Modification of the basic skeleton of the isolated compounds from the plants by incorporating bioactive scaffolds further enhances the activity of parent compounds. The first semisynthetic drug used in clinics was aspirin which is an acetyl derivative of salicylic acid obtained from plant species. Some significant examples of semisynthetic drugs from plant natural products are summarized in Table **1A.1**.

Table 1A.1: Semisynthetic drugs from plant derived natural products

Natural Drug Lead	Semisynthetic Drug	Class of Compound	Medicinal Use
Artemisinin	Arteether	Sesquiterpenoid	Antimalarial
	Artemether	Sesquiterpenoid	Antimalarial
Atropine	Ipratropium bromide	Tropane alkaloid	Asthma
	Triotropium bromide	Tropane alkaloid	Pulmonary Disease
Captothecin	Irinotecan	Quinoline alkaloid	Anticancer
	Topotecan	Quinoline alkaloid	Anticancer
(+)-cytisine	Varenicline	Quinizolidine alkaloid	smoking cessation
Khellin	Chromolyn sodium	Chomine derivative	Bronchodilator
Leptospermone	Nitisinone	β-triketone	Hereditary tyrosinemia
Morphine	Apomorphine	Benzyltetrahydro isoquinoline alkaloid	Parkinson's disease
Podophyllotoxin	Etoposide	Lignan	Anticancer
	Teniposide	Lignan	Anticancer
Quinine	Chloroquinine	Quinoline alkaloid	Antimalarial
Theophylline	Aminophylline	Purine alkaloid	Antiasthma
Vinblastine	Vinorelbine	Indole alkaloid	Anticancer

1A.4.1.2. Microbial Derived Secondary Metabolites

Microorganisms also regarded as the valuable source of bioactive natural products. The microbial secondary metabolites specifically characterized with special features such as their unique molecular skeleton and interaction with the environment and were known to possess variety of biological activities includes antimicrobial, inhibition of enzymes, anticancer, immune-suppressive, antiparasitic, plant growth stimulation etc.

The discovery of penicillin initiated the exploration of bioactive secondary metabolites from microorganisms. Microorganisms include *actinobacteria, Bacillus* and *Pseudomonas* species significantly contributed towards the identification various class of antibiotic agents. Most of the antibiotics we used today are derived from microorganisms. In addition microorganisms provided potent chemotherapeutic agents that have been evaluated through clinical trials. Approved anticancer agents from microorganism include actinomycin D, anthracycline, epirubicin etc. Besides the antifungal, anti-inflammatory and immunosuppressive activity, Rapamycin also showed excellent anticancer potential. Natural products from fungal sources also provided various therapeutic agents. Enzyme inhibitor wortmannin is a fungal furanosteroid derivative of *Penicillium funiculosum.* Several top selling biopharmaceuticals from microorganisms include an insulin analog insulin glargine (Lantus®) derived from the gram-negative bacteria *E. coli* and the pneumococcal vaccines (Prevnar® family) were derived from *Staphylococcal pneumoniae* and *Corynebacterium diphtheria* [6, 27].

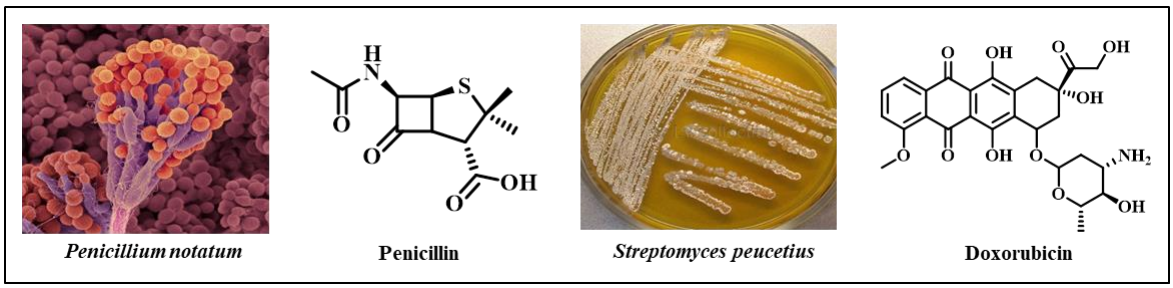

Figure 1A.3: Important microbial derived drugs

1A.4.1.3. Marine Derived Secondary Metabolites

The marine environment is characterized by unique groups of organisms represents the source of a wide range of fascinating structures. It covers around 70% of the earth's surface with huge variety of ecosystem having enormous biodiversity. Marine organisms including bacteria, fungi, sponges, algae, mollusks, coelenterates and echinoderms are the rich source of novel structural classes of secondary metabolites and played a prominent role in the development of numerous drugs. Amongst sponges represents the most described group with bioactive secondary metabolites due to their chemical defense mechanisms. Lichens and lower plants, such as the *Bryophyta* species, have produced hundreds of bioactive compounds. Terpenoids and its derivatives were the important class of compounds produced by algae species [28]. Marine natural products displayed variety of biological activities such as antiviral, anticancer, antifungal

analgesic, antibacterial, anti-inflammatory etc. which make them promising source of drug discovery and development.

The exploration of novel chemical structures from marine organisms started in 1950 by the isolation of first active nucleosides spongouridine and spongothymidine from the Carribean sponge *Cryptothya crypta* by Bergmann and coworkers. These two compounds showed anticancer and antiviral potential and inspired from their structure two new synthetic antivirals cytarabine and vidarabine were developed which in turn revealed the tremendous potential of marine natural products for the development of new drugs. Discovery of such novel compounds with potent activity from sponges encouraged the exploitation of marine sources as drug leads. Discodermolide is another example of marine derived agent from *Discodermia dissolute* having excellent anticancer potential with same mechanism of action to that of paclitaxel [29].

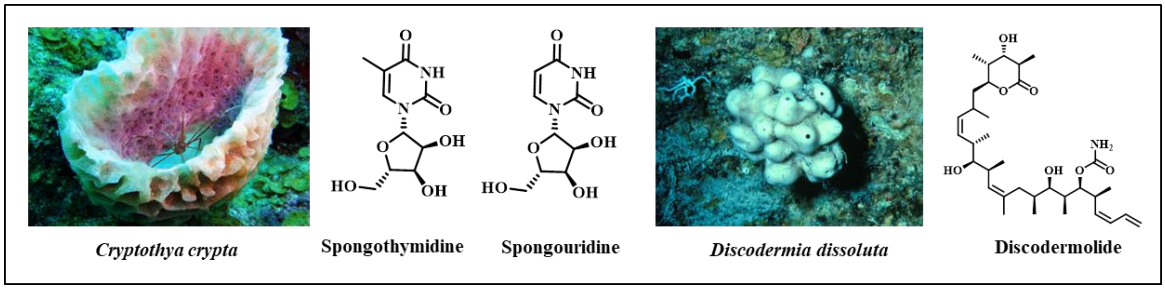

Figure 1A.4: Important marine derived natural products

Ziconotide (Prialt®) was the first FDA approved marine derived semisynthetic non-narcotic analgesic drug. It is the synthetic equivalent of a conopeptide originating from a marine species of the cone snail genus *Conus* [2]. Some of the semisynthetic FDA approved drugs derived from marine species [30] are summarized in Table **1A.2**.

Table 1A.2: Semisynthetic FDA approved drugs derived from marine species

Name of the Drug	Class of Compound	Marine Source	Medicinal Use
Ziconotide	Peptide	Cone snail	Chronic pain
Cytarabine	Nucleoside	Sponge	Anticancer
Vidarabine	Nucleoside	Sponge	Antiviral
Trabectedin	Alkaloid	Tunicate	Anticancer
Brentuximab vedotin	Antibody drug conjugate	Mollusk	Anticancer
Eribulyn mesylate	Macrolide	Sponge	Anticancer

Omega-3-acid methyl ester	Omega-3-fatty acid	Fish	Anticancer
Ecteinascidin 743	Tetrahydro isoquinoline alkaloid	Tunicate	Anticancer
Lubrinectidin	Tetrahydro isoquinoline alkaloid	Tunicate	Anticancer

1A.4.1.4. Animal Derived Secondary Metabolites

Animals were also contributed several therapeutically active secondary metabolites. Indigenous knowledge played a remarkable role in exploration of therapeutic agents from animal sources. Several bioactive agents were developed from the body parts and metabolic produce (including excrements and corporal secretions), venoms and toxins of animals. Teprotide, extracted from the venom of a Brazilian viper *Bothrops jararaca* has led to the development of acetylcholinisterase inhibitor cilazapril and antihypertensive agent captopril [31]. The analgesic epibatidine was isolated from the skin of an Ecuadorian poisonous frog (*Ameerega bilinguis)* which is ten times more potent than morphine and led to the development of novel class of pain killers [32-33]. Another significant discovery was isolation of exendin-4 from the venom of the Gila monster (*Heloderma suspectum*) which formed the basis for the development of the extenatide polypeptide, Byetta® effective against type 2 diabetes. In 2009, another peptide liraglutide a very close relative of human GLP-1 was approved against type 2 diabetes. In contrast to plants and microorganisms, the structural diversity of natural product from animals seems to be limited [2].

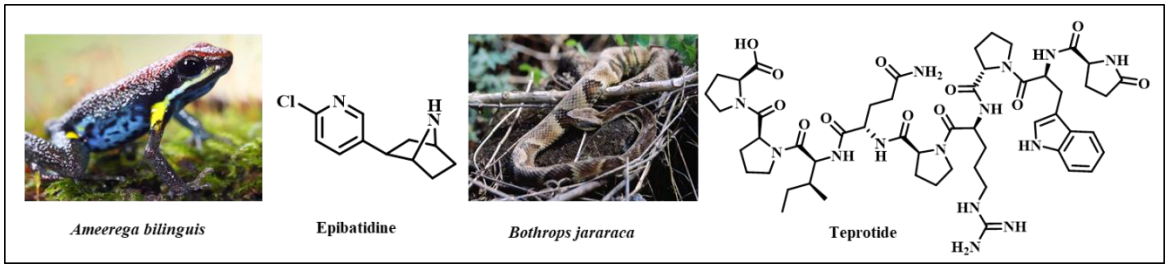

Figure 1A.5: Important animal derived natural products

1A.4.2. Biosynthetic Pathway

Biosynthesis is a multi-step, enzyme catalyzed process wherein simple substrate compounds are converted or modified, or joined together to form more complex products in living organisms through numerous intermediates. Sometimes an intermediate is simultaneously acted as a precursor for another part of the biosynthetic pathway. This

process often consists of several metabolic pathways. Primary metabolites and secondary metabolites are synthesized in plants as a result of metabolic process. The rate of biosynthesis is substantially higher in plants and in microbes resulting diverse natural products. Plants maintain an equilibrium between biosynthetic and degradation pathways to retain the carbon and energy flow for growth and development. The biosynthetic pathways derived from various precursors of primary metabolism including amino acid, nucleic acid etc. [34]. Distinct class of secondary metabolites including alkaloids, phenolic compounds, flavonoids, terpenes and steroids were produced as a result of various biosynthetic pathways. A general schematic representation of biosynthetic pathway is given in Fig. **1A.6**.

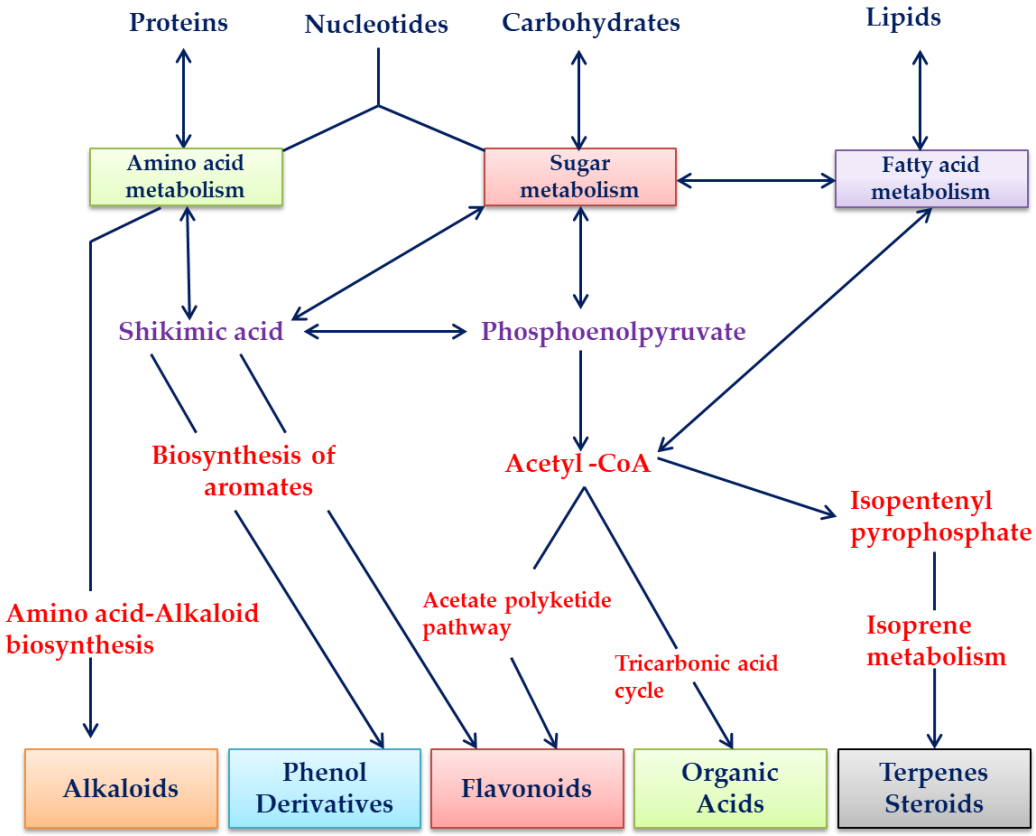

Figure 1A.6: Schematic representation of biosynthetic pathways

The major precursors for the biosynthesis are derived from protein (amino acids), carbohydrate (sugars) and lipid (fatty acid) metabolism and it consists of several pathways incudes shikimic pathway, acetate pathway, mevalonate and deoxyxylulose phosphate pathway etc. Several intermediates derived from the above mentioned pathways include shikimic acid, acetyl coenzyme A (acetyl-CoA), mevalonic acid and 1-deoxyxylulose 5-phosphate also functions as precursor for the biosynthesis of other

secondary metabolites. Shikimate is biosynthesized from two metabolites derived from the pentose phosphate cycle and glycolysis such as D-erythrose-4-phosphate and phosphoenolpyruvate. Addition of a C_3 unit from phosphoenolpyruvate converts shikimate to chorismate. Further the chorismate act as precursor for the biosynthesis of aromatic amino acids (L-phenylalanine, L-tyrosine and L-tryptophan). Most of the aromatic compounds including flavonoids, phenols, alkaloids etc. were derived from the aromatic amino acid precursor. Acetyl-CoA is another significant metabolite resulted from the glycolysis and also through the β-oxidation of fatty acids which are involved in the biosynthesis of several distinct classes of metabolites such as phenylpropanoids, flavonoids, terpenes and lignans. In addition Acetyl-CoA is also used to synthesize organic acids in the tricarboxylic acid cycle. Organic acids also act as precursors in the biosynthetic process. The biosynthesis of terpenes can proceed via two different pathways including the mevalonate and methylerythritol-phosphate pathway. Synthesis of basic skeleton of secondary metabolites is influenced by several chemical reactions and enzymatic conditions so the basic knowledge about the biosynthetic pathways of natural compounds is essential for the targeted manipulation of these pathways in biotechnology [35].

1A.4.3. Chemical Structure

Natural products are characterized with chemically diverse structural templates. The major class of secondary metabolites resulted from biosynthesis include flavonoids, alkaloids, phenylpropanoids, terpenes and glycosides (Fig. **1A.7**). Aliphatic compounds, tannins, polyketides, oxygen hetrocycles, benzofuranoids etc. are the other structurally diverse natural products. Natural products entails of diverse complex molecular structures of high molar mass with specific structural features. The basic skeleton of compounds are generally rigid and consist of rings (aromatic/aliphatic), more than one stereo centres, higher number of unsaturation, various functional groups, higher number of hydrogen bond donors/acceptors, several flexible carbon-carbon single bonds etc. Moreover, large number of secondary metabolites contains heteroatom such as nitrogen and oxygen in plenty. All these facts about the natural products make it necessary to classify them into various sub-classes according to the chemical characteristics for a better and clear understanding.

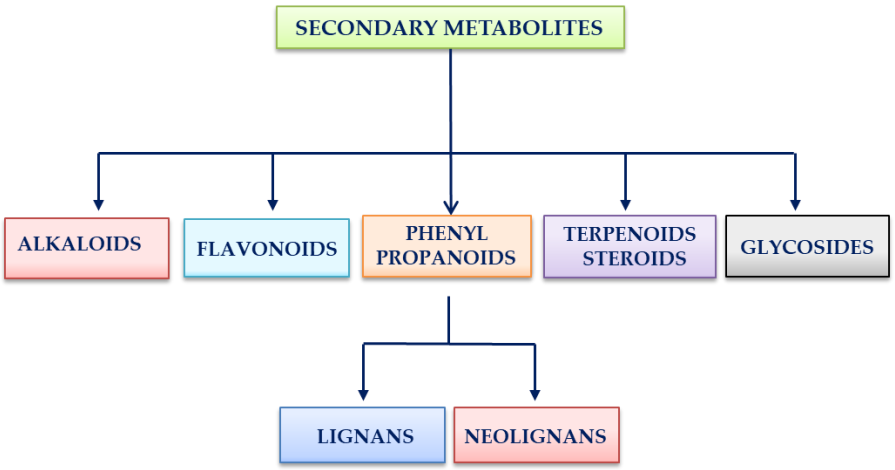

Figure 1A.7: Major chemical class of secondary metabolites

1A.4.3.1. Alkaloids

Alkaloids represents the most diverse groups of secondary metabolites found in living organisms with a wide variety of chemical structures containing hetero cyclic nitrogen compounds biosynthesized from amino acids such as phenylalanine, tyrosine, tryptophan, ornithine and lysine. In 1819 the German Scientist Carl F. W. Meissner introduced the word alkaloid which is derived from an Arabic word 'Al-quali' means ashes of plants. Alkaloids whose name originally comes from "alkali-like" and have basic properties connected with heterocyclic tertiary nitrogen atom. Plants were found to be the primary source of alkaloids along with plants microbial world, animals and marine organisms also have significant contribution. Alkaloids have extensive uses in human life as diet ingredients, supplements and pharmaceuticals and have been used for hundreds of years. Alkaloids are known to possess anti-inflammatory, anticancer, analgesics, local anesthetic and pain relief, neuropharmacologic, antimicrobial, antifungal etc. activities. Enormous biological activity of the alkaloids makes them always a potential candidate for the development of new drugs. Alkaloids can be classified into numerous types according to its molecular skeleton, botanical origin and biosynthetic precursor. Generally based on structures alkaloids belong to different classes include indoles, quinolines, isoquinolines, pyrrolidines, pyridines, pyrrolizidines, tropanes, terpenoids and steroids. Several plant species were found to be the rich source of alkaloids. Based on the botanic origin they are categorized into *Papaver* (opium), *Cinchona*, *Rauvolfia*, *Catharanthus*, *Strychnos*, *Ergot*, *Cactus* and *Solanum* alkaloids. Some important examples of alkaloids are morphine, strychnine, atropine, colchicine, ephedrine, quinine and nicotine [36-41].

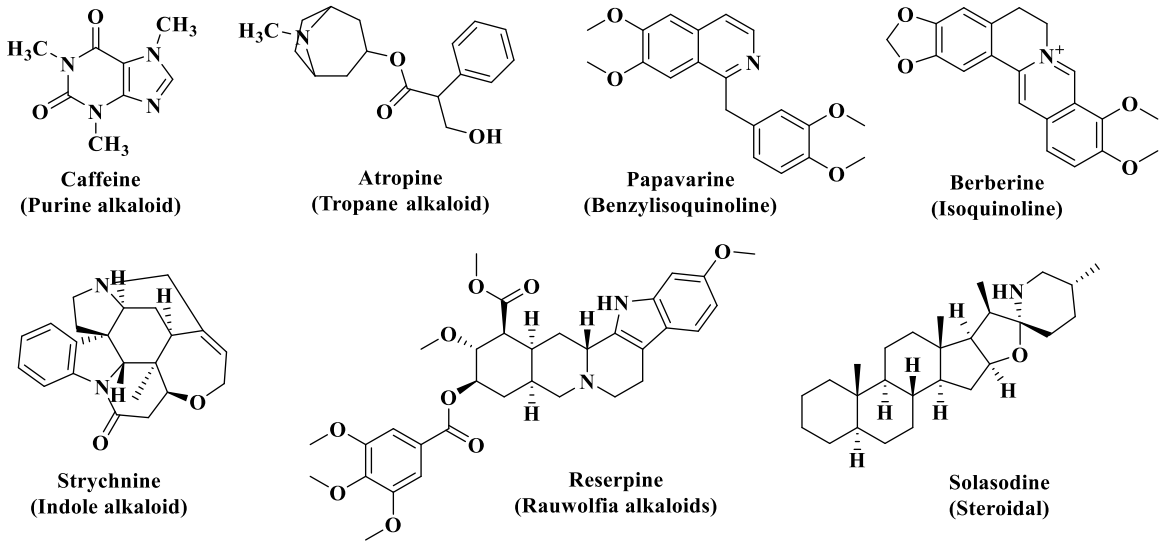

Figure 1A.8: Structures of various classes of alkaloids

1A.4.3.2. Flavonoids

Flavonoids are ubiquitously occurring polyphenolic compounds represent the huge class of abundant secondary metabolites. Flavonoids are of enormous interest because of its tremendous bioactive potential and are widely utilized as dietary supplements, nutraceuticals, medicinal and in cosmetic formulations. Flavonoids are credited with wide spectrum of pharmacological actions like antioxidant, antiviral, antibacterial, anti-inflammatory, anticancer, antidiabetic, cardioprotective and anti-allergic potentials. They are biosynthesized from the phenyl propanoid metabolic pathway. The backbone of flavonoids was resulted from the combination of 4-coumaroyl-CoA (from phenylalanine) and malonyl-CoA. Chalcones are the main intermediate in biosynthesis and is also the precursor of all subgroups of flavonoids. Flavonoids can be classified according to their biosynthetic origin. Flavonoid structure consists of a phenylpropanoid C_6-C_3 backbone is extended with three C_2 units which form a second aromatic ring. This basic C_6-C_3-C_6 skeleton of flavonoids can be modified by hydroxylations, methylations, prenylation and by aryl migration in the case of isoflavonoids. Based on the structural characteristics flavonoids are categorised into flavones, flavanones, flavonols, flavan-3-ols, flavan-4-ols isoflavones and anthocyanidines. The structural features include, the way in which the rings (**B** & **C**) are connected, the degree of saturation, oxidation and hydroxylation of the **C** ring (Fig. **1A.9**) [42-45].

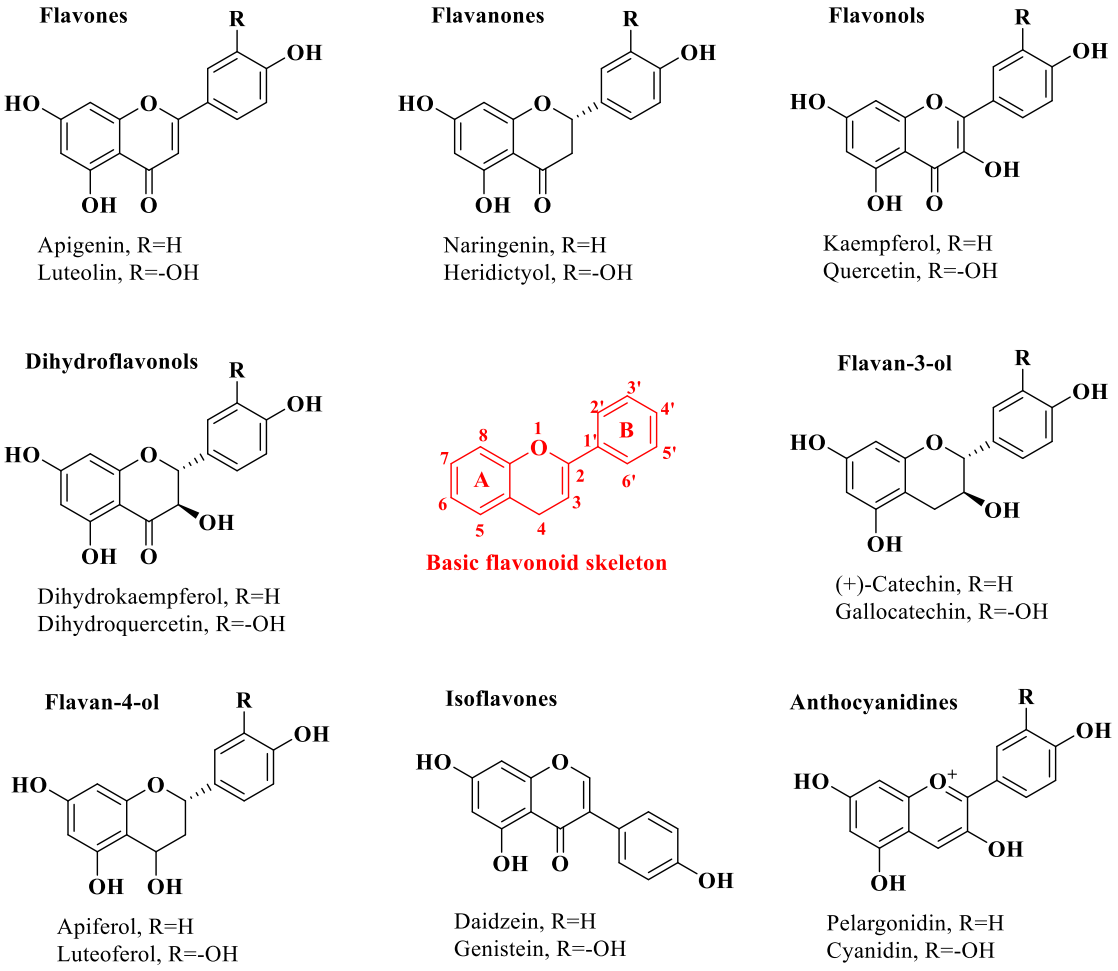

Figure 1A.9: Structures of flavonoids

Flavones, the important sub group of flavonoids usually occurs in glycosylated form and is dispersed in leaves, fruits and vegetables. Flavanones (dihydroflavones) consist of a saturated C ring and are found in citrus fruits, possess antioxidant, anti-inflammatory, blood lipid-lowering and cholesterol-lowering potential. Saturated C ring makes them chiral at the C_2 position. The chirality implies the B-ring is twisted relative to the A-C rings and is not planar like the conjugated flavones. Flavanols are the building blocks of proanthocyanins found to exhibit wide range of health benefits like antioxidant potential and reduced risk of vascular disease. Isoflavonoids are huge and very distinctive subgroup of flavonoids having broad biological potential. Anthocyanins are pigments responsible for colour in plants, flowers and fruits and help to attract pollinators and seed dispersers. These different classes of flavonoid based pharmaceutical agents played a vital role in the treatment of various infectious and degenerative diseases [46-47].

1A.4.3.3. Phenylpropanoids

Phenylpropanoids are structurally diverse group of phenylalanine-derived metabolites found in plants. The term "phenylpropanoid" is sometimes referred as compound bearing a 3-carbon chain attached to 6-carbon aromatic ring (C_6-C_3 skeleton). Cinnamic or *p*-coumaric acids are the biosynthetic precursor of phenylpropanoids and this class of compound comprises of cinnamic acid derivatives, lignin, lignans, phenylpropenes and coumarins [48].

Lignans and neolignans are wider group of secondary metabolites derived from the shikimic acid biosynthetic pathway through the oxidative coupling of phenylpropanoids that originates from the metabolism of L-phenylalanine. Starting monomers in the biosynthesis of lignans and neolignans include the *p*-coumaric acid its derivative aldehydes and alcohols [49].

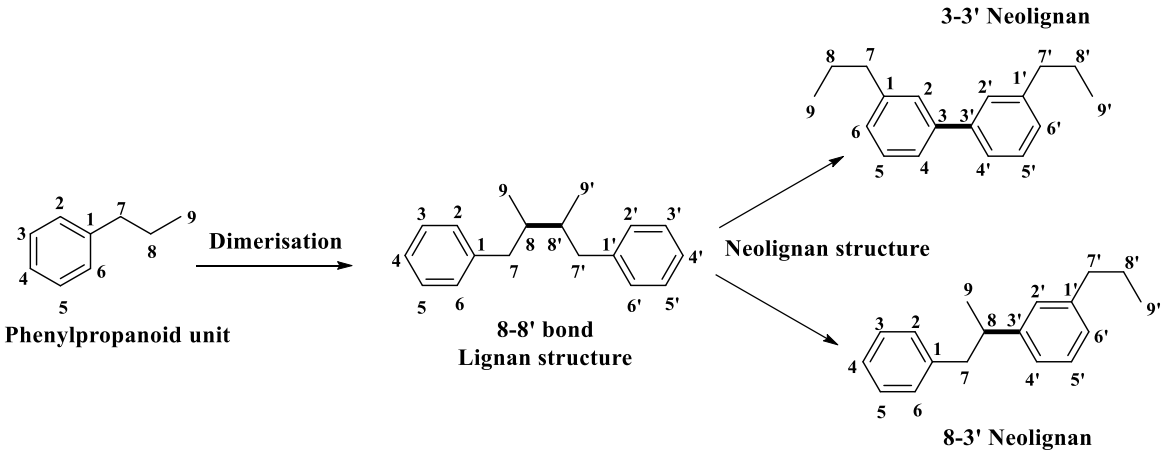

Figure 1A.10: General scheme for lignan and neolignan synthesis

Lignans and neolignans are dimeric structures formed by a *β*, *β*'- linkage between two phenylpropanoid units with a different degree of oxidation in the side-chain and a different substitution pattern in the aromatic moieties (Fig. **1A.10**). Compounds that contains two phenylpropanoid monomers linked by a bond between carbons **C_8** and **$C_{8'}$**, are referred as "lignans". If such bond is missing and is replaced by any other type of connection including the oxygen etheric linkage, the compounds are referred to as "neolignans". Lignans are classified according their structural arrangements including carbon skeletons, cyclisation and attachment of oxygen in the skeleton into eight groups such as arylnaphthalene, aryltetralin, dibenzocyclooctadiene, dibenzylbutane, dibenzylbutyrolactol, dibenzylbutyrolactone, furan and furfuran. Owing to their enormous biological activities including antioxidant, antiviral, anticancer, insecticidal, estrogenic,

antiviral, anti-hypersensitive, anti-inflammatory etc. they were widely employed in medicine and also provided structural templates for drug development. Podophyllotoxin (non-alkaloid toxin), a major constituent of the roots and rhizomes of *Podophyllum peltatum* is a well-known example of lignan and it gathered adequate attention in terms of its antiviral & anticancer potential. Podophyllotoxin with D-glucose derivatives such as etoposide (Etopophos®) and teniposide (Teniposide®) were the FDA approved drugs for clinical use against various type of cancer. Arctigenin from *Arctium lappa* found to possess antioxidant, anti-inflammatory, anti-proliferative, and antiviral activity and its glucose conjugate arctiin also have similar properties (Fig. **1A.11**) [50-51].

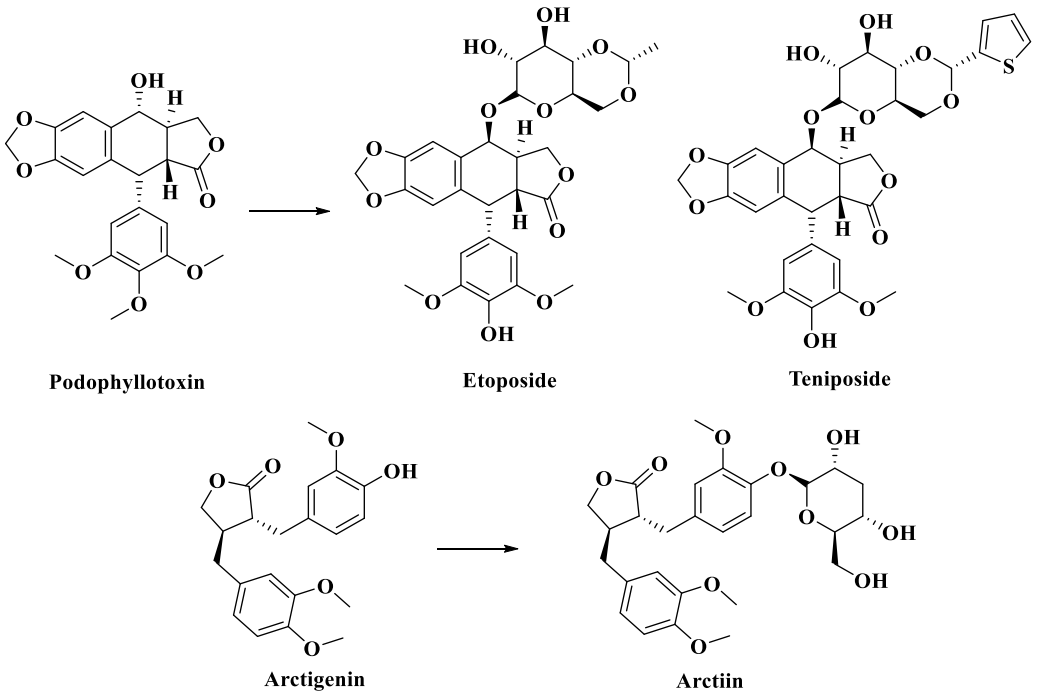

Figure 1A.11: Lignan compounds and its glucose derivatives

1A.4.3.4. Terpenoids

Terpenoids are prominent class of secondary metabolites in plants comprises of more than 40,000 different structures. The five carbon isoprene units are the building blocks of terpenoids and based on the number of isoprene units present in the structure they are classified into hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}) and polyterpenes ($C_{>40}$). Terpenoids are derived from biosynthetically through mevalonic acid pathway and 2C-methyl-D-erythritol-4-phosphate pathway. Terpenes are defined as the compounds with simple hydrocarbon skeleton, whereas terpenoids constitutes the modified group of terpenes in

which the carbon skeleton is reformed by substitution of functional groups, oxidation or rearrangement. Terpenoids gained significant pharmaceutical value since the ancient time itself due to their broad spectrum of biological activities. Terpenoids possessed enormous therapeutic potential include antioxidant, antimicrobial, anti-hyperglycemic, anti-inflammatory, antioxidant, antiparasitic, immunomodulatory etc. Among terpenoids, monoterpenes were specifically well studied for their antiviral property. Terpenoids are widely explored in clinics to cure variety of diseases including cancer, inflammation and infectious diseases. The wide array of structures and functionalities of natural terpenoids provide an excellent pool of molecules for the development of new drugs against various ailments [52-55].

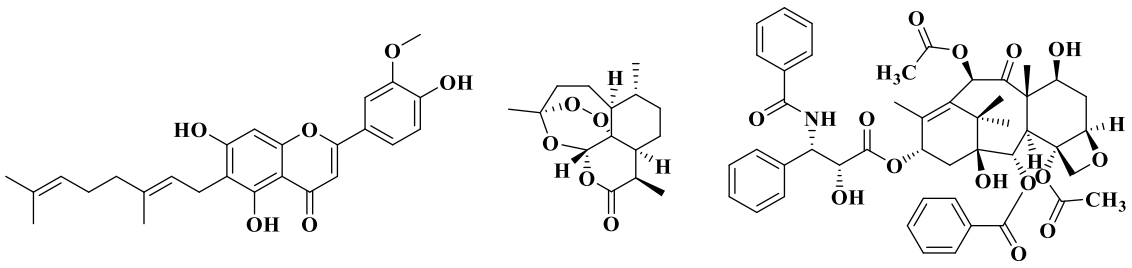

Figure 1A.12: Medicinally important terpenoids

1A.5. Recent Advances in Natural Products Derived Drug Development

The legacy of natural products for drug discovery continues even today. Innovative techniques are employed for the drug discovery approach from natural scaffolds to afford therapeutically active agents. The improved efficacy and pharmacokinetic potential, fewer side effects associated with natural scaffolds always make them a promising candidate for drug development. Synthetically derived drugs cannot achieve these characteristics all the time. Advancement of drug resistance mechanisms, toxicity and long term side effects of the existing drugs urge the development of new alternatives. Synergistic combination of the extra pure natural products or extracts along with the existing drugs attracted the great deal of attention to compete the devastating diseases and is found to be very effective to achieve the desired therapeutic potential. Semisynthetic modification of active natural products by incorporating several structural templates make them more drug like scaffold with enormous structural diversity. The advents of genomics, biomarker identification, target identification etc. are the new frontier in natural product derived drug development.

Application of nanotechnology by creating natural products nanoparticle hybrids for targeted drug delivery also achieved in recent times [56-58].

Recent history of FDA approved drugs (Fig. **1A.13**) from pure natural products or its derivatives highlights the importance of natural products in drug discovery and in pharmaceutics. Every year a prominent number of novel drugs are derived from natural lead scaffolds. Besides the plants other sources especially marine world, animals, microorganisms are also mined for new therapeutic agents.

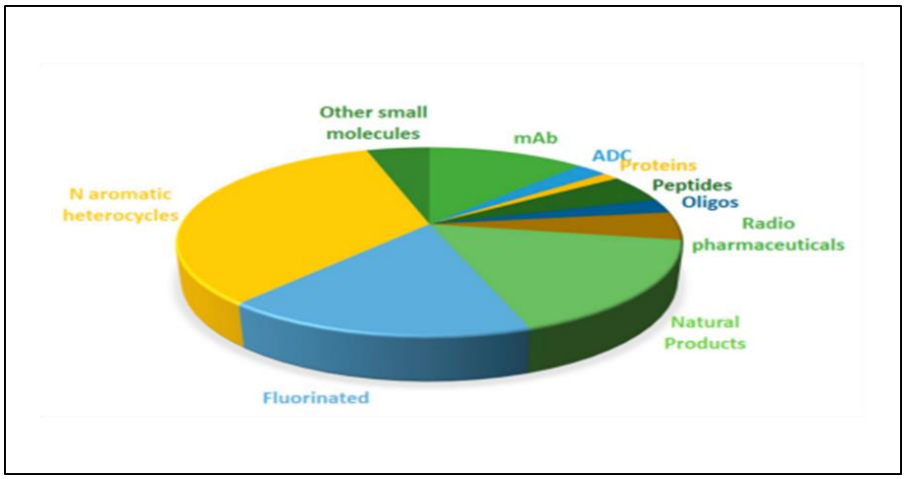

Figure 1A.13: Classification of FDA approved drugs in 2020 based on their Structure

In 2019 seven natural product inspired drugs were approved by FDA for infectious diseases, cancer, Parkinson's diseases, postpartum depression etc. Lefamulin (Xenleta^{TM}) is an antibiotic to treat community acquired bacterial pneumonia belongs to the family of pleuromutilin, which is derived from the fungus *Clitopilus passeckerianus.* Natural products provide a master class in medicinal chemistry in 2020. In this regard, two steroids, and one triglyceride, sugar, nucleoside, nucleotide, and two complex molecules with several chiral centers and cyclic structures have been approved by the FDA. Artesunate (Artesunate^{TM}) and lurbinectedin (Zepzelca^{TM}) are both semi-synthetic derivatives of two natural products approved for severe malaria and metastatic small cell lung cancers respectively. Lurbinectedin is structurally similar to trabectedin (Yondelis^{TM}) used for the treatment of advanced soft-tissue sarcoma and ovarian cancer, which was isolated from the sea squirt *Ecteinascidia turbinate*. Natural products and its hybrids, analogues and inspired molecules from natural products as well as superior structure functionalization approaches can continue to provide novel drug leads for human health care [59-60].

1A.6. Conclusion and Present Work

From the previous discussions, it is evident that natural products remain a promising avenue for the discovery of scaffolds with high structural diversity and various bioactivities which plays a pivotal role in novel drug development. The advent of new diseases caused challenges in pharmaceutics which in turn demanded the identification of new bioactive agents to tackle it. Synthetic approaches for the novel drug development are tedious and expensive which in turn prioritizes the utilization of natural products as an indispensable component in the development of new drug. Traditional knowledge based drug discovery from natural sources reemerged as a new strategy in therapeutics. Drug efficacy, potency, selectivity and pharmacokinetic parameters can be improved by making necessary structural changes in the known compound. Derivatives of natural products or natural product inspired drug development acquired great interest rather than the mere use pure natural products. Exploration of such lead chemical structures that can be used as a template for new drugs can overcome the existing drug resistant mechanisms and can provide enhanced therapeutic care. Substantially a lower percentage of natural resources were exploited for their hidden potential.

In this regard, we were interested to explore the rare and endemic floristic diversity of Western Ghats for the identification of promising candidate for drug development against infectious and lifestyle diseases. Our studies focused on the renowned myristicaceae family and the unexplored wild nutmeg species of the *myristica* genus along with the traditionally important species *Hyptis capitata*. In addition our efforts are directed towards the scientific validation of a well-known Ayurvedic medicine *Nayopayam Kwatha* based on the marker phytoconstituents present in the individual herbs of the polyherbal formulation.

We have selected the rare, endemic wild nutmeg species of the Agastyamala Biosphere Reserve located in the Southern Western Ghats. In chapter **2**, we describe bioprospecting of the two unexplored wild nutmeg species. Part A deals with the detailed phytochemical analysis of the aerial parts of the endangered Wild nutmeg species *Myristica beddomei subsp. spherocarpa* W.J. de Wilde and explains chemotaxonomic significance of novel flavonoids and its antiproliferative potential against different cancer cell line. The species is one among the three sub species of *Myristica beddomei* and is only reported from Agastyamala Biosphere Reserve. No studies on its ecology, phytochemistry and pharmacology were done so far but its restricted distribution is reported. Tribal communities were utilized this species as a forest produce. Part B

describes the phytochemical investigation of the newly identified wild nutmeg species from Agastiamala Biosphere Reserve. *Myristica trobogarii* is a new species nutmeg reported after 150 years (2020) from this region. It shares several morphological similarities with other wild nutmeg species. Since it is newly identified we were eager to study the phytoconstituents and biological activity of this particular species. Biological potential of this species is largely unknown so initially we have attempted the extract level screening against antibacterial and anticancer potential.

Chapter **3** describes the *in vitro* antidiabetic potential of bioactives from the two nutmeg species. Part A deals with the phytochemical investigation of the aerial parts of *Myristica malabarica* in detail along with the inhibitory potential of isolated malabaricones against digestive enzymes along with antiglycation properties. *M. malabarica* was found to possess various biological activities but its antidiabetic potential was not explored. In part B we describe the antidiabetic potential of lignans isolated from the mace of the common nutmeg *Myristica fragrans*. We also discuss the phytochemical aspects of the species in order to compare it with other wild nutmeg species.

Antibiotic resistance has currently emerged as an unmet looming threat, as a consequence of the exhaustive use of antibiotics, their unprescribed availability and over consumption. Medicinal plants have played a significant role in the treatment of infectious diseases and have long back history. Owing to their enormous potential, fewer side effects and cost-effectiveness of herbal drugs have gathered much attention among researches. In this context exploration of new antibacterial agents from natural resources having the ability to compete the multi-drug resistance is of utmost importance. Herein Chapter **4** describes the antibacterial efficacy of malabaricones against multi drug resistant *Staphylococcus aureus* infections. *In vitro* and *in vivo* studies of malabaricone B including cytotoxicity assessments; time-kill studies, biofilm inhibition and post-antibiotic effect determine its antibacterial potency.

Part A of chapter **5** discusses the phytochemical reinvestigation of the traditionally important species *Hyptis capitata.* It is also known as false ironwort, belongs to lamiaceae family credited with variety of medicinal uses especially the wound healing property and its leaves are extensively employed in traditional medicine to cure skin infections. We describe the antibacterial susceptibility of the isolated compounds against ESKAPE pathogen panel. Part B deals with the scientific validation and standardization protocol development of an Ayurvedic formulation *Nayopayam Kwatha*. Standardization of herbal

formulations is inevitable in order to ensure its purity, quality, safety and efficacy. One of the major challenges associated with herbal based formulation is its acceptability in modern medicine due to the lack of standardization. In view of this we mainly focused on the scientific validation of the formulation based on the marker constituent present in the individual herbs. We describe in detail the phytochemical analysis of the individual herbs of this formulation along with its quantification in three different drug ratios based on the isolated marker constituents with the aid of HPLC. The study highlights the importance of developing standardization protocol of the Ayurvedic formulations for quality control.

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Myristicaceae Species of Western Ghats: An Overview

1B.1. Introduction

Western Ghats region of Kerala is endowed with rich floristic diversity of rare and endemic species and are distributed among renowned plant families. Myristicaceae is one among the huge flowering plant family ubiquitous in southern Western Ghats. Myristicaceae acquired immense attention in terms of species richness and the medicinal importance of its species. Myristicaceae species were known under the sole genus *Myristica*. *Myristica* with five species and two subspecies are endemic to India include *Myristica fragrans* and its wild relatives; *Myristica malabarica, Myristica magnifica, Myristica beddomei* and *Myristica andamanica*. Amongst, Southern Western Ghats constitutes *M. fragrans, M. malabarica, M. magnifica, M. beddomei* and the newly identified species *M. trobogarii* [1-2].

The 'nutmeg' family nested within Magnoliales order taxonomically placed in between the Annonaceae and Lauraceae [3]. It is a primitive angiosperm family native to Africa, Asia, Pacific islands and the America comprised of approximately 500 species and 21 genera [4]. Myristicaceae is widely distributed in the subtropical and tropical regions of the world, especially in Asia. This is a very homogenous group distinctly separated from other families and is characterized by species that are highly endemic to the Western Ghats area of India that have significant ecological and ethnobotanical importance. Myristicaceae often place within the top 10 most important families particularly in terms of their species abundance and diversity [5]. Myristicaceae has a broad pantropical distribution and the species are found in different types of forests (swamp, humid, submontane etc.) normally occurs the heights up to 2100 m. Myristicaceae is florally uniform family, the flowers show few evolutionary trends; especially the pistillate flowers and the species share several common features worldwide. Generally they are shade-tolerant, evergreen, aromatic trees with pink or red resinous juice in the bark and the fruits are dehiscent, have brightly coloured arils.

Myristica, *virola*, *Knema, Horsfieldia, Gymnacranthera* are the important genus of this family. *Myristica* is species-rich in New Guinea and diminishes to the west. *Virola* is the major component of the Amazon forests and *Knema* is found in Southeast Asia and Malaysia [6-7]. Among the 21 genera (Table **1B.1**) India represents *Myristica*,

Horsfieldia, *Endocomia*, *Gymnacranthera* and *Knema*. Altogether the 15 species were scattered in the evergreen forests of India (Western Ghats, Andaman and Nicobar Islands and Meghalaya). Three major genera found within the Southern Western Ghats are Gymnacranthera (1 species), Myristica (4 species) and Knema (1 species).

Table 1B.1: Different genus of myristicaceae family

Bicuiba	*Gymnacranthera*	*Osteophloeum*
Brochoneura	*Haematodendron*	*Otoba*
Cephalosphaera	*Horsfieldia*	*Paramyristica*
Coelocaryon	*Iryanthera*	*Pycnanthus*
Compsoneura	*Knema*	*Scyphocephalium*
Doyleanthus	*Mauloutchia*	*Staudtia*
Endocomia	*Myristica*	*Virola*

1B.2. Myristica Swamps

Myristica swamps, the highly threatened and shattered fresh water ecological unit endemic to Western Ghats. It spread across the Ghats but usually located in foothills, valleys and along the rivulet and have high watershed value. In 1960, Krishnamoorthy reported Myristica swamps for the first time as a distinct habitation of plants and animals from Travancore. Myristica swamps were located in the forests and hill regions of the southern Western Ghats. The unusual biodiversity region possesses unique species composition and behaves like islands even though surrounded by forest vegetation [8].

Figure 1B.1: Myristica swamps

Myristica swamps, as their name indicates are dominated by the species of ancient family Myristicaceae along with many rare, endemic and economically important threatened plant species. *Gymnacranthera canarica* and *Myristica magnifica* are exclusively found in myristica swamps which thrive in the waterlogged condition. *M. malabarica* is generally distributed in the evergreen forests but also an important species of swamps [9].

Another distinctive feature of plants of myristica swamps include the presence of specialized above ground roots (breathing roots, Fig. **1B.2**). Plants in swamps consist of such roots for the survival. The species diversity of swampy vegetation includes pandanus and calamus along with the members of widespread family, zingiberaceae, urticaceae, aroideae and acanthaceae.

Figure 1B.2: Breathing roots of plants in myristica swamps

1B.3. Myristica Genus

Myristica Gronov is the largest genus comprised of more than 70 plants, dispersed in India and Sri Lanka along with several worldwide regions [11]. Amongst 40 known species of *Myristica* 34 endemics are located in Indonesia which is considered as the native of this genus. Only a few species are found in India and their habitat varies from the sea level to altitude in the Western Ghats and Himalayas. Important species of the genus are summarized in Table **1B.2**.

Table 1B.2: Myristicaceae species and their distribution

SL. NO	Myristica Species	Distribution
1	*M. alba*	Maluku Islands of Indonesia
2	*M. ampliata*	Queensland &Australia
3	*M. andamanica*	India
4	*M. arfakensis*	West Papua (Indonesia)
5	*M. argentea*	New Guinea
6	*M. atrescens*	Papua New Guinea
7	*M. basilanica*	Philippines
8	***M.beddomei***	India
9	*M. brachypoda*	Papua New Guinea
10	*M. brevistipes*	Papua New Guinea
11	*M. buchneriana*	New Guinea
12	*M. byssacea*	Papua New Guinea
13	*M. ceylanica*	Sri Lanka.
14	*M. cinnamomea*	Sumatra, Peninsular Malaysia, Singapore & Borneo
15	*M. coacta*	Papua New Guinea
16	*M. colinridsdalei*	Philippines
17	*M. conspersa*	West Papua (Indonesia)
18	*M. corticata*	Borneo
19	*M. crassa*	Peninsular Malaysia, Singapore & Borneo
20	***M. dactyloides***	Sri Lanka, India
21	*M. dasycarpa*	Papua New Guinea
22	*M. depressa*	Sumatra, Peninsular Malaysia &Borneo
23	*M.devogelii*	Sulawesi in Indonesia
24	*M. elliptica*	Sumatra, Peninsular Malaysia, Singapore & Borneo
25	*M. extensa*	Borneo
26	*M. fasciculate*	Papua New Guinea
27	***M. fatua***	India
28	*M. filipes*	Papua New Guinea
29	*M. fissurata*	Maluku, Indonesia

30	*M. flavovirens*	West Papua (Indonesia)
31	***M. fragrans***	China, Taiwan, Indonesia, Malaysia, Caribbean, India, Sri Lanka & South America
32	*M. frugifera*	Philippines
33	*M. gigantea*	Sumatra, Peninsular Malaysia & Borneo
34	*M. gillespieana*	Fiji
35	*M. globosa*	Papua New Guinea, Solomon Islands & Australia
36	*M. grandifolia*	Fiji
37	*M. guadalcanalensis*	Solomon Islands
38	*M. guatteriifolia*	Indonesia, Malaysia, Myanmar, Philippines & Vietnam
39	*M. guillauminiana*	Fiji & Solomon Islands
40	*M. hollrungii*	Papua New Guinea
41	*M. inaequalis*	West Papua (Indonesia)
42	*M. incredibilis*	Papua New Guinea
43	*M. iners*	Singapore & Borneo
44	*M. inundata*	Papua New Guinea
45	*M. irya*	Burma, India, Malaysia, Papua New Guinea, Singapore, Solomon Islands, Sri Lanka, Thailand & Vietnam
46	*M. kalkmanii*	New Guinea
47	*M. kjellbergii*	Sulawesi in Indonesia
48	*M. lasiocarpa*	Papua New Guinea
49	*M. leptophylla*	Papua New Guinea
50	*M. longipetiolata*	Philippines
51	*M. lowiana*	Sumatra, Singapore, Peninsular Malaysia & Borneo
52	*M. macrantha*	Fiji
53	*M. magnifica*	India
54	*M. maingayi*	Sumatra, Peninsular Malaysia & Singapore
55	***M.malabarica***	India
56	*M. maxima*	Peninsular Malaysia, Singapore & Borneo
57	*M. mediterranea*	New Guinea
58	*M. millepunctata*	West Papua (Indonesia)

59	*M. nana*	Papua New Guinea
60	*M. olivacea*	Papua New Guinea
61	*M. ornata*	Papua New Guinea
62	*M. ovicarpa*	Papua New Guinea
63	*M. pachycarpidia*	Papua New Guinea
64	*M. papillatifolia*	New Guinea
65	*M. petiolata*	Solomon Islands
66	*M. perlaevis*	Maluku in Indonesia
67	*M. philippensis*	Philippines
68	*M. pilosella*	Papua New Guinea
69	*M. pilosigemma*	Philippines
70	*M. polyantha*	Papua New Guinea
71	*M. psilocarpa*	Manus Island & Papua New Guinea
72	*M. pubicarpa*	Maluku Islands in Indonesia
73	*M. pygmaea*	Papua New Guinea
74	*M. robusta*	Maluku Islands in Indonesia
75	*M. sangowoensis*	Maluku in Indonesia
76	*M. sarcantha*	West Papua (Indonesia)
77	*M. schlechteri*	Papua New Guinea
78	*M. simulans*	Papua New Guinea
79	*M. sinclairii*	Papua New Guinea
80	*M. sogeriensis*	Papua New Guinea
81	*M. succadanea*	Maluku Islands in Indonesia
82	*M. tamrauensis*	West Papua (Indonesia)
83	*M. teijsmannii*	Java in Indonesia
84	*M. trianthera*	Java in Indonesia
85	*M. ultrabasica*	Sulawesi in Indonesia
86	*M. verruculosa*	West Papua (Indonesia)
87	*M. xylocarpa*	Solomon Islands
88	*M. yunnanensis*	Southern Yunnan, China, northern Thailand & in ThanhHoa Province, Vietnam

1B.4. *Myristica fragrans*

Myristica fragrans, commonly recognized as 'nutmeg' ('Jathikka' in Malaylam) is the most famous member of the myristicaceae family native to Banda Islands in the Moluccas (earlier known as spice island) of Eastern Indonesia and widely cultivated in tropical regions, especially in Indonesia, Malaya, Sri Lanka and India. In India it is found in Kerala, Tamil Nadu, Assam and Meghalaya [12]. It thrives at 700-4500 m elevations having high rainfall and an optimum temperature [13]. The species is well known for its spices, nutmeg and mace which are extensively used in various food cuisines as well as in traditional system of medicine to cure various ailments.

Figure 1B.3: *M. fragrans* fruit

1B.4.1. Morphology

Myristica fragrans is an evergreen, aromatic, dioecious tree grows up to 18-20 m high has a soft, smooth brown-red bark flakes off in thin layers or large plates. It is a lofty tree with slender branches. The leaves are elliptic-lanceolate, acuminate. Flowers are unisexual, inconspicuous, yellow, waxy, fleshy and bell-shaped and small. The fruit is comprised of a single seed, typically with a ruminate endosperm, covered to various degrees by a fatty white-reddish aril and is similar in size of an apricot, when ripens it consist of hard seeds (nutmeg). The seed is covered by crimson-red colored thread like arils (mace).

1B.4.2. Medicinal Uses

Myristica fragrans gained huge interest due to its extensive medicinal applications and is widely employed in traditional medicine in the Middle East and Asia. In Western medicine nutmeg employed as medication for stomach problems and act as stimulant and carminative. It is also used to cure intestinal catarrh and colic, headaches, diarrhea, vomiting, nausea, fever, asthma, heart disease, liver and spleen disorder, insomnia, menorrhagia and bad breath [13]. Decoctions of the leaves are commonly used to relief

flatulence and intestinal spasm. The mace is used as a stomach tonic and for healing headache and migraine [14]. Mace of the species was found to be an excellent agent against rheumatism (Indonesian folk medicine) and employed as a spice to flavor the food. It also possesses anthelmintic, antiseptic etc. activities. In addition the essential oil of nutmeg found to have analgesic, anti-rheumatic and anti-inflammatory properties. Nutmeg also served as the major ingredient of several Ayurvedic preparations.

1B.4.3. Phytochemical Constituents

Terpenoids, phenolics, lignan compounds, protein, starch and volatile components are the major phytoconstituents of nutmeg [15-17]. A study of preliminary phytochemical screening of the of various extracts (DCM, methanol, chloroform etc.) of the dried seeds and leaves of *M. fragrans* revealed that DCM and aqueous extracts contains majority of the secondary metabolites include alkaloids, saponins, phenols, triterpenoids, anthraquinones, glycosides, flavonoids, coumarins, anthocyanins, chalcones, diterpenes, quinones, tannins and steroids [18-24].

Essential oil of the nutmeg was extensively studied to explore its active constituents and medicinal uses. Essential oil composition of nutmeg constitutes >80% monoterpenes, >5% monoterpene alcohols, >5% aromatics and other minor components. Sabinene, myristicin, safrole and elemicin constitutes 80% of both nutmeg and mace oil. The oil from nutmeg cultivated in Grenada found to have considerable amount of β-pinene, α-pinene and sabinene (40%-50%) and are low in safrole and myristicin whereas oil from nutmeg cultivated in Indonasia and other regions of South East Asia have higher amount of myristicin [25]. GC-MS analysis of nutmeg and mace oil revealed that α-pinene, β-pinene and sabinene were the major constituent of nutmeg (77.38%) and mace (60.76%). The concentration of myristicin and elimicin was very high in Indian nutmeg oils [26]. The characteristic flavor of nutmeg can be attributed to the presence of myristicin and elemicin. Mace and nutmeg oil was found to contain ~1.9% and ~0.3% safrole respectively, which has been suspected to be carcinogenic. Retention indices of the composition of the volatile oil extracted by hydro-distillation from nutmeg pericarp were also reported. The oil was found to contain monoterpenes (60%), monoterpene alcohols (29%) and several other constituents. The components are similar in both nutmeg and mace oils but differ substantially in their concentrations [27]. Another study of nutmeg essential oil by GC and GC-MS revealed that monoterpene hydrocarbons (89%) constituted the main fraction of nutmeg oil in which α-pinene (28%), β-pinene (27%) and sabinene (16%) were the main components [28]. Off-line solid phase

extraction (SPE-C18) and GC/MS analysis identified sabinene (21.38%), 4-terpineol (13.92%) and myristicin (13.57%) were the major constituents of essential oil [29]. Pal *et. al*. identified twenty eight compounds representing 95.9% of the oil from the fruits of the species [30]. Supercritical fractioned extraction of volatile and fixed oil of *M. fragrans* revealed the presence of high content of myristicin (32.8%), sabinene (16.1%), *α*-pinene (9.8%) etc. [31]. Helen *et. al*. reported the GC MS analysis of the essential oil of leaves of the nutmeg and identified the presence of *β*-pinene (22.69%), *α*-pinene (14.06%), *α*-thujen (13.93%) and p-menth 1-en-4-ol (10.53%) [32]. The essential oil isolated from the seeds of *M. fragrans* from Nigeria was found to contain sabinene (49.09%), *α*-pinene (13.19%), *α*-phellandrene (6.72%) and terpinen-4ol (6.43%) as major constituents by GC and GC-MS analysis [33]. The essential oil study of Banda nutmeg reported that myristicin constitutes the major component along with monoterpenes [34]. Physical characterization and essential oil properties of West Sumatra mace and nutmeg seeds were reported. The main constituents identified from the oil include *α*-pinene, sabinene, *β*-pinene, myristicin, eugenol etc. [35]. Isolation of myristicin from nutmeg oil by sequential distillation method was reported [36]. Recently Matulyte *et al.* carried out the GC-MS analysis of essential oil from *M. fragrans* seeds by using magnesium aluminometasilicate as excipient [37].

Lignans, neolignans, phenylacyl phenols, diarylpropanoids are the major secondary metabolites associated with *Myristica fragrans*. Diarylpropanoid type compounds have been isolated from the hexane extract of seed (nutmeg) and aril (mace) and reported the presence of two different structural types analogous to the *β*-O-4 ether and 2, 3-dihydrobenzofuran types of dilignols [38]. Hattori *et al*. reported the isolation of two cyclic dilignols, three acyclic dilignols and a new acylic dilignol derivative guaiacin from the methanol extract of the mace [39]. The same group in 1987 reported the isolation of tetrahydrofuran lignans, named fragransins A2, B1, B2, B3, C1 & C2 from the methanol extract of the aril of *M. fragrans* along with nectandrin B and verrucosin [40]. Woo *et al*. reported a new lignan, a macelignan from the aril of the species [41]. Hattori *et al*. in 1988 isolated some compounds for the first time from the aril of *Myristica fragrans* which include 3-(3,4,5-trimethoxyphenyl)-2-(E)-propen-1-ol, 3-(3-methoxy-4,5-methylenedioxyphenyl)-2-(E)-propen-1-ol, 2,3-dihydro-7-methoxy-2-(3,4-dimethoxyphenyl)-3methyl-5-(1-(E)-propenyl) benzofuran, fragransol-C, fragransol-D, 2,3-dimethyl-1,4-bis-(3,4-methylenedioxyphenyl) butan-1-ol, myristicanol-A and myristicanol-B [42]. Hada *et al*. reported eight neolignans and five lignans from the

phenolic fraction of the mace include, erythro-2-(4''-allyl-2'',6''-dimethoxyphenoxy)-l-(3',4',5'-trimethoxyphenyl) propan-1,3-diol, threo-2-(4''-allyl-2''-methoxyphenoxy)-1-(4'-hydroxy-3'-methoxyphenyl) propan-1-ol, threo-l-(4'-hydroxy-3'-methoxyphenyl)-2-(2''-methoxy-4''-(l'''(E)-propenyl) phenoxy) propan-l-ol and its erythro form, threo-1-(4'-hydroxy-3'-methoxyphenyl)-1-methoxy-2-(2''-methoxy-4''-(l'''-(E)propenyl) phenoxy)-propane and its erythro form, fragransol-A, fragransol-B, fragransin D1, fragransin D2 , fragransin D3, fragransin El and the known compound austrobailignan-7 [43]. Orabi *et al*. reported two phenyacylphenols, malabaricone B and C from the ethanol extract of the mace [44]. Cho *et al*. isolated three lignans from the methanol extract of *M. fragrans* seeds and identified as erythro-austrobailignan-6, meso-dihydroguaiaretic acid and nectandrin-B [45]. Lee *et al*. isolated several lignans included macelignan, machilin F, nectandrin B, safrole, licarin A, licarin B, myristargenol, and meso-dihydroguaiaretic acid from the ethanol extract of the seeds [46]. Duan *et al*. reported the isolation and identification of two new phenolic compounds (−)-1-(2,6-dihydroxyphenyl)-9-[4-hydroxy-3-(p-menth-1-en-8-oxy)phenyl]-1-nonanone and (7 R, 8R)-7, 8-dihydro-7-(3, 4-dihydroxyphenyl)-3′-methoxy-8-methyl-1′-(E-propenyl) benzofuran as well as 10 known analogues: (+)-erythro-(7S, 8R)-$\Delta^{8'}$-7-acetoxy-3, 4, 3′,5′-tetramethoxy-8-O-4′-neolignan, (7S, 8S, 7′R, 8′S)-4, 5′-dihydroxy-3, 3′-dimethoxy-7, 7′-epoxylignan, (+)-erythro-(7S, 8R)-$\Delta^{8'}$-4, 7-dihydroxy-3, 3′, 5′-trimethoxy-8-O-4′neolignan-8′-ene, (+)-erythro- (7S, 8R)$\Delta^{8'}$-7-dihydroxy-3 4, 5, 3′, 5′-pentamethoxy-8-O-4′-neolignan-8′-ene, (2R)-3-(3, 4, 5-trimethoxyphenyl)-1, 2-propanediol, (2R)-3-(5-methoxy-3,4-methylenedioxyphenyl)-1,2-propanediol, (2R)-3-(3,4-methylenedioxyphenyl)-1,2-propanediol, (1R,2R)-1-(4-hydroxy-3-methoxyphenyl)-1, 2-propanediol, 1-(2,6-dihydroxyphenyl)-9-(4-hydroxyphenyl)-1-nonanone and 1-(2,6-dihydroxy phenyl)-9-(3,4-dihydroxyphenyl)-1-nonanone [47]. Min *et al*. reported the isolation of (8R,8'S)-7'-(3',4'-methylenedioxyphenyl)-8, 8'-dimethyl-7-(3,4-dihydroxyphenyl)-butane, meso-mono methyl dihydroguaiaretic acid, (+)-guaiacin, (7S,8'R,7'R)-4, 4'-dihydroxy-3, 3'dimethoxy-7', 9-epoxylignan and 7-(4-hydroxy-3-methoxyphenyl)-7-(3,4-methylenedioxyphenyl)-8, 8-lignan-7-methyl ether from the methanol extract of the seeds [48]. Cuong *et al.* reported the isolation of new phenolic compounds such as ((7S)-8'-(benzo [3',4'] dioxol-1'-yl)-7-hydroxypropyl) benzene-2,4-diol, ((7S)-8'-(4'-hydroxy-3'-methoxyphenyl)-7-hydroxypropyl) benzene-2,4-diol & ((8R,8'S)-7-(4-hydroxy-3-methoxyphenyl)-8'-methylbutan-8-yl)-3'-methoxybenzene- 4', 5'-diol along with other known compounds from the seeds of *M. fragrans* [49]. Hou *et al.* isolated licarin-B, dehydrodiisoeugenol,

malabaricone B, malabaricone C, *β*-sitosterol, and daucosterol from the ethylacetate extract of the seeds of nutmeg [50]. Cao *et al.* reported the isolation of five new 8-O-4' type neolignans, named myrifralignan A–E, together with five known analogues from the seeds [51]. In 2014 Fransis *et al.* carried out the phytochemical analysis of fruit pericarp and from the acetone extract isolated a neolignan, erythrosurinamensin and a diaryl phenyl propanoid, virolane for the first time along with previously known steroids, other lignans and neolignans [52]. Thuong *et al*. reported four lignans, meso-dihydroguaiaretic acid, macelignan, fragransin A2 and nectandrin B from the seeds of *Myristica fragrans* (Vietnamese nutmeg) [53]. Cho *et al*. recently reported the isolation of five phenylpropanoids; a benzofuran neolignan, two 8-O-4'-neolignans, and five tetrahydrofuran lignans from the methanol extract of *Myristica fragrans* seeds [54]. Acuna *et al*. reorted a new acyclic bis phenylpropanoid and four previously known phenolic compounds including (S) 1-(3, 4, 5-trimethoxyphenyl)-2-(3-methoxy-5-(prop-1-yl) phenyl) propan-1-ol, α-[1-(2, 6-dimethoxy-4-(2-propen-1-yl) phenoxy) ethyl]-3, 4dimethoxy-1-acetate, odoratisol A, 4-[(2S, 3S)-2, 3-dihydro-7-methoxy-3-methyl-5(1E)-1-propenyl-2-benzofuranyl]-2, 6-dimethoxy,1, 3-benzodioxate-5-methanol,α-[1-[2, 4-(2-propenyl) phenoxy] ethyl]-acetate, licarin C, 2, 3-dihydro-7-methoxy-3-methyl-5-(1E)-1-yl-2-(3, 4, 5-trimethoxy phenyl) by the bioassay-guided fractionation of the aril of *M. fragrans* [55]. In 2016 Ginting *et al*. reported the isolation of a flavonoid compound dihydrocaempferol from the methanol extract of the leaves of the nutmeg [56]. Recently Fransis *et al*. carried out the phytochemical analysis of the stem bark of *M. fragrans* and isolated bis-aryl dimethyl tetrahydrofuran lignans, such as grandisin [(7S, 8S, 7′S, 8′S)-3, 3′, 4, 4′, 5, 5′-hexamethoxy-7, 7′, 8, 8′-lignan] and (7S, 8S, 7′R, 8′R)-3, 3′, 4, 4′, 5, 5′-hexamethoxy-7, 7′, 8, 8′-lignan along with important lignans and neolignans, licarin A, licarin B, odoratisol A, (2S, 3R)-7-methoxy-3-methyl-5-((E)-prop-1-enyl)-2-(5-methoxy-3, 4-methylenedioxyphenyl)-2, 3-dihydrobenzofuran, elemicin, fragransin B1, raphidecursinol B, erythro-(7S, 8R)-$\Delta^{8'}$-4, 7-dihydroxy-3, 5, 3′-trimethoxy-8-O-4′-neolignan, erythro-(7S, 8R)-$\Delta^{8'}$-7-hydroxy-3, 4, 3′,5′-tetramethoxy8-O-4′-neolignan, surinamensin and *β*-sitosterol [57]. The structures of the reported compounds from the species are given in Fig. **1B.4**.

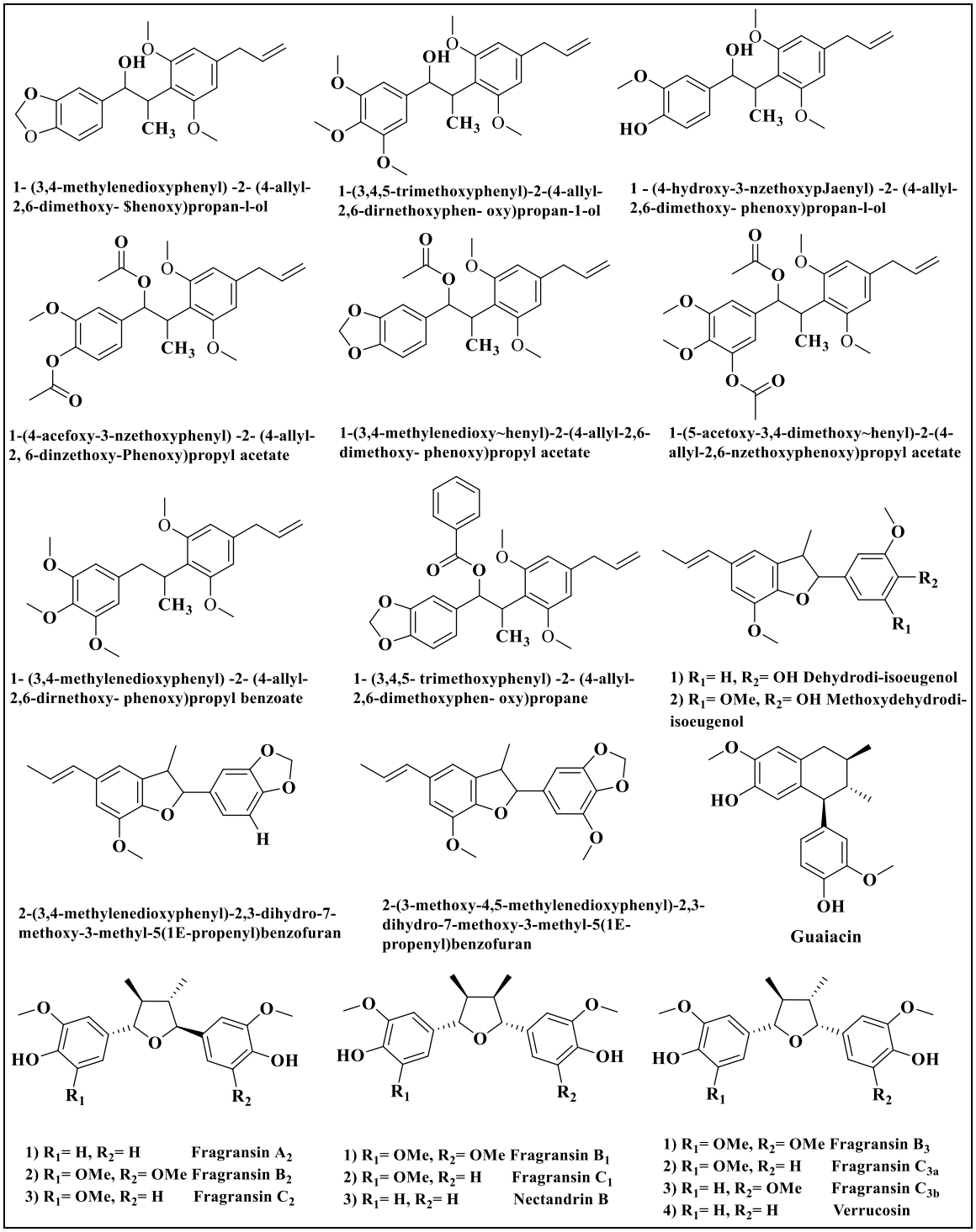

Figure 1B.4: Reported compounds from *M. fragrans*

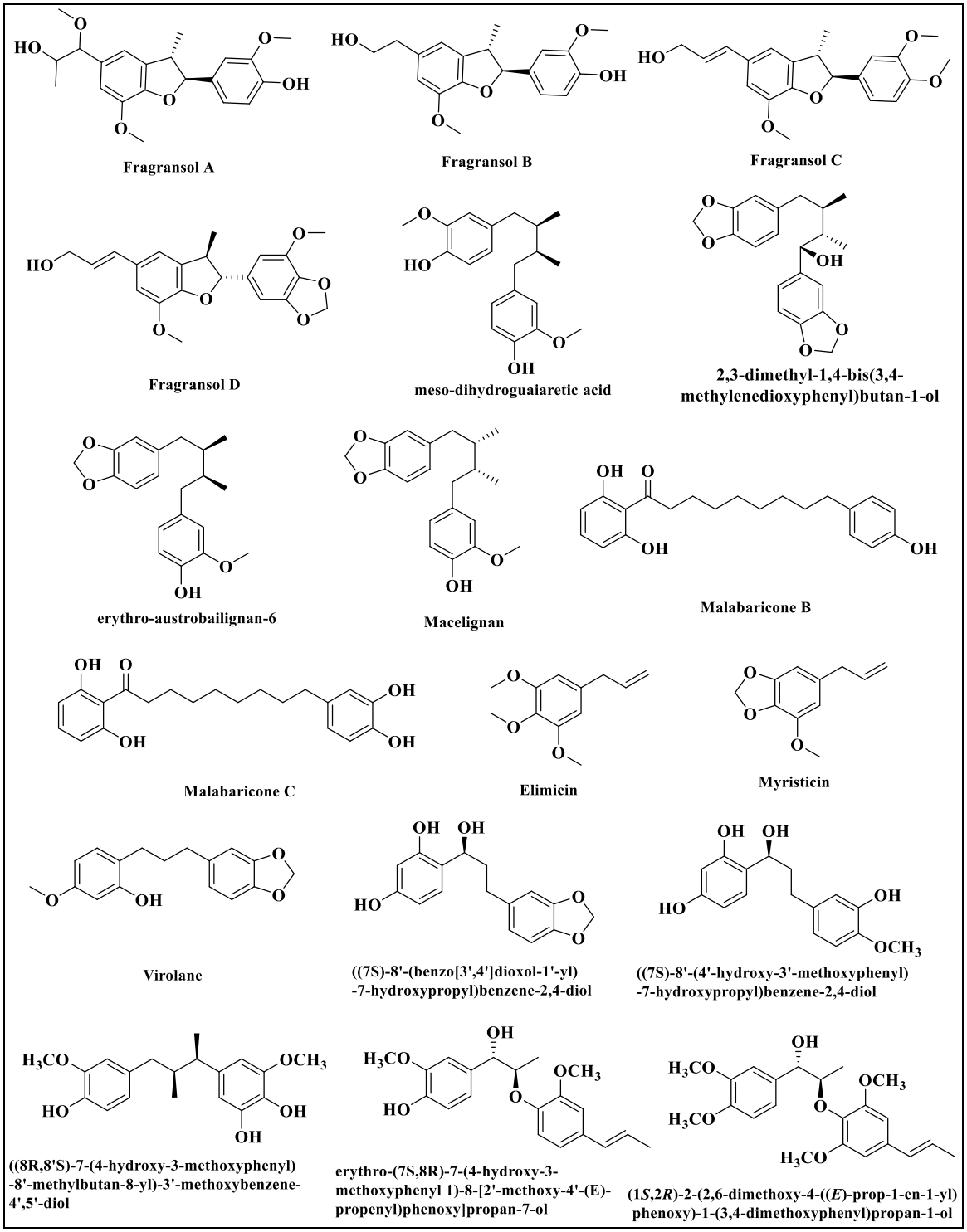

Figure 1B.4: Continued…

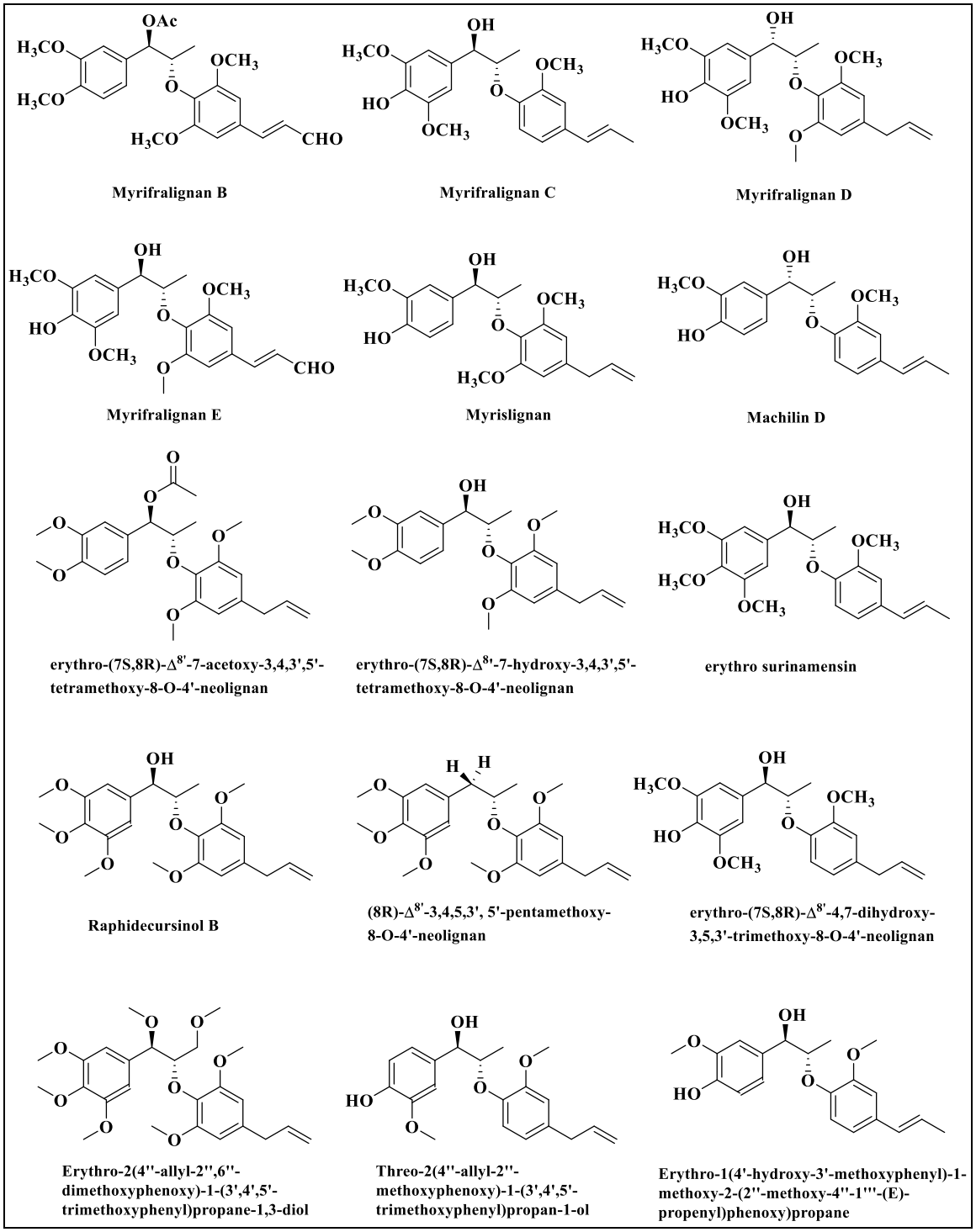

Figure 1B.4: Continued…

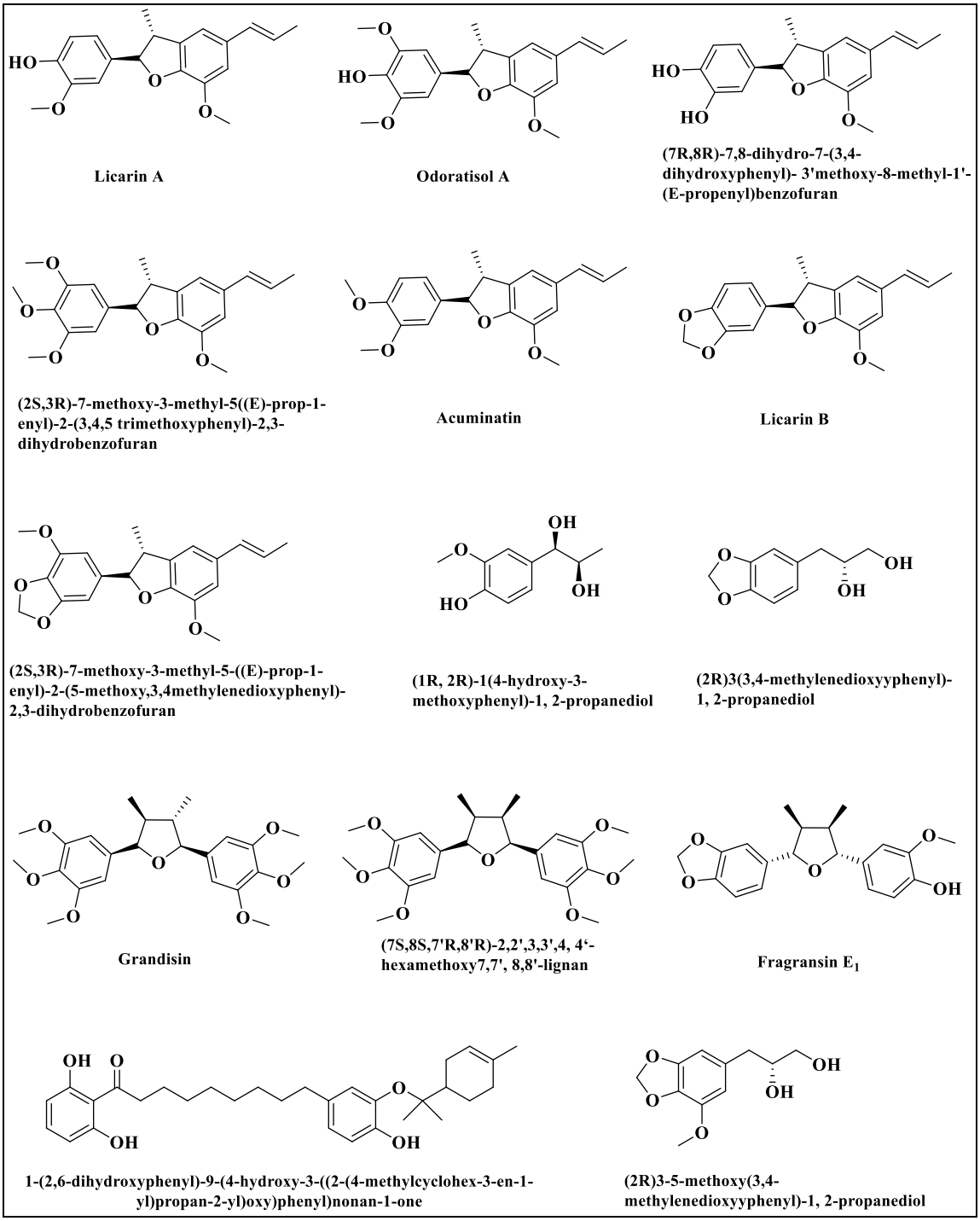

Figure 1B.4: Continued…

1B.4.4. Pharmacological Activities

Myristica fragrans is known to possess variety of pharmacological activities. Most of the pharmacological activities credited with nutmeg can be attributed to the components present in the species. Essential oil obtained from the nutmeg was also found to have different pharamacological activities including antioxidant, anticancer, antitermite, cytotoxicity and locomotor inhibitory activities [28-32]. The nematicidal activity of *Myristica fragrans* against *Meloidogyna incognita* was reported by Gotke *et al.* and revealed that mace oil was the most effective antimicrobial agent followed by nutmeg oil [58].

1B.4.4.1. Antimicrobial Activity

M. fragrans (nutmeg and mace) was known to inhibit the growth of several disease causing pathogens including gram positive and negative bacteria [59]. Dehydroxy- isoeugenol and 5′-methoxy dehydro-di-isoeugenol isolated from the mace reported to have antibacterial potential against *Streptococcus mutants* [39]. Malabaricone B and C obtained from the mace demonstrated strong antimicrobial potential against *S. aureus* and *C. albicans* while methylation and reduction of these resorcinols resulted in lower activity [44]. Alcoholic extracts of nutmeg showed antimicrobial properties [60]. Antifungal properties of essential oil, phenyl propanoids and 8-O-4′ neolignans were reported [61-63]. Takikawa *et al*. reported the antimicrobial activity of nutmeg seed extract against *Escherichia coli O157* [64]. Aqueous extract of nutmeg reported to have bactericidal activity against *Helicobacter pylori* [65]. Methanol extract of *M. fragrans* was found to be effective against multi-drug resistant *Salmonella typhi* [66]. Antimicrobial activity of nutmeg was reported in *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 10536) and *Saccharomyces cerevisiae* (ATCC 9763) [67]. Goncalves *et al*. reported the *in vitro* anti-rotavirus activity of nutmeg seeds [68]. Macelignan isolated from *M. fragrans* exhibited biofilm inhibition potential against *Streptococcus sanguis* and *Actinomyces viscosus* [69]. Lignans isolated from the seeds including erythro-austrobailignan-6, meso-dihydroguaiaretic acid and nectandrin-B showed *in vitro* and *in vivo* potential against several bacterial strains [45]. Recently Simamora *et al*. reported the antibacterial potential of methanol and ethylacetate extract of Indonesian nutmeg against *S. aureus* ATCC 25922 [70]. Iyer *et al.* reported the *in vitro* antibacterial activity of the seeds of nutmeg against *Candida albicans* [71].

1B.4.4.2. Antidiabetic Activity

Macelignan isolated from *M. fragrans* reported to have the ability to enhance the insulin sensitivity, PPAR activity and suggested that it can be a potential candidate for the treatment of type 2 diabetes [72]. Lestari *et al*. reported the *in vitro* and *in vivo* antidiabetic potential of methanol extract of the seeds of the nutmeg. *In vitro* PPAR activity of nutmeg seeds was measured using a GAL4/PPAR chimera [73]. Shyni *et al*. studied the cellular effect of Licarin B on PPARγ and insulin signaling pathways in 3T3-L1 preadipocytes and the molecular mechanism of the compound was explained by *in vitro* and *in silico* methods [74]. Recently, antidiabetic activity of methanol and ethylacetate extract of the mace of *M. fragrans* was investigated using an *in vitro* α-glucosidase inhibitory assay. The results revealed that both the extracts exhibited good inhibition against α-glucosidase having IC_{50} values much lower than the standard acarbose [70].

1B.4.4.3. Antioxidant Activity

Myristica fragrans is best known for its antioxidant potential because of the presence of phenolic compounds. Antioxidant activity of phenylpropanoids isolated from the nutmeg was reported [75]. Mace of the species and its constituents were reported to possess excellent antioxidant potential [76-77]. Antioxidant activity of monoterpenoid such as terpinene-4-ol, alpha-terpineol and 4-allyl-2, 6-dimethoxyphenol from nutmeg seed extract was reported [78]. Hou *et al*. reported the antioxidant activity of Malabaricone C isolated from the mace [79]. Kwon *et al*. identified new LDL antioxidant agents from the methanol extract of the seeds and identified macelignan as potent LDL antioxidant [80]. Assa *et al*. reported the antioxidant activity of methanol extract of the flesh, seed and mace of nutmeg and revealed that methanol extract of seed has the best ability to scavenge free radicals showed by DPPH and FRAP tests, while the flesh extract has the best ability in ferrous ion chelating activity [81]. Recently Sethi and Dahiya reported the antioxidant potential of *Myristica fragrans* in albino rabbits [82].

1B.4.4.4. Cytotoxicity and Anticancer Activity

Myristicin from the nutmeg possessed cytotoxic activity against human nueroblastoma cells [83]. Chirathaworn *et al.* observed that the methanolic extract of *M. fragrans* induces apoptosis of Jurkat leukemia T cell line through SIRT1 mRNA down regulation [84]. Prakash and Gupta *et al.* reported the *in vitro* efficacy of ethanol extract of *M. fragrans* seeds against different human cancer cell lines (Colon502713, Colo205,

Hep-2, A-549, OVCAR-5 & PC-5) [85]. Extracts of nutmeg was also found to suppress the growth of human lymphoid leukemic cells Molt 4 B [86].

1B.4.4.5. Anti-inflammatory Activity

Anti-inflammatory activities of myristicin and trimyristin were well studied [87-90]. Essential oil from the nutmeg also possessed excellent anti-inflammatory activity [91] by efficient inhibition of prostaglandin synthesis in rat kidney [92]. Recently Sethi and Dhahiya reported the anti-inflammatory activity of *M. fragrans* in albino rabbits. Significant anti-inflammatory and anti-lipid peroxidative effect was observed in animals who received supplementation of *Myristica fragrans* along with high fat diet induced oxidative stress [82].

1B.4.4.6. Hypolipidaemic & Hypocholesterolemic Effect

Ram *et al*. reported the hypolipidaemic effect of ethanol extract of *M. fragrans* on experimentally induced hyperlipidaemia in albino rabbits [93]. Sharma *et al.* reported that activity of seed extracts [94]. Later Capasso *et al.* reported the anti-ulcerogenic activity of eugenol from the nutmeg seeds [95].

In addition *M. fragrans* also reported to have hepatoprotective, memory enhancing & antidepressant activities [96-99].

1B.5. *Myristica malabarica*

Myristica malabarica Lam. generally known as "Malabar Wild Nutmeg, Rampatri or Bombay mace" (In Malayalam; Kattujathikka, Ponnampoovu, Kottappannu) is a red-listed, endemic species [100-102] found in evergreen forests of Western Ghats of South India [103]. It is one of the characteristic species in 'Myristica swamps', having swamp and lowland forest habitat. The tree is perennial with greyish black color trunks. The aril is commonly called as '**ponnampu**' (golden flowers) has its own economic value as a raw drug, and is used as febrifuge, cooling, expectorant and as adulterant for the *Myristica fragrans* (commercial Nutmeg). Mace of the species is widely used in Ayurveda for the treatment of fever, bronchitis, cough and burning sensation and seed fat is for rheumatism, myalgia, sprains and sores. The plant is considered as one among the highly traded top twenty medicinal plants in India and also has medicinal value [104-105]. It is the major ingredient of several Ayurvedic (Indian medicine) preparations like '*Baladir taila*' and '*Muthu Marunthu*' (used for the treatment of indolent ulcers and rheumatism) [106] and '*pasupasi*'. In traditional medicine it is used for indigestion, ulcers, wounds, as rejuvenator in treating inflammation, cough, diarrhea, dropsy, liver

disorders, paralysis and urinary calculi [107] also used in bronchitis, fever, burning sensation, to relieve pain in muscles, sprains and sores [108].

1B.5.1. Morphology

Myristica malabarica Lam. is a perennial, dioecious tree about 25 m tall with stilt roots. Bark is greenish-black, smooth; blaze reddish. Leaves are simple, alternate, oblong and distichous. Flowers are yellow and unisexual. Fruits are egg shaped with obtuse seed and having yellow coloured aril (mace).

Figure 1B.5: *M. malabarica* fruit, seed and mace

1B.5.2. Phytochemical Constituents

Myristica malabarica was found to contain various phytoconstituents including diarylnonanoids (Malabaricones A-D), isoflavones, tannins etc. Previous phytochemical studies of the fruit rinds of *Myristica malabarica* Lam. revealed the occurrence of several terpenes, flavones, four novel phenylacyl phenols (Malabaricones A-D) [108] and an aryl tetradecanoid. In 2016, the same group [109] also reported the isolation of an aryl cyclohexyl nonanoid from the methanol extract of the rind. In addition, a lignan malabaricanol and an isoflavone, biochanin, long chain *α*, *ω*-diarylnonanoids, malabaricones A–D have been isolated from the mace of *Myristica malabarica* [110]. In 2000 Talukdar *et al*. reported the isolation of a new isoflavone along with two known isoflavones, a rare *α*-hydroxydihydrochalcone and a 1, 3-diarylpropanol from the heart wood of *M. malabarica* [111]. Earlier studies on essential oil constituents of leaves of *Myristica malabarica* from south Kerala revealed that they are predominated with *β*-caryophyllene (27.3%), α-humulene (13.8%), *α*-copaene (11.5%) and *δ*-cadinene (5.4%) [112]. Later Zachariah *et al*. in 2008 reported that the oil contained *β*-caryophyllene (20.15%) and *α*-humulene (10.17%) as major constituents along with other minor constituents (monoterpenes) [113] by GCMS analysis of leaf oil. The phytochemicals isolated from *Myristica malabarica* are shown in Fig. **1B.6**.

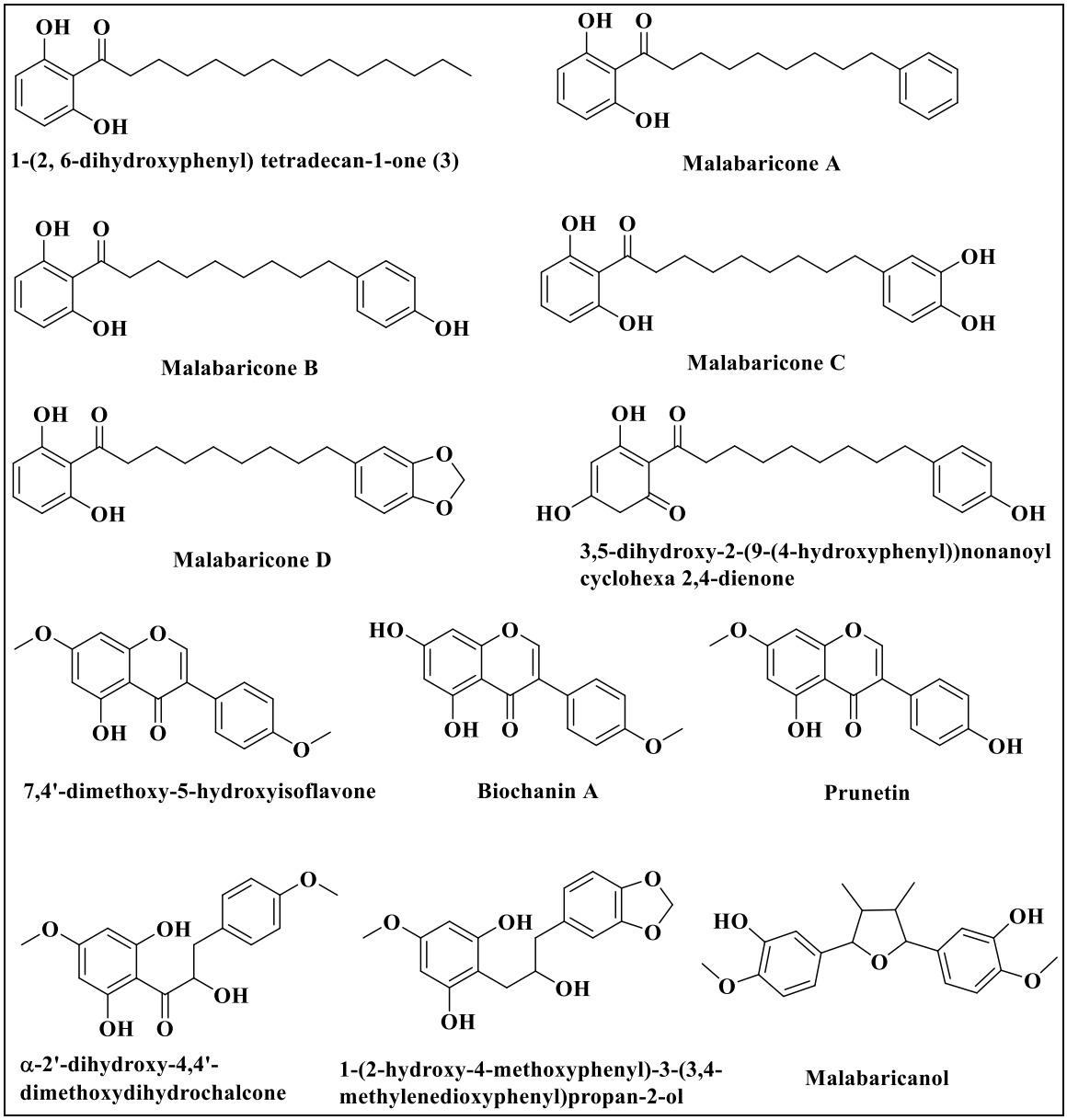

Figure 1B.6: Phytochemicals isolated from *M. malabarica*

1B.5.3. Pharmacological Activities

Myristica malabarica Lam. is known to possess a wide spectrum of pharmacological activities owing to their high phenolic content. Major pharmacological activities credited with the species include hepatoprotective, antithrombotic [114], anti-carcinogenic [115-116], anti-leishmanial [117], antiulcer [118-119], anti-proliferative [120-124], anti-inflammatory [116], anti-quorum sensing [125] and anti-thrombotic [95, 126-127] properties. Several researchers reported variety of pharmacological activities exhibited by the methanol extract of the fruit rind of *M. malabarica* which include antifungal [128], antioxidant [126-130], antidiabetic [131] properties. *M. malabarica* seeds (resin) were also exhibited antioxidant potential [132]. The chemo preventive action

of mace on methyl cholanthrene induced carcinogenesis in the uterine cervix of mice has also been reported [133].

1B.6. *Myristica beddomei* King

Myristica beddomei King., a wild relative of nutmeg commonly known as 'bitter nutmeg' is an endangered species [134] endemic to the evergreen forests and semi evergreen forests of South Western Ghats, Kerala, India and Srilanka [135]. It is mainly distributed from 1,000 to 1,500 m in the mid-elevation evergreen forests of Western and Eastern Ghats [136] indicating the altitude specificity of the species. In Kerala the major populations are found in the districts of Wayanad, Palakkad and Thiruvananthapuram and also in Pathanamthitta, Malappuram, Thrissur, Kannur, Kollam and Idukki. Its local names in Malayalam include Adakkapayin, Chithirapoovu, Kattujathi, Kothappayin, Painpoo, Pasupathi, Pathiripoovu etc.

Figure 1B.7: *M .beddomei* fruit

1B.6.1. Morphology

M. beddomei is a dominant, sub-canopy species. It is an evergreen dioecious tree with blackish green surface and growing up to 25 m high. The heartwood is light reddish-brown. Flowers are unisexual and having white colour. It has a single hard seed covered by an orange-yellow aromatic protein-rich aril.

1B.6.2. Medicinal Uses

Fruit pericarp is a rich source of nutritional compounds. Wild nutmeg and mace of the species is used for medicinal and industrial applications [137]. The aril is used as a spice and having medicinal values. It has been used to treat '*vata*', '*kapha*' disorders, increase digestive power and prevent constipation.

1B.6.3. Phytochemical Constituents

M. beddomei is a less explored species of myristicaceae family. So far there is no detailed study on the phytochemical screening of the compound was done apart from the

essential oil study of the leaves and rind. In 2008, Zachariah *et.al.* studied the essential oil constituents of the leaves of *Myristica beddomei* by GCMS technique and reported that the leaf oil of the plant contained mono (48%) and sesquiterpenes (35%) in higher amount. α-pinene (19.59%), β-caryophyllene (14.63%) and β-pinene (12.46%) are the major constituents in the oil [113]. Vidya *et.al*. in 2015 carried out the essential oil composition, total phenolic and anthocyanin, antioxidant and nutritional analysis of pericarp of *Myristica beddomei* and identified β-caryophyllene (25.41 %) is the major constituent of essential oil of pericarp. The results revealed that the methanolic extract of pericarp showed higher amount of phenols and polyphenols. The total anthocyanin content was found to be 33.064 mg/100 g [138]. In 2016, Pandey *et al.* identified seventeen phytoconstituents for the first time from the ethanol extract of different fruit parts (mace, seed and pericarp) of *Myristica beddomei* using efficient and sensitive HPLC-QTOF-MS/MS analysis. Malabaricone B, gigantenone A, myristic acid and trimyristin were identified from the ethanol extract using NMR technique according to their detectable concentration in different fruit parts of *Myristica* species [124]. Preliminary phytochemical screening of the ethanol and water extract of the plant revealed the presence of different classes of compounds include sterols, alkaloids, glycosides, tannins, phenols, flavonoids, amino acids and proteins [139].

1B.6.4. Pharmacological Activities

Pharmacological activity of the species is not that much explored. Antioxidant activity of the ethanol extract of the pericarp is reported and showed that the higher scavenging ability of the extract against radicals is due to the presence of higher phenolic and anthocyanin compounds [138]. Recently *in vitro* anti-proliferative potential of different fruit parts (pericarp, mace and seed) of *M. beddomei* was reported in five human cancer cell lines (A549, DLD-1, DU145, FaDu and MCF-7) and was found to possess excellent activity.

1B.7. *Myristica magnifca (Myristica fatua. var magnifica)*

Myristica magnifica, a wild relative of *Myristica fragrans*, belongs to Myristicaceae family is native to Maluku, Lesser Sunda Island, Philippines, Samoa and Vanuatu and commonly found in fresh water swampy evergreen forests up to 600 m. According to IUCN, 2000, it is an endangered [134] species endemic to the Western Ghats (Agasthyamalai) and Central Malanad and it is also distributed in Southern Western Ghats regions. It is one of the characteristic species of *Myristica* swamp forest. The species is commonly recognized as "Long Nutmeg", "Wild Nutmeg" or "Mountain

Nutmeg" and locally known as "Kottapannu", "Churapayin", "Kotthapanu" and "Kothapayin".The tree is having restricted distribution in myristica swamps and also employed in medicinal applications [137]. The seeds were used in traditional medicine for the treatment of headaches and other sicknesses.

1B.7.1. Morphology

Myristica magnifica is an evergreen tree with a narrow, open, relatively sparse crown; it can grow up to 37 m tall generally found in humid, swampy rainforest up to 800 m elevation. The tree is dioecious with greyish-purplish trunk often with aerial roots and often produces stilt roots especially when growing in swampy soils. Nut and arils are smaller than nutmeg. The fresh nut is faintly aromatic but loses its aroma on drying and storage. The nuts are occasionally used as a condiment, though they have little commercial value. The seeds are ellipsoid to oblong and 30-35 mm long. Leaves are simple, alternate, distichous and petiole 2-4 cm long. Flowers are unisexual, dioecious, urceolate. The red coloured resin present in the bark of the species is used as dye to give permanent brown stain.

Figure 1B.8: *M. magnifca* fruit

1B.7.2. Phytochemical Constituents

In 2016, Pandey *et al*. identified seventeen compounds from the ethanol extract of different fruit parts of *Myristica magnifica* using efficient and sensitive HPLC-QTOF-MS/MS analysis and the compounds Malabaricone A, B, C and licarin A were identified by using NMR technique [124]. Megawati *et al*. in 2017 reported the isolation of methyl 3, 4-dihydroxybenzoate from the methanol extract of the bark of *M. magnifica* using chromatographic methods [140]. The same group also reported the two resorcinol compounds, malabaricone C and malabaricone B from the methanol extract of the bark of *M. fatua* [141]. Fajriah *et al.* isolated novel cytotoxic lignan and diarylnonanoid

derivatives from the methanol extract of the leaves of *Myristica fatua* [142]. Recently, Prabha *et al*. reported the isolation of six known acylphenols including one novel compound from the DCM extract of the stem bark of *M. fatua*. The compounds include (1-(2-hydroxy-6-methoxyphenyl) tetradecan-1-one, 1-(2,6-dihydroxyphenyl) tetradecan-1-one, malabaricone A, 1-(2-hydroxy-6-methoxyphenyl)-9-(4-hydroxyphenyl) nonan-1-one, malabaricone B and malabaricone C [143]. Recently the same group also reported the isolation of promalabaricone B and trimyristin from the DCM extract of the seeds of *M. fatua* [144]. The compounds isolated from the species are given below.

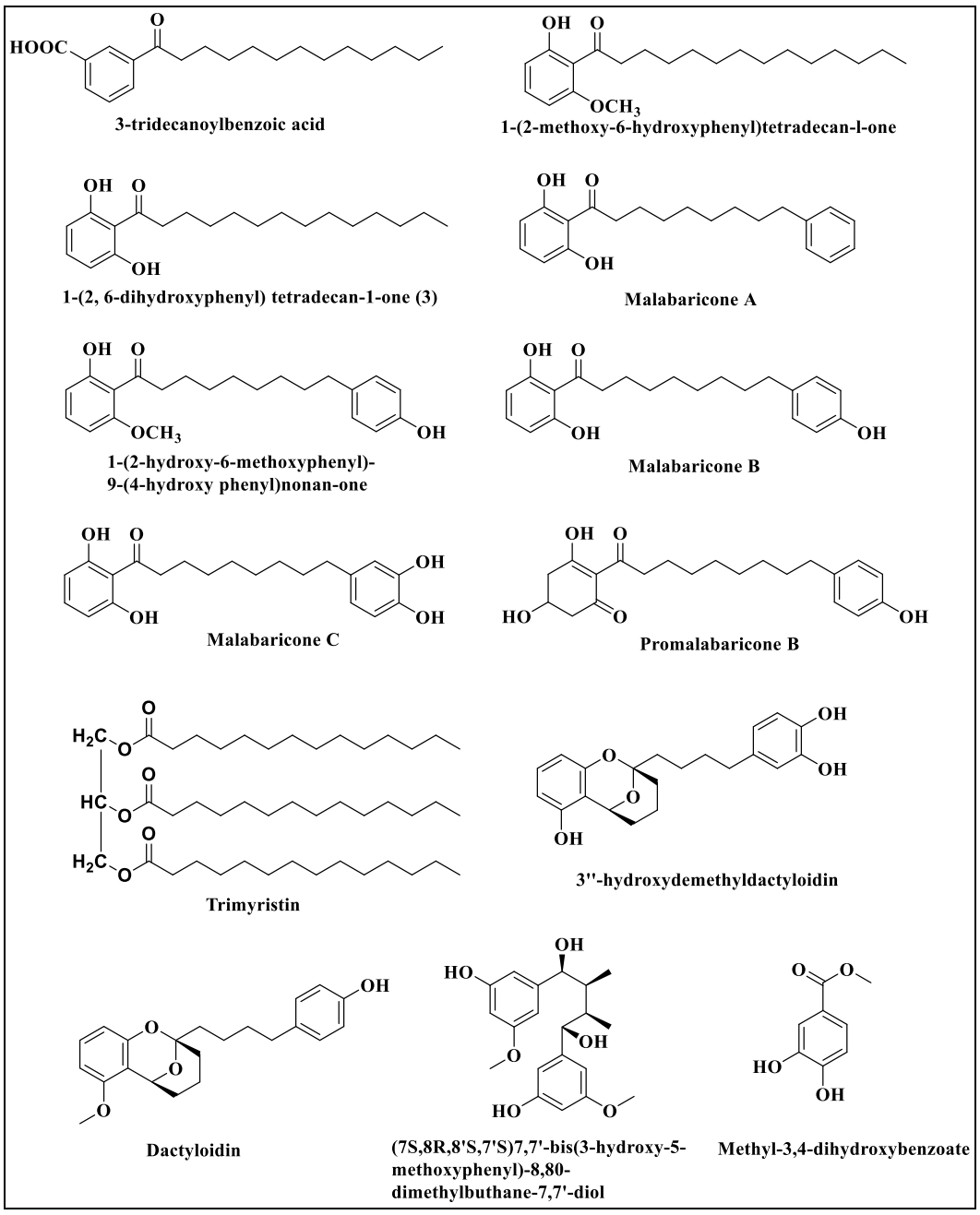

Figure 1B.9: Compounds reported from *M. magnifica*

1B.7.3. Pharmacological Activities

M. fatua was found to possess various pharmacological activities. Anti-mycobacterial activity of DCM and methanol extract of aril and almond of *M. fatua* was reported against *Mycobacterium bovis* BCG [145]. Methanol and DCM extract of stem and aril of *M. fatua* reported to have anti-parasitic and nematicidal activities [146]. Viveka *et al*. reported the antioxidant and antibacterial activity of different extract of the plant parts. Antibacterial activity was screened against different gram positive and negative bacteria [147]. Tantry *et al*. was also reported the antioxidant and antibacterial activity of methanolic and aqueous seed extracts of the plant [148]. *In vitro* anti-proliferative activity of different fruit parts (pericarp, mace and seed) of the species was reported against five human cancer cell lines (A549, DLD-1, DU145, FaDu and MCF-7) [124]. Methyl 3, 4-dihydroxybenzoate from *M. fatua* reported to have antioxidant and antidiabetic activities through DPPH free radicals scavenger and α-glucosidase inhibition activities [140]. The resorcinol compounds were reported from the plant for their *in vitro* cytotoxic activity against the breast carcinoma cancer cell lines MCF-7 [141]. Two novel compounds from the leaves of the plant were also reported for their cytotoxicity against MCF-7 [142]. Prabha *et al*. reported the antidiabetic potential of different acylphenols from *M. fatua* [143-144].

1B.8. *Myristica dactyloids*

M. dactyloides commonly known as 'wild nutmeg or bitter nutmeg' and 'kattujathikka, Panthapayin, Pattapannu' in Malayalam is endemic to mid to high elevation (850– 1400 m) zone forests of Sri Lanka and South Western Ghats of India and a characteristic species of wet evergreen forests of Western Ghats of South India [149]. It is indigenous in Africa, Indo Malaysian region and cultivated all over India. The species is considered as endangered/ vulnerable according to IUCN 2003 because of the overharvesting, forest degradation and habitat loss [150]. *Myristica dactyloides* is often the only representative of the family found in the heavily fragmented, high elevation evergreen forest communities [151-153]. It is one of the dominant species of myristica swamps. The tree is a local source of wood and extensively harvested for its fruits which are traded locally and nationally for its medicinal uses.

1B.8.1. Morphology

Myristica dactyloides is an evergreen, dioceous tree that can grow up to 20 m tall and having stilt roots. Bark is brownish; leaves are simple, alternate and distichous. Flowers are unisexual, dioecious, and white; produced at the end of the branches. It

produces globose fruits with orange red aril [136]. Plants take around 30 years from seedlings to starting to produce seeds.

Figure 1B.10: *Myristica dactyloides* tree, fruits, seed and mace

1B.8.2. Medicinal Uses

Myristica dactyloides is an indigenous medicinal plant and used very commonly in the management of various ailments including diabetes, bronchitis, constipation, diarrhea, nausea, vomiting, chronic bowel complaints, ulcers, splenic disorders, rheumatism, asthma, skin infections etc. It is useful as tonic for the heart and brain and also in general debility [154]. The fruits are used in Ayurveda and Siddha systems of medicine. The fruits are traded in the name of "Jaiphal". The bark and the leaves are boiled and the liquid used as a gargle in the treatment of throat infections. Seeds and arils are the most commonly used parts of the plant as traditional medicine for treating intestinal worms and bowel disorders and often as an alternative to *Myristica fragrans* (common nutmeg) [155]. Seed paste prepared with water is administered orally to cure dysentery. The aril combined with dried ginger is used to check diarrhea. The aril is also used to treat coughs, bronchitis, burning sensations, inflammation of joints, skin disorders, liver disorders etc. In addition the species used as preservative agent and exhibit good antioxidant activities.

1B.8.3. Phytochemical Constituents

The plant was found to be enriched with phenolic constituents. Tillekeratne *et al*. reported myoinositol, a hexitol from the methanol extract of the bark *of M. dactyloides* [156]. Cooray *et al.* in 1987 isolated and reported six acylresorcinols namely 1-(2, 6-dihydroxyphenyl)-9-(4-hydroxy-3-methoxyphenyl)nonan-1-one, 1-(2,6-dihydroxyphenyl) tetradecan-1-one and malabaricones A-D from the acetone extract of the seeds of *M. dactyloides* [157]. Kumar *et al.* reported four new aryl alkanones from the DCM extract of the stem bark of *M. dactyloides* namely 1-(2-methoxy-6-hydroxyphenyl) tetradecan-1-one, 1-(2-methoxy-6-hydroxyphenyl)-9-(3,4'-methylenedioxyphenyl)-

nonanone, 1-(2,6-dihydroxyphenyl)-tetradecan-1-one and 1-(2-methoxy-6-hydroxyphenyl)-9-(4´-hydroxyphenyl)-nonan-1-one along with the known acylresorcinols malabaricone A, B and D [158]. Phytochemical investigation of the hot hexane extract of the stem bark of *Myristica dactyloides* has resulted in the isolation of two new lignans, rel-(8S,8'R)-dimethyl-(7S,7'R)-bis(3,4-methylenedioxyphenyl)tetrahydrofuran and rel-(8R,8'R)-dimethyl-(7S,7'R)-bis(3,4-methylenedioxyphenyl) tetrahydrofuran, a new diaryl alkanone, 1-(2,6-dihydroxyphenyl)-9-(4-hydroxy-3-methoxyphenyl) nonan-l-one, sitosterol and six other previously reported aryl alkanones [159]. The same group in 1997 reported the isolation of a new lignan rel.(8S,8'S)-bis-(3,4-methylenedioxy)-8,8'-neolignan from the hexane extract from the stem bark of *Myristica dactyloides* and two more lignans, rel.(8S,8'R)-dimethyl-(7S,7'R)-bis(4-hydroxy-3-methoxyphenyl) tetrahydrofuran and rel. (8S,8'S)-dimethyl-(7S,7'S)-bis-(4-hydroxy-3-methoxyphenyl) tetrahydrofuran from the DCM extract [160]. In 1998 Herath *et al.* reported the isolation of dactyloidin from the hot DCM extract of the root bark of *Myristica dactyloids* [161]. Koperuncholan *et al.* reported the preliminary phytochemical screening of the root bark, stem bark and leaf extracts of *Myristica dactyloids* using different solvents such as petroleum ether, chloroform, and ethanol and identified the presents of secondary metabolites such as steroids, triterpenoids, reducing sugar, sugars, phenolic compounds, catechins, flavonoids, saponins and tannins [162]. Subha *et al.* reported the preliminary phytochemical screening of the petroleum ether, n-hexane, chloroform, acetone, alcohol and aqueous extracts of the leaves of *M. dactyloides* and identified the presence of alkaloids, tannins, saponin, flavonoids, amino acid, steroids, glycosides and carbohydrates. Presence of alkaloids, glycosides, steroids flavonoids was confirmed by thin layer chromatography [154]. Recently, Bhavani *et al.* reported the presence of 11 phytochemicals in the ethanol extract of the seeds of *Myristica dactyloides* by GC-MS analysis [163]. Rajiv *et al.* reported the preliminary screening of phytochemicals and FT-IR analysis of fruit extracts of *Myristica dactyloids* using different solvents such as methanol, ethanol, petroleum ether, ethylacetate and water. The results revealed the presence of alkaloids, steroids, flavonoids, phenolic compounds, proteins, carbohydrates, cardio glycosides and saponins in methanolic extract when compared to other solvent extracts. FT-IR analysis showed the presence of different functional groups such as carboxylic acid, aromatics, alkanes, alcohols, phenols, aliphatic amines, alkenes and amine groups in the fruit extracts [164].

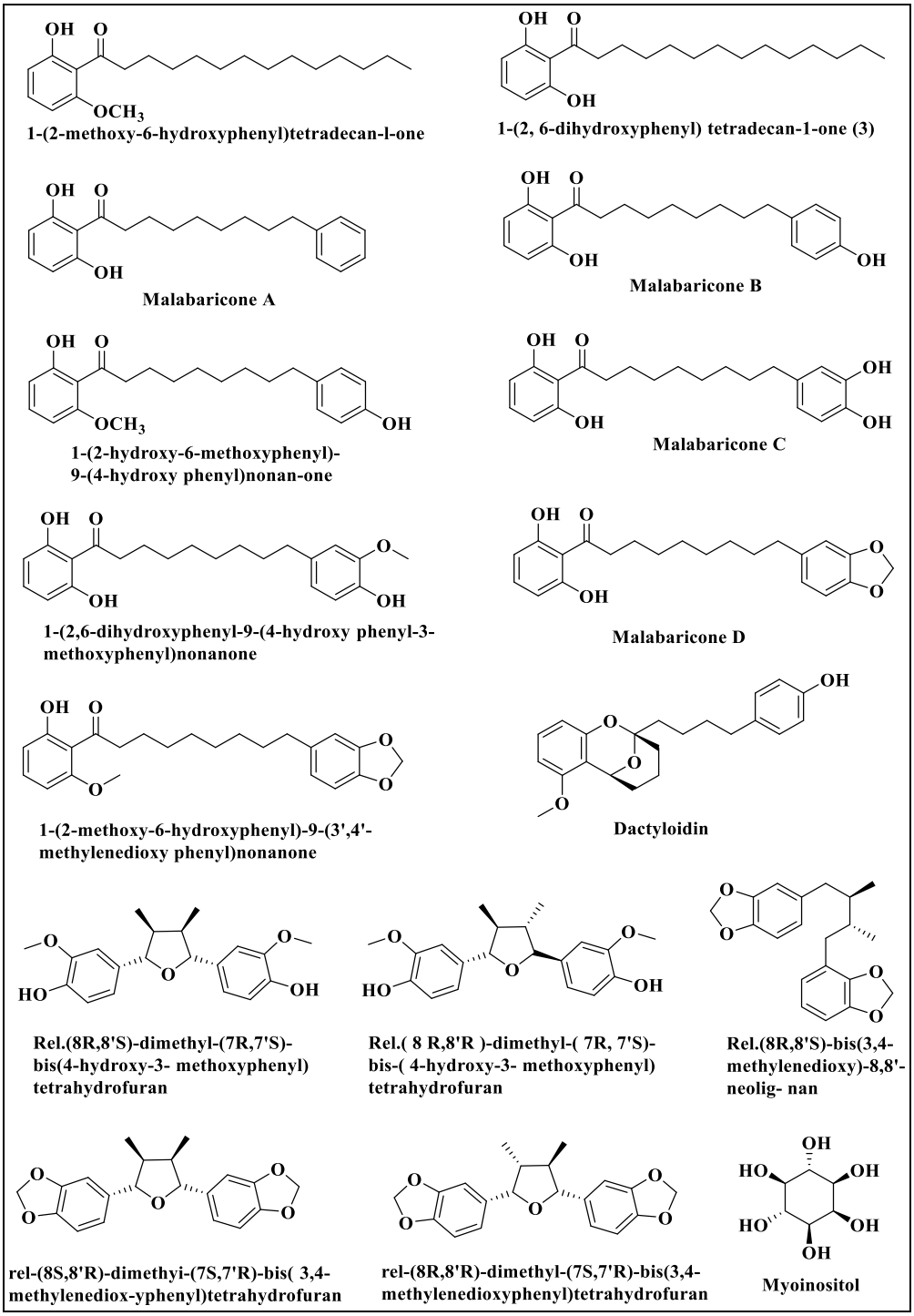

Figure 1B.11: Reported compounds from *M. dactyloids*

1B.8.4. Pharmacological Activities

Myristica dactyloids reported to have pharmacological actions like aromatic, sedative, stimulant, carminative, spasmolytic, antiemetic or exigenic. Antibacterial and antifungal activities in petroleum ether, chloroform, and ethanol extracts of root bark, stem bark and leaves of *M. dactyloides* were reported. Inhibitory activitiy of the petroleum ether, chloroform and ethanol extracts of the root bark, stem bark and leaves

were recorded for both gram-positive and bacteria gram-negative bacteria. Gram-positive were highly inhibitory than gram-negative bacteria. *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Aeromonas hydrophila* showed significant inhibitory activity to the ethanol extract. Antifungal activity of the root bark extract showed the highest activity than stem bark and leaves [162]. In 2015 Ajish *et al.* reported the antimicrobial, anthelmintic and antioxidant activities of petroleum ether, ethylacetate and methanol extract of the stem bark of *Myristica dactyloides.* Antimicrobial activities of the different extract were screened against different gram-positive and bacteria gram-negative bacteria. Methanol extract reported to have higher antibacterial activity when compared to other extracts against gram positive bacteria than gram negative bacteria. Anthelmintic activity of different extracts was screened against Indian earthworm *Pheretima Posthuma* among them petroleum ether extract exhibited significant antifungal activity. DPPH radical scavenging activity was measured by the DPPH antioxidant assay and showed that petroleum ether extract having higher antioxidant activity [165]. Recently Bhavani *et al.* reported the antimicrobial and antioxidant activities of ethanol extract of the seeds of *Myristica dactyloides*. The antimicrobial activity of the ethanol extract was determined against of *E. coli*, *Klebsiella pneumonia*, *Streptococcus sp,* and *Staphylococcus aureus.* Ethanol extract of *Myristica dactyloides* seeds showed strong antibacterial and antioxidant activity [163].

1B.9. Conclusion

Myristicaceae, one among the renowned top ten families of flowering plants is credited with species richness. In India the family represents five genera includes *Myristica*, *Horsfieldia*, *Endocomia*, *Gymnacranthera* and *Knema*. Amongst *Myristica*, the specious genus with 5 species and 2 subspecies is endemic to India. In Southern Western Ghats (Agastyamala Biosphere Reserve) *Myristica* genus comprised of five species *viz.* the true nutmeg *M. fragrans* along with its wild relatives such as *M. malabarica*, *M. beddomei*, *M. magnifica* and the newly identified *M. trobogarii. Myristica beddomei* consists of three sub species includes *M. beddomei* subsp. beddomei, *M. beddomei* subsp. sphaerocarpa W.J. de Wilde and *M. beddomei* subsp. ustulata. The wild nutmeg species are red listed and endemic and most of them are unexplored. The wild nutmeg species thrives normally at high altitude and some species at myristica swamps. Limited distribution on high altitude, dense forest and strict phenology made research about this species difficult. Most of the wild nutmeg species are used as a forest produce. Nutmegs and wild nutmegs are a rich reservoir of bioactive molecules and are used in traditional

medicines. Fruits of *M. malabarica* largely consumed by tribal population and were employed in several Ayurvedic preparations also it is used as adulterant for true nutmeg. Morphology, Phytochemical and pharmacological aspects the species found in Agastyamala Biosphere Reserve were included in this study and it will contribute towards its conservation and the application of its bioactive constituents in pharmaceutics.

1B.10. References

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Chemotaxonomic Significance of Novel Flavonoids from the Unexplored & Endangered Wild nutmeg Species *Myristica beddomei* subsp. *spherocarpa* W.J. de Wilde

2A.1. Introduction

Western Ghats, the treasure trove of biodiversity represents a distinct biogeographic zone in India characterized by highly diverse and unique flora, fauna and topography. It is designated as one among the eight "hottest hotspot" of biodiversity region in the world due to its species richness and high incidence of endemism. Environmental heterogeneity, undulating topographical features, gradient climate are responsible for species richness. Most of its area has a natural cover of forests ranging from evergreen to moist and dry-deciduous types. The highest diversity was attributed to its southern tip Kerala with high statured, rich tropical rain forests. It is estimated that the floristic diversity of Kerala is gifted with around 4500 species of flowering plants, of which 1600 species are rare and endemic to the region distributed among the well-known plant families. In addition Western Ghats are enriched with a number of wild relatives of cultivated plants, including nutmeg, pepper, cardamom, plantain etc. along with medicinal plants endemic to it. Amongst a wide variety of medicinally important species were utilized in traditional and folk medicine [1-3].

Agasthyamala Biosphere Reserve is the biodiversity hotspot region located in the southernmost end of Western Ghats distributed in 3,500.36 km² of which 1828 km² is in Kerala and 1672.36 km² is in Tamil Nadu considered as the unique genetic reservoir of several important plant species. It includes tropical wet evergreen forests, South Western Ghats moist deciduous forests, Shola and montane rain forests. It contains flagship families of plant kingdom and is the habitat for 2,000 varieties of medicinal plants amongst around 50 species are rare and endangered. In addition the ancient tribal community *Kanikaran* lives in Agasthyamala region. Agastyarkoodam, the highest peak 1868 m in the Western Ghats is the abode of rare flora and fauna and even wild animals. Agasthyamalai hills, the core of the Reserve is considered as one of the five centers of plant diversity and endemism in India by IUCN. Neyyar, Peppara and Shenduruny wild life sanctuaries were located in this region which is known for their unique eco diversity [4-6].

Figure 2A.1: Agastyarkoodam of Agasthyamala Biosphere Reserve

Myristicaceae is one among the huge family of flowering plants commonly found in Western Ghats, gathered attention in terms of the medicinal importance of the variety of plant species nested within this family. Diversity among the species and its abundance make them a unique family. In India, the family represents five genera, *Horsfieldia* Willd. *Endocomia* Wilde, *Gymnacranthera* Warb., *Knema* Lour. and *Myristica* Gronov. of which latter three are distributed in evergreen forests of the Western Ghats [7]. *Myristica*, the representative genus of myristicaceae family is more specious and constitutes 175 species worldwide [8]. Amongst India signifies only five species *viz. M. andamanica* Hook. F, *M. beddomei* King, *M. fragrans* Houtt., *M. magnifica* Bedd. and *M. malabarica* Lam [9]. Of which, *M. beddomei, M. magnifica* and *M. malabarica* are strictly endemic to the Western Ghats and *M. fragrans* is native to Mollucas Islands and under cultivation in India [7, 9]. Most of the wild nutmeg species of this genus are still unexplored.

Myristica beddomei King (Chithirapoovu, Pathiripoovu in Malayalam) commonly known as "bitter nutmeg'' is an endangered wild nutmeg species [10] endemic to the evergreen forests (up to 1300m altitude) and semi evergreen forests of South Western Ghats of Kerala, possess three subspecies *M. beddomei* subsp. *beddomei, M. beddomei* subsp. *ustulata* and *M. beddomei* subsp. *spherocarpa.* Apart from the other two species, *M. beddomei* subsp. *spherocarpa* not gained adequate attention. No studies on taxonomy, ecology, population structure of this species is done so far. But its restricted distribution was reported by Wilde [11]. It thrives only at high altitude (> 900 m) and is reported only from Agasthyamala Biosphere Reserve. *M. beddomei* and its sub species were less

investigated members of *myristica* genus. Few chemical reports have been published regarding the preliminary studies of the related sub species (Chapter **1B.6.3**).

Taxonomic description of the species: Tree, evergreen, 20–35 m tall, dioecious; exudate orange red coloured, viscous. Leaves alternate, petiolate; lamina 8–13 × 4–6 cm, broadly lanceolate, apex acute to acuminate, margin undulate, base rounded, adaxially dark green, gland-dotted, abaxially glaucous, glabrous; midvein prominent, lateral veins not prominent, 13-15 paired. Male inflorescence 2-3 flowered, axillary umbel; peduncle erect, 2 cm long, cylindrical, scurfy tomentose. Flowers caducous, urceolate, long than broad, 4.1-4.3 × 2.3–3.0 mm, brownish yellow, pedicellate; pedicel 7–10 mm long, pubescent; bracteole 2.1–3.0 × 2.2–3.1 mm, ovate, acute at apex, appressed to the base of male flower, dorsally convex, brownish yellow, densely pubescent outside, persistent. Perianth fleshy, lobes 3, equal, acute, re-curved outward, brownish yellow, densely pubescent outside; staminal column 3–5 mm long, cylindrical, pilose up to anthers from base; anthers 12-13. Female inflorescence 2 flowered, axillary umbel; peduncle erect, 3 mm long, stout, cylindrical, puberulous. Flowers urceolate, 4.2-6.1 × 4–5 mm, yellow, persistent, pedicellate; pedicel up to 5 mm long, stout, pubescent; perianth fleshy, lobes 3, equal, acute, re-curved outward, scabrid outside; stigma short, bilobed; ovary ovate, 2–3 mm, densely tomentose. Fruits usually solitary, rarely in pairs, dehiscent, 2.5–4 × 3.5–5 cm, ovoid to sub globose, brown, apex obtuse, base round, longitudinal suture on both sides, scurfy pubescent; rind fleshy, soft, 7–10 mm thick, brown; seed 3–5 × 2.5–4 cm, oblong, black, arillate; partially covering, aril yellow turns to orange red, lacerated. Different aerial parts of the species including leaf, fruits and seeds are depicted in Fig. **2A**.**2**.

This tree has been used as a root stock for the vegetative propagation of *M. fragrans* [12]. The tree is economically important for its wild nutmeg and mace which is used for both medicinal and industrial purposes [13]. Fruit pericarp and seed is used as vermicidal, stomach ache and health tonic. The mace, seed and pericarp are used in Ayurveda, siddha and unani and widely utilized in South India as substituent or adulterant of nutmeg (*M. fragrans)* products. Reddish grey wood is moderately hard and useful for boxes of tea, match and for splints. Fruit rind and seeds were found to possess anti-proliferative and antioxidant activity [14].

Figure 2A.2: Aerial parts (Leaf, fruit, rind, and seed) of *M. beddomei* subsp. *spherocarpa*

2A.2. Aim and Scope of the Present Work

Bioactive natural products have enormous economic importance in terms their pharmacological application. Nutmeg, considered as an important spice of Kerala which is extensively studied. Along with nutmegs, some of the wild nutmeg varieties are also distributed in the forests of South Western Ghats of Kerala. Most of the wild relatives of nutmeg are endemic to Western Ghats and are red listed. Taxonomic and ecological studies of such rare and endangered plant species will contribute much towards its conservation. Apart from that the chemoprofiling of the unexplored species provide a detailed knowledge about the bioactive phytoconstituents present in it. So the identification of such species and their detailed study is important in order to preserve their biodiversity. Wild nutmegs have high medicinal, economic and conservative values than the ordinary nutmegs and are widely used by the tribes to cure various ailments.

M. beddomei subsp. *spherocarpa* a sub species of *M. beddomei* is one among the unexplored species of Myristicaceae family. Due to limited distribution on high altitude, dense forest and strict phenology made research about this species difficult. Morphologically, this subspecies shows noticeable variation from other subspecies of *M. beddomei*. Thus a detailed chemical investigation will aid to solve taxonomical problems of these particular taxa. Apart from the preliminary studies of the related sub species no reports are available for this particular species. It necessitates the detailed

studies on phytochemical as well as pharmacological aspects of the plant along with the exploration of bioactive constituents from it. Herein in the present work we establish a complete chemoprofiling of the different aerial parts of the species along with the *in vitro* cytotoxicity of the isolates against various cancer cell lines.

2A.3. Extraction, Isolation and Characterization of Phytochemicals from the Different Fruit Parts of *M. beddomei*

2A.3.1. Collection of Plant Material

Fruits of *Myristica beddomei* were collected from Chemunji Hills, Bonaccadu region of Thiruvananthapuram District, Kerala, India in April 2019. The plant material was authenticated by Dr. Mathew Dan, Senior Scientist, Plant Genetic Resource Division KSCSTE-Jawaharlal Nehru Tropical Botanic Garden & Research Institute, Palode, Thiruvananthapuram Kerala, India and a voucher specimen [***Myristica beddomei*, Chemunji Hills, Bonaccadu, Trivandrum, Kerala, April, 2019, Govind, *91046* (TBGT)**] was deposited in the herbarium of Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Trivandrum in Kerala State. A geographical map of the species is given in Fig. **2A.3**.

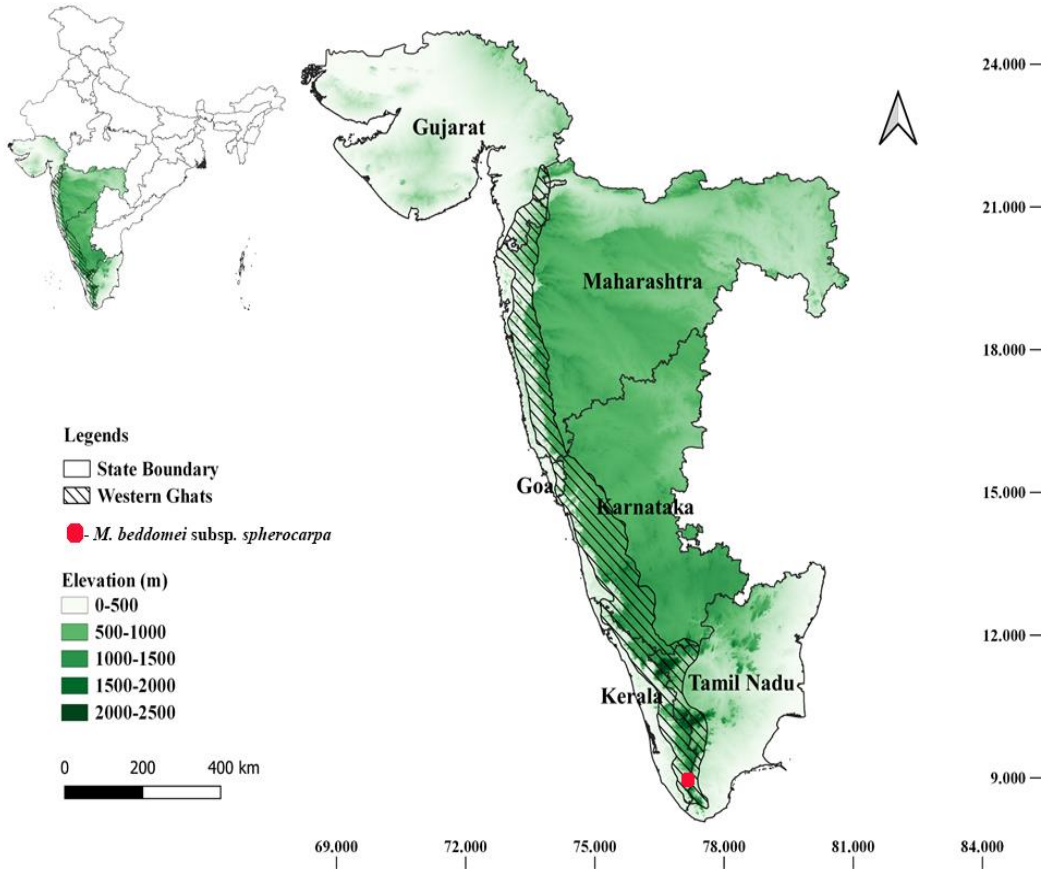


Figure 2A.3: Distribution and collection site of *M. beddomei* subsp. *spherocarpa*

2A.3.2. Extraction of the Fruit Rinds of *M. beddomei*

Initially rinds (pericarp) were separated from the fruits and dried in an air oven maintained at 50 ^{0}C for three days. About 800 g of the dried material was then powdered mechanically and subjected to extraction with hexane at room temperature (5 L × 3 days). The extract was then filtered and concentrated under reduced pressure using Heidolph rotary evaporator to yield approximately 10 g of the hexane extract. The residue obtained after extraction using hexane was further subjected to extraction using DCM at room temperature (5 L × 3 days) to afford 25 g of DCM extract. The residue was then extracted successively with acetone (5 L× 3 days) and ethanol (5 L× 3 days) and finally with water afforded 50 g, 19 g and 16 g extracts respectively.

2A.3.2.1. Identification of Volatile Components from the Various Extracts of Rind

In order to get an overall idea about the valuable phytochemicals present in the rare species, initially we have carried out the GC-MS analysis. Extracts of the fruit rinds (Hexane, DCM and Acetone) were subjected to GCMS analysis in order to find out the volatile components present in it. GCMS analysis revealed the presence of several hydrocarbons and phenylacyl phenol type compounds. The list of various compounds identified from the extracts of fruit rinds are given in Table **2A.1A-C**.

Table 2A.1A: GCMS analysis of Hexane extract of the rind

Peak	Ret. time	Area%	Height%	Compound name
1	21.422	1.28	1.75	δ-cadinene
2	21.531	1.31	1.10	4-isopropyl-1,6-dimethyl-1,2,3,7,8,8a-hexahydronaphthalene
3	20.897	1.44	1.27	1β, 4β, 10β -guaia-5,11-diene
4	50.928	1.45	0.87	Tetrapentacontane
5	19.882	1.66	2.38	α-humulene
6	39.948	1.80	1.62	Hexatriacontane
7	42.445	1.81	1.83	Heneicosane
8	25.955	1.83	2.20	Farnesol isomer B
9	56.073	2.14	2.25	Stigmasta-5,22-dien-3-ol, (3.beta.,22e)-
10	17.826	2.98	4.13	α-copaene
11	10.133	3.30	1.82	linalool L
13	31.370	3.53	2.06	l-(+)-ascorbic acid 2,6-dihexadecanoate
14	49.443	4.70	3.58	phen-1,3-diol, 2-tetradecanoyl-
15	52.923	5.78	4.79	Malabaricone A
16	18.994	6.37	7.89	bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-, (E)-(1R,9S)-(-)-
17	44.316	1.21	0.80	2,6,10,15,19,23-hexamethyl-tetracosane

Table 2A.1B: GCMS analysis of DCM extract of the rind

Peak	Ret. Time	Area%	Height%	Compound Name
1	47.950	1.16	1.18	1,3,5-trisilacyclohexane
2	48.891	1.34	1.04	Octadecanoic acid, 2,3-dihydroxypropyl ester
3	45.335	1.43	1.74	2'-hydroxy-4'-methoxyacetophenone, tert-butyldimethylsilyl ether
4	21.142	1.54	1.83	Phenol, 2,4-bis(1,1-dimethylethyl)-
5	50.673	1.55	1.75	3-hydroxy-4-methylbenzaldehyde
6	43.817	1.78	2.20	1H-Indole-3-ethanamine
7	44.206	2.06	1.53	Dianhydroglucitol, TBS 2X
8	44.559	2.46	2.21	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
9	46.368	2.49	2.20	Phosphine sulfide, triphenyl-
10	57.933	2.62	2.04	3-[(2-hydroxy-3-methyl-benzoyl)-hydrazono]-n-(2-methoxy-5-methyl-phenyl)-butyramide
11	56.990	3.79	2.03	Promalabaricone B
12	22.407	4.38	5.11	1,6,10-dodecatrien-3-ol, 3,7,11-trimethyl-
13	53.082	8.48	9.61	3-[(2-hydroxy-3-ethyl-benzoyl)-hydrazono]-N-(2-methoxy-5-methyl-phenyl)-butyramide
14	49.348	11.70	10.29	Phen-1,3-diol, 2-tetradecanoyl-
15	52.891	12.05	14.34	Malabaricone A
16	52.838	22.57	19.87	(4-benzo[1,3]dioxol-5-ylmethyl-Piperazin-1-yl)-(3,5-Dinitro-Phenyl)-methanone

Table 2A.1C: GCMS analysis of Acetone extract of the rind

Peak	Ret. Time	Area%	Height %	Compound Name
1	56.640	1.13	1.51	2-methylhexacosane
2	22.416	1.34	2.56	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-
3	52.902	4.26	5.78	Malabaricone A
4	49.389	4.67	4.58	Phen-1,3-diol, 2-tetradecanoyl-
5	56.885	5.61	5.64	γ-sitosterol
6	53.056	7.36	17.13	1,10-Epoxy-4-methoxy-7-methyl-8-(3,4-methylenedioxyphenyl)-spiro(5,5)undeca-1,4-dien-3-one
7	52.776	11.41	28.7	(4-benzo[1,3]dioxol-5-ylmethyl-piperazin-1-yl)-(3,5-dinitro-phenyl)-methanone
8	16.356	22.62	3.37	Resorcinol

2A.3.2.2. Isolation and Characterization of Phytochemicals from the Rind

TLC analysis of the extracts of the rinds was carried out initially. After that About 25 g of DCM extract was subjected to silica gel (100-200 mesh) column chromatographic separation using hexane, hexane-EtOAc gradient and EtOAc afforded 23 fraction pools based the similarity in TLC. Similarly 50 g of acetone extract was also subjected to column chromatography with 100-200 mesh sized silica gel using hexane, hexane-EtOAc gradient and EtOAc afforded 63 fraction pools. All the fraction pools on repeated column chromatographic separation resulted in the isolation of following compounds. A schematic representation of the entire isolation procedure is given below.

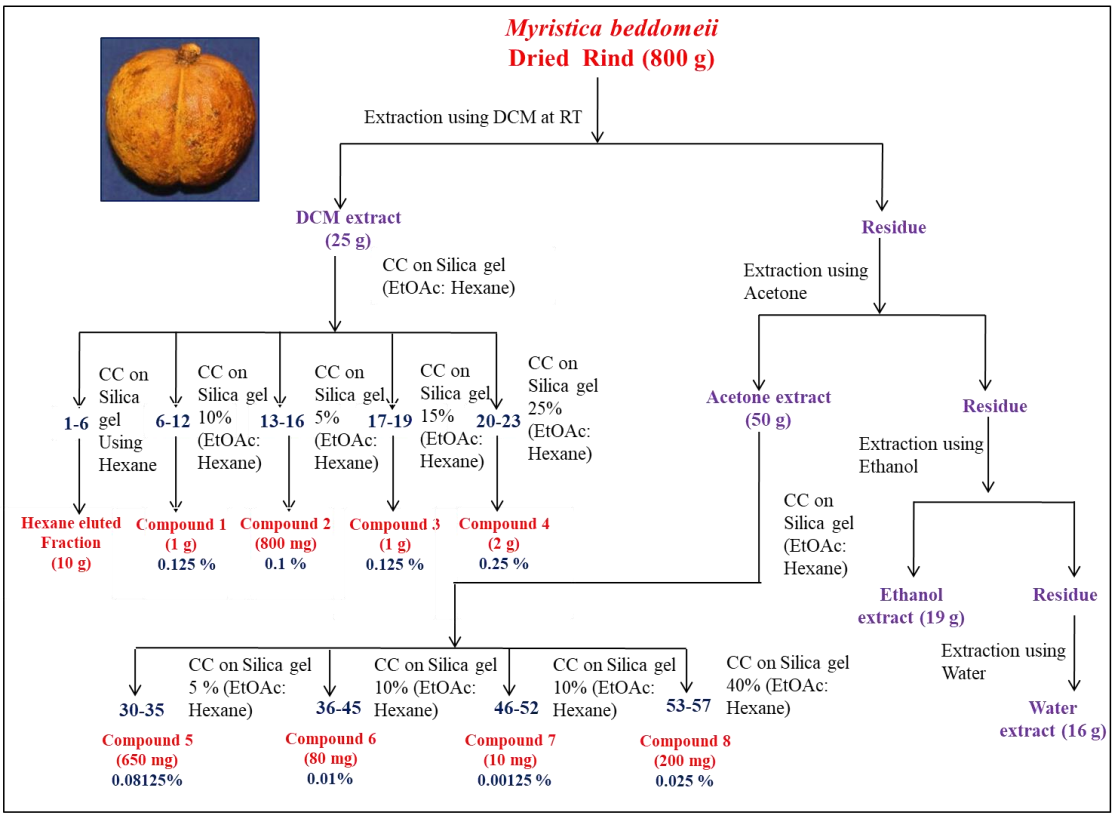
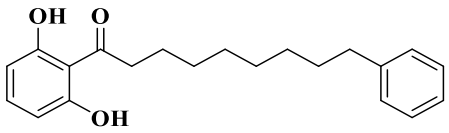


Figure 2A.4: Schematic representation of extraction and isolation procedure

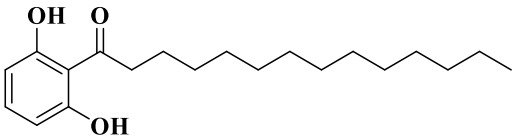
Fraction pools 6-12 obtained from the column chromatographic separation of DCM extract showed the presence of an intense UV active spot. On charring in Enholm yellow solution the TLC turned into intense pink colour. Purification of the fraction by CC on silica gel using 10% ethyl acetate-hexane solution afforded compound **1** as pale yellow crystalline solid. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be malabaricone A [15]. The IR spectrum of the compound showed absorptions at 3254 and 1635 cm^{-1} suggesting the presence of hydroxyl as well as carbonyl groups. Presence of

carbonyl group was further confirmed from a peak obtained at δ 208 ppm in the ^{13}C NMR spectrum. In the ^{1}H NMR spectrum a triplet at δ 3.17 ppm integrating for two protons could be attributed to $-CH_2$ group adjacent to carbonyl carbon. Two aromatic protons adjacent to hydroxyl group resonated at δ 6.43 ppm as doublet with a coupling constant of 8.5 Hz. A broad singlet at δ 11.3 ppm integrating for two protons indicates the presence of two phenolic hydroxyl groups. Six down ward peaks in DEPT 135 spectrum ranging from 24.4-44.4 ppm suggests the presence of six $-CH_2$ groups. The mass spectrum of the compound gave a molecular ion peak at 349.17833 which is the $[M+Na]^+$ peak.



Malabaricone A
Compound **1**

Compound **2** was isolated from the fraction pool 13-16 after column chromatographic separation using 5% ethyl acetate-hexane solution. It was obtained as UV active pale yellow crystalline solid. The IR spectrum of the compound showed absorptions at 3450 and 1624 cm^{-1} indicates the presence of hydroxyl as well as carbonyl groups. In the ^{1}H NMR spectrum a broad singlet at δ 11.30 ppm integrating for two protons indicates the presence of two hydroxyl groups. A peak obtained at δ 208 ppm in the ^{13}C NMR spectrum corresponds to carbonyl group. DEPT 135 spectrum revealed the presence of seven $-CH_2$ groups. ^{1}H NMR spectrum showed the presence of a methyl group resonated at δ 0.73 ppm. In the ^{13}C NMR spectrum a peak obtained at δ 13.44 ppm confirmed the presence of methyl group. The two aromatic protons adjacent to hydroxyl group resonated at δ 6.29 ppm as doublets with a coupling constant of 8 Hz. The remaining aromatic protons resonated as a triplet at δ 7.12 ppm. The mass spectrum of the compound gave a molecular ion peak at 343.22436 which is the $[M+Na]^+$ peak. Finally ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be 1-(2, 6-dihydroxyphenyl) tetradecan-1-one [16].



1-(2, 6-dihydroxyphenyl) tetradecan-1-one
Compound **2**

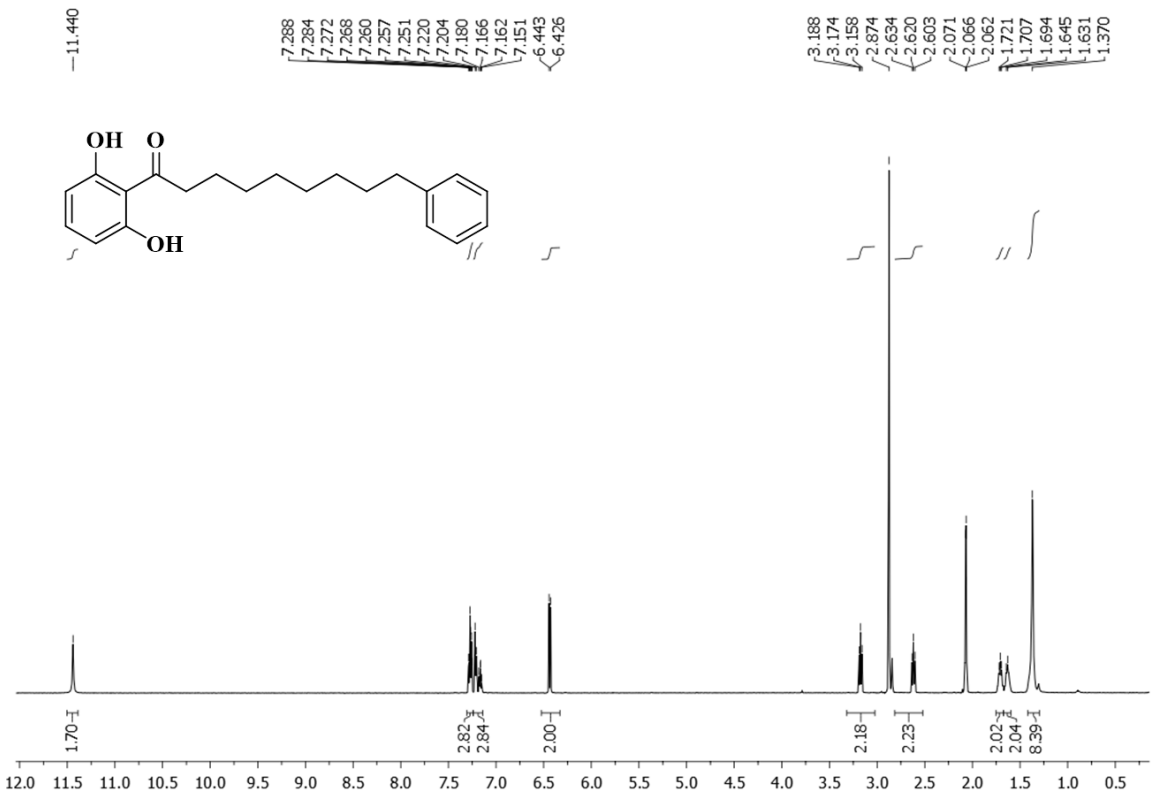


Figure 2A.5: ^{1}H NMR spectrum of malabaricone A

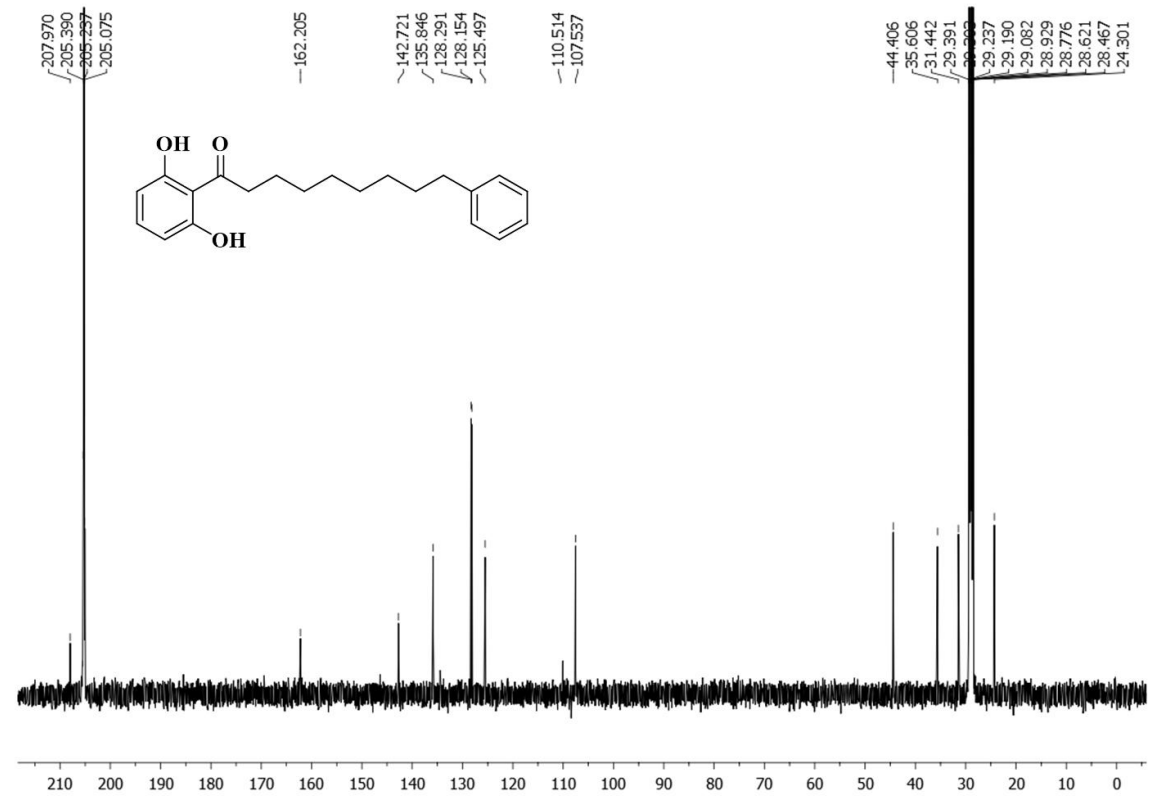


Figure 2A.6: ^{13}C NMR spectrum of malabaricone A

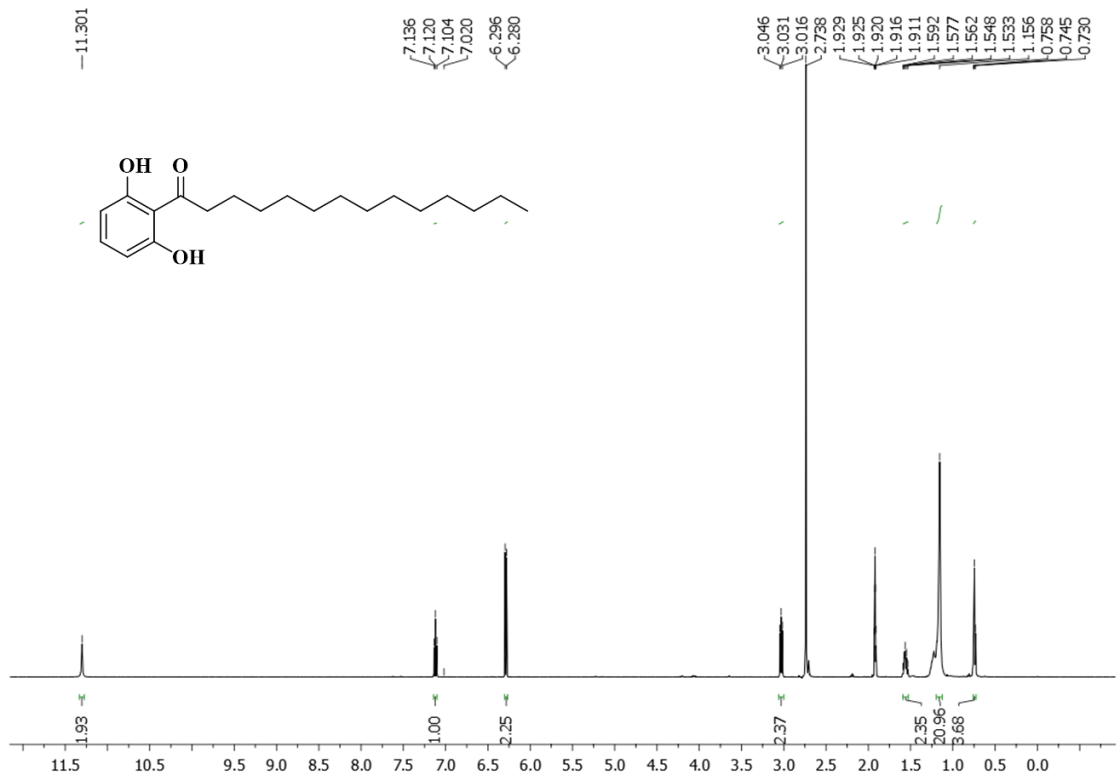


Figure 2A.7: ^{1}H NMR spectrum of 1-(2, 6-dihydroxyphenyl) tetradecan-1-one

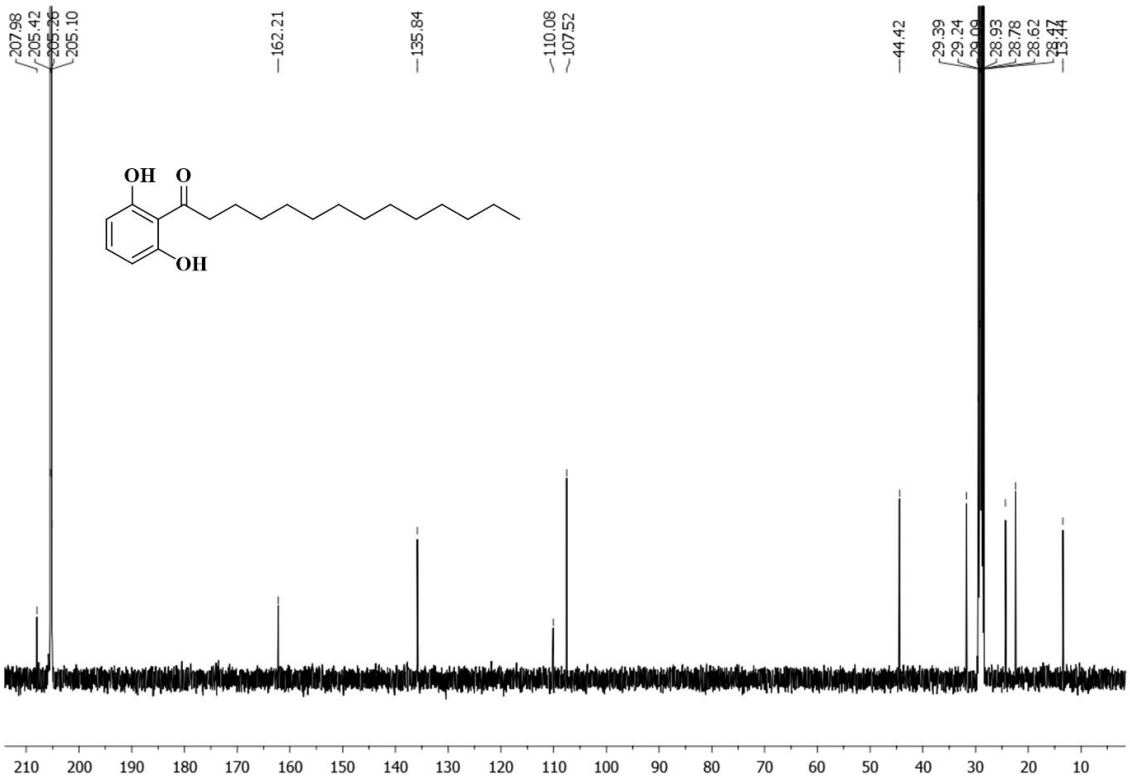
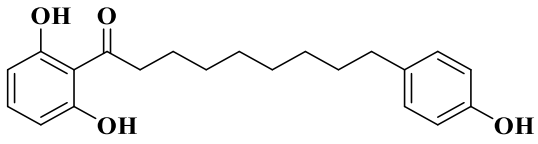


Figure 2A.8: ^{13}C NMR spectrum of 1-(2, 6-dihydroxyphenyl) tetradecan-1-one

Fraction pool 17-19 indicates presence of a UV active spot upon charring the TLC in Enhlom yellow solution it turns into intense pink colour. These fractions on CC separation using 15% ethyl acetate-hexane solution afforded the compound as pale yellow solid. The IR spectrum of the compound showed absorptions at 3260 and 1635 cm^{-1} indicates the presence of hydroxyl and carbonyl groups. In the ^{1}H NMR spectrum two broad singlets resonated at δ 11.37 ppm integrating for two protons and δ 8.00 ppm integrating for one proton confirms the presence of three hydroxyl groups. Presence of carbonyl group can be confirmed from the peak at δ 208 ppm in the ^{13}C NMR spectra. DEPT 135 spectra gave six downward peaks corresponds to $-CH_2$ groups. In the ^{1}H NMR spectrum two doublets at δ 6.87 and 6.67 ppm with a coupling constant of 8.5 Hz integrating for two protons each represents the aromatic *ortho* coupled protons of second phenyl ring. The mass spectrum of the compound gave a molecular ion peak at 365.17291 which is the $[M+Na]^{+}$ peak. By comparing various spectral details of the compound and literature reports the compound **3** was identified as malabaricone B [15].



Malabaricone B
Compound **3**

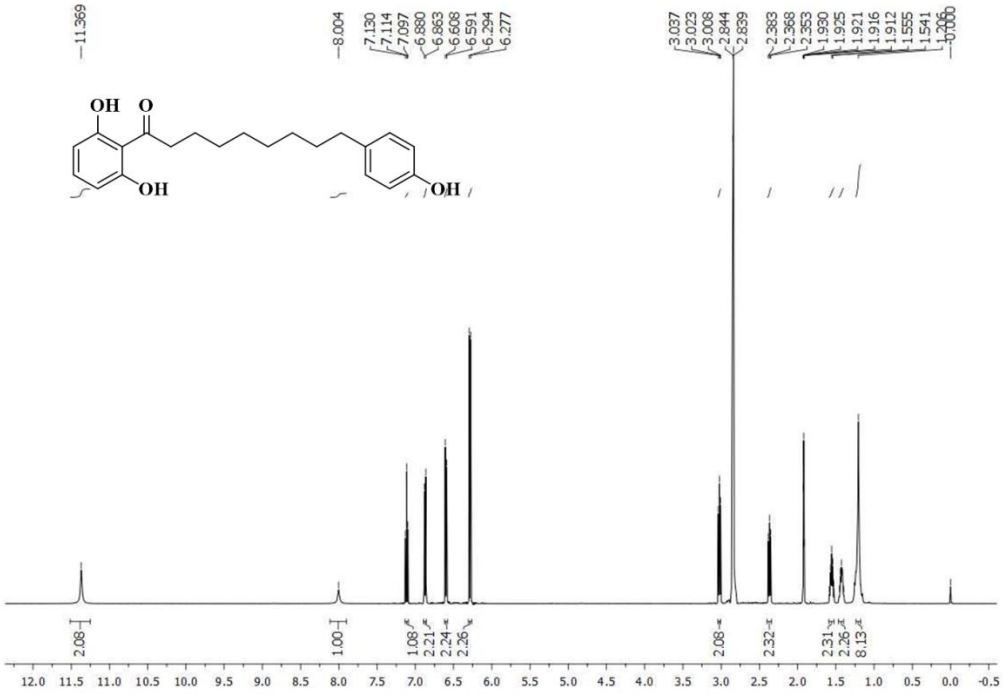


Figure 2A.9: ^{1}H NMR spectrum of malabaricone B

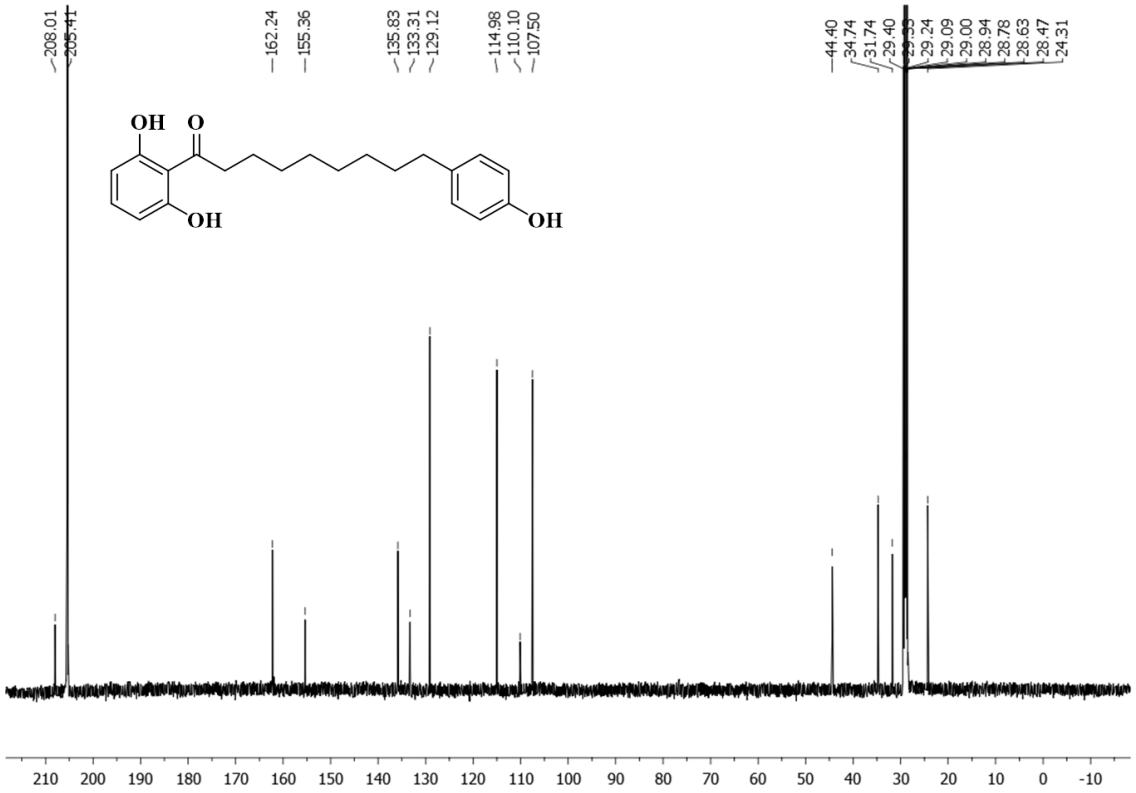
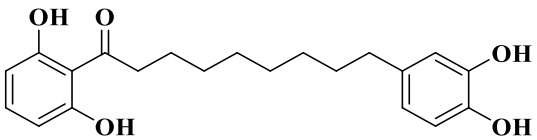


Figure 2A.10: ^{13}C NMR spectrum of malabaricone B

Compound **4** was obtained as a UV active yellow solid from the fraction pool 20-23 after column chromatographic separation using 25% ethyl acetate-hexane solution. Spectral data of the compound **4** is similar to that of compound **3** suggests the presence of a phenyl acyl phenol moiety. The IR spectrum of the compound showed absorptions at 3347 and 1623 cm^{-1} indicates the presence of hydroxyl and carbonyl groups. Presence of carbonyl group can be confirmed from the peak at δ 208 ppm in the ^{13}C NMR spectrum. In the ^{1}H NMR spectrum two broad singlets resonated at δ 11.37 and 8.00 ppm integrating for two protons each confirmed the presence of four hydroxyl groups. DEPT 135 spectrum revealed the presence of six -CH_2 groups. The mass spectrum of the compound gave a molecular ion peak at 381.16727 which is the $[M+Na]^+$ peak. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be malabaricone C [15].



Malabaricone C
Compound **4**

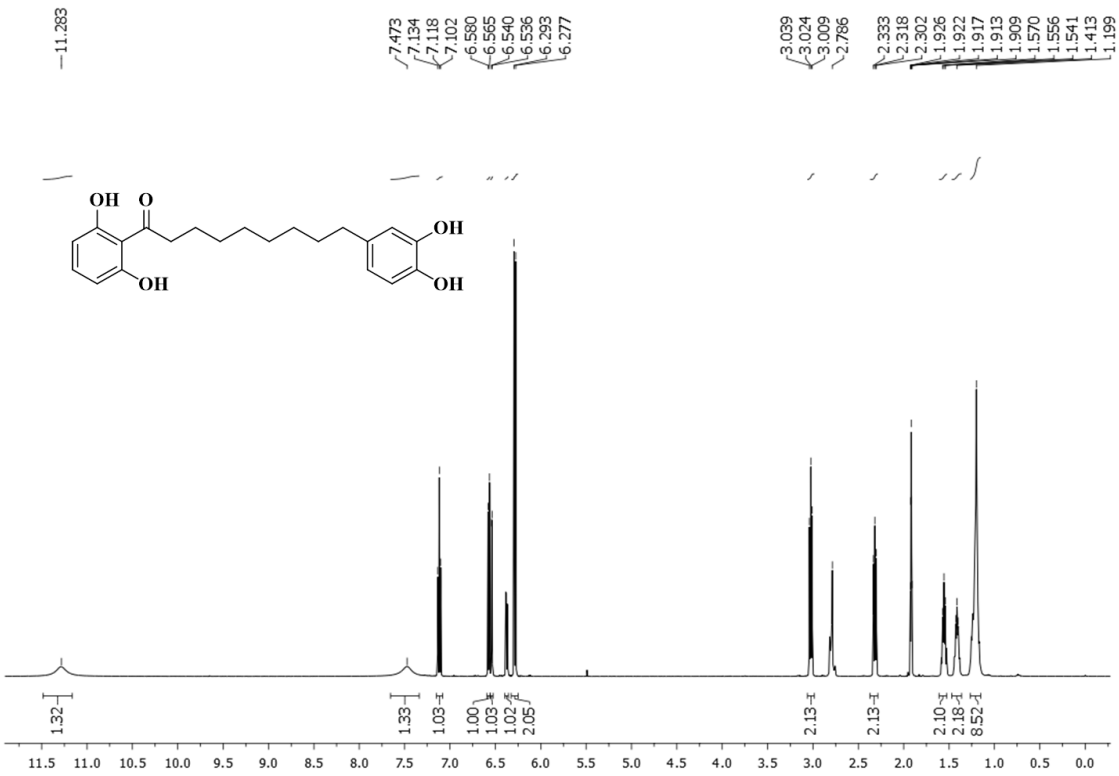


Figure 2A.11: ^{1}H NMR spectrum of malabaricone C

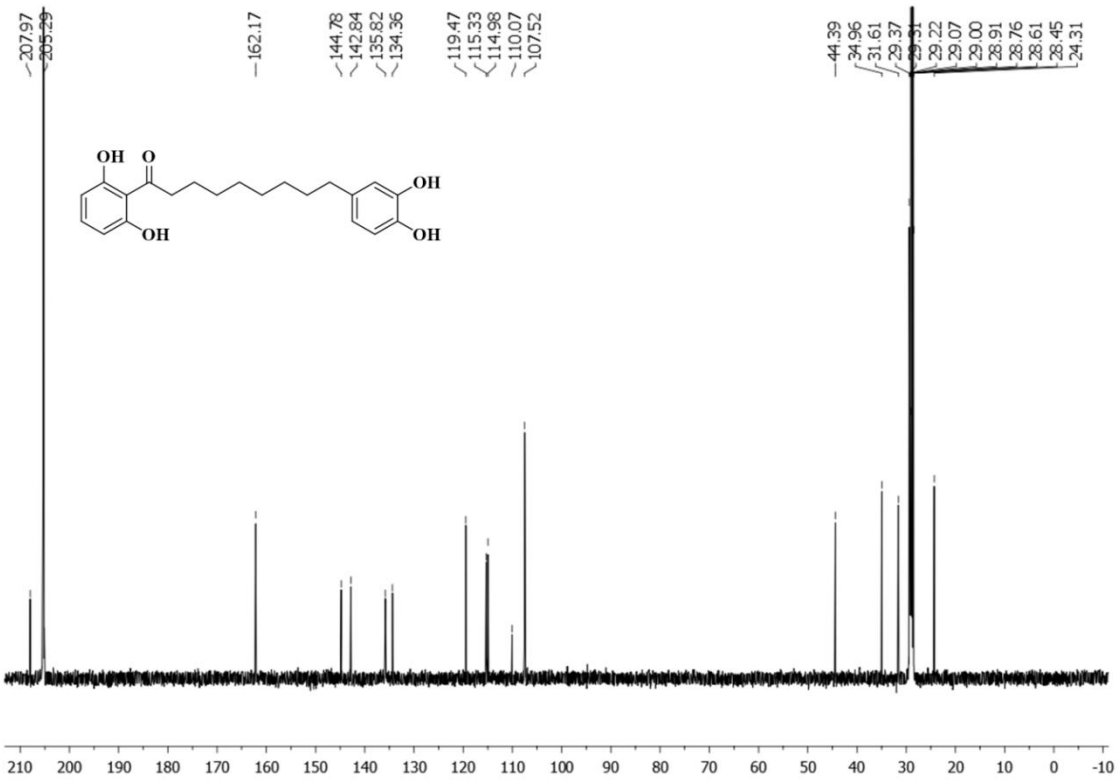
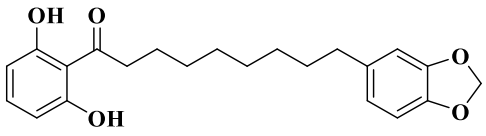


Figure 2A.12: ^{13}C NMR spectrum of malabaricone C

Fraction pool 30-35 of acetone extract showed the presence of an intense UV active spot. The TLC turned into red colour on charring in Enhollm yellow solution. The fraction on CC separation using 5% ethyl acetate-hexane afforded compound **5** as white powder. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be malabaricone D [15]. In the IR spectra, the carbonyl stretching frequency was obtained at ν_{max} of 1622 cm^{-1}. Carbonyl carbon of ketone resonated at δ 207.9 ppm in ^{13}C NMR spectrum. ^{1}H NMR spectrum showed a triplet at δ 3.02 ppm integrating for two protons indicating the presence of -CH_2 group adjacent to carbonyl group. A broad singlet at δ 11.33 ppm integrating for two protons indicates the presence of two hydroxyl group. Two aromatic protons adjacent to hydroxyl groups resonated at δ 6.28 ppm as doublet with a coupling constant of 8.5 Hz. Presence of a sharp singlet at δ 5.79 ppm, integrating for two protons confirmed the presence of methylene dioxy group. In ^{13}C NMR spectrum methylene dioxy group resonated at δ 100.7 ppm. In DEPT-135 spectrum six -CH_2 were identified as six downward peaks in between δ 44.4-28.9 ppm. The mass spectrum of the compound gave a molecular ion peak at 393.16763 which is the $[M+Na]^{+}$ peak.



Malabaricone D
Compound **5**

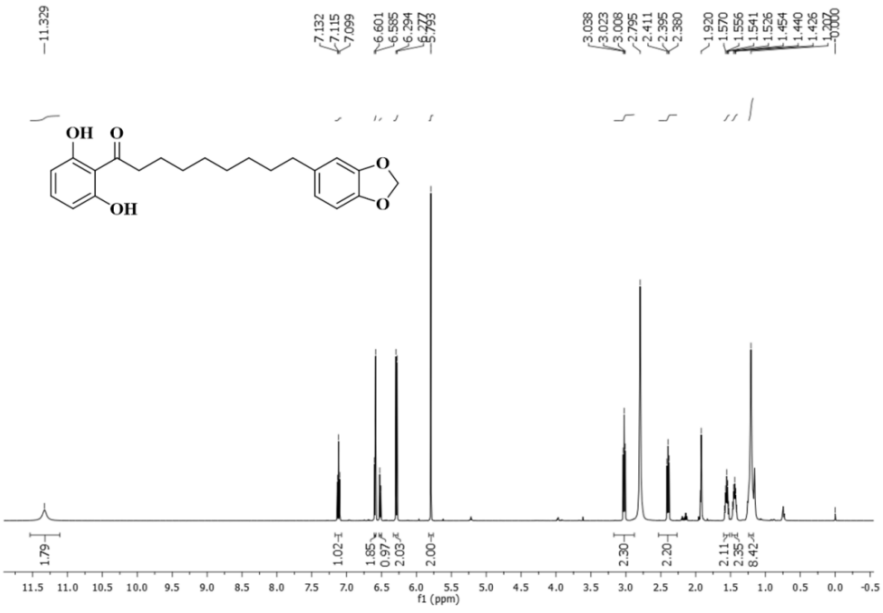


Figure 2A.13: ^{1}H NMR spectrum of malabaricone D

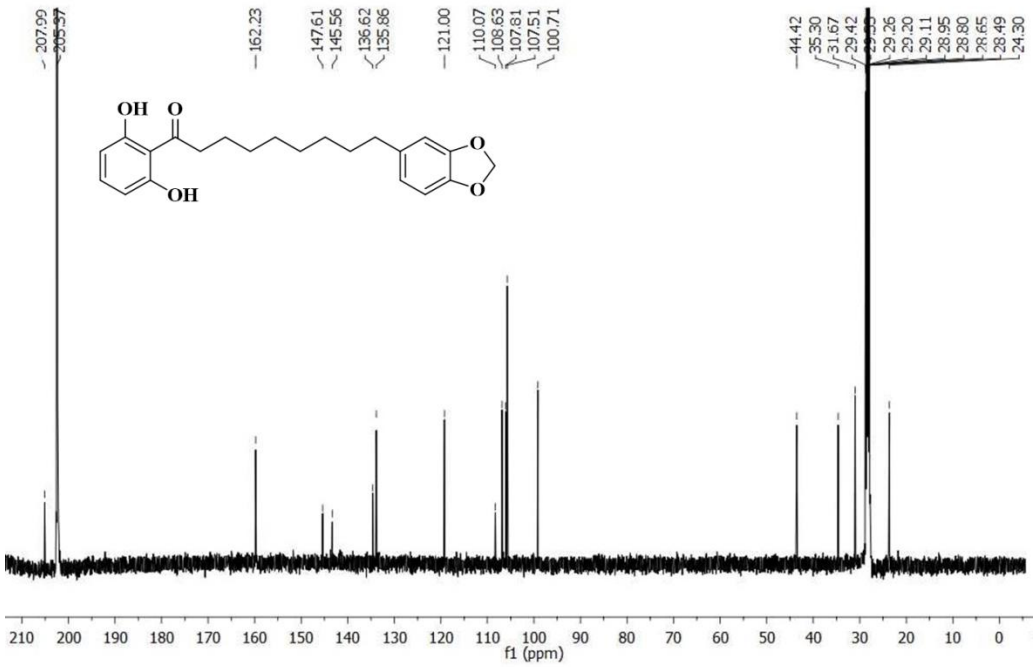
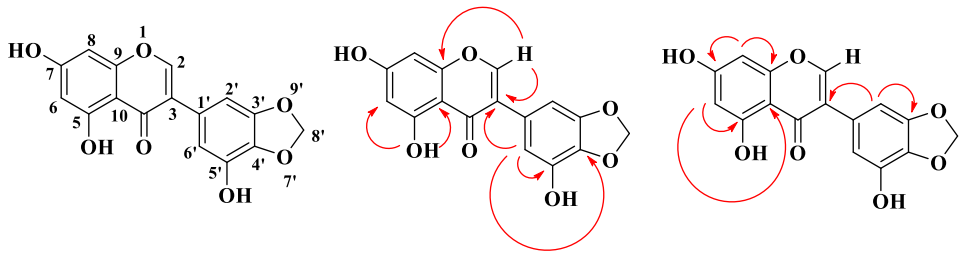


Figure 2A.14: ^{13}C NMR spectrum of malabaricone D

Fractions 36-45 of acetone extract showed the presence of a UV active spot. On charring in MC Gill solution the TLC turned into intense blue colour. It was then subjected to CC on silica gel using 20-25% ethyl acetate-hexane solution afforded the compound **6** as white powder. The IR absorption bands at 3390, 3371 and 1649 cm^{-1} specifies the presence of hydroxyl and carbonyl carbon functionalities. The ^{1}H NMR spectrum displayed three broad singlets at (δ_H 12.86, 9.70, 8.57 ppm) confirmed the presence of three hydroxyl groups. The one proton singlet at (δ_H 12.86 ppm) without carbon correlation in HMQC clearly indicates the presence of an *enolisable* hydroxyl group and the characteristic proton resonance of an isoflavone skeleton [17] was observed at (δ_H 8.02 (1H, s, H-2) ppm. Besides the ^{1}H NMR also exhibited two sets of doublets at [δ_H 6.29 (1H, d, *J*=2 Hz, H-8), δ_H 6.16 (1H, d, *J*=2 Hz, H-6) ppm] and [δ_H 6.64 (1H, d, *J*=1 Hz, H-2'), δ_H 6.57 (1H, d, *J*=1 Hz, H-6') ppm] indicate the presence of *meta* coupled aromatic protons of the isoflavone and another two proton singlet at δ_H 5.86 ppm for the methylenedioxy group. The ^{13}C NMR spectrum of the compound showed sixteen carbon resonances comprising of a carbonyl carbon (δ_C 180.5 ppm), a methylenedioxy carbon (δ_C 101.2 ppm), *olefinic* carbons (δ_C 153.9 and 122.9 ppm) and aromatic carbons (δ_C 164.2, 163.0, 158.1, 148.9, 140.6, 134.4, 125.1, 112.3, 105.2, 101.7, 99.0, 93.6 ppm). ^{1}H NMR, ^{13}C NMR and HMBC correlation data of the compound **6** is summarized in Table **2A.2**. The HMBC spectra displayed a long range ^{1}H-^{13}C correlation of H-2 to carbonyl carbon

(C-4), C-9 and C-3. The hydroxyl group at position 5 showed correlation with C-5, C-10 and C-6. The protons H-6 is correlated with C-5 and also has long range correlation with C-8 & C-10. Similarly H-8 has correlations with C-7/C-9 and C-6/C-10. The proton H-6' also has notable long range correlation with C-3 &C-2'. In addition ^{1}H-^{1}H COSY correlations of aromatic protons was also observed (Fig. **2A.15**). The molecular formula was assigned as $C_{16}H_{10}O_7$ on the basis of HRESIMS (positive-ion mode) ion peak at m/z 337.03302 $[M+Na]^+$ (calcd for $C_{16}H_{10}O_7Na$, 337.0427) together with NMR data. The structure was elucidated by the detailed 2D NMR analysis (COSY, HMQC and HMBC). To the best of our knowledge, there were no reports on the structure shown below (Fig. **2A.15**) and this molecule can be considered as a novel one.



5,7-dihydroxy-3-(5'-hydroxybenzo[*d*](7',9')-dioxol-1'-yl)-4*H*-chromen-4-one
Compound **6**

Figure 2A.15: Some selected HMBC interactions are represented in red curves

Table 2A.2: NMR data of the compound **6**

Position	**^{1}H NMR** (500MHz, CD_3COCD_3) δ in ppm	**^{13}C NMR** (125 MHz, CD_3COCD_3) δ in ppm	**HMBC Correlations**
2	8.02 (s, 1H)	153.9	158.1, 122.9,180.5, 125.1
3	-	122.9	
4	-	180.5	
5	12.86 (s,1H, -OH)	163.0	163.02, 105.2, 99.0
6	6.16 (d, *J*=2 Hz,1H)	99.0	163.02, 105.2, 93.6
7	9.70 (s, 1H, -OH)	164.2	
8	6.29 (d, *J*=2 Hz, 1H)	93.6	164.2, 158.1, 105.2, 99.0
9	-	158.1	
10	-	105.2	
1'	-	125.1	
2'	6.64 (d, *J*=1 Hz, 1H)	112.3	140.6, 134.4, 122.9, 101.7
3'	-	140.6	
4'	-	134.4	
5'	8.57 (s, 1H, -OH)	148.9	
6'	6.57 (d, *J*=1 Hz, 1H)	101.7	148.96, 134.4, 122.9, 112.3
8'	5.86 (s, 2H, -O-CH_2-O-)	101.2	134.4

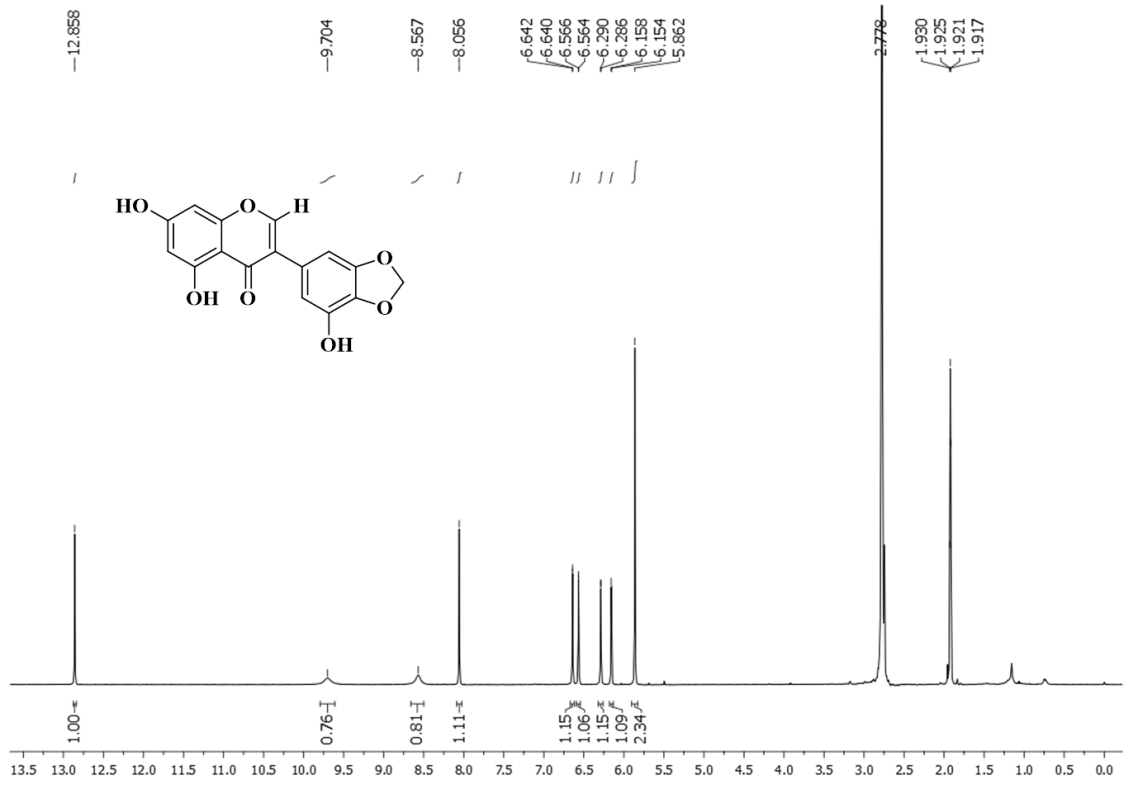


Figure 2A.16: ^{1}H NMR spectrum of Compound 6

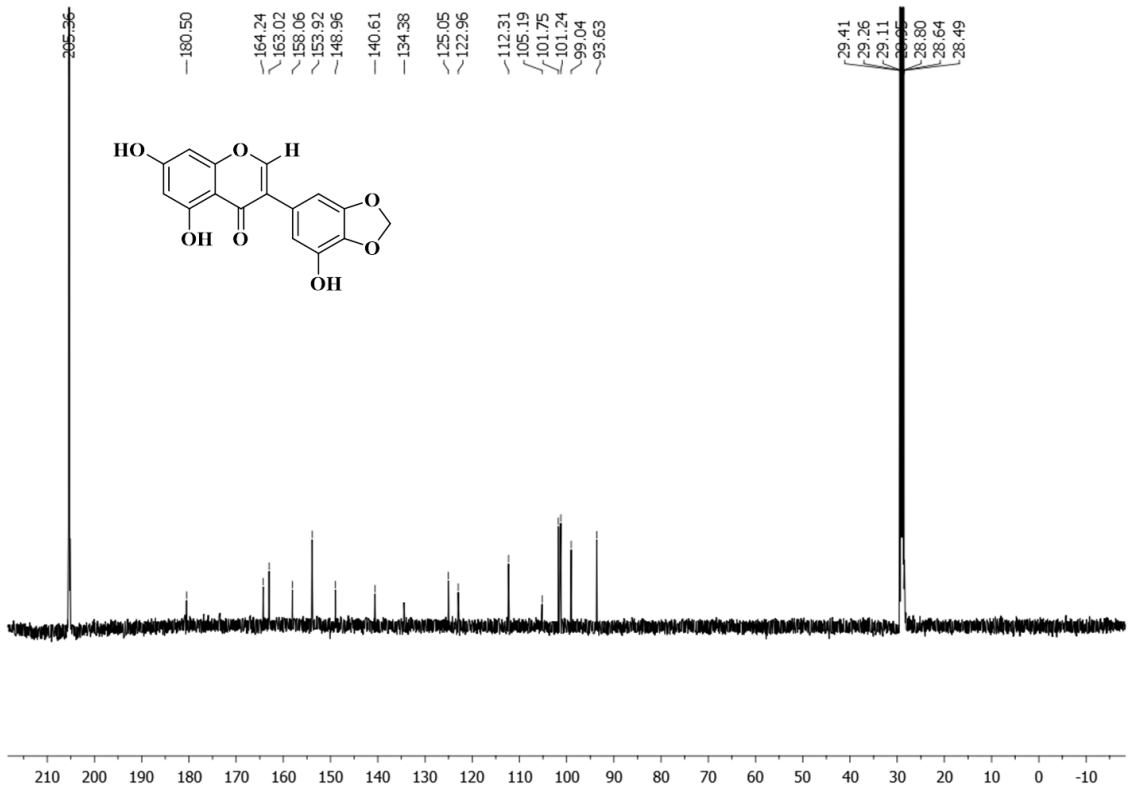


Figure 2A.17: ^{13}C NMR spectrum of Compound 6

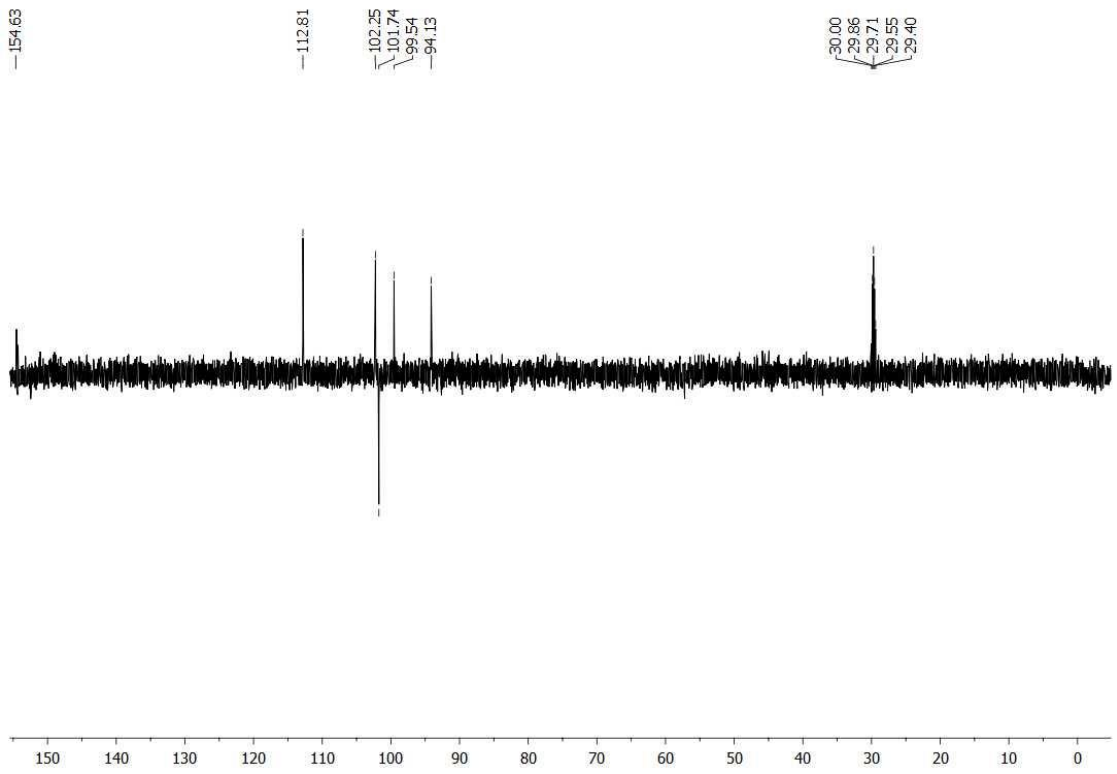


Figure 2A.18: DEPT 135 NMR spectrum of Compound 6

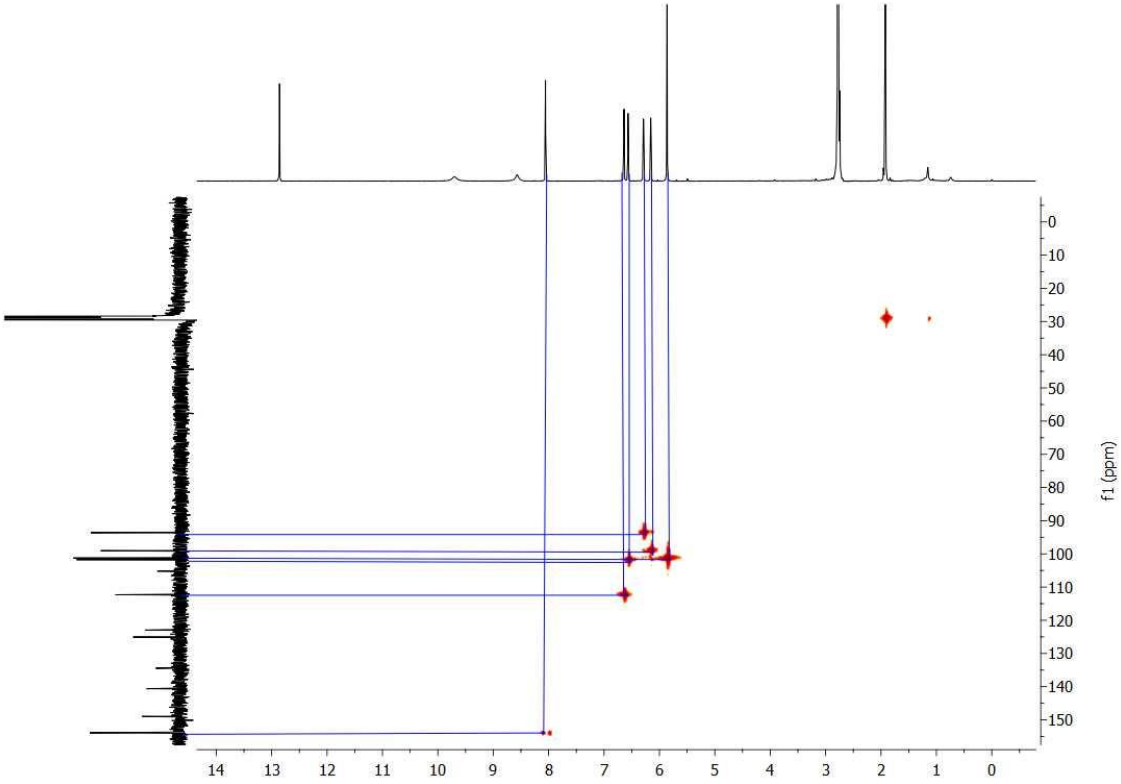


Figure 2A.19: HMQC correlations of Compound 6

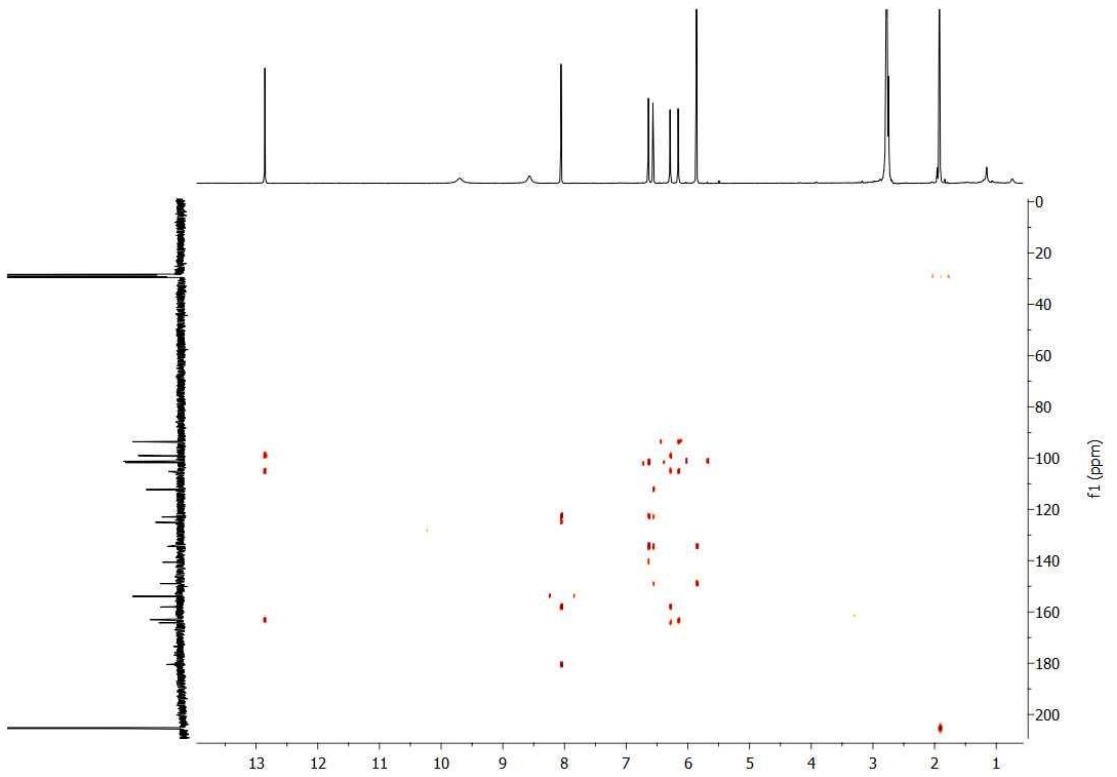


Figure 2A.20: HMBC correlations of Compound 6

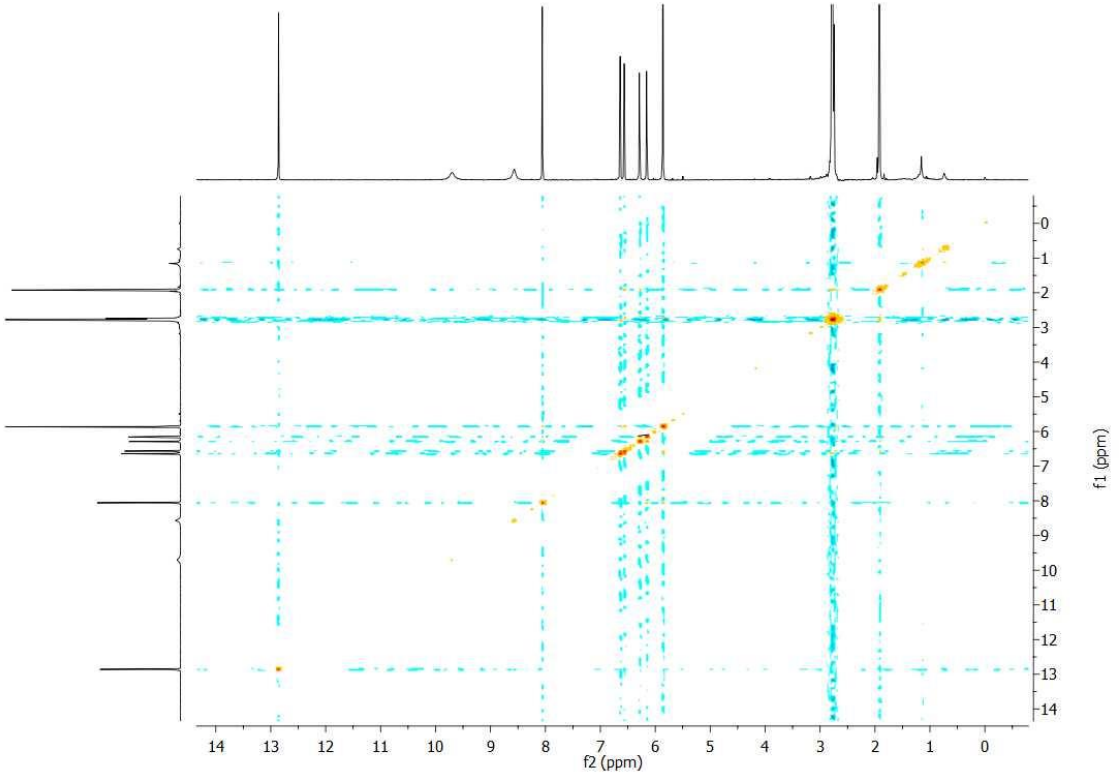
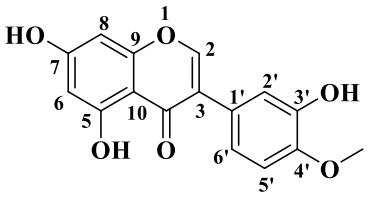


Figure 2A.21: ^{1}H-^{1}H correlations of Compound 6

Compound **7** was obtained as UV active white solid from the fraction pool 46-52 of acetone extract after column chromatographic separation using 15% ethyl acetate-hexane solution. Spectral data of the compound **7** was similar to that of compound **6** suggests the presence of an isoflavanoid moiety. In the ^{1}H NMR spectrum three broad singlets at δ 12.91, 9.70 and 7.60 ppm integrating for one proton each indicate the presence of three hydroxyl groups. A sharp one proton singlet at δ 8.05 ppm with corresponding carbon resonated at δ 153.7 ppm is one of the characteristic peaks of isoflavanoid moiety. The three proton sharp singlet at δ 3.75 ppm indicates the presence of a -OCH_3 group and the methoxy carbon resonated at δ 55.4 ppm. The two doublets at δ 6.29 and 6.16 ppm integrating for one proton each with a coupling constant of 2 Hz having carbons at δ 93.6 and 99.0 ppm indicate the presence of a *meta* coupled aromatic protons. In the ^{1}H NMR spectrum a doublet at δ 6.87 ppm with a coupling constant of 8 Hz suggests the presence of an *ortho* coupled proton. The two protons at δ 7.00 (d, J=2.5Hz), and 6.92 (dd, J_1=8.5Hz, J_2=2Hz) ppm clearly suggest an *ortho* coupled aromatic system, along with a *meta* coupled proton. The mass spectrum of the compound gave a molecular ion peak at 323.05375 which is the $[M+Na]^+$ peak. By comparing all the spectral data and literature reports the compound **7** was identified as 3'-hydroxy biochanin A [18].



3'-hydroxy biochanin A
Compound **7**

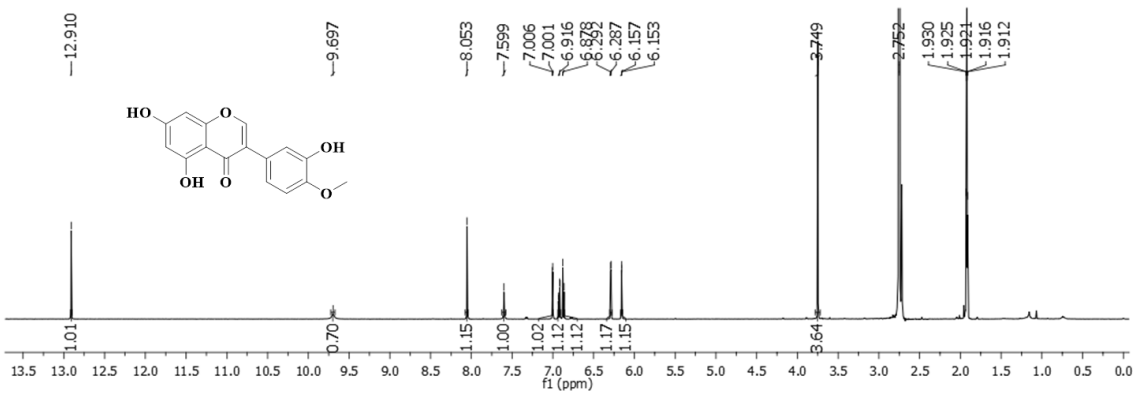


Figure 2A.22: ^{1}H NMR spectrum of 3'-hydroxy biochanin A

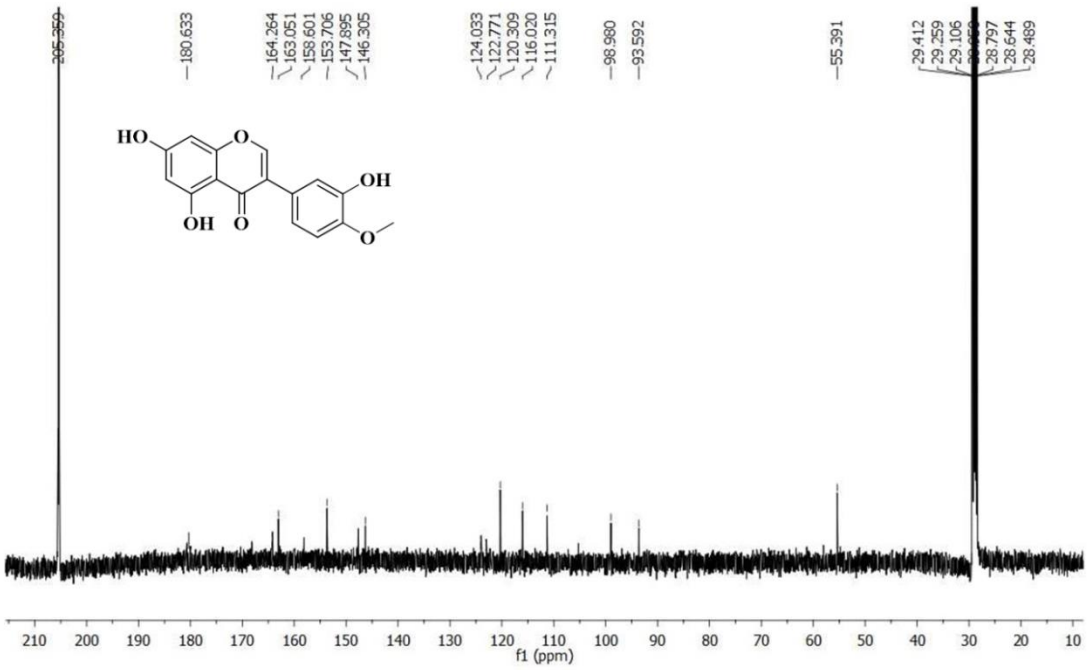
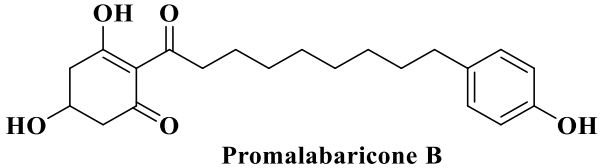


Figure 2A.23: ^{13}C NMR spectrum of 3'-hydroxy biochanin A

Fraction pool 53-57 from the acetone extract showed the presence of an intense UV active spot. On charring in Enholm yellow solution the TLC turned into intense red colour. It was then subjected to CC on silica gel using 40% ethyl acetate-hexane solution afforded the compound **8** as pale yellow crystalline solid. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be promalabaricone B [19]. ^{1}H NMR spectrum showed a triplet at δ 3.00 ppm integrating for two protons indicating the presence of -CO-CH_2 group. Peaks at δ 204.8 and 197.3 ppm in the ^{13}C NMR spectrum confirmed the presence of two carbonyl carbons. Two aromatic protons (12 and 14) adjacent to phenolic hydroxyl group resonated at δ 6.75 ppm as doublet with a coupling constant of 8.5 Hz. Remaining two aromatic protons (11 and 15) resonated at δ 7.02 ppm as doublet with a coupling constant of 8.5 Hz. A multiplet at δ 4.40 ppm integrating for one proton could be attributed to the -CH proton of the -CH-OH. In DEPT-135 spectrum -CH_2 were identified as eight downward peaks in between δ 46.8-24.6 ppm. The mass spectrum of the compound gave a molecular ion peak at 383.18257 which is the $[M+Na]^+$ peak.



Promalabaricone B
Compound **8**

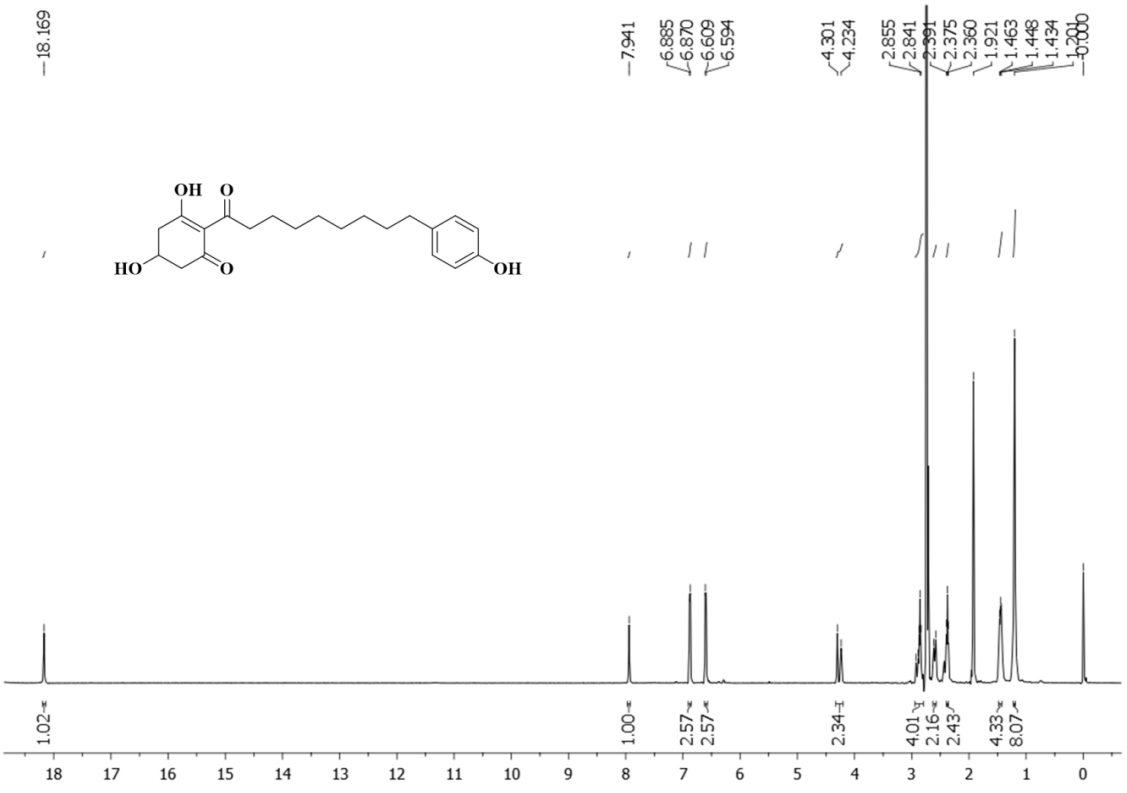


Figure 2A.24: ^{1}H NMR spectrum of promalabaricone B

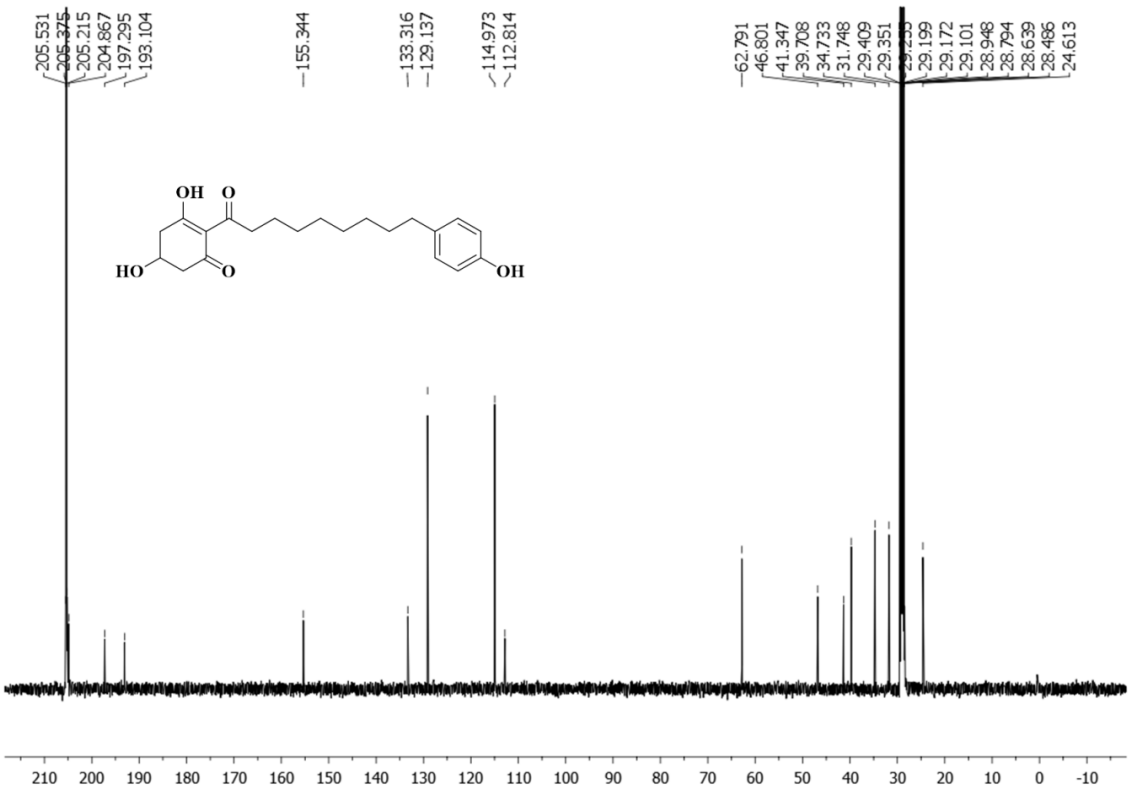


Figure 2A.25: ^{13}C NMR spectrum of promalabaricone B

2A.3.3. Extraction of the Seeds of *M. beddomei*

Seeds of *M. beddomei* were separated from the fruit and dried in an air oven maintained at 45-50 °C for five days and coarsely powdered. Around 800 g of dried seed was extracted with 5 L hexane at room temperature for 3 days. The extract was then filtered and concentrated under reduced pressure afforded 20 g of hexane extract. The residue was again extracted with DCM (3 L x 3 days). Removal of solvent under reduced pressure gave 55 g of crude DCM extract. The procedure was repeated and the residue was further subjected to successive extraction with acetone, ethanol and finally with water to obtain 50 g, 16 g and 10 g of acetone, ethanol and water extract respectively.

2A.3.3.1. Isolation and Characterization of Phytochemicals from the Seed

About 55 g of the DCM extract was subjected to silica gel (100-200 mesh) column chromatography. The column was eluted successively with gradient mixtures of hexane and ethyl acetate of increasing polarities and finally with pure ethyl acetate. A total of 66 fractions of 400 ml each were collected and based on the similarities in TLC they were pooled in to 13 fraction pools.

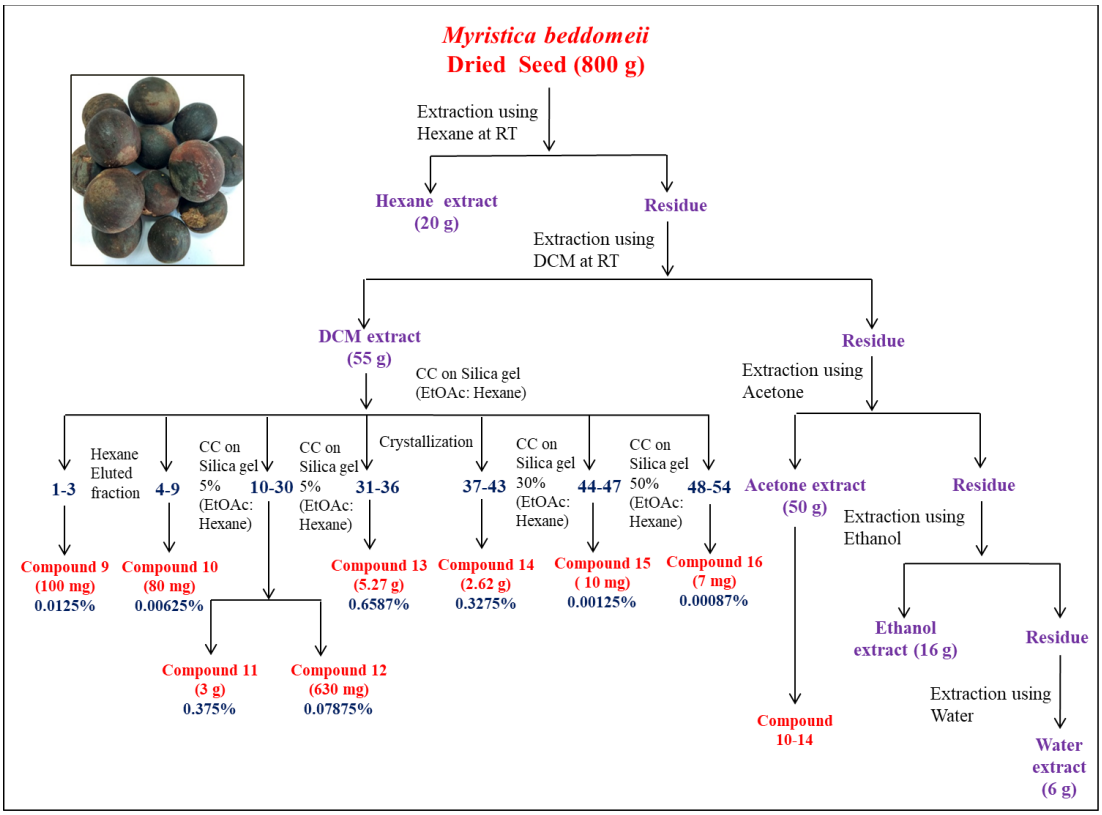
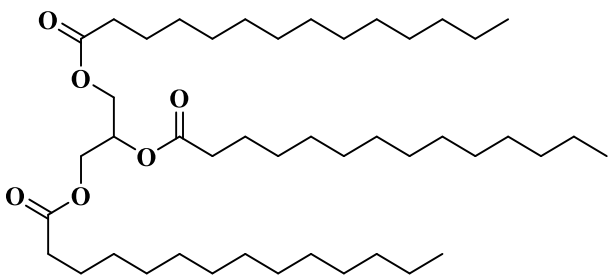


Figure 2A.26: Schematic representation of extraction and isolation procedure

Similarly around 50 g of the acetone extract afforded 15 fraction pools after column chromatographic separation using hexane, hexane-ethylacetate mixtures of increasing polarity. The fraction pools obtained were subjected to repeated column chromatographic separation and crystallization techniques led to the isolation of following compounds. A schematic representation of the entire extraction and isolation procedure is given in Fig. **2A.26**.

Fraction pool 1-3 on crystallization using hexane afforded compound **9** as white amorphous solid. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be a triglyceride trimyristin [20]. IR spectrum of the compound showed strong absorptions between 2955-2850 cm^{-1} and 1735 cm^{-1} suggested the presence of alkane and ester group. The presence of ester group was further confirmed from the peak obtained at δ 172.5 ppm in the ^{13}C NMR spectrum. In the ^{1}H NMR spectrum a triplet at δ 2.32 ppm integrating for six protons confirmed the presence of three -CH_2 attached to carbonyl group. DEPT 135 spectrum confirmed the presence of ten -CH_2 groups resonated in the region of δ 22.5-61.8 ppm. In the ^{1}H NMR spectrum aliphatic protons resonated at δ 1.62-1.34 ppm. A multiplet δ 5.38- 5.36 ppm indicates the presence of -CH proton in between three ester moiety. A triplet at δ 0.89 ppm integrating for nine protons suggests the presence of three methyl groups. The mass spectrum of the compound gave a molecular ion peak at 745.63288 which is the $[M+Na]^{+}$ peak.



Trimyristin
Compound **9**

Compound **10** was obtained as pale yellow crystalline solid after column chromatographic separation of the fraction 4-9 using 5% ethyl acetate-hexane solution. Detailed spectral analysis confirmed that the compound is similar to that of compound **2** which was obtained earlier from the fruit rinds.

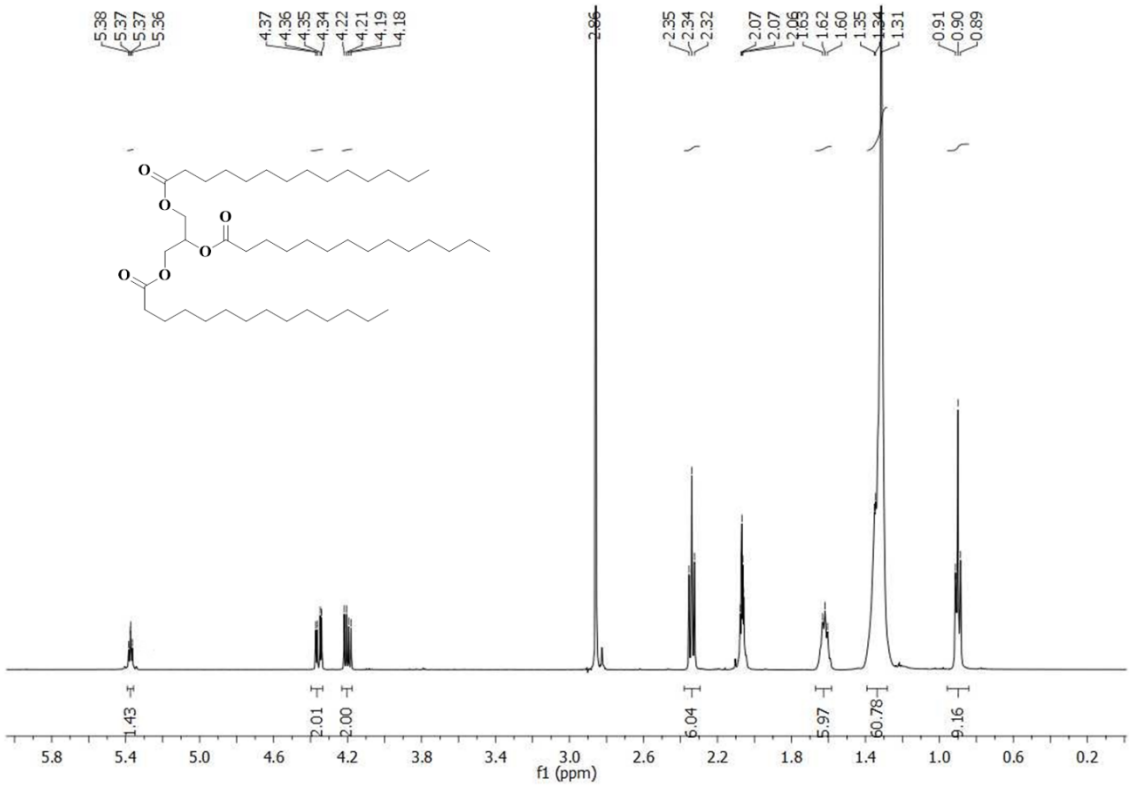


Figure 2A.27: ^{1}H NMR spectrum of trimyristin

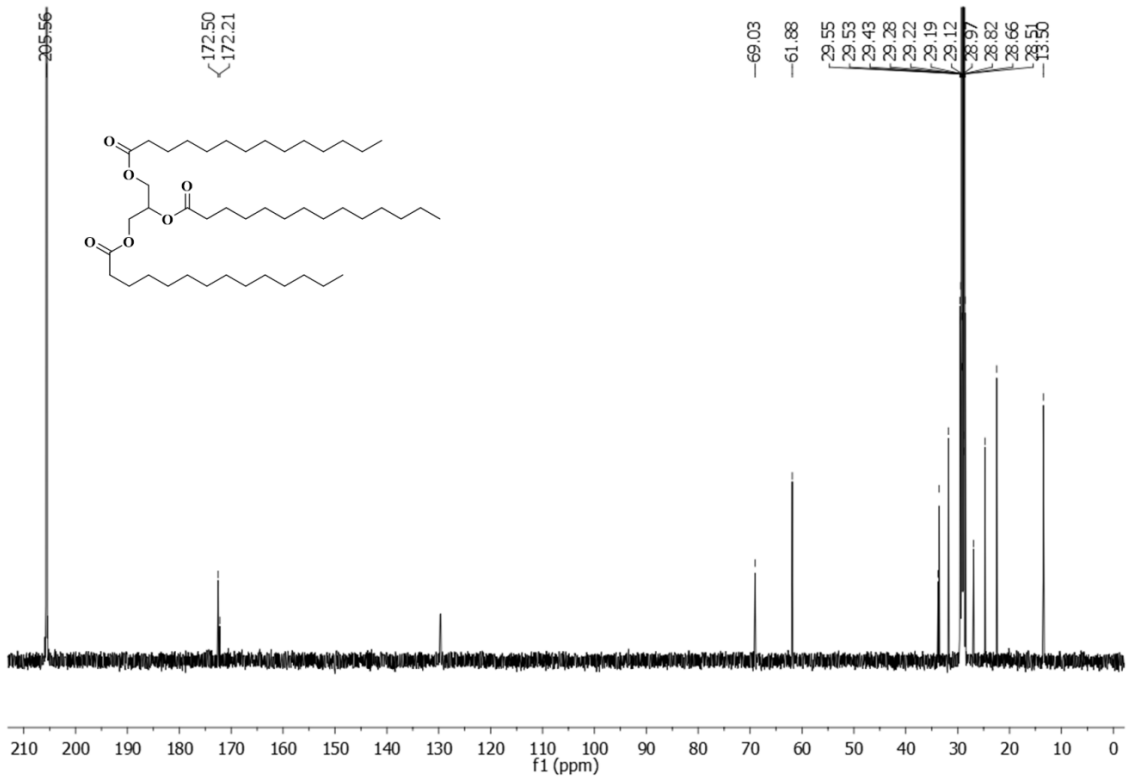


Figure 2A.28: ^{13}C NMR spectrum of trimyristin

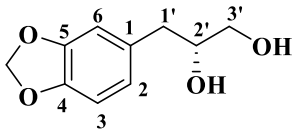
Fraction pool 10-30 showed the presence of two UV active spots having almost similar R_f value. On charring in Enhollm yellow solution both the spot in the TLC become intense red colour. CC separation using 5% ethyl acetate-hexane solution afforded the compound **11** as pale yellow crystals. Based on the spectral analysis, the compound was identified as malabaricone A, similar to compound **1** isolated earlier from the fruit rind. Compound **12** was afforded as white powder after eluting the column with 5% ethyl acetate-hexane solution which was also found to similar to that of compound **5** (Malabaricone D) isolated from the fruit rinds.

Fraction pool 31-36 showed the presence of an intense UV active spot. On charring in Enholm yellow solution the TLC turned into intense pink colour. Crystallization using DCM-hexane afforded the compound as pale yellow solid. It exhibited similar NMR characteristics of the compound **3**. The compound **13** was further confirmed as malabaricone B by TLC co-spotting with authentic sample.

Compound **14** was afforded as yellow solid from the fraction pool 37-43 by column chromatographic technique on silica gel by eluting 30% ethyl acetate-hexane. By comparing the spectral data and TLC co-spotting with an authentic sample the compound was identified as malabaricone C similar to that of compound **4** isolated from the fruit rinds.

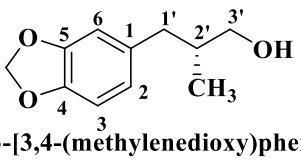
Fraction pool 44-47 showed the presence of an intense UV active spot, while charring in Enhollm yellow solution the TLC turned into intense blue colour. Column chromatographic separation of this fraction using 15% ethyl acetate-hexane solution afforded the compound **15** as colorless liquid. In the ^{1}H NMR spectra a sharp singlet at δ 5.92 ppm integrating for two protons indicates the presence of -O-CH_2-O- group. In the ^{13}C NMR spectrum methylene dioxy group resonated at δ 100.8 ppm. Presence of three -CH_2 groups is confirmed from the three downward peaks appeared at δ 100.8, 60.3, 25.9 ppm in the DEPT 135 spectrum. The two diasterotopic protons of position 3' resonated as two doublet of doublet at δ 3.78 and 3.58 ppm. The other two protons at position 1' appeared as one proton multiplet at δ 2.77 and 2.63 ppm and the corresponding carbon obtained at δ 35.9 ppm in the ^{13}C NMR spectrum. In the ^{1}H NMR spectra a single proton doublet at δ 6.71 ppm with a coupling constant of 7.5 Hz indicates the presence of an *ortho* coupled proton. The one proton doublet of doublet displayed at δ 6.60 ppm indicates the presence of an aromatic proton having both *ortho* and *meta* coupling (Position 2). The -CH protons of the carbon bearing a hydroxyl group appeared as a multiplet at δ 1.84-1.83 ppm. A one proton broad singlet at δ 3.26 ppm confirmed the

presence of a hydroxyl group. The mass spectrum of the compound gave a molecular ion peak at 197.07356 which is the $[M+H]^+$ peak. Finally the compound was identified as (2R)-3(3, 4-methylenedioxyphenyl) 1, 2-propanediol based on the spectral analysis [21-22].



(2R)-3(3, 4-methylenedioxyphenyl) 1, 2-propanediol
Compound **15**

Compound **16** was isolated as a UV active blue coloured liquid from the fraction pools 48-54 (50-60%) using column chromatographic separation. Spectral data of the compound showed similarities in basic structure with compound **15** the only difference in functional group at position 2. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be 2R'-methyl-3-[3, 4-(methylenedioxy) phenyl] 1'-propanol [23]. In the ^{1}H NMR spectra a sharp three proton singlet at δ 1.30 ppm indicates the presence of a -CH_3 group and the methyl carbon appeared at δ 24.1 ppm in the ^{13}C NMR spectrum. In the ^{1}H NMR spectra a sharp singlet at δ 5.92 ppm integrating for two protons indicates the presence of -O-CH_2-O- group. In the ^{13}C NMR spectrum methylene dioxy group resonated at δ 100.8 ppm. Presence of three -CH_2 group was confirmed from the three downward peaks at δ 36.9, 43.7 and 100.8 ppm in the DEPT 135 NMR spectra. In the ^{1}H NMR spectra a two proton doublet at δ 2.68 ppm indicates the presence of -CH_2 proton attached to benzene ring. The remaining two diasterotopic protons appeared at δ 3.84 (d, J=12.5 Hz, 1H) and δ 3.34 (dd, J_1=12.5 Hz, J_2=4.5 Hz 1H) ppm. A one proton multiplet at δ 1.60 ppm indicates the presence of -CH proton (position 2') and the corresponding carbon appeared at δ 43.7 ppm. The carbon bearing hydroxyl group obtained at δ 60.5 ppm in the ^{13}C NMR spectrum. . The mass spectrum of the compound gave a molecular ion peak at 195.0943 which is the $[M+H]^+$ peak.



2'R-methyl-3-[3,4-(methylenedioxy)phenyl]1'-propanol
Compound **16**

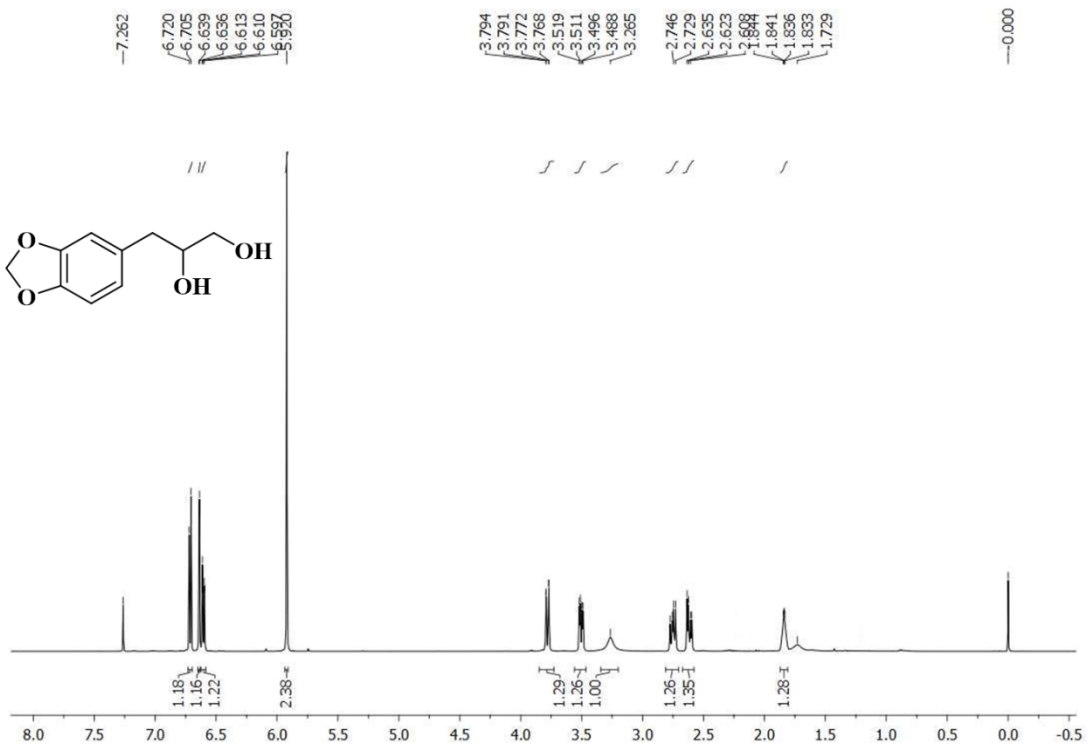


Figure 2A.29: ^{1}H NMR spectrum of (2R)-3(3, 4-methylenedioxyphenyl) 1, 2-propanediol

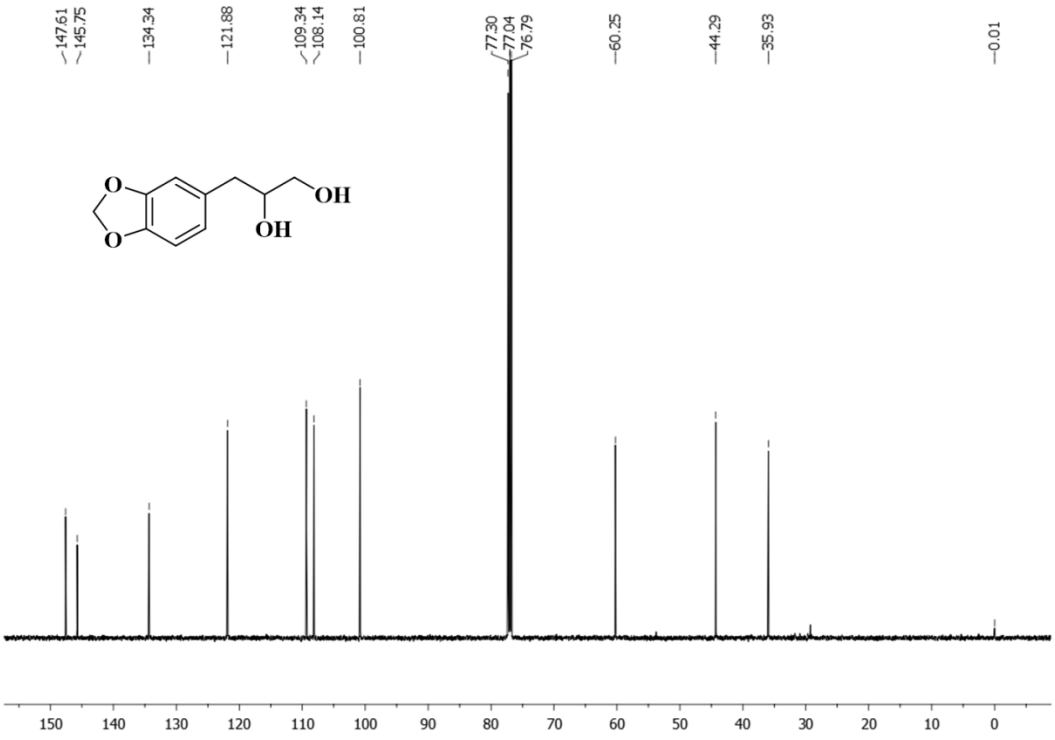


Figure 2A.30: ^{13}C NMR spectrum of (2R)-3(3, 4-methylenedioxyphenyl) 1, 2-propanediol

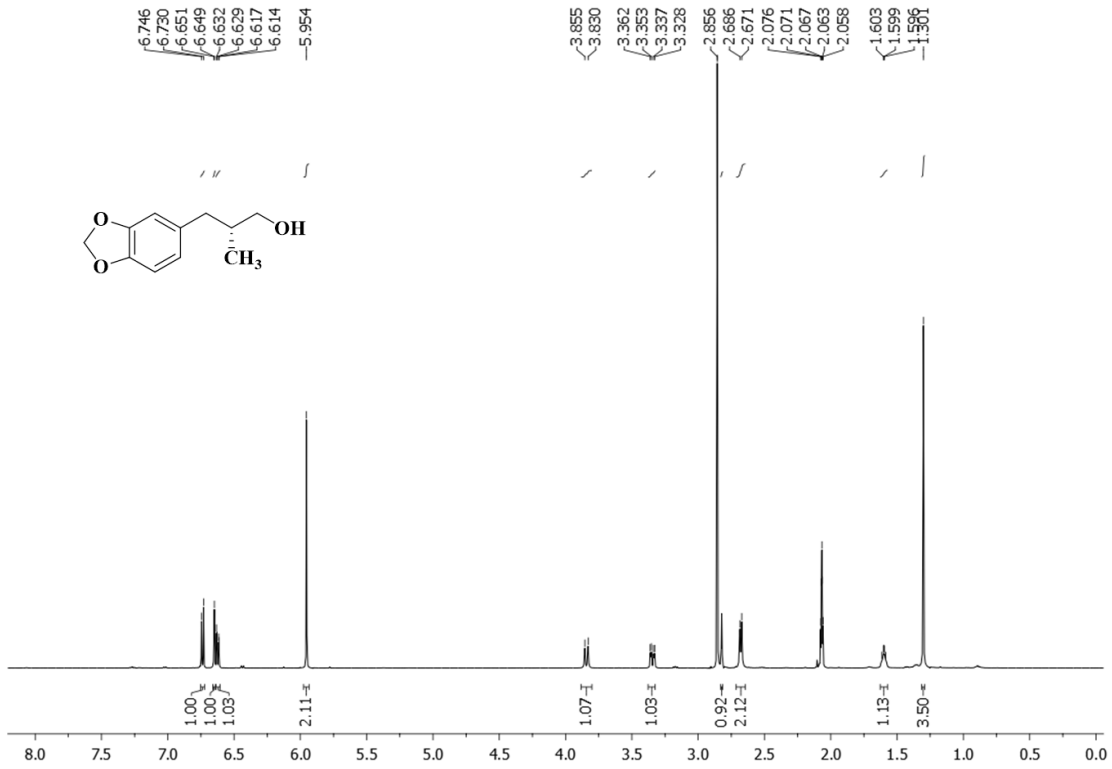


Figure 2A.31: ^{1}H NMR spectrum of 2'R-methyl-3-[3,4-(methylenedioxy) phenyl] 1'-propanol

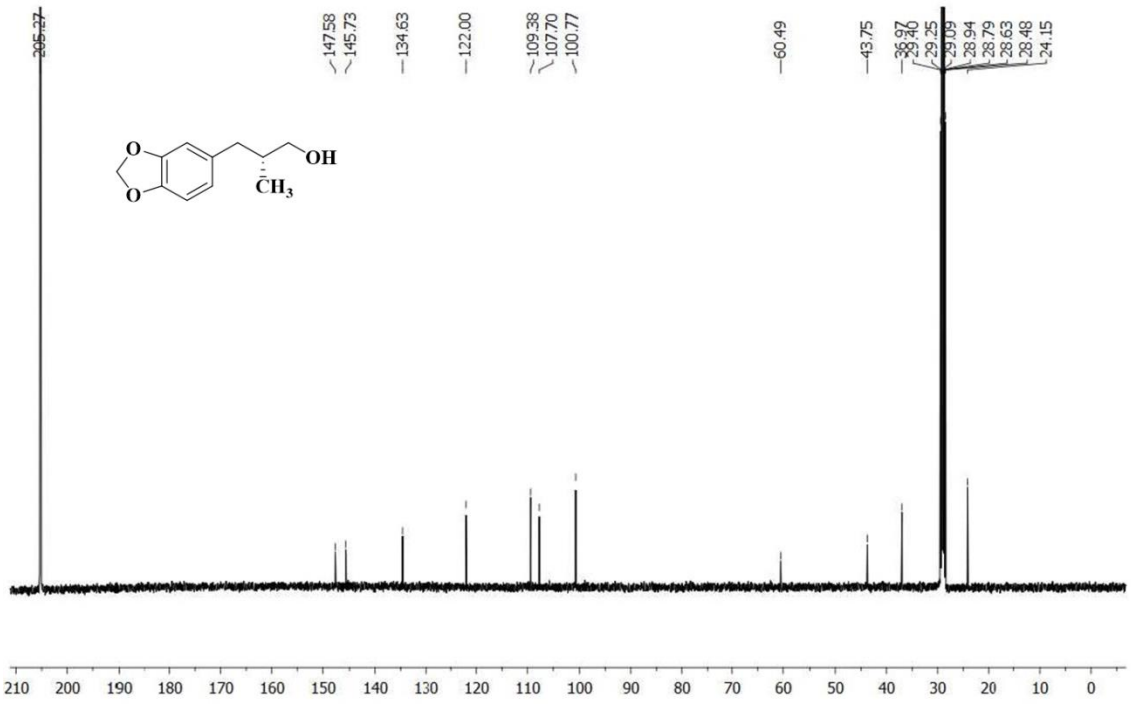


Figure 2A.32: ^{13}C NMR spectrum of 2'R-methyl-3-[3,4-(methylenedioxy) phenyl] 1'-propanol.

2A.3.4. Extraction of the Stem bark of *M. beddomei*

Initially the collected stem bark were cut into small pieces and dried in an air oven maintained at 45-50 ^{0}C. The dried material was then coarsely powdered and about 600 g of powdered material was subjected to extraction using 2.5 L DCM for three days. The extract was then filtered and concentrated under reduced pressure using a rotary evaporator afforded 14 g of DCM extract. Using the residue the entire extraction process was repeated with acetone followed by ethanol and finally with water in order to obtain 20 g of acetone, 10 g of ethanol and 8 g of water extract.

2A.3.4.1. Isolation and Characterization of Phytochemicals from the Stem bark

Based on the similarities in the TLC analysis of DCM and acetone extract both were combined and subjected to column chromatographic separation. An aliquot of the combined extract was sub divided by column chromatographic separation using hexane, hexane-ethyl acetate mixtures of varying polarity resulted in fourteen fraction pools. All the fraction pools were again subjected to repeated column chromatographic separation and purification with hexane-ethyl acetate polarities for the isolation of active components.

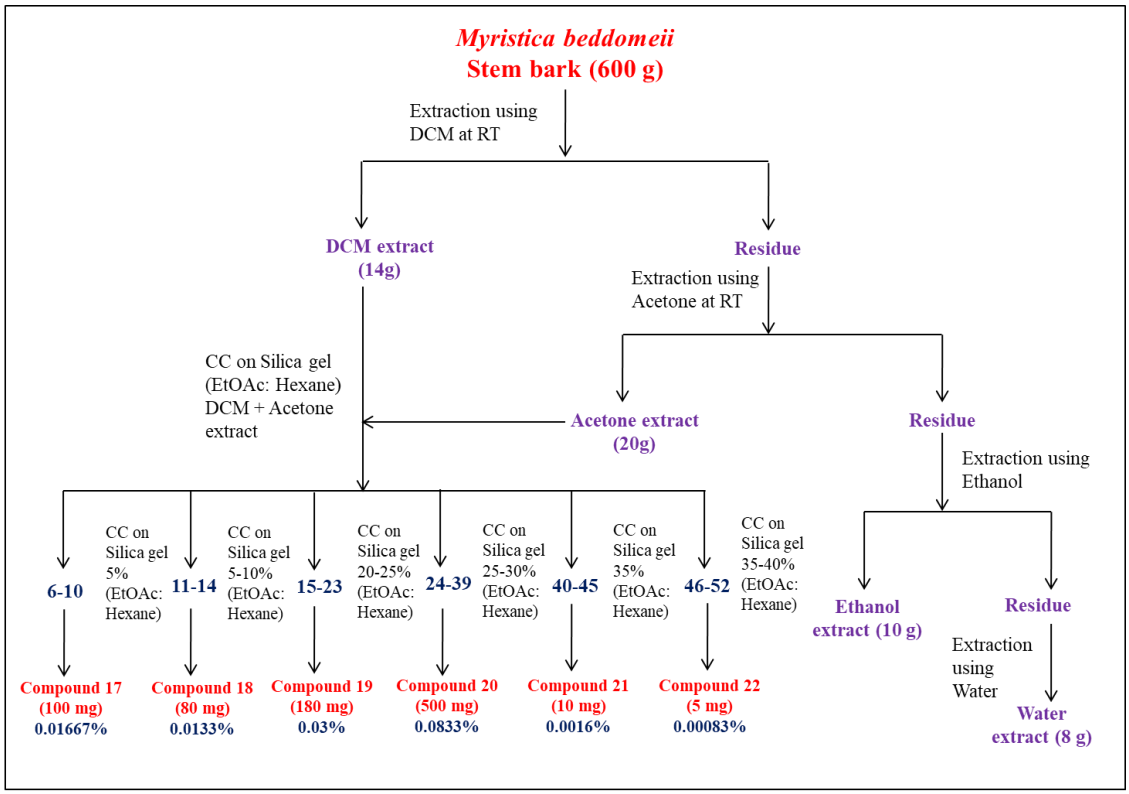
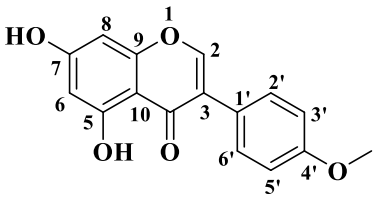


Figure 2A.33: Schematic representation of extraction and isolation procedure

Compound **17-20** were isolated from the fractions of extract and identified as malabaricone A, malabaricone D, malabaricone B and malabaricone C respectively which was also isolated from the fruit rinds and seeds.

Fraction pool 40-45 showed the presence of an intense UV active spot. On charring in Enholm yellow solution the TLC turned into intense red colour. After column chromatographic purification using 30-35% ethyl acetate- hexane afforded the compound as white powder. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound **21** was confirmed to be Biochanin A [24]. In the ^{1}H NMR spectrum, a sharp singlet at δ 8.20 ppm integrating for one proton indicating the proton of the B ring of flavonoid moiety. It is one of the characteristic peaks. Two aromatic protons (3' and 5') adjacent to methoxy group resonated at δ 6.43 ppm as doublet with a coupling constant of 2 Hz. Remaining two aromatic protons of the ring (2'and 6') resonated at δ 7.55 ppm as doublet. Presence of two sharp singlets at δ 9.82 and 13.01 ppm integrating each for one proton indicate the presence of two phenolic hydroxyl groups. A sharp singlet at δ 3.85 ppm integrating for three protons confirmed the presence of a methoxy group. Presence of carbonyl group is confirmed from the ^{13}C NMR which is resonated at δ 181.6 ppm. The mass spectrum of the compound gave a molecular ion peak at 285.07595 which is the $[M+H]^{+}$ peak.



Biochanin A
Compound **21**

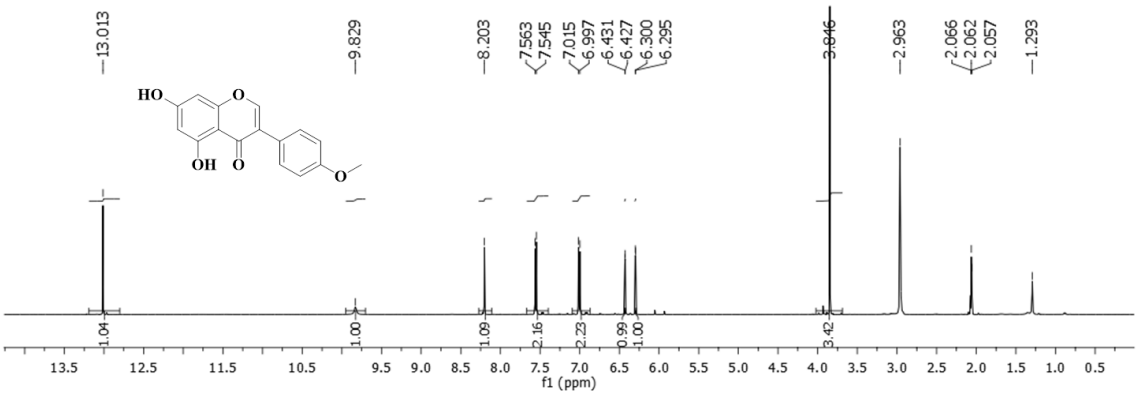


Figure 2A.34: ^{1}H NMR spectrum of biochanin A

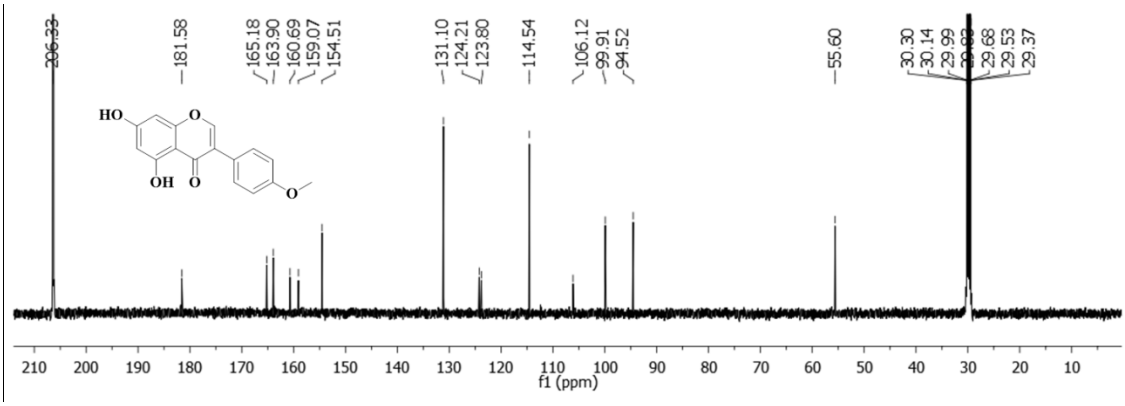


Figure 2A.35: ^{13}C NMR spectrum of biochanin A

Compound **22** afforded as a pale yellow solid from the fraction pool 46-52 after column chromatographic purification using 35-40% ethyl acetate-hexane. The molecular formula $C_{15}H_{14}O_5$ was established on the basis of HRESIMS (positive ion mode) molecular ion obtained at m/z 274.27429 $[M]^+$ (calcd for $C_{15}H_{14}O_5$, 274.2720). The IR spectrum showed characteristic absorption for hydroxyl group (3178 cm^{-1}), hydrogen bonded hydroxyl group (2885 cm^{-1}), carbonyl groups (1634 cm^{-1}) and aromatic moiety (1459 cm^{-1}). UV spectrum absorption maxima of the compound at 240 and 289 nm revealed a *β*-hydroxydihydrochalcone skeleton [25-26]. A sharp one proton singlet at (δ_H 12.04 ppm) in the ^{1}H NMR spectrum confirms the presence of an intra-molecularly hydrogen bonded hydroxyl group. The ^{1}H NMR also displayed two doublet of doublets at [δ_H 3.09 (1H, dd, J_1=13 Hz, J_2=3 Hz H-2a) and δ_H 2.83 (1H, dd, J_1=17 Hz, J_2=3 Hz H-2b) ppm] indicates the two *diasterotopic* protons of the methylene group. The -CH proton of position **3** obtained as a doublet of doublets at δ_H 5.43 (1H, dd, J_1=13 Hz, J_2=3 Hz H-3) ppm. Besides the presence of an aromatic ring with 2, 4, 6-tri substitution confirmed from the signals at δ_H 6.01 (2H, s) ppm. The protons of the second aromatic ring obtained at [δ_H 7.45-7.42 (4H, m) and 7.41-7.39 (1H, m) ppm]. The ^{13}C NMR spectrum of the compound exhibited fifteen carbon resonances including a carbonyl carbon (δ_C 195.8 ppm), a methylene carbon (δ_C 43.3 ppm), carbon bearing a hydroxyl group (δ_C 79.2 ppm) and aromatic carbons (δ_C 164.8, 164.3, 163.2, 138.3, 128.9, 128.9, 126.2, 103.2, 96.8, 95.5 ppm). In addition the DEPT 135 spectrum confirms the presence of a methylene carbon at δ_C 43.3 ppm. ^{1}H NMR, ^{13}C NMR and HMBC correlation data of the compound **2** is summarized in Table **2A.3**.

The connectivity of the protonated carbons C_2-C_3 was established from ^{1}H-^{1}H COSY (Fig. **2A.36**) interactions. In addition ^{1}H-^{1}H COSY correlations of aromatic protons was also observed. The key HMBC correlations (Fig. **2A.36**) between H-2 and C-

1, H-2 and C-3, H-2 and C-1"as well as correlations between H-3 and C-1'' suggest that a *β*-hydroxyl carbonyl moiety is flanked between two aromatic rings. The compound appears to be the first report on the occurrence of a *β*-hydroxydihydrochalcone in *M. beddomei*. *β*-hydroxydihydrochalcones are a rare subclass of flavonoids. Only few examples of these types from natural resources are reported, while *α* and *β*-dihydroxyhydrochalcones are still less common [27-28]. Compound showed a specific roation $[\alpha]^{25.5}_{D}$ +106.6 (c 0.1277, $CHCl_3$). The compound appears to be the first report on the occurrence of a *β*-hydroxydihydrochalcone in *M. beddomei*.

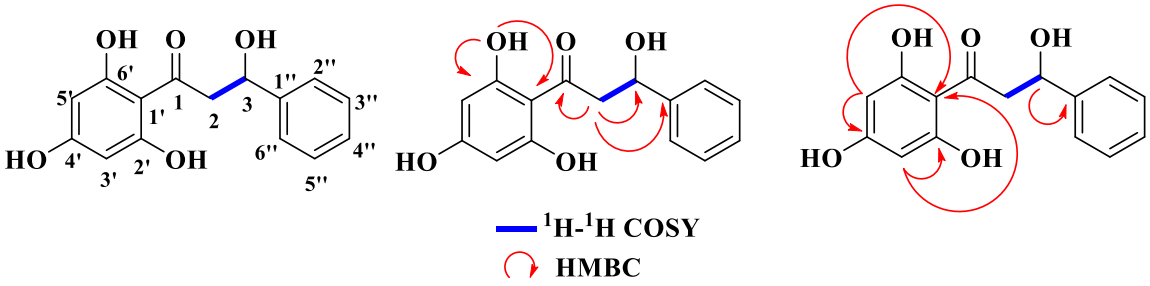


Figure 2A.36: Some selected HMBC and ^{1}H-^{1}H COSY interactions of Compound **22**

Table 2A.3: ^{1} H and ^{13}C NMR data of the Compound **22** (*δ* in ppm, *J* in Hz)

Position	δ_H (mult. *J*, Hz) (500 MHz, $CDCl_3$)	δ_C (125 MHz, $CDCl_3$)	HMBC Correlations
1		195.8	
2	3.09, dd, (13, 3) 2.83, dd, (17, 3)	43.3	195.8, 79.2, 138.3
3	5.43, dd, (13, 3)	79.2	138.3, 128.9
1'		103.2	
2'	12.04 (s, 1H)	164.3	103.2, 164.3
3'	6.01 (s,1H)	96.8	164.2, 103.2
4'		164.8	
5'	6.01 (s,1H)	95.5	163.2, 103.2
6'		163.2	
1''		138.3	
2''	7.45-7.42 (m, 4H)	128.9	138.3
3''	7.41-7.39 (m, 1H)	128.9	
4''		126.2	
5''		128.9	
6''		128.9	138.3

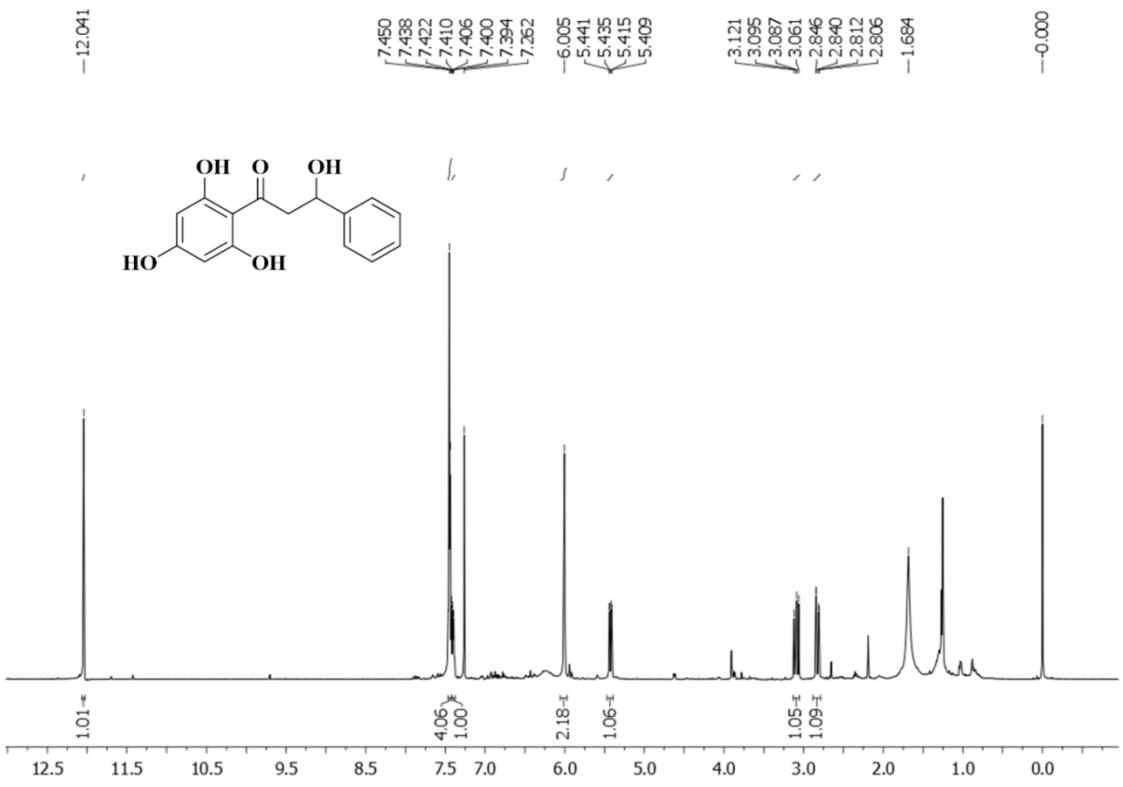


Figure 2A.37: ^{1}H NMR spectrum of (3(S)-hydroxy-3-phenyl-1-(2',4',6'-trihydroxyphenyl)propan-1- one

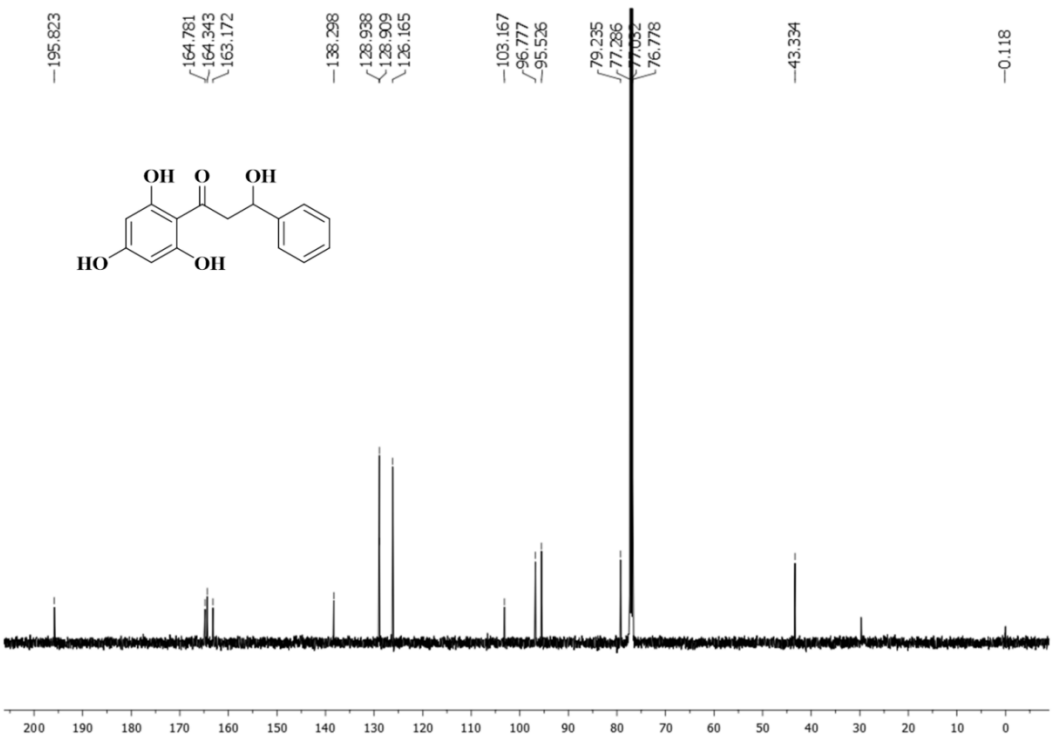


Figure 2A.38: ^{13}C NMR spectrum of (3(S)-hydroxy-3-phenyl-1-(2',4',6'-trihydroxyphenyl)propan-1- one

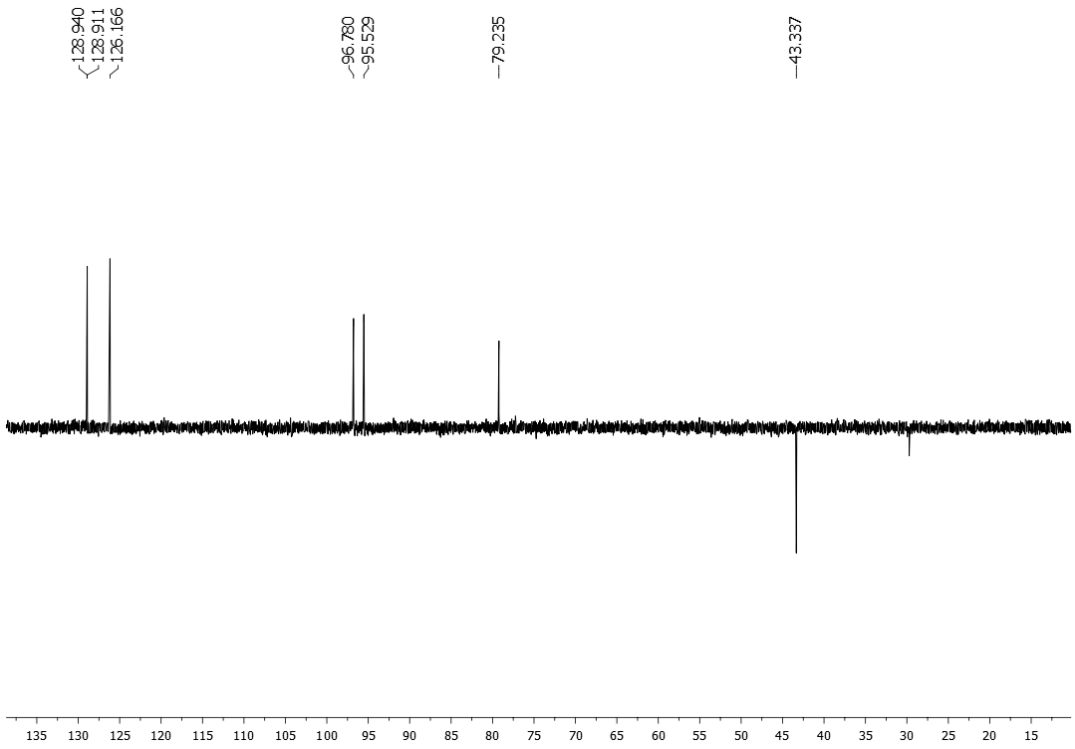


Figure 2A.39: DEPT 135 NMR spectrum of (3(S)-hydroxy-3-phenyl-1-(2',4',6'-trihydroxyphenyl)propan-1- one

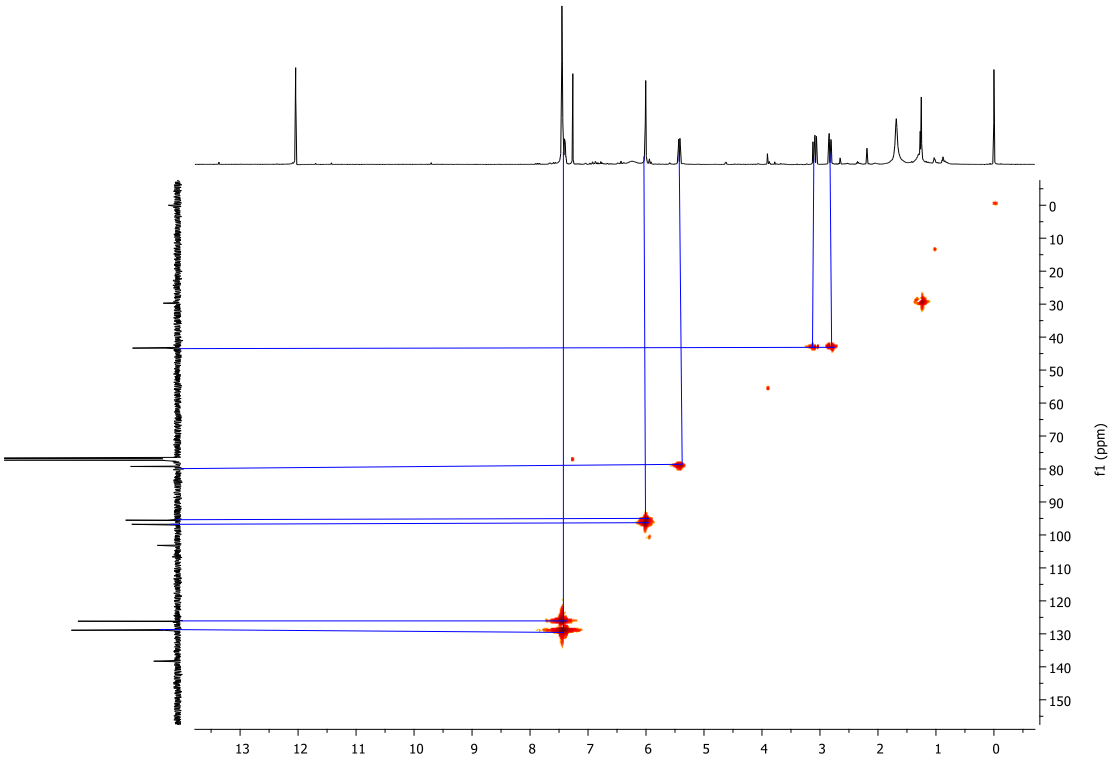


Figure 2A.40: HMQC Correlations of (3(S)-hydroxy-3-phenyl-1-(2',4',6'-trihydroxyphenyl)propan-1- one

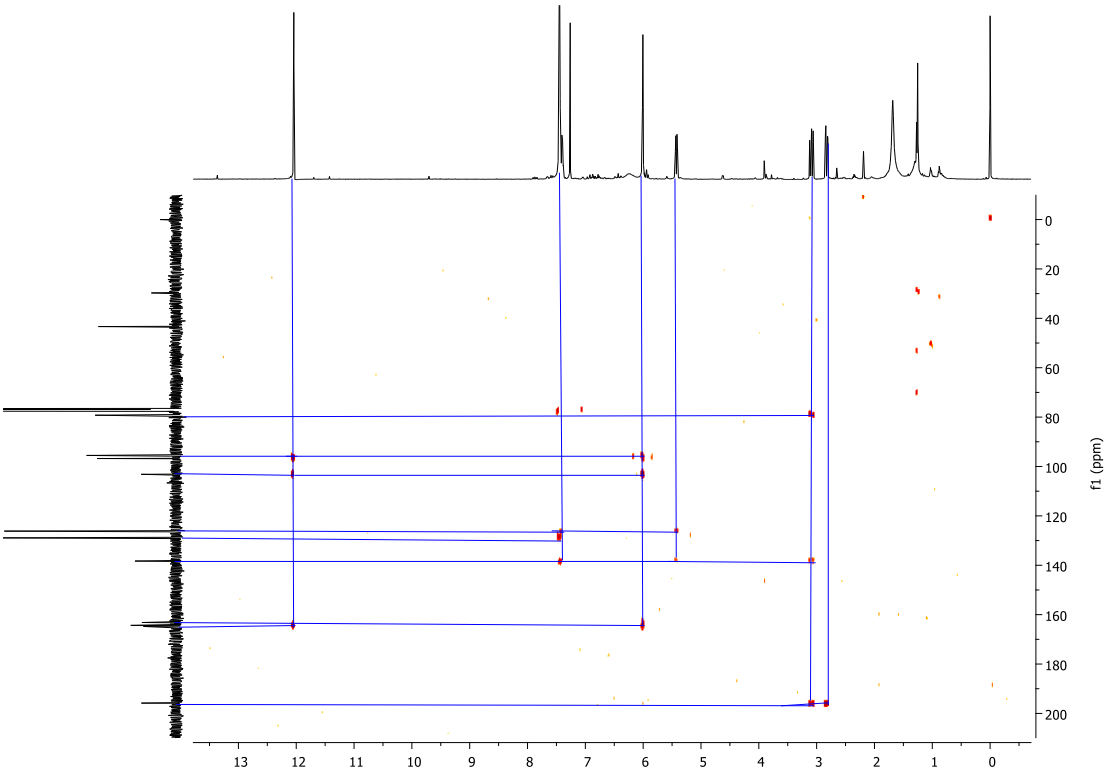


Figure 2A.41: HMBC Correlations of (3(S)-hydroxy-3-phenyl-1-(2',4',6'-trihydroxyphenyl)propan-1- one

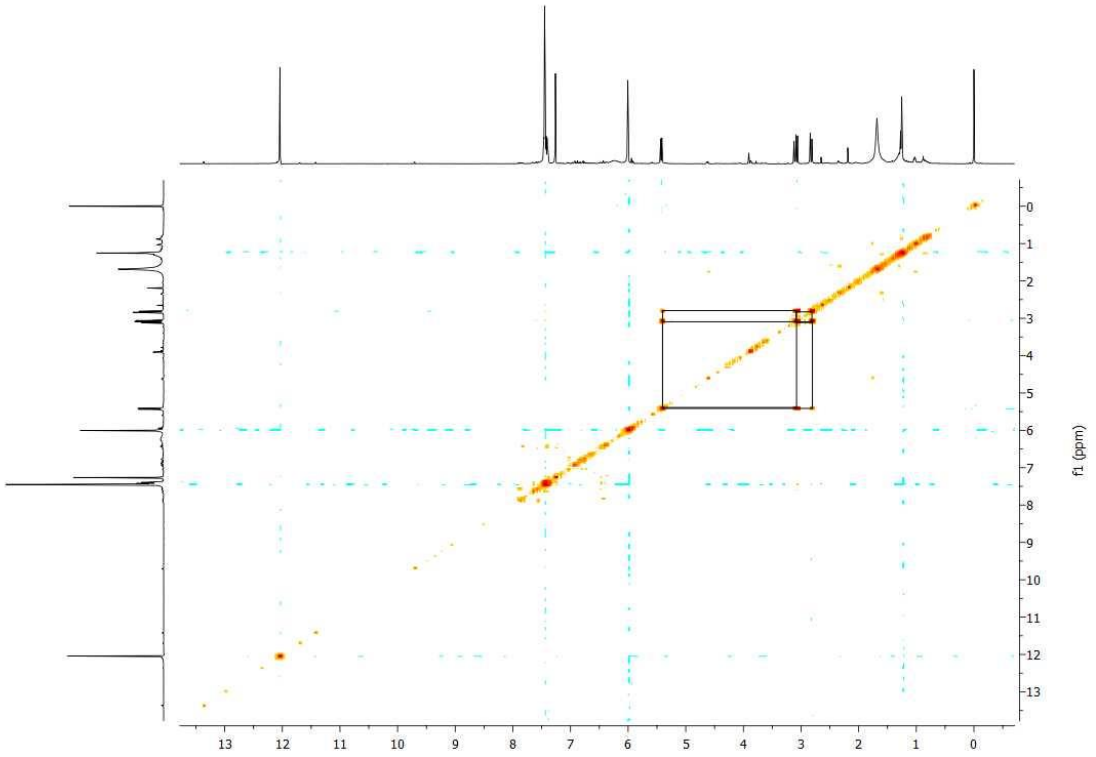


Figure 2A.42: H^1-H^1 COSY Correlations of (3(S)-hydroxy-3-phenyl-1-(2',4',6'-trihydroxyphenyl)propan-1- one

The UV and ECD spectra of compound **22** was measured (Fig. **2A.43**) and the ECD spectra exhibited negative Cotton effects at 255-305 nm and positive Cotton effects at 305−366 nm .The absolute configuration of the *β*-stereo center was deduced through circular dichroism (CD) spectrum. Based on the comparison of experimental and theoretical CD spectrum (Fig. **2A.44**) the compound was found to have (S) configuration at the *β* carbon.

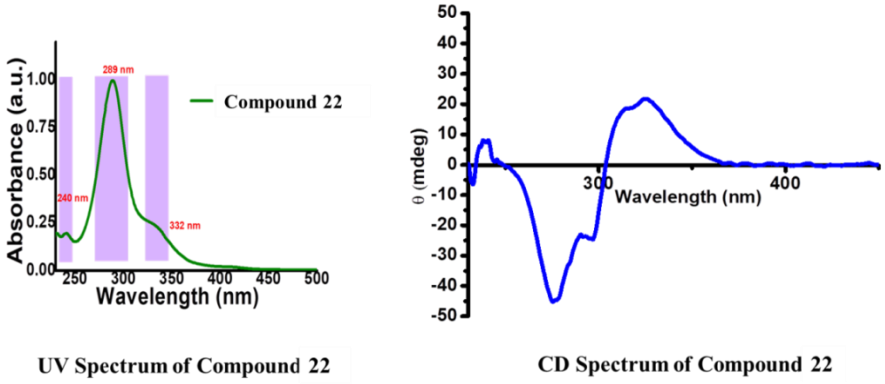


Figure 2A.43: UV and CD spectrum Compound **22**

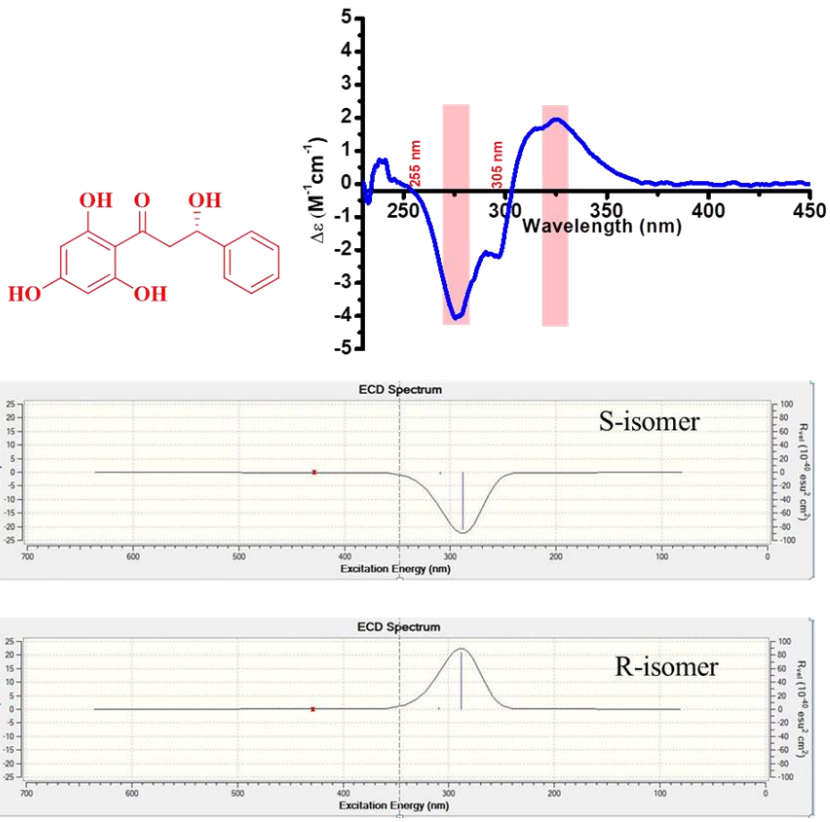


Figure 2A.44: Experimental and theoretical CD spectrum of Compound **22**

The optimized structure of the compound with **S** configuration was depicted in Fig. **2A.45**.

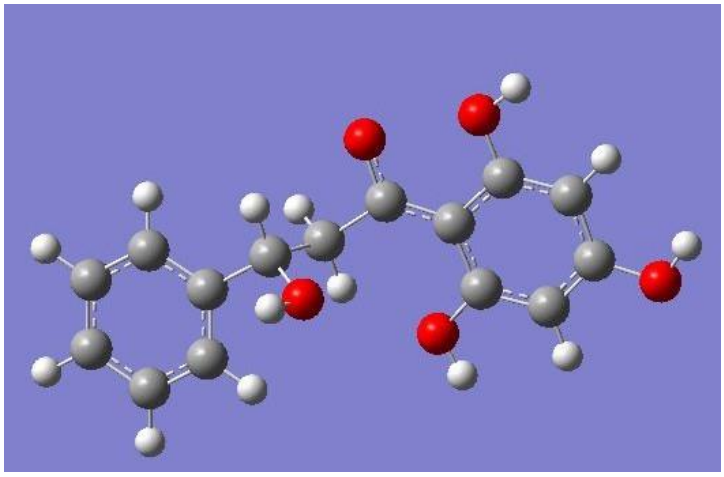


Figure 2A.45: Optimized structure of S configuration of Compound **22**

2A.4. *In vitro* Screening of Antiproliferative Potential

Naturally derived compounds have been widely utilized for targeting cancer cells in the cancer chemotherapy. Most of the currently used anticancer agents are originated from various natural scaffolds and are found to be more effective and less toxic. So there is an ongoing interest to find out the novel anticancer agents from various natural products. In this regard we have evaluated the *in vitro* anti-proliferative potential of various phytochemicals obtained from the species *M. beddomei*. The major phytoconstituents of the species include phenylacylphenols (Malabaricones), flavonoids, triglcerides and phenyl propanoids. Malabaricones are the unusual class of compounds abundantly present in the myristicaceae family endowed with numerous biological activity. Literature suggests that they can act as potential drug lead against various cancer type including breast cancer, melanoma, leukemia, lung cancer, liver cancer etc. and found to be more active against breast cancer. In the present work we have determined the *in vitro* anti-proliferation potential of isolated compounds against human adenocarcinoma cell lines MCF-7 and MDAMB-231 along with normal cell WI 38 by 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay and the results are summarized in (Table **2A.4**.) in terms of their IC_{50} values. In the case of malabaricones no studies on anti-proliferation against the cell line MDA-MB-231 was reported. Among the tested compounds malabaricones A-D showed promising activity in both cell lines but showed moderate toxicity in normal cells. The IC_{50} was comparable or better than that of

standard Doxorubicin. Trimyristin (9) efficiently inhibited MCF-7 while promalabaricone B (both cell) and partensein (MDAMB 23) showed moderate toxicity.

Table 2A.4: *In vitro* cytotoxicity assay result showing IC_{50} values of compounds

Compound Name	IC_{50} (µM) 24 Hrs			IC_{50} (µM) 48Hrs		
	MCF-7	MDA-MB -231	WI 38	MCF-7	MDA-MB -231	WI 38
Malabaricone A	15.46	28.58	25.37	27.38	27.90	41.23
Malabaricone B	22.92	14.67	35.13	28. 32	8.65	75.76
Malabaricone C	36.25	31.25	>100	48.63	9.85	>100
Malabaricone D	20.58	32.87	31.80	35.32	15.94	42.83
Trimyristin	34.90	>100	>100	>100	>100	>100
Partensein	>100	97.29	>100	>100	74.55	>100
Promalabaricone B	74.41	86.12	>100	70.15	83.59	>100
1-(2,6-dihydroxyphenyl) tetradecan-1-one	>100	>100	>100	>100	41.35	>100
5,7-dihydro 3-(7-hydroxy benzol [d] [1,3] dioxol 5-yl) 4H chromen-4-one	-	>100	>100	>100	>100	>100
Biochanin A	-	>100	>100	>100	>100	>100
Doxorubicin	>100	31.90				

2A.5. Conclusion

Myristica, the representative genus of myristicaceae family is endowed with diverse variety of species and subspecies. In our present study, we have successfully carried out the detailed chemo-profiling of the unexplored wild nutmeg sub species of *Myristica beddomei*. We have identified thirteen phytoconstituents including two novel molecules from the aerial parts (fruits and stem bark) of *M. beddomei* subsp. *spherocarpa* for the first time. Malabaricones, isoflavones, phenyl propanoid derivatives and triglyceride are the different class of phytoconstituents present in the species. *β*-hydroxydihydrochalcones are reported for the first time from myristicaceae family. The two new compounds identified from the species will serve as potential chemotaxonomic markers for the species and could be used to distinguish from the other species of myristica genus. In addition the selected compounds were assessed for their *in vitro*

cytotoxicity against human adenocarcinoma cell lines (MCF-7 and MDA-MB-231). Amongst malabaricones showed promising activity in both the cell lines with moderate cytotoxicity.

2A.6. Experimental Section

2A.6.1. General Methods

Commercially available top graded chemicals were employed in the study. Solvents were used after purification by standard methods. Analytical thin layer chromatography (TLC) was performed on Merck silica gel (60 F_{254} aluminium sheets) and the visualization was effected with UV and/or by staining with Enholm yellow/ Mc-Gill solution. Silica gel (100-200 mesh & 230-400 mesh) & Hexane/EtOAc gradient solvent system were used for gravitational column chromatographic separation. Melting point was determined on a Fisher Johns melting point apparatus and is uncorrected. The NMR (Nuclear magnetic resonance) spectra were recorded on Bruker AMX 500 spectrometer ($CDCl_3$ & CD_3COCD_3 as solvents). Chemical shifts were expressed as δ in ppm units (parts per million) downfield from tetramethyl silane (TMS, δ 0.0 ppm). Multiplicities were reported as; s (singlet), brs (broad singlet), d (doublet), t (triplet), m (multiplet), dd (doublet of doublet). Coupling constants were expressed as *J* value in Hz. Thermo Scientific Exactive Mass Spectrometer was used to record high resolution mass spectra under electron spray ionization (ESI/HRMS) at 61800 resolution. IR spectra were recorded on Bruker Alpha FT-IR spectrometer and specific rotation was recorded using Jasco P-2000 polarimeter. The electronic absorption spectra were recorded on a Shimadzu UV-2600 UV-Vis scanning spectrophotometer equipped with peltier thermostatic cell holders. Circular dichroism (CD) measurements were performed on JASCO 810 spectrometer equipped with peltier thermostatic cell holders. CD spectra were recorded as function of **'*θ*'** in millidegrees, and the value is then converted into **'*Δε*'** according to the general equation **$\Delta\varepsilon = \theta / (33982cl)$** in which **'*Δε*'** is the difference in the molar absorptivity for oppositely polarized light in M^{-1}/cm, **'*c*'** is the concentration in M and **'*l*'** is the path length in cm. Theoretical ECD spectra were recorded using time dependent density functional theory (TDDFT) calculations by using B3LYP/6311++G**\\.

2A.6.2. GCMS Profiling

The GCMS phytochemical profiling of different extracts of *Myristica beddomei* was performed by using GCMS-TQ8030 Shimadzu instrument. The extract of the different fruit parts were dissolved in acetone and 1µL of sample was injected to a GC equipped with a MS and a medium polar capillary column Rxi-5Sil MS (30m X 0.25mm

I. D, 0.25µm). The oven program had an initial temperature of 60 ^{o}C for 2 min, which is then subsequently raised to different temperatures (200 ^{o}C for 2 min at the rate of 5 ^{o}C/m, 220 ^{o}C for 1min at the rate of 3 ^{o}C /min) and finally attained a temperature of 250 ^{o}C at the rate of 6 ^{o}C /min for 7minutes with a total run time of 50 minutes. Helium was the carrier gas (purity 99.999% at a flow rate of 1mL/ min) with 250 ^{o}C as the detector and the injector temperature. The sample was injected in the split less mode. The ion energy of 70 eV was used for the electron impact ionization (EI) mode. The mass (m/z) scanning range was 100-1000. Identification of the volatile components was achieved by analyzing the spectral matches with the reference compounds in the NIST & WILEY library. The relative amounts of individual components were expressed as percentage peak areas relative to total peak area.

2A.6.3. Cell culture and Treatment Condition

For the study the human breast adenocarcinoma cell line MCF-7 & MDA-MB-231 were procured from ATCC (American Type Culture Collection, Manassas, VA, USA) and the cell line WI-38 (lung fibroblast) was kindly gifted from Indian Institute of Chemical Biology (CSIR-IICB, Kolkata, India). Cells were maintained at 37 °C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 5% CO_2. Cytotoxicity experiments were performed using the cultured cells. Cells were cultured in glass-bottom, 96-well plates, T25 flasks and T75 flasks.

2A.6.4. MTT Assay

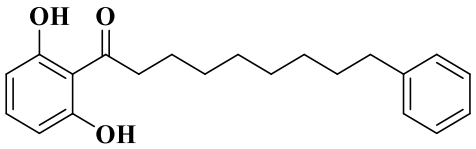
The growth inhibition capacity of all compounds was determined using MTT assay in cancer cell and normal cell lines initially using the previously reported methods [29]. In MTT assay cell viability was measured on the basis of the tetrazolium salt cleavage by mitochondrial dehydrogenase. The absorbance of formazan crystals resulted from the enzymatic reaction was measured at 570 nm using a BioTek Power Wave XS microplate spectrophotometer. Initially the cells were seeded in 96 well plates and incubated for 24 h. after that test compounds in desired concentration was added and incubated again for 24 and 48 h. Further MTT was added and incubated finally the formed crystals were dissolved in DMSO for measuring absorbance. The concentration corresponds to 50% inhibition of cell growth was also measured.

2A.7. Spectral Data

Compound 1 [Malabaricone A]

Fraction pool 6-12 after CC separation on silica gel using 10% ethyl acetate-hexane solution afforded the compound **1** as pale yellow crystalline solid. Spectral data of

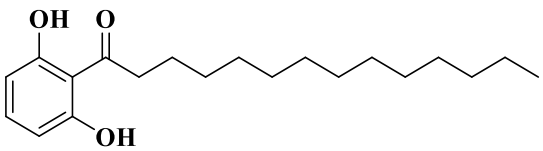
the compound indicated the presence of phenyl acyl phenol moiety. Finally a detailed spectral study of this compound and in comparison to the literature values the compound **1** was confirmed to be the malabaricone A.



Molecular formula	:	$C_{21}H_{26}O_3$
Melting point	:	80-82 ^oC
FT-IR (neat, ν_{max}, cm^{-1})	:	3254 (OH), 2925, 2850, 1635 (C=O), 1600, 1471, 1246, 1038, 966, 876
^{1}H NMR (500MHz, CD_3COCD_3)	:	δ 11.44 (s, 2H, 2-OH), 7.28 (dd, J_1=2Hz, J_2=8Hz, 2H), 7.26 (t, J=3Hz, 1H), 7.21 (d, J=8Hz, 2H,), 7.16 (dd, J_1=1.5Hz, J_2=7Hz, 1H), 6.43(d, J=8.5Hz, 2H), 3.17 (t, J=7Hz, 2H), 2.62 (t, J=7Hz, 2H), 1.74-1.67 (m, 2H), 1.66-1.62 (m, 2H), 1.37 (s, 8H, 4 CH_2) ppm
^{13}C NMR (125MHz, CD_3COCD_3)	:	δ 208.0 (C=O), 162.2, 142.7, 135.9, 128.3, 128.1, 125.5, 110.2, 107.5, 44.4, 35.6, 31.4, 29.4, 24.3 ppm
HR-ESIMS	:	349.17833 $[M+Na]^+$

Compound 2 [1-(2, 6-dihydroxyphenyl) tetradecan-1-one]

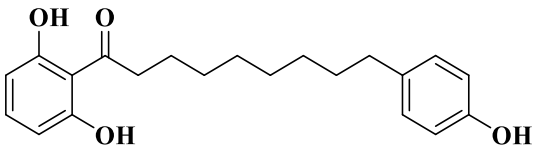
Fraction pool 13-16 showed the presence of a highly UV active spot. Column chromatographic separation of the fraction on 100-200 mesh silica gel using 15% ethyl acetate-hexane solution as eluent afforded the compound **2** as pale yellow crystalline solid. The molecule showed an intense red color, and was detected in the TLC with bare eyes. Various studies of this compound and in comparison to the literature values the compound **2** was confirmed as 1-(2, 6-dihydroxyphenyl) tetradecan-1-one.



Molecular formula	:	$C_{20}H_{32}O_3$
Melting point	:	91-92 °C
FT-IR (neat, ν_{max}, cm^{-1})	:	3450, 2927, 2852, 1624, 1601, 1448, 1243, 1028, 963, 782 and 720
^{1}H NMR (500MHz, CD_3COCD_3)	:	δ 11.30 (s, 2H, 2-OH), 7.12 (t, *J*=8Hz, 1H, H-18), 6.29 (d, *J*=8Hz, 2H, H-17,19), 3.03 (t, *J*=7.5Hz, 2H, H-2), 1.59-1.53 (m, 2H, H-3), 1.16 (s, 20H, H-4-13), 0.74 (t, *J*=6.5Hz, 3H, CH_3) ppm
^{13}C NMR (125MHz, CD_3COCD_3)	:	δ 208.0 (C=O), 162.2, 135.8, 110.1, 107.5, 44.4, 31.7, 29.5, 24.3, 22.4, 13.4 ppm
HR-ESIMS	:	321.24256 $[M+Na]^+$

Compound 3 [Malabaricone B]

Compound **3** was isolated as pale yellow solid from the fraction pool 17-19 after CC separation on silica gel using 20% ethyl acetate-hexane solution. Spectral data of the compound indicated the presence of phenyl acyl phenol moiety as the basic skeleton. Presence of carbonyl group and hydroxyl group were confirmed from the IR as well as NMR spectrum. DEPT-135 NMR spectrum confirmed presence of six -CH_2 group. Finally by analyzing all the spectral data the compound was identified as malabaricone B.



Molecular formula	:	$C_{21}H_{26}O_4$
Melting point	:	110 – 112 ^oC
FT-IR (neat, ν_{max}, cm^{-1})	:	3260 (OH), 2920, 2850, 1635 (C=O), 1600, 1471, 1246, 1038, 966, 876
^{1}H NMR (500MHz, CD_3COCD_3)	:	δ 11.37 (s, 2H, 2-OH), 8.00 (s, 1H), 7.11 (t, *J*=8Hz, 1H), 6.87 (d, *J*=8.5Hz, 2H), 6.60 (d, *J*=8.5Hz, 2H), 6.29 (d, *J*=8.5Hz, 2H), 3.02 (t, *J*=7Hz, 2H), 2.37(t, *J*=7.5Hz, 2H), 1.56-1.53 (m, 2H), 1.46-1.40 (m, 2H,

H-8)1.21 (s, 8H, 4 CH_2) ppm

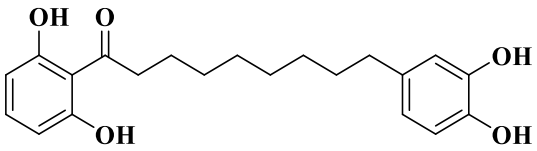
^{13}C NMR : δ 208.0 (C=O), 162.2, 155.4, 135.8, 133.3, 129.1, 115.0, 110.1, 107.5, 44.4, 34.7, 31.7, 29.2, 24.3 ppm

(125MHz, CD_3COCD_3)

HR-ESIMS : 365.17291 $[M+Na]^+$

Compound 4 [Malabaricone C]

Fraction pool 20-23 on repeated purification with column chromatography by using 25% ethyl acetate in hexane afforded the compound as UV active yellow crystalline solid. The IR spectrum of the compound showed absorptions at 3347 and 1623 cm^{-1} indicates the presence of hydroxyl and carbonyl groups. Presence of carbonyl group is also confirmed from the peak at δ 208 ppm in the ^{13}C NMR spectrum. Detailed spectral analysis of the compound and in comparison with literature reports the compound was confirmed as Malabaricone C.



Molecular formula : $C_{21}H_{26}O_5$

Melting point : 122-124 ^oC

FT-IR (neat, ν_{max}, cm^{-1}) : 3347, 2962, 2849, 2349, 1865, 1623, 1592, 1517, 1432, 1340, 1246, 1120

^{1}H NMR : δ 11.45 (s, 2H, OH-17, 21), 7.63 (s, 2H, OH-12, 13), 7.24 (t, *J*=8Hz, 1H, H-19), 6.70 (d, *J*=8Hz, 1H, H-14), 6.67 (d, *J*=2.5Hz, 1H, H-11), 6.50 (dd, J_1=2Hz, J_2=8Hz, 1H, H-15), 6.42 (d, *J*=8Hz, 2H, H-18, 20), 3.15 (t, *J*=8Hz, 2H, H-2), 2.44 (t, *J*=7.5Hz, 2H, H-9), 1.67-1.55 (m, 2H, H-3), 1.54-1.29 (m, 2H, H-8), 1.28 (s, 8H, 4 CH_2) ppm

(500MHz, CD_3COCD_3)

^{13}C NMR : δ 208.1 (C=O), 162.3, 144.9, 142.9, 135.9, 134.3, 119.4, 115.3, 115.0, 110.1, 107.4,

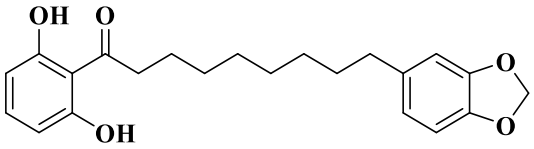
(125MHz, CD_3COCD_3)

44.4, 34.9, 31.7, 29.4, 24.3 ppm

HR-ESIMS : 381.16666 $[M+Na]^+$

Compound 5 [Malabaricone D]

Fraction pool 30-35 obtained from the acetone extract, on column chromatographic separation using 5% ethyl acetate-hexane solution afforded compound **5** as white powder. Presence of a sharp singlet at δ 5.79 ppm, integrating for two protons confirmed the presence of methylene dioxy group. In ^{13}C NMR spectrum methylene dioxy group resonated at δ 100.7 ppm. IR spectrum of the compound indicated the presence of carbonyl and hydroxyl groups. Finally the compound was identified as malabaricone D based on the various spectral data.



Molecular formula : $C_{22}H_{26}O_5$

Melting point : 68-70 ^oC

FT-IR (neat, ν_{max}, cm^{-1}) : 2924, 2843, 1622 (C=O), 1588, 1492, 1449, 1380, 1362, 1329, 1293, 1249, 1176, 1124, 1091, 1037, 961,938,917, 872, 840, 790, 749, 717, 657

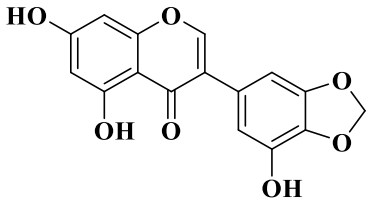
^{1}H NMR (500MHz, CD_3COCD_3) : δ 11.33 (s, 2H, -OH), 7.12 (t, *J*=8.5Hz, 1H), 6.59 (d, *J*=8 Hz, 1H), 6.52 (d, *J*=7.5Hz, 1H), 6.29 (d, *J*=8.5Hz, 2H), 5.79 (s, 2H, -O-CH_2-O-), 3.02 (t, *J*=7.5Hz, 2H), 2.40 (t, *J*=8Hz, 2H), 1.58-1.52 (m, 2H), 1.45-1.41 (m, 2H), 1.21 (s, 8H, 4 CH_2) ppm

^{13}C NMR (125MHz, CD_3COCD_3) : δ 207.9 (C=O), 161.1, 147.4, 145.3, 136.8, 135.6, 121.1, 110.0, 108.9, 108.4, 108.0, 100.7(-O-CH_2-O), 44.8, 35.6, 31.7, 29.4, 29.3, 29.0, 24.4 ppm

HR-ESIMS : 393.16763 $[M+Na]^+$

Compound 6 [5,7-dihydroxy-3-(5'-hydroxybenzo[d](7',9')-dioxol-1'-yl)-4H-chromen-4-one]

Fractions 36-45 of acetone extract after CC on silica gel using 20-25% ethyl acetate-hexane solution afforded the compound **6** as white powder. Spectral details of the compound suggest that it is an isoflavone. In the ^{1}H NMR spectrum three broad singlets at δ 12.86, 9.70, 8.57 ppm integrating one proton each confirmed the presence of three hydroxyl groups. A sharp two proton singlet at δ 5.86 ppm indicates the presence of a -O-CH_2-O- group. ^{13}C NMR spectrum gave the carbon resonances of carbonyl carbon, *olefinic* carbon, methylene dioxy carbon along with aromatic carbons. The compound was identified as a novel isoflavone based on the spectral data.

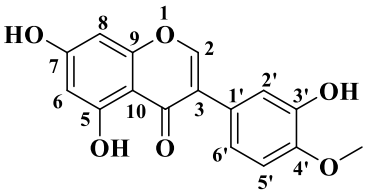


Molecular formula	:	$C_{16}H_{10}O_7$
Melting point	:	270-274 ^{o}C
FT-IR (neat, ν_{max}, cm^{-1})	:	3281, 2928, 1649, 1579, 1452, 1375, 1260, 1184, 1154, 1074, 928, 823,754, 704
^{1}H NMR (500MHz, CD_3COCD_3)	:	δ 12.86 (s,1H, -OH), 9.70 (s, 1H, -OH), 8.57 (s, 1H, -OH), 8.02 (s, 1H), 6.64 (d, *J*=1 Hz, 1H), 6.57 (d, *J*=1 Hz, 1H), 6.29 (d, *J*=2 Hz, 1H), 6.16 (d, *J*=2 Hz,1H), 5.86 (s, 2H, -O-CH_2-O-) ppm
^{13}C NMR (125MHz, CD_3COCD_3)	:	δ 180.5, 164.2,163.0, 158.1, 153.9, 148.9, 140.6, 134.4, 125.1, 122.9, 112.3, 105.2, 105.2, 101.2, 99.0, 93.6 ppm
HR-ESIMS	:	337.03302 $[M+Na]^+$

Compound 7 [3'-hydroxy biochanin A]

Fraction pool 46-52 of acetone extract after column chromatographic separation using 15% ethyl acetate-hexane solution resulted in the isolation of compound **7** as UV active white solid. Spectral data of the compound indicated the presence of an

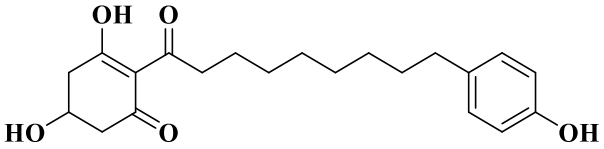
isoflavonoid moiety. In the ^{1}H NMR spectra the three proton sharp singlet at δ 3.75 ppm indicated the presence of a -OCH_3 group and the methoxy carbon resonated at δ 55.4 ppm. Presences of three hydroxyl groups are observed at three broad singlets at δ 12.91, 9.70 and 7.60 ppm in the ^{1}H NMR spectrum. From the literature reports, the structure of the molecule was confirmed as 3'-hydroxy biochanin A (Partensein), and it was well supported by HRMS data in which $[M+Na]^+$peak was obtained at 323.05375.



Molecular formula	:	$C_{16}H_{12}O_6$
Melting point	:	244-246 ^{o}C
FT-IR (neat, ν_{max}, cm^{-1})	:	3749, 3295, 2856,2359, 1654, 1506, 1266, 1171, 1126, 1015, 818,767, 646
^{1}H NMR (500MHz, CD_3COCD_3)	:	δ 12.91 (s, 1H, OH-5), 9.70 (s, 1H, OH-7), 8.05 (s, 1H, H-2), 7.60 (s, 1H, H-3'), 7.00 (d, J=2.5Hz, 1H), 6.92 (dd, J_1=2Hz, J_2=8.5Hz, 1H), 6.87 (d, J=8Hz, 1H), 6.29 (d, J=2.5Hz, 1H), 6.16 (d, J=2Hz, 1H), 3.75 (s, 3H, -OCH_3) ppm
^{13}C NMR (125MHz, CD_3COCD_3)	:	δ 180.9 (C=O), 164.3 (C-7), 162.7, 157.7, 154.2, 148.5, 146.7, 124.7, 123.4, 120.0, 116.2, 111.2, 105.1, 99.0, 93.6, 55.4 ppm
HR-ESIMS	:	323.05375 $[M+Na]^+$

Compound 8 [Promalabaricone B]

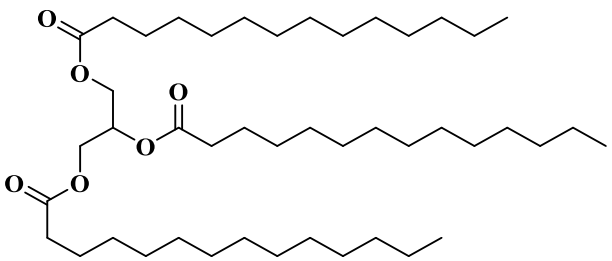
Compound **8** was isolated as pale yellow powder from the acetone extract fraction 53-57, via silica gel column chromatography with 40% of ethyl acetate in hexane. DEPT-135 spectrum confirmed the presence of eight -CH_2 groups as eight downward peaks in between δ 46.8-24.6. Carbonyl carbon was observed at δ 204.8 ppm in the ^{13} C NMR spectrum. Detailed analysis of the various spectral data and in comparison with the literature reports the compound was identified as promalabaricone B.



Molecular formula	:	$C_{21}H_{28}O_5$
Melting point	:	100-102 ^oC
FT-IR (neat, ν_{max}, cm^{-1})	:	3743, 3243, 2912, 2849, 1638, 1542, 1512, 1441, 1363, 1237, 1071, 847, 819, 757,719
^{1}H NMR (500MHz, CD_3COCD_3)	:	δ 18.12 (s, 1H, -OH), 7.94 (s, 1H, -OH), 6.88 (d, *J*=7.5 Hz, 2H), 6.60 (d, *J*=7.5Hz, 2H), 4.30 (brs, 1H,-OH), 4.23 (s, 1H), 2.85 (t, *J*=7.5Hz, 2H), 2.71 (s, 2H), 2.60 (d, *J*=17Hz, 2H), 2.38 (t, *J*=8Hz, 2H) 1.45 (m, 4H), 1.20(s, 8H, 4 CH_2) ppm
^{13}C NMR (125MHz, CD_3COCD_3)	:	δ 204.8 (C=O), 197.3 (C=O), 193.1 (C-17), 155.3 (C-13), 133.3 (C-10), 129.1 (C-11,15), 115.0 (C-12,14), 112.8 (C-16), 62.8 (C-19), 46.8 (C-20), 41.4 (C-18), 39.7 (C-2), 34.7 (C-7), 31.7 (C-8), 29.2 (CH_2), 24.6 (CH_2) ppm
HR-ESIMS	:	383.18399 $[M+Na]^+$

Compound 9 [Trimyristin]

Compound 9 was obtained as white amorphous solid from the fraction pool 1-3 of DCM extracts of the seeds after crystalliasation in hexane. IR spectrum of the compound showed strong absorptions between 2955 cm^{-1}-2850 cm^{-1} and 1735 cm^{-1} suggested the presence of alkane and ester group. DEPT 135 spectrum confirmed the presence of ten -CH_2 groups resonated in the region of δ 22.5-61.8 ppm. Carbonyl carbon of ester group was observed at δ 172.5 ppm in the ^{13}C NMR spectrum. Based on the spectral analysis and in comparison with literature reports the compound was identified as a triglyceride trimyristin.



Molecular formula	:	$C_{45}H_{86}O_6$
Melting point	:	37-39^oC
FT-IR (neat, ν_{max}, cm^{-1})	:	2917, 2852, 1737, 1465, 1387, 1251, 1171, 1114, 894,718
^{1}H NMR (500MHz, $CDCl_3$)	:	δ 5.38-5.36 (m, 1H, H-30), 4.36 (dd, J_1=4Hz, J_2=12Hz, CH_2-29), 4.20 (dd, J_1=4Hz, J_2=12Hz, CH_2), 2.34 (t, J=7Hz, 6H, CH_2- 2,16), 1.62 (t, J=7.5Hz, 6H, CH_2- 3,17), 1.34 (s, 60H, CH_2-30), 0.89 (t, J=6.5Hz, 9H, 3-CH_3) ppm
^{13}C NMR ($CDCl_3$)	:	δ 172.5, 172.2, 69.0, 61.9, 29.6, 29.5, 28.7, 28.5, 22.5, 13.5 ppm
HR-ESIMS	:	745.63288 $[M+Na]^+$

Compound 10 [1-(2, 6-dihydroxyphenyl) tetradecan-1-one]

Fraction pool 4-9 obtained from the DCM extract of the seeds after column chromatographic separation using 5% ethyl acetate-hexane solution afforded 80 mg of yellow crystalline solid. Spectral details of the compound was similar to that of compound **2** (1-(2, 6-dihydroxyphenyl) tetradecan-1-one) previously isolated from the fruit rinds.

Compound 11 [Malabaricone A]

Compound **11** was obtained as pale yellow crystals from the fraction pool 10-18 of the DCM extract of the seeds after column chromatographic separation using 5% ethylacetate-hexane solution. Detailed spectral analysis of the compound revealed that it is similar to that of compound **1**.

Compound 12 [Malabaricone D]

Fraction pool 26-30 showed the presence of an intense UV active spot which on charring in Enholm yellow solution TLC turned into intense pink colour. Column chromatographic separation of the fraction using 5% ethyl acetate-hexane solution

afforded the compound **12** as white powder. Based on the spectral studies of this compound and in comparison to the literature values the compound was confirmed to be malabaricone D isolated earlier from the rind.

Compound 13 [Malabaricone B]

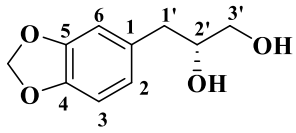
Compound **13** was isolated as pale yellow solid from the fraction pool 31-36 using column chromatographic separation with 25% ethyl acetate-hexane which was same that of compound **3**.

Compound 14 [Malabaricone C]

Fraction pool 37-43 when subjected to crystallization using DCM-hexane afforded the compound as yellow solid. Spectral data of the compound is similar to that of compound **4** which was obtained earlier from the rind.

Compound 15 [(2R)-3(3, 4-methylenedioxyphenyl) 1, 2-propanediol]

Compound **15** was identified as UV active colour less liquid from the fraction pool 44-47 (50-60%) of DCM extract of the seed after column chromatographic separation using 100-200 mesh silica gel using 25 % ethyl acetate-hexane solution. Detailed analysis of the spectral data of the compound and in comparison with the literature reports the compound was confirmed as (2R)-3(3, 4-methylenedioxyphenyl) 1, 2-propanediol.



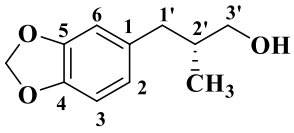
Molecular formula	:	$C_{10}H_{12}O_4$
Melting point	:	82-84 ^oC
FT-IR (neat, ν_{max}, cm^{-1})	:	3293, 2885, 1607, 1501, 1439, 1241, 1188,1035, 927, 864
^{1}H NMR (500MHz, $CDCl_3$)	:	δ 6.71 (d, J=7.5Hz, 1H, H-3), 6.64 (d, J=1.5Hz, 1H, H-6), 6.60 (dd, J_1=1.5Hz, J_2=11Hz, 1H, H-2), 5.92 (s, 2H, -O-CH_2-O), 3.78 (dd, J_1=1.5Hz, J_2=11Hz, 1H, H-3'), 3.50 (dd, J_1=4Hz, J_2=11.5Hz, 1H, H-3'), 3.26 (brs, 1H, -OH), 2.75 (dd, J_1=9Hz, J_2=14Hz, 1H, H-1'), 2.62 (dd, J_1=6Hz, J_2=13.5Hz, 1H, H-1'), 1.84-1.83 (m, 1H,

H-2') ppm

^{13}C NMR (125MHz, $CDCl_3$)	:	δ 147.6 (C-5), 145.8 (C-4), 134.3 (C-1), 121.9 (C-2), 109.3 (C-6), 108.1 (C-3), 100.8 (-O-CH_2-O-), 60.3 (C-3'), 44.3 (C-2'), 35.9 (C-1') ppm
HR-ESIMS	:	197.07356 $[M+H]^+$

Compound 16

Fraction pool 48-54 (50-60%) after column chromatographic separation afforded the compound **16** as pale yellow soild. In the ^{1}H NMR spectra a sharp singlet at δ 5.92 ppm integrating for two protons indicates the presence of -O-CH_2-O- group. The detailed spectral analysis of the compound and in comparison with the literature report the compound was confirmed as 2R'-methyl-3-[3, 4-(methylenedioxy) phenyl] 1'-propanol.

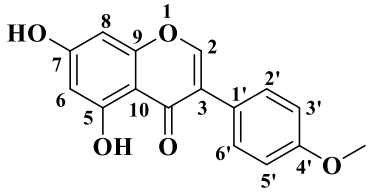


Molecular formula	:	$C_{11}H_{14}O_3$
Melting point	:	72-74 ^oC
FT-IR (neat, ν_{max}, cm^{-1})	:	3351, 2921, 1494, 1442, 1371, 1248, 1216, 1088, 1039, 925, 807
^{1}H NMR (500MHz, CD_3COCD_3)	:	δ 6.74 (d, J=8Hz, 1H, H-3), 6.65 (d, J=1Hz, 1H, H-6), 6.62 (dd, J_1=7.5Hz, J_2=1.5Hz, 1H, H-2), 5.95 (s, 2H, -O-CH_2-O), 3.84 (d, J=12.5Hz, 1H, H-3'), 3.34 (dd, J_1=12.5Hz, J_2=4.5Hz, 1H, H-3'), 2.82 (brs, 1H, -OH), 2.68 (d, J=7.5Hz, 2H, H-1'), 1.60 (m, 1H, H-2'), 1.30 (S, 3H, -CH_3) ppm
^{13}C NMR (125MHz, CD_3COCD_3)	:	δ 147.6 (C-5), 145.7 (C-4), 134.6 (C-1), 122.0 (C-2), 109.4 (C-6), 107.7 (C-3), 100.8 (-O-CH_2-O-), 60.5 (C-3'), 43.7 (C-2'), 36.9 (C-1') , 24.1 (CH_3) ppm

HR-ESIMS : 195.0943 $[M+H]^+$

Compound 21

Fraction pool 40-45 after CC separation on 100-200 mesh silica gel using 35% ethyl acetate- hexane solution afforded the compound as white powder. Spectral data of the compound indicated the presence of a flavonoid moiety. Further detailed spectral analysis and in comparison with the literature reports the compound was identified as biochanin A.



Molecular formula : $C_{16}H_{12}O_5$

Melting point : 215-216 ^oC

FT-IR (neat, ν_{max}, cm^{-1}) : 3300, 2950, 1640

^{1}H NMR (500MHz, CD_3COCD_3) : δ 13.01 (s, 1H, OH-5), 9.80 (brs, 1H, OH-7), 8.20 (s, 1H, H-2), 7.55 (d, *J*=9Hz, 2H, H-2', 6'), 7.01 (d, *J*=9Hz, 2H, H-3', 5'), 6.43(d, *J*=1.5Hz, 1H, H-8), 6.30(d, *J*=1.5Hz, 1H, H-6), 3.85 (s, 3H, -OMe) ppm

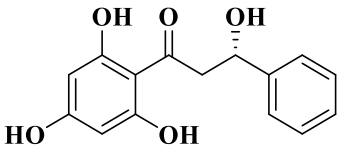
^{13}C NMR (125MHz, CD_3COCD_3) : δ 181.6 (C=O), 165.2 (C-7), 164.0 (C-5), 160.7 (C-9), 159.1 (C-4'), 154.5 (C-2), 131.1 (C-2', 6'), 124.2 (C-1'), 123.8 (C-3), 114.5 (C-3',5'), 106.1 (C-10), 99.9 (C-6), 94.5 (C-8), 55.6 (-OMe) ppm

HR-ESIMS : 285.07595 $[M+H]^+$

Compound 22 [(3(S)-hydroxy-3-phenyl-1-(2', 4', 6'-trihydroxyphenyl) propan-1-one]

Fraction pool 46-52 after column chromatographic purification using 35-40% ethyl acetate-hexane afforded compound as a pale yellow solid. The IR spectrum showed characteristic absorption for hydroxyl group (3178 cm^{-1}), hydrogen bonded hydroxyl

group (2885 cm^{-1}), carbonyl groups (1634 cm^{-1}) and aromatic moiety (1459 cm^{-1}). The structure of the compound was identified on the basis of various spectroscopic analyses.



Molecular formula	:	$C_{15}H_{14}O_5$
Melting point	:	158-160 ^oC
FT-IR (neat, v_{max}, cm^{-1})	:	3178, 2885, 1634, 1459, 1341, 1273, 1150, 1087, 976, 888, 715
^{1}H NMR (500MHz, $CDCl_3$)	:	δ 12.04 (s,1H, -OH), 7.45-7.42 (m, 4H), 7.41-7.39 (m, 1H), 6.01 (s, 2H), 5.43 (dd, J_1=13 Hz, J_2=3 Hz 1H), 3.09 (dd, J_1=13 Hz, J_2=3 Hz 1H), 2.83 (dd, J_1=17 Hz, J_2=3 Hz 1H) ppm
^{13}C NMR (125MHz, $CDCl_3$)	:	δ 195.8, 164.8, 164.3, 163.2, 138.3, 128.9, 128.9, 126.2, 103.2, 96.8, 95.5, 79.2, 43.3 ppm
HR-ESIMS	:	274.27429 $[M+H]^+$

2A.8. References

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Chemoprofiling of the Newly Discovered Wild nutmeg Species *Myristica trobogarii* Govind & M. Dan

2B.1. Introduction

Exploration of biodiversity for the identification of new plant species is of enormous interest. Western Ghats always fascinates us with its hidden beauty of biodiversity. Floristic diversity of Western Ghats is still remains as a mystery. All the plants possess certain specific characteristic features that make them unique. The species of the same genus is having some common features along with diversities. Identification of ecologically significant species especially from its endemic habitat forms the basis of its conservation [1]. The discovery of new endemic species will contribute the species diversity of that region. Species of Myristica genus shows great variability in Southern Western Ghats and include wild species that are not explored.

Myristica trobogarii Govind & M. Dan is a newly discovered wild nutmeg species from Southern Western Ghats belongs to myristicace family. It was identified from Sankily forest of Agasthyamala Biosphere Reserve that spread across Kerala for the first time after 124 years. It is endemic to this region and thrives at 300-500 m altitude. Morphologically the species is similar in many aspects to other three species reported earlier from the Western Ghats includes *Myristica beddomei*, *Myristica magnifica* and *Myristica malabarica* [2]. It has larger leaves and flowers with more stamens when compared to other species and the fruits are bigger, egg-shaped (glabrous) and having a yellow mace (Fig. **2B.1**).

Figure 2B.1: Fruits and mace of *Myristica trobogarii*

Taxonomic description of the species: exudate bright blood red coloured, watery. Leaves 26-30 cm & 10-14 cm, elliptic to oblonglateral veins adaxially prominent, 15–17 paired. Male inflorescence 16–18 flowered. Flowers caducous, urceolateyellowish-brown, Perianth fleshy, lobes 3 (rarely 4), equal, acute, re-curved outward, yellowish-brown, densely pubescent outside; staminal column 3.2–3.5 mm long, cylindrical, with a shallow groove just below anthers, glabrous; anthers 14, with an apiculus white cushion. Female inflorescence 2–3 flowered, Flowers urceolate, yellowish-green, persistent, perianth fleshy, lobes 3, equal, acute, re-curved outward, scabrid outside; stigma short, bilobed; ovary ovate, 1.5–3 mm, glabrous. Fruits usually in pairs, rarely solitary, dehiscent, 6–8 × 4.5–6 cm, ovoid, pale yellow turning orange–yellow on maturity, apex mucronate, base cordate, longitudinal suture on one side, glabrous; rind fleshy, soft, 2–2.4 cm thick, orange–yellow; seed 4–5 × 3–3.5 cm, oblong, black, arillate; aril yellow, lacerated.

The uses of these wild nutmegs are largely unknown. But they were collected as forest produce and used as adulterants of the true nutmeg.

2B.2. Aim and Scope of the Present Work

Western Ghats is the genetic reservoir of wild relatives of numerous plant species. The floristic diversity of endemic wild aromatic plants is still unknown. Many researches are ongoing to reveal the hidden potential of many hitherto unexplored species. *M. trobogarii* is the newly discovered wild nutmeg species from the Western Ghats after 124 years. Since the species is newly identified there is an urge to explore its phytochemistry and biological potential. In this regard we were interested to investigate the phytochemical constituent of the species along with its bioactivity. Initially our efforts are directed towards the phytochemical investigation of leaves and stem bark of the species. The medicinal uses of the species are largely unidentified so we were also aimed to evaluate the extract level biological activity of the species. The bioactivity assessment of plant extracts for its ability to inhibit proliferation of cancerous cells will help in its utilization as a therapeutic agent. Preliminary cytotoxic effects of leaf and stem bark extracts (Hexane, DCM, Acetone, Ethanol & Water) of *M. trobogarii* was tested against human cervical cancer cell line HeLa in order to investigate its anticancer potential. In addition we have recognized the antibacterial efficacy of the extracts against various gram positive and negative pathogens. Herein in the present work we will establish the phytoconstituents of the aerial parts of the species along with its cytotoxic and antibacterial potential.

2B.3. Extraction, Isolation and Characterization of Phytochemicals from the Stem bark & Leaves of *M. trobogarii*

2B.3.1. Collection of Plant Material

The leaves and stem bark of the species were collected from Sankily forest of Agastyamala Biosphere Reserve region of Kollam district, Kerala in September 2020. The authenticity of the plant material was evaluated by the plant taxonomist, Dr. Mathew Dan, KSCSTE-JNTBGRI, Palode, Thiruvananthapuram and a voucher specimen [***Myristica trobogarii,* Kerala: Kollam district, Agasthyamala Biosphere Reserve, Sankily forest, Sep 2020, *Govind* 81763 *(TBGT)***] was deposited in the herbarium of of the institute. A geographical map of the species is given in Fig. **2B.2**.

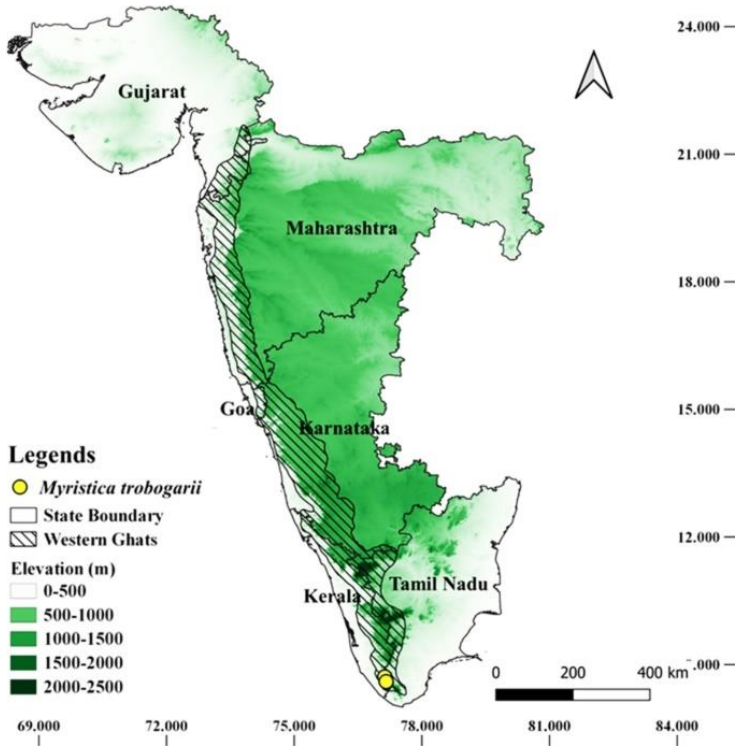


Figure 2B.2: Distribution and collection site of *M. trobogarii*

2B.3.2. Extraction of the Stem bark of *M. trobogarii*

Around 1.7 kg of stem bark was collected and dried in an air oven maintained at 50 ^{0}C for 3-6 days. The dried material was then crushed and powdered mechanically. Around 900 g of the powdered material was subjected to extraction using hexane at room temperature (5 L × 3 days). The extract was then filtered and concentrated under reduced pressure using Heidolph rotary evaporator to yield 5 g of the hexane extract. The residue was then extracted sequentially with acetone (5 L × 3 days), Ethanol (3 L × 3 days) and

finally with water at room temperature afforded 37 g, 12 g & 6g of the corresponding crude extract.

2B.3.2.1. Identification of Volatile Components from the Various Extracts of Stem bark

Since the species is newly discovered initially we have identified volatile components present in various extract (Hexane, Acetone, and Ethanol) of the stem bark through GC MS analysis. The list of various volatile compounds identified from the extracts is given in Table **2B.1A-C**.

Table 2B.1A: GCMS analysis of Hexane extract

Peak	Retention time	Area%	Height%	Compound name
1	33.552	1.17	1.09	Heptadecanoic acid
2	44.402	1.22	1.61	2-hydroxy-1-(hydroxymethyl)ethyl ester-hexadecanoic acid
3	26.404	1.27	2.59	Eicosane
4	42.052	1.30	1.01	Triacontyl heptafluorobutyrate
5	47.336	1.45	1.45	3-penten-2-one, 4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1-yl)-, (E)-
6	48.402	1.72	1.89	Tricosane
7	45.582	1.83	2.45	Adamantane-1-carboxylic acid (2-methyl-4-thiocyanato-phenyl)-amide
8	48.990	2.01	1.74	22-tricosenoic acid
9	28.488	3.18	5.81	2-pentadecanone, 6,10,14-trimethyl-
10	36.369	5.84	3.93	Octadecanoic acid
11	31.443	6.16	7.91	l-(+)-ascorbic acid 2,6-dihexadecanoate
12	31.160	7.45	4.23	Tetracosane
13	44.775	7.76	8.83	Diisooctyl phthalate
14	35.847	9.57	5.95	9-octadecenoic acid, (E)-
15	35.664	10.14	4.53	9,1-octadecadienoic acid (Z,Z)-
16	31.350	13.19	6.97	Octadecane

Table 2B.1B: GCMS analysis of Acetone extract

Peak	Retention time	Area%	Height%	Compound name
1	32.007	1.36	1.03	Phthalic acid, butyl 3-methylbutyl ester
2	33.341	1.51	1.45	Phthalic acid, hex-3-yl isobutyl ester
3	45.174	1.61	1.50	2'-hydroxy-4'-methoxyacetophenone, tert-butyldimethylsilyl ether

4	31.600	1.65	1.26	bis(2-pentyl)-phthalic acid ester
5	21.025	1.84	2.25	Phenol, 2,4-bis(1,1-dimethylethyl)-
6	44.712	2.43	2.47	1,2-benzenedicarboxylic acid, 1,2-bis(2-ethylhexyl) ester
7	45.569	2.48	2.00	Adamantane-1-carboxylic acid (2-methyl-4-thiocyanato-phenyl)-amide
8	47.539	2.86	1.51	Hexatriacontane
9	30.876	3.11	2.79	Phthalic acid, di(2-methylbutyl) ester
10	48.402	3.52	2.26	Tricosane
11	30.133	7.62	8.97	Phthalic acid, isobutyl 2-pentyl ester
12	28.827	7.79	8.57	1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester
13	31.170	11.70	11.53	Phthalic acid, butyl 2-pentyl ester
14	29.786	23.78	26.45	Dibutyl phthalate

Table 2B.1C: GCMS analysis of Ethanol extract

Peak	Retention time	Area%	Height%	Compound name
1	23.261	1.18	1.98	β-selinene
2	31.174	1.20	1.52	Phthalic acid, butyl 2-pentyl ester
3	35.055	1.32	1.52	Methyl stearate
4	23.523	1.46	2.31	12-oxabicyclo[9.1.0]dodeca-3,7-diene, 1,5,5,8-tetramethyl-, [1R-(1R*,3E,7E,11R*)]-
5	44.710	1.49	1.68	1,2-benzenedicarboxylic acid, 1,2-bis(2-ethylhexyl) ester
6	36.804	1.57	2.05	Octadecanoic acid, ethyl ester
7	26.230	1.67	1.73	2,6,10-cycloundecatrien-1-one, 2,6,9,9-tetramethyl-, (E,E,E)-
8	34.340	1.93	1.96	9-octadecenoic acid, methyl ester, (E)-
9	34.160	2.08	1.99	9,12-octadecadienoic acid (Z,Z)-, methyl ester
10	29.790	2.09	3.10	Dibutyl phthalate
11	48.395	2.10	0.95	Tricosane
12	19.773	2.31	3.79	α-humulene
13	22.402	2.47	0.99	3',5'-Dimethoxyacetophenone
14	44.398	2.55	1.72	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
15	45.550	2.80	2.42	Adamantane-1-carboxylic acid (2-methyl-4-thiocyanato-phenyl)-amide
16	48.153	3.20	1.22	9-octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-

17	47.555	4.05	2.57	Tetracontane
18	30.172	4.21	5.59	Hexadecanoic acid, methyl ester
19	16.047	5.37	1.61	2-methoxy-4-vinylphenol
20	35.890	5.45	6.43	Ethyl (9Z,12Z)-9,12-octadecadienoate
21	36.069	5.71	5.86	Ethyl oleate
22	42.036	5.82	3.19	Dotriacontane
23	4.204	6.31	16.41	2-pentanone, 4-hydroxy-4-methyl-
24	31.671	6.90	9.17	Hexadecanoic acid, ethyl ester
25	45.455	6.96	4.76	Malabaricone A
26	48.990	8.14	3.24	γ-Tocopherol

2B.3.2.2. Isolation and Characterization of Phytochemicals from the Stem bark

About 37 g acetone extract was fractionated over silica gel (100-200 mesh) column chromatography by gradient elution with Hexane/ EtOAc afforded 28 fraction pools based the similarity in TLC. All the fraction pools on repeated column chromatographic separation resulted in the isolation of following compounds. A schematic representation of the entire isolation procedure is given below (Fig. **2B.3**).

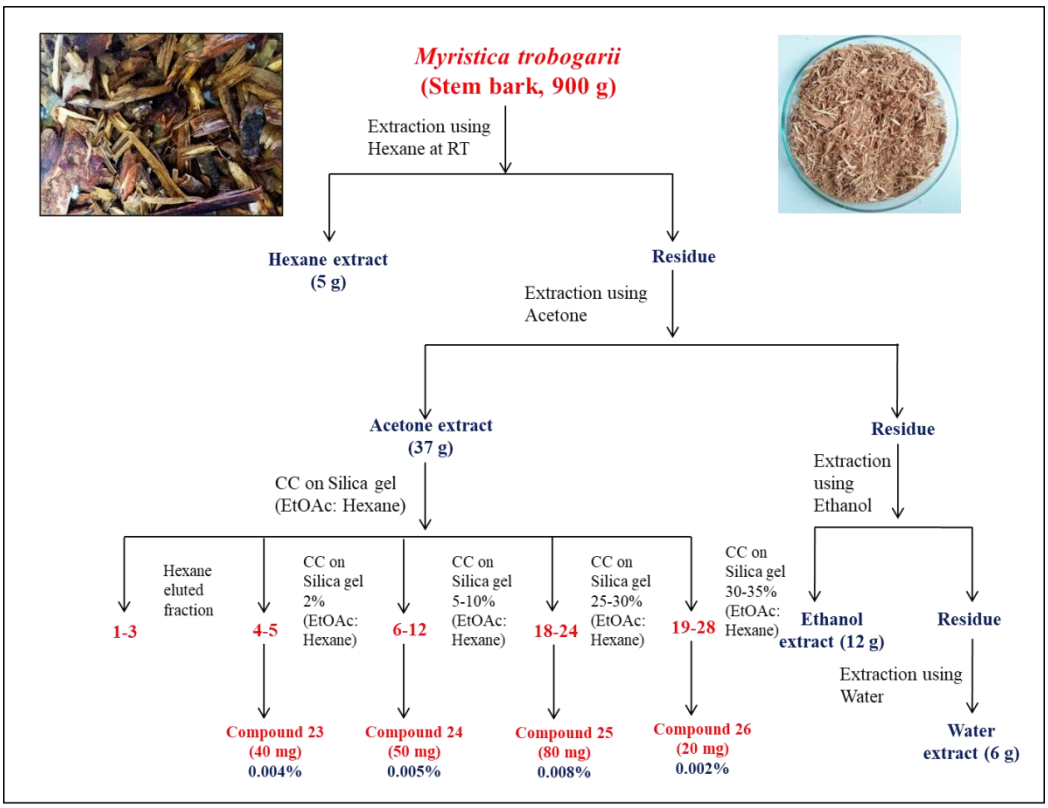
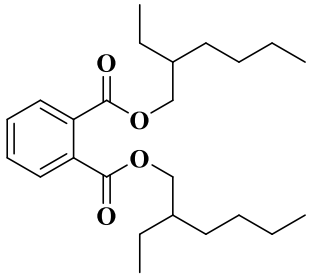


Figure 2B.3: Schematic representation of extraction and isolation procedure

Compound **23** was isolated as a UV active colourless liquid after CC separation of fraction pool 4-5 using 2% ethyl acetate-hexane. The ^{1}H NMR displayed two sets of

doublet of doublets at [δ_H 7.62 (dd, J_1=3Hz, J_2=6Hz, 2H, H-2, 5) & 7.52 (dd, J_1=3Hz, J_2=5.5Hz, 2H, H-3, 4) ppm] indicates the aromatic protons. A four proton multiplet at δ 4.08 ppm in ^{1}H NMR indicates the presence of methylene groups attached to oxygen atom. Besides a triplet observed at δ 0.77 ppm can be attributed to methyl group. The ^{13}C NMR spectrum of the compound showed eleven carbon resonances comprising of a carbonyl carbon of ester (δ_C 167.1 ppm), aromatic carbons (δ_C 132.6, 131.1, 128.8 ppm) along with aliphatic carbons (δ_C 67.4, 38.8, 30.3, 23.6, 22.7, 13.5 & 10.5 ppm). In the DEPT-135 spectrum five downward peaks at (δ_C 67.4, 30.3, 23.6, 22.7, 13.5 ppm) confirmed the presence of methylene groups. The mass spectrum of the compound gave a molecular ion peak at 391.28360 which is the $[M+H]^+$ peak. Finally based on the spectral data (NMR & HRMS) analysis, the compound was confirmed as bis (2-ethylhexyl) phthalate [3].



bis (2-ethylhexyl) phthalate
Compound **23**

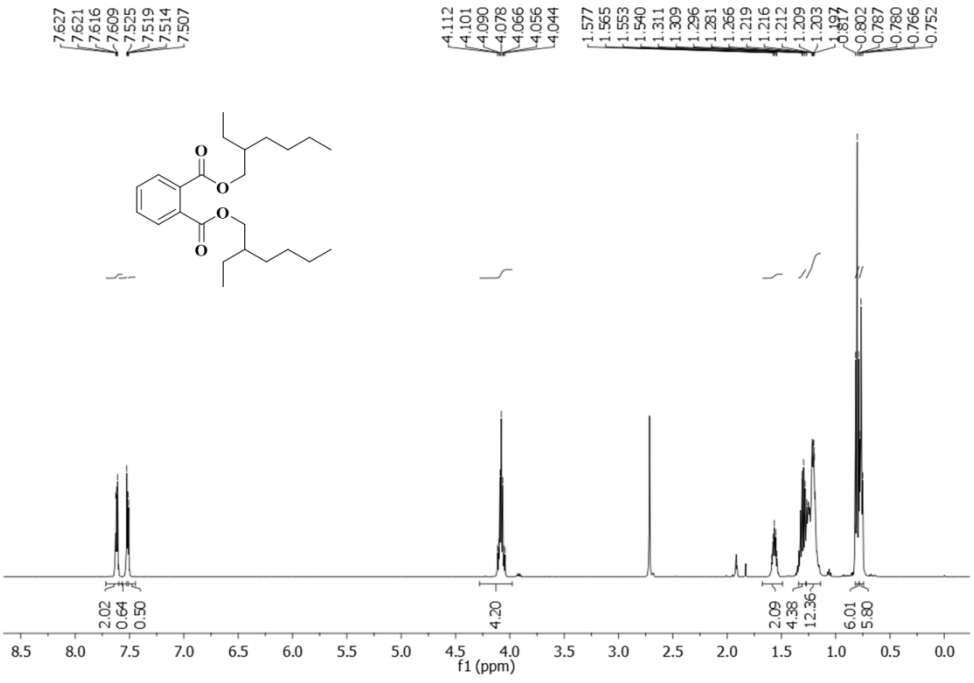


Figure 2B.4: ^{1}H NMR spectrum of bis (2-ethylhexyl) phthalate

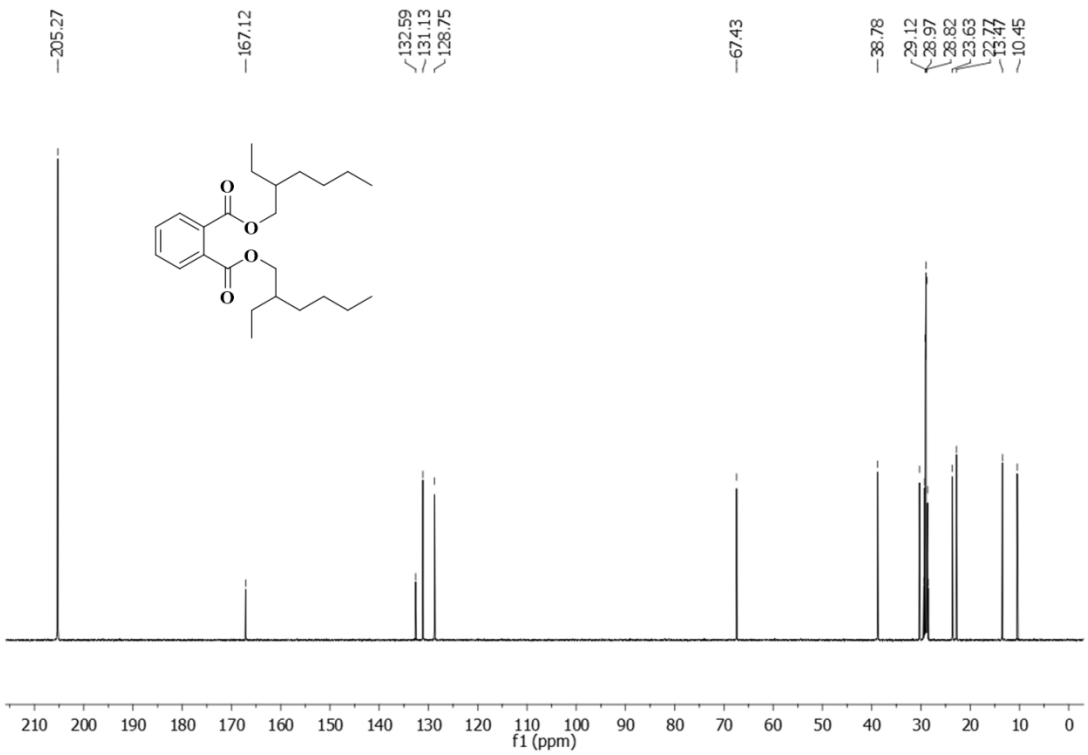


Figure 2B.5: ^{13}C NMR spectrum of bis (2-ethylhexyl) phthalate

Compunds **24-26** were identified as malabaricone A, biochanin A & malabaricone C respectively which was previously isolated from the species *M. beddomei* (Chapter **2A**: Compound **2, 7** & **4**). These compounds were isolated from the fraction pool 6-28 after CC separation with gradient elution of ethyl acetate-hexane (5: 9.5 to 3.5: 6.5 v/v). Amongst biochanin A was found to be the major phytoconstituent of the species with a weight percent of 0.008.

2B.3.3. Extraction of the Leaves of *M. trobogarii*

Around 3 kg of the fresh leaves was collected and dried in an air oven maintained at 50 ^{o}C for 3-6 days. Around 850 g of the powdered material was extracted with hexane at room temperature (5 L × 3 days). The extract was then filtered and concentrated under reduced pressure using Heidolph rotary evaporator afforded 6 g of the hexane extract. The residue was then sequentially extracted with acetone (5 L × 3 days), Ethanol (3 L × 3 days) and finally with water at room temperature gave 37 g, 12 g & 5g of the corresponding crude extract.

2B.3.3.1. Identification of Volatile Components from the Leaf Extracts

Different extracts of the leaves (Hexane, Acetone & Ethanol) was evaluated by GC MS analysis in order to identify the volatile components of the leaves of the species. Major constituents of leaves include the long chain aliphatic hydrocarbons along with

phenolic compounds. The lists of various compounds identified from the extracts are given in Table **2B.2A-C**.

Table 2B.2A: GCMS analysis of Hexane extract

Peak	Retention time	Area%	Height%	Compound name
1	10.045	1.12	0.66	3,7-(dimethyl)decane
2	16.695	1.28	0.98	4-methyl- dodecane
3	17.706	1.33	1.79	α-copaene
4	28.464	1.39	2.00	6,10,14-trimethyl-2-pentadecanone
5	45.330	1.41	0.91	Malabaricone A
6	23.468	1.56	1.45	Asarone
7	44.706	1.57	1.87	Bis (2-ethylhexyl) phthalate
8	29.763	1.66	2.12	7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
9	21.500	1.81	0.87	8-heptyl- pentadecane
10	41.437	1.90	1.64	((3aS,6S,6aR,9aS,9bR)-6a-hydroxy-6-methyl-3-methylene-2,9-dioxodecahydroazuleno[4,5-b]furan-9a(4H)-yl)methyl 2-methylpropanoate
11	22.866	2.07	2.52	(-)-5-oxatricyclo[8.2.0.0(4,6)] dodecane,,12-trimethyl-9-methylene-, [1R-(1R*,4R*,6R*,10S*)]-
12	36.070	2.11	1.83	Octadecanoic acid
13	18.857	2.22	2.86	Caryophyllene
14	8.798	2.41	0.69	5-ethyl-2-methyloctane
15	16.208	2.63	3.30	2,6,10,14-(tetramethyl) hexadecane
16	40.807	2.66	2.49	4,8,12,16-Tetramethylheptadecan-4-olide
17	22.745	2.70	3.33	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aα.,4aβ.,7β.,7aβ.,7bα.)]
18	30.736	2.87	3.33	Tetrapentacontane
19	25.434	2.94	3.71	Docosane
20	29.856	2.96	3.46	Octadecane
21	14.944	3.05	3.75	4,6-(dimethyl) dodecane
22	21.614	3.07	4.25	Hexadecane
23	20.501	3.29	4.44	Eicosane
24	34.604	4.44	4.30	Phytol

Table 2B.2B: GCMS analysis of Acetone extract

Peak	Retention time	Area%	Height%	Compound name
1	33.064	1.04	1.18	Dibutyl phthalate
2	16.702	1.05	0.76	4-methyl- dodecane
3	49.441	1.19	0.51	Phen-1,3-diol, 2-tetradecanoyl-
4	20.509	1.31	1.98	Eicosane
5	19.773	1.45	2.04	α-humulene
6	16.215	1.53	1.94	Hexadecane, 2,6,10,14-tetramethyl-
7	14.947	1.70	1.95	4,6-dimethyl- dodecane
8	17.717	1.71	2.27	Copaene
9	28.834	1.86	2.61	1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester
10	28.878	1.86	3.00	2-hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-
11	30.140	2.11	2.74	Phthalic acid, isobutyl 2-pentyl ester
12	29.253	2.99	4.54	2-hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-
13	31.177	3.00	3.62	Phthalic acid, butyl 2-pentyl ester
14	29.799	5.38	7.18	Dibutyl phthalate
15	34.614	5.65	5.75	Phytol isomer
16	28.389	8.80	12.70	Neophytadiene
17	18.881	8.88	11.60	Caryophyllene
18	10.288	9.58	0.90	1-butanol, 3-methyl-, formate
19	15.772	13.31	2.77	Resorcinol

Table 2B.2C: GCMS analysis of Ethanol extract

Peak	Retension time	Area%	Height%	Compound name
1	29.794	0.89	2.06	Dibutyl phthalate
2	30.174	0.99	1.67	Hexadecanoic acid, methyl ester
3	35.895	1.12	1.88	Ethyl (9z,12z)-9,12-octadecadienoate
4	19.784	2.02	4.73	α-humulene
5	36.812	2.28	3.74	Octadecanoic acid, ethyl ester
6	36.078	3.11	4.44	Ethyl oleate
7	4.209	4.44	13.67	2-pentanone, 4-hydroxy-4-methyl-
8	50.096	11.03	4.20	Dotriacontane
9	10.838	19.95	4.42	1-butanol, 3-methyl-, formate
10	34.636	20.14	31.63	Phytol isomer
11	24.580	21.67	4.45	Ethyl- α- d-glucopyranoside

2B.3.3.2. Isolation and Characterization of Phytochemicals from the Leaves

About 40 g acetone extract of the leaves was fractionated over silica gel (100-200 mesh) column chromatography by gradient elution with Hexane/ EtOAc afforded 18 fraction pools based the similarity in TLC. All the fraction pools on repeated column chromatographic separation resulted in the isolation of following compounds. A schematic representation of the entire isolation procedure is given below (Fig. **2B.6**).

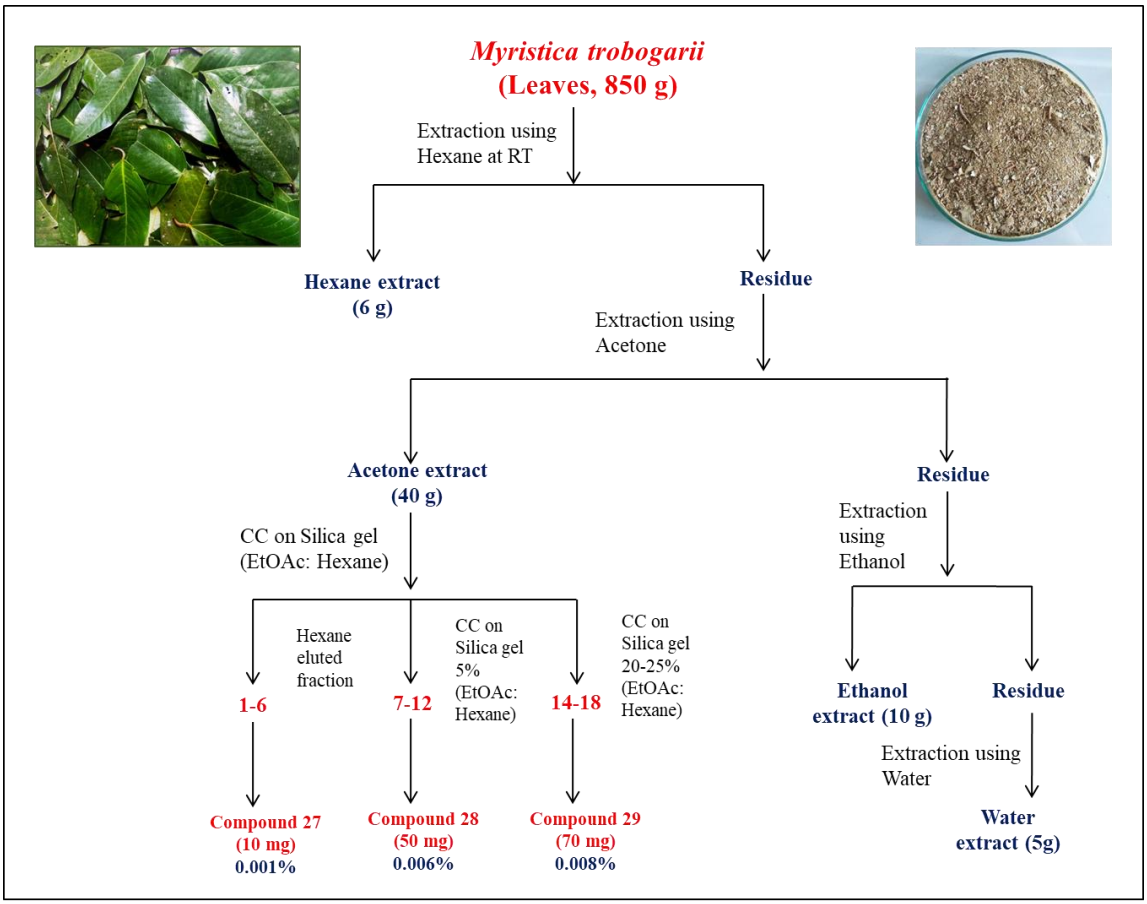


Figure 2B.6: Schematic representation of extraction and isolation procedure

Compound **27** was isolated as a white viscous solid from the hexane eluted fractions 1-6 of the acetone extract during column chromatography. The compound was identified as a triglyceride trimyristin which was previously isolated from the species *M. beddomei* (Chapter **2A**: Compound **9**). Compounds **28** & **29** were identified as malabaricone A and biochanin A similar to compounds 24 & 25 isolated from the stem bark of the species.

2B.4. Extract Level Biological Assays

Biological activities of the newly identified species are largely unknown. Generally wild nutmeg species of myristicaceae family found to possess various

biological activities including anticancer, antibacterial, antioxidant etc. To demonstrate the potential biological activity we have evaluated *in vitro* cytotoxicity and antibacterial potential of the extract of the leaves and stem bark of the species.

2B.4.1. *In vitro* Cytotoxicity Studies

Cervical cancer is the third most severe malignancy leading to increased morbidity & mortality in women worldwide. Due to increased drug resistance and side effects of existing drugs imparted difficulties in curing the cervical cancer. Several studies emphasized the role of plant derived chemotherapeutic agents to inhibit the early stages of carcinogenesis [4-5]. Herbal extracts were found to be effective in inhibiting the growth of cancer cells. Evaluation of cytotoxicity is an important step towards the establishment of any cancer chemotherapeutic, and hence the cytotoxicity of the plant extracts was evaluated on HeLa cell line by MTT assay. Extract of the leaves of *M. trobogarii* include hexane (MTLE-1), DCM (MTLE-2), acetone (MTLE-3), Ethanol (MTLE-4) and water (MTLE-5) along with clinically used doxorubicin were assessed across a wide range of concentrations (5µm to 100 µg/mL) for 24, and 48 h. All the tested compounds displayed cytotoxicity, especially at higher concentrations. Calculation of IC_{50} value revealed that MTLE-4 is most appropriate and can be used as a cytotoxic agent at lower concentrations.

Table 2B.3: IC_{50} value of Leaf extracts at different concentration on 24 h incubation

Extract Code	IC_{50} value (µg/mL)				
	5 µg	**10 µg**	**20 µg**	**50 µg**	**100 µg**
MTLE-1	14.63	15.91	20.06	43.36	73.98
MTLE-2	15.913	16.139	26.244	66.440	84.012
MTLE-3	10.030	12.670	13.348	49.548	72.775
MTLE-4	17.87	33.31	79.47	82.24	83.43
MTLE-5	17.87	33.31	79.47	82.24	83.43
Doxorubicin	41.766	46.164	47.950	53.373	59.623

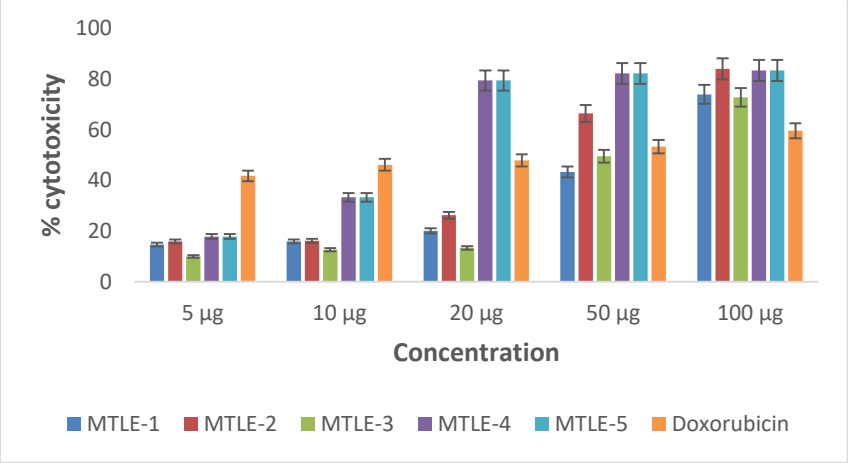


Figure 2B.7: Graphical representation of cytotoxicity of leaf extracts at different concentration on 24 h incubation

Table 2B.4: IC_{50} value of Leaf extracts at different concentration on 48 h incubation

Extract Code	IC_{50} value (µg/mL)				
	5 µg	**10 µg**	**20 µg**	**50 µg**	**100 µg**
MTLE-1	17.59	21.05	29.50	62.01	88.79
MTLE-2	9.74	10.42	12.59	74.42	91.57
MTLE-3	3.40	5.02	8.27	64.61	85.48
MTLE-4	40.32	73.42	88.52	90.53	91.60
MTLE-5	13.82	15.15	15.96	62.59	87.59
Doxorubicin	66.376	69.995	74.485	76.677	80.692

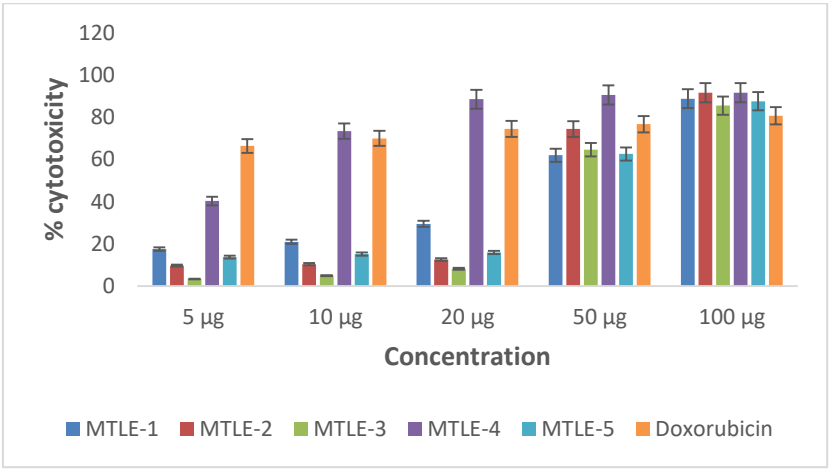


Figure 2B.8: Graphical representation of cytotoxicity of leaf extracts at different concentration on 48 h incubation

Similarly, cytotoxicity was estimated for stem bark extracts MTSE-1, MTSE-2, MTSE-3, MTSE-4 and MTSE-5 by using doxorubicin as a positive control across a wide range of concentrations (5 µm to 100 µg/mL) for 24, and 48 h. Here also a concentration dependent increase in the cytotoxicity was observed for all the extracts tested. The inhibition of the growth of the cancerous cells by *M. trobogarii* extract can be conceivably due to the several bioactive components present in them. The studies revealed that the species *M. trobogarii* possess anticancer potential.

Table 2B.5: IC_{50} value of stem bark extracts at different concentration on 24 h incubation

Extract Code	IC_{50} value (µg/mL)				
	5 µg	**10 µg**	**20 µg**	**50 µg**	**100 µg**
MTSE-1	59.25	62.20	67.91	72.15	71.55
MTSE-2	30.55	35.29	52.78	80.82	86.34
MTSE-3	13.25	27.60	34.63	48.60	77.43
MTSE-4	13.63	16.39	16.58	25.50	72.01
MTSE-5	9.26	11.87	37.33	76.80	80.97
Doxorubicin	41.76	46.16	47.95	53.373	59.62

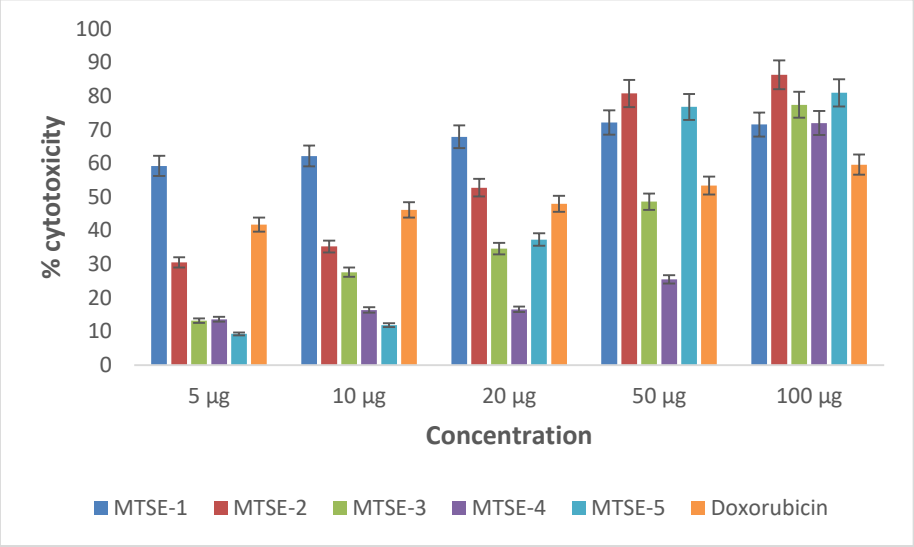


Figure 2B.9: Graphical representation of cytotoxicity of stem bark extracts at different concentration on 24 h incubation

Table 2B.6: IC_{50} value of stem bark extracts at different concentration on 48 h incubation

Extract Code	IC_{50} value (µg/mL)				
	5 µg	10 µg	20 µg	50 µg	100 µg
MTSE-1	27.64	32.97	35.50	48.64	53.46
MTSE-2	0.10	0.85	12.25	77.19	79.85
MTSE-3	5.64	7.55	8.15	40.52	71.36
MTSE-4	24.64	16.97	9.78	51.20	86.55
MTSE-5	10.23	39.19	73.05	88.58	90.41
Doxorubicin	66.376	69.995	74.485	76.677	80.692

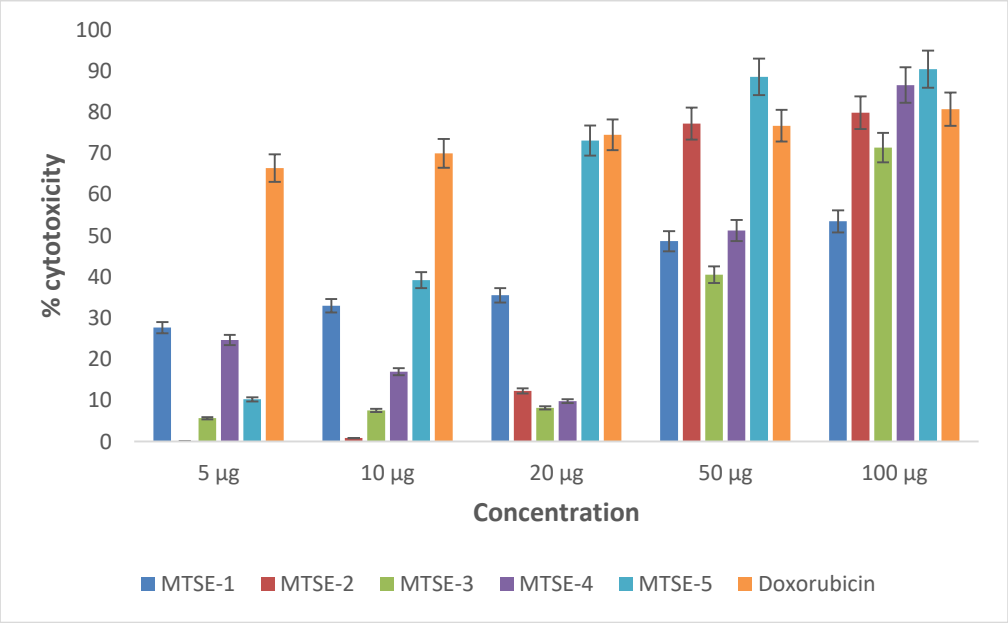


Figure 2B.10: Graphical representation of cytotoxicity of stem bark extracts at different concentration on 48 h incubation

2B.4.2. *In vitro* Antibacterial Studies

Infectious diseases are real threat to human lives worldwide. Plant extracts were proved to be potentially effective against various disease causing bacteria and were utilized in traditional medicine especially in skin diseases from ancient time itself. Discovery of antibiotics were reformed the treatment of bacterial diseases. Natural products from various sources provided bioactive scaffolds for the development potential drug leads against bacterial diseases [6]. Unfortunately the increased resistance over the bacteria against existing antibiotics created a looming threat in the treatment and it

necessitates the development new active antibacterial agents that are capable of overcoming the resistance. In this regard we were interested in investigating the new species for its antibacterial potential. Initially *in vitro* screening of the extract level antibacterial efficacy of the species was carried out by determining the antibiotic susceptibility against gram positive and negative pathogens.

2B.4.2.1. Antibiotic Susceptibility of the Extracts

Antibiotic susceptibility testing of the various extracts (Hexane, DCM, acetone, ethanol and water) of the leaves and stem bark was performed by determining the Minimum Inhibitory Concentration (MIC) according to standard CLSI guidelines [7] by broth microdilution assay. Extracts of the species were screened for their antibacterial potential against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Klebsiella pneumoniae* BAA-1705, *Acinetobacter baumannii* BAA-1605 and *Pseudomonas aeruginosa* ATCC 27853 [ESKAP pathogen panel]. Levofloxacin the standard antibiotic is used as the positive control and results are summarized in Table **2B.7**.

Table 2B.7: MIC of extracts against ESKAPE panel

SL. NO	Extract Code	Minimum Inhibitory Concentration (µg/mL)				
		E. coli **ATCC 25922**	***S. aureus*** **ATCC 29213**	***K. pneumoniae*** **BAA 1705**	***A. baumannii*** **BAA 1605**	***P. aeruginosa*** **ATCC 27853**
1	MTLE-1	>64	>64	>64	>64	>64
2	MTLE-2	>64	32	>64	>64	>64
3	MTLE-3	>64	32	>64	>64	>64
4	MTLE-4	>64	>64	>64	>64	>64
5	MTLE-5	>64	>64	>64	>64	>64
6	MTSE-1	>64	>64	>64	>64	>64
7	MTSE-2	>64	8	>64	>64	>64
8	MTSE-3	>64	64	>64	>64	>64
9	MTSE-4	>64	>64	>64	>64	>64
10	MTSE-5	>64	>64	>64	>64	>64
Levofloxacin		0.03	0.25	64	8	1

MIC value of the extracts of leaf and stem bark revealed that the species is specifically active against gram positive bacteria *Staphylococcus aureus* in ATCC 29213

strain and was found to be inactive in gram negative strains. Amongst the extracts tested both the DCM and acetone extract of the species showed significant activity in *S. aureus* strain. DCM extract of the stem bark showed excellent activity with an MIC value of 8 µg/mL whereas DCM extract of the leaves showed an MIC value of 32 µg/mL which was slightly lower compared to stem bark extract. The activity of these extracts can be attributed to the bioactive components present in the respective extracts. Further detailed studies are needed to explore the antibacterial efficacy of this species.

2B.5. Conclusion

Herein, in the present work we have studied the newly identified species *M. trobogarii* of myristicaceae family from Agastyamala Biosphere Reserve. Chemoprofiling of the leaves and stem bark of the species led to the isolation of five compounds for the first time from the species. In addition extract level biological activities of the species is also evaluated. *In vitro* cytotoxicity was evaluated in cervical cancer cell line HeLa along with antibacterial potential against ESKAPE pathogen panel. The extracts showed significant cytotoxicity against HeLa cell line and were also found to be active against the gram positive bacteria *S. aureus*. The potential activity exhibited by the extracts can be attributed to the presence of bioactive phytochemicals in it.

2B.6. Experimental Section

2B.6.1. General Methods

Different analytical techniques were used for the characterization of isolated compounds. The NMR spectrum was recorded on Bruker AMX 500 spectrometer with acetone-d_6. Chemical shifts were expressed in δ ppm downfield from TMS (δ 0.0) and the coupling constant as *J* value in Hz. Multiplicities were expressed as ‘s’ for singlet, ‘brs’ for broad singlet, ‘d’ for doublet, ‘t’ for triplet, ‘m’ for multiplet and ‘dd’ for doublet of doublet. Mass spectra were recorded by using Thermo Scientific Exactive Mass Spectrometer (ESI/HRMS at 61800 resolution). IR spectra were recorded on Bruker Alpha FT-IR spectrometer. GC MS analyses of the extracts were carried out using GCMS-TQ8030 Shimadzu instrument as described in Chapter **2A**.

2B.6.2. Cell Culture and Treatment Condition

The HeLa cell lines (human cervical cancer) was procured from American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained DMEM with 10% fetal bovine serum and 5% CO_2 at 37 °C. Cells were cultured in glass-bottom, 96-well plates, T25 flasks and T75 flasks, for the purpose of cytotoxicity experiments.

2B.6.3. MTT Assay

MTT assay was used to determine the growth inhibition capacity of all the extracts in cancer cell and in normal cell lines using reported procedures [8]. The detailed experimental procedure is described in Chapter **2A**.

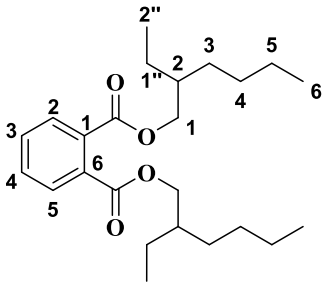
2B.6.4. Antibiotic Susceptibility Testing against ESKAP Pathogen Panel

The effect of plant extracts (Stem bark & leaves) in inhibiting the bacterial growth was assessed by determining the minimum inhibitory concentration (MIC) according to standard CLSI guidelines in which levofloxacin as the standard antibiotic. Various concentrations of the extracts were evaluated against gram positive and negative pathogenic bacterial strains. Three replicate measurements were taken for each extract. The detailed procedure of antibacterial testing is included in Chapter **4**.

2B.7. Spectral Data

Compound 23 [bis (2-ethylhexyl) phthalate]

Compound **23** was obtained as a UV active colourless liquid from the fraction pool 4-5 after CC separation using 2% ethyl acetate-hexane eluent. The IR spectrum showed characteristic absorption for carbonyl carbon of ester (1723 cm^{-1}), aromatic carbons (C-H & C=C) and -C-H aliphatic (2927–2860 cm^{-1}). The compound was identified as bis (2-ethylhexyl) phthalate based on the spectroscopic data and in comparison with literature.



Molecular formula	:	$C_{24}H_{38}O_4$
FT-IR (neat, ν_{max}, cm^{-1})	:	3070, 2927, 2860, 1773,1598, 1462, 1273
^{1}H NMR (500MHz, CD_3COCD_3)	:	δ 7.62 (dd, J_1=3Hz, J_2=6Hz, 2H, H-2,5), 7.52 (dd, J_1=3Hz, J_2=5.5Hz, 2H, H-3,4), 4.11-4.04 (m, 4H, CH_2-1), 1.59-1.54 (m, 2H, H-3'), 1.31-1.26 (m, 4H, H-1''), 1.22-1.19 (m, 12H, 6-CH_2), 0.80 (t, J=7.5Hz, 6H, 2-CH_3-2''), 0.77 (t, J=7Hz, 6H, 2-

		CH_3-7') ppm
^{13}C NMR (125MHz, CD_3COCD_3)	:	δ 167.1(C=O), 132.6 (C-3,4), 131.1 (C-1,6), 128.8 (C-2,5), 67.4 (C-2'), 38.8 (C-3'), 30.3 (C-4'), 23.6 (C-1''), 22.7 (C-5'), 13.5 (C-7'), 10.5 (C-2''0) ppm
HR-ESIMS	:	391.28360 $[M+H]^+$

Since the compounds **24-29** were earlier isolated from the species *Myristica beddomei* characterization and Spectroscopic data of the compounds were described in Chapter **2A**.

2B.8. References

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Bioprospecting of the Different Fruit Parts of *Myristica malabarica* Lam & *In Vitro* Antidiabetic Potential of Malabaricones

3A.1. Introduction

The wild nutmeg species, *Myristica malabarica* nested within the myristicaeae family is a red listed endemic species generally distributed in sub-tropical evergreen forests up to 1000 m altitude and in myristica swamps of Southern Western Ghats. Traditionally the species is used as a medicine and as a spice in food. Tree is economically important for its wild nutmeg and mace and the fruits are extensively exploited for its aril locally. The fruit rind of the specie is used as an exotic spice in Indian cuisine [1]. The plant has also been recognized in traditional medicine for its ability to defeat against cancer. Commonly the species is used as an adulterant of the true nutmeg *M. fragrans*. *M. malabarica* was endorsed with wide range of pharmacological activities including anticancer, anti-inflammatory, antioxidant, antimicrobial etc. [Chapter **1B.5**].

Figure 3A.1: Different fruit parts *M. malabarica*

Taxonomic description of the species: Tree, evergreen, 20–35 m tall, dioecious; main trunk 90–130 cm in diameter, bark rough, brown, exudate orange red coloured, viscous,

branching usually whorled. Leaves alternate, petiolate; petiole 7-12 mm long, shallowly grooved, glabrous; lamina 10-15× 5–7 cm, narrowly lanceolate, apex acute, margin entire, base obtuse, adaxially dark green, gland-dotted, abaxially glaucous, glabrous; midvein prominent, lateral veins adaxially prominent, 10–12 paired. Male inflorescence 5-6 flowered, axillary umbel; peduncle erect, 2-4 cm long, cylindrical, green, scabrid. Flowers caducous, urceolate, 4.5–6 × 2.3–3.5 mm, yellow, pedicellate; pedicel 7–14 mm long, scabrid; bracteole 1.2–3 × 2–2.5 mm, ovate, acute at apex, appressed to the base of male flower bud, dorsally convex, brown, densely scabrid outside, early caducous. Perianth fleshy, lobes 3, equal, acute, re-curved outward, yellow, scabrid outside; staminal column 3.2–4 mm long, cylindrical, pilose at base with golden hairs; anthers 8-10. Female inflorescence 1-2 flowered, axillary umbel; peduncle erect, 5-10 mm long, stout, cylindrical, green, scabrid. Female flowers urceolate, 3.5-5 × 3–4 mm, yellow, pedicellate; pedicel up to 4-5 mm long, stout, loosely scabrid; perianth fleshy, lobes 3, equal, acute, scabrid outside; stigma short, bilobed; ovary ovate, 1.5–3 mm, densely tomentose. Fruits usually solitary, rarely in pairs, dehiscent,4.5–7 × 3.5–6 cm, ovoid, brown, apex obtuse, base round, longitudinal suture on both sides, densely pubescent; rind fleshy, soft, 1.5 cm thick, brown; seed 4–6 × 3–3.5 cm, oblong, black, arillate; aril orange red, lacerated.

3A.2. Aim and Scope of the Present Work

Wild nutmegs, the less explored species of flowering plants plays significant role in the ecological and environmental factors of evergreen and swamp forests. They are endemic, threatened and economically important plants of the Western Ghats. Since the seeds of the species are widely employed in commercial and therapeutic applications, the species is highly exploited. Most of the natural habitat of *M. malabarica* has been fragmented due to human activities leading to decline in their natural populations *M. malabarica*, a wild relative of nutmeg was found to contain an unusual class of biologically important compounds called phenylacylphenols. Varieties of biological activities displayed by these compounds are impressive but it is not that much explored. In this context, the present study of wild nutmeg of evergreen and swamp forests in Kerala part of Western Ghats (Agasthyamala Biosphere Reserve) is carried out to identify the phytochemical constituents of different parts of the fruit (rind, seed and mace) and to explore their pharmacological relevance. Herein the present study aims the isolation phenylacylphenols from the species and the evaluation of their antidiabetic potential.

3A.3. Extraction, Isolation and Characterization of Phytoconstituents from the Fruits of *M. malabarica*

3A.3.1. Collection of Plant Material

Fruits of *Myristica malabarica* were collected in April 2017 from Chemunji Hills of Agasthyamalai biosphere reserve, Thiruvananthapuram District, Kerala, India. Collected fruits were analyzed by Dr. Mathew Dan (Plant taxonomist, JNTBGRI, Palode, Trivandrum) in order to validate its authenticity and a voucher specimen [***Myristica malabarica*, Chemunji Hills, Trivandrum, Kerala, April, 2017, Govind, *83442* (TBGT)]** was deposited in the herbarium of the same Institute. A geographical map of the species is given in Fig. **3A.2**.

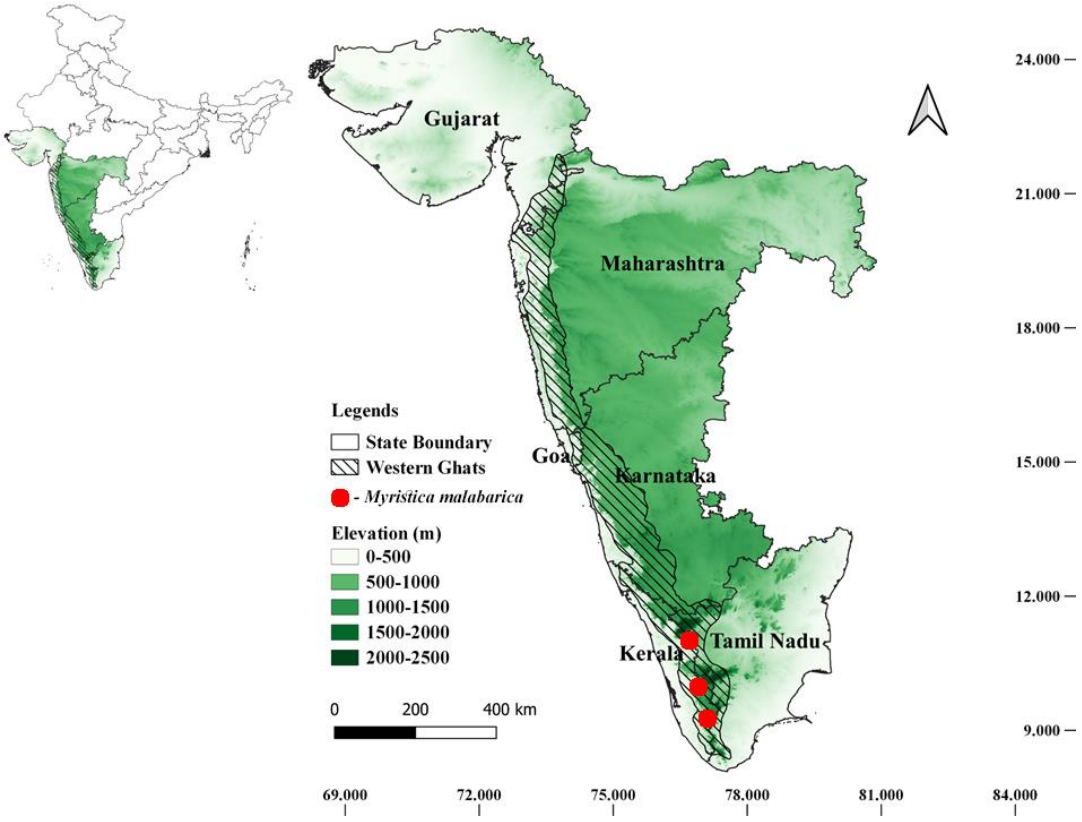


Figure 3A.2: Distribution and collection site of *M. malabarica*

3A.3.2. Extraction of the fruit rinds of *M. malabarica*

Rinds were separated from the fruits and air dried. Coarsely powdered dry fruit rinds (670 g) of *M. malabarica* was extracted DCM (2 L x 3days) at room temperature. The extract was then filtered and concentrated under reduced pressure using Heidolph rotary evaporator afforded 25 g of DCM extract. The residue was then sequentially extracted with acetone (2.5 L x 3days), ethanol (2.5 L x 3days) and water (2.5 L x 3days) to afford crude extracts (12 g of acetone, 15 g of ethanol and 10 g water).

3A.3.2.1. Isolation and Characterization of Compounds from the Fruit Rind

About 25 g of DCM extract was subjected to silica gel (100-200 mesh) column chromatographic separation with gradient elution of Hexane/EtOAc (100:0 to 0:100, v/v) afforded 34 fraction pools based the similarity in TLC. Similarly 12 g of acetone extract was also fractionated over silica gel (100-200 mesh) column chromatography using hexane, hexane-EtOAc gradient and EtOAc afforded 12 fraction pools. These fractions on repeated column chromatographic separation and crystallization resulted in the isolation of following compounds. A schematic representation of the entire isolation procedure is given below.

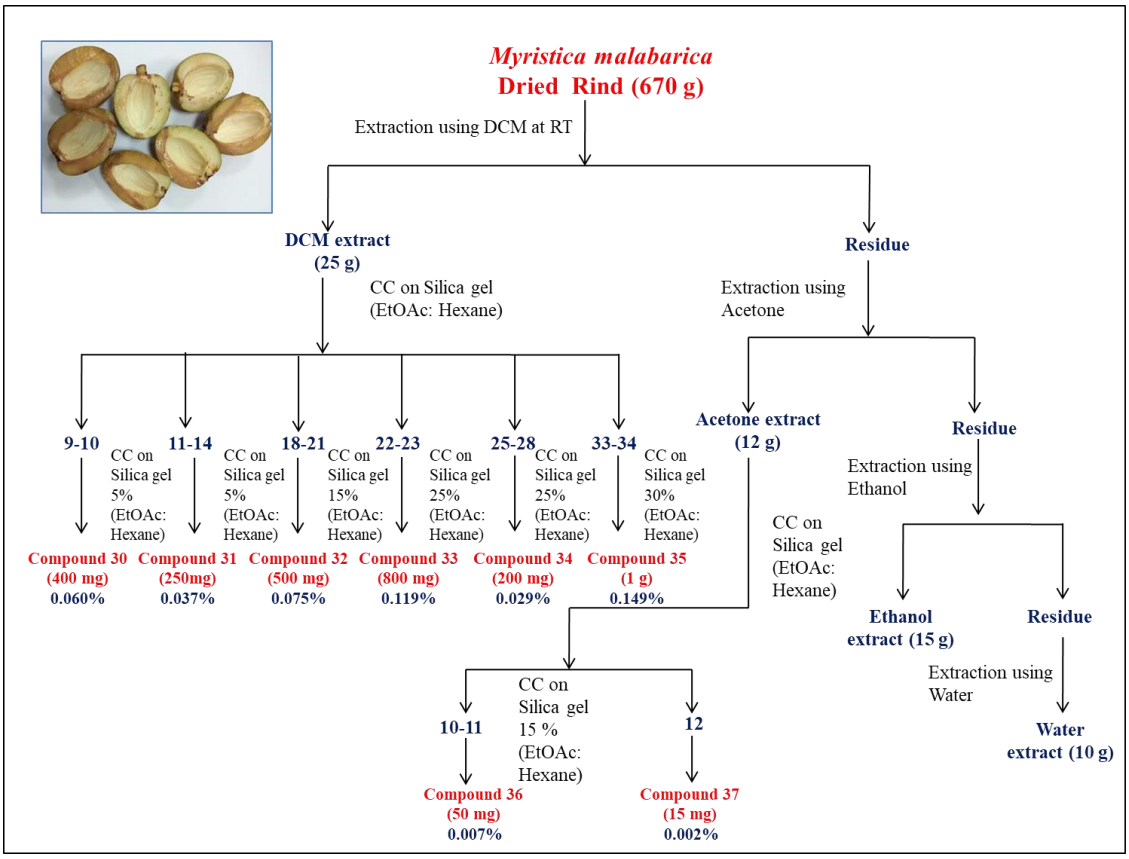


Figure 3A.3: Schematic representation of extraction and isolation procedure

Compounds **30-36** obtained from the fruit rinds of *M. malabarica* were characterized based on the comparison of spectral data with literature and were found to be similar to the compounds previously isolated from *M. beddomeii* (Chapter **2A**). Fraction pool 9-10 showed the presence of an intense UV active spot. On charring in Enholm yellow solution the TLC turned into intense pink colour. CC separation of the fraction on silica gel using 5% ethyl acetate-hexane afforded the compound **30** as white

crystalline solid and was identified as 1-(2, 6-dihydroxyphenyl) tetradecan-1-one similar to the previously isolated compound **2**.

Compound **31** was isolated as a white powder after CC separation of the fraction pool 11-14 by eluting the column with 5% ethyl acetate-hexane and was confirmed as malabaricone D similar to the previously isolated compound **5**.

Fraction pool 18-21 after CC separation using 15% ethyl acetate-hexane gave the compound **32** as pale yellow crystalline solid and was identified as malabaricone A similar to the previously isolated compound **1**.

CC separation of fraction pool 22-23 on silica gel by elution with 20% ethyl acetate-hexane solution afforded the compound **33** as pale yellow solid which was identified as malabaricone B similar to compound **3**.

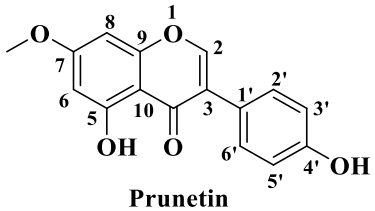
Compound **34** was afforded as pale yellow crystalline solid from the fraction pool 25-28 after CC separation on silica gel using 25% ethyl acetate-hexane as eluent and was confirmed as promalabaricone B similar to compound **8**.

Fraction pool 33-34 showed the presence of an intense UV active spot. On charring in Enholm yellow solution the TLC turned into intense red colour. It was then subjected to CC separation on silica gel using 30% ethyl acetate-hexane afforded the compound **35** as pale yellow crystalline solid and was confirmed as malabaricone C similar to the previously isolated compound **4**.

Similarly around 12 g of the acetone extract of the rind was fractionated over 100-200 mesh sized silica gel by gradient elution with Hexane/EtOAc (100:0 to 0:100, v/v) afforded fractions based on the similarities in TLC. Fraction pool 10-11 on CC separation using 15% ethyl acetate-hexane eluent afforded compound **36** as white powder. Based on the spectral analysis and a comparative study with the literature, the compound was confirmed as Biochanin A which is similar to compound **22** previously isolated from *M. beddomei*.

Fraction pool 12 from the acetone extract of the rind showed an intense UV active spot. On charring in Enholm yellow solution the TLC turned into intense pink colour. It was then subjected to CC on silica gel using 15% ethyl acetate-hexane solution afforded the compound **37** as white powder. Based on the various spectral analysis and comparative study with literature reports the compound was identified as prunetin. ^{1}H NMR displayed a sharp singlet at δ 8.23 (s, 1H, H-2) ppm for the proton of the B ring of isoflavonoid moiety. It is one of the characteristic peaks. The one proton singlet at δ 12.86 ppm without carbon correlation in HMQC clearly indicates the presence of an

enolisable hydroxyl group and the one proton broad singlet at δ 8.68 ppm corresponds to the hydroxyl group at position 4'. Besides the ^{1}H NMR also exhibited two sets of doublets at δ [6.56(d, *J*=2.5Hz, 1H, H-8) & 6.36 (d, *J*=2.5Hz, 1H, H-6)] ppm for the two aromatic protons (6 and 8) adjacent to methoxy group and another two sets of doublet at δ [7.47 (d, *J*=8.5Hz, 2H, H-2',6') & 6.91 (d, *J*=8.5Hz, 2H, H-3',5')] ppm for the aromatic protons. A sharp singlet at δ 3.85 ppm integrating for three protons confirmed the presence of a methoxy group. Position of the methoxy group was confirmed from the HMBC correlations by comparing the spectrum of biochanin and prunetin [2]. ^{13}C NMR gave carbon resonance including carbonyl carbon (δ 181.7 ppm), methoxy carbon (δ 56.4 ppm), carbon bearing hydroxyl group (δ 163.5, 158.5 ppm), olefinic carbon (δ 154.5 ppm) and aromatic carbons. The mass spectrum of the compound gave a molecular ion peak at 285.07595 which is the $[M+H]^{+}$ peak.



Prunetin
Compound **37**

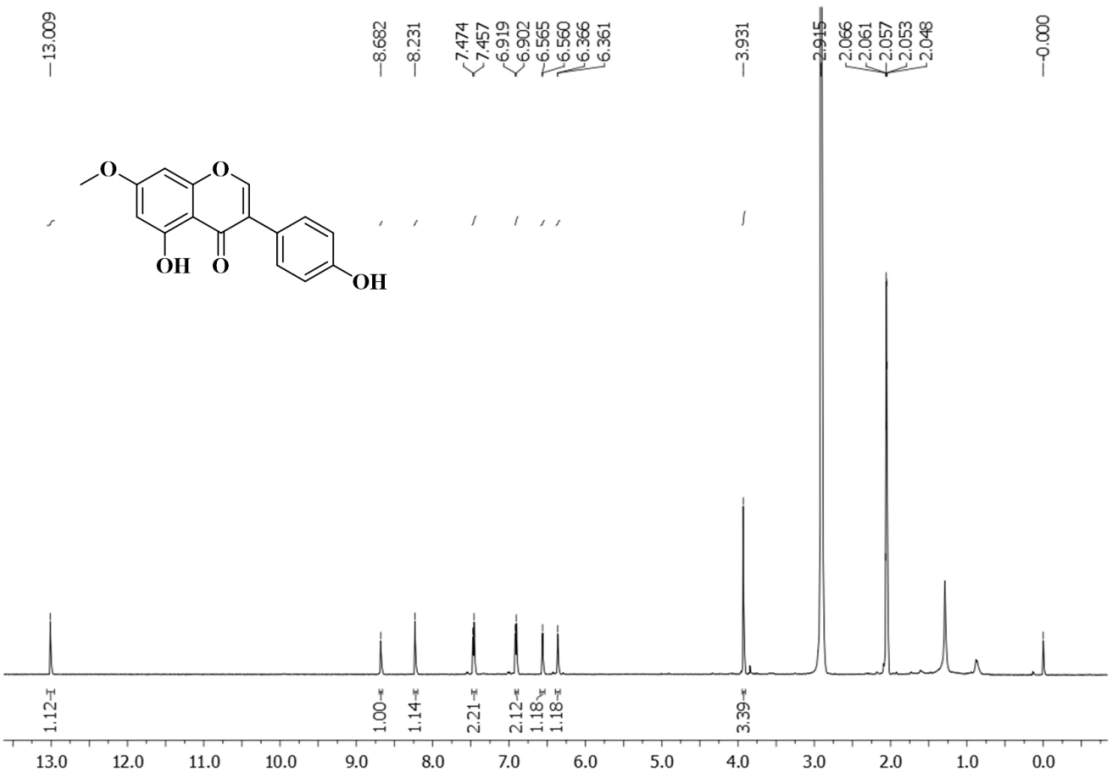


Figure 3A.4: ^{1}H NMR spectrum of prunetin

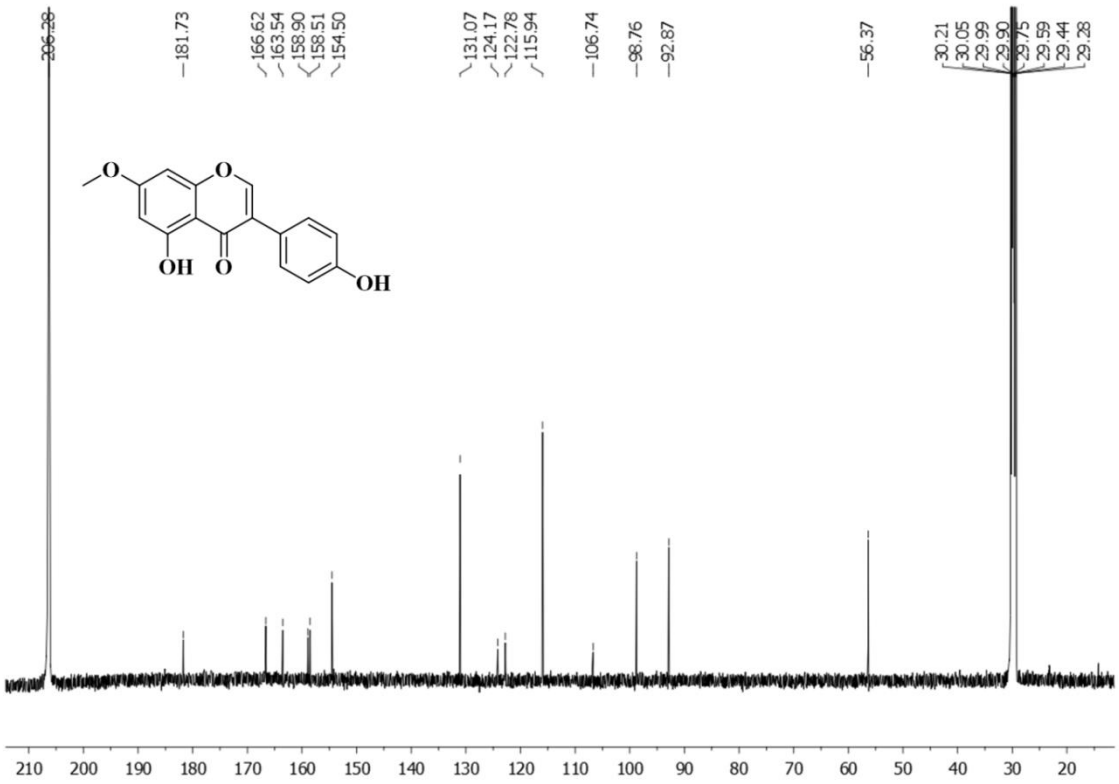


Figure 3A.5: ^{13}C NMR spectrum of prunetin

3A.3.3. Extraction of the Seeds of *M. malabarica*

Seeds were dried in an air oven maintained at 50 ^{o}C for an hour, and then powdered mechanically. About 500 g of powdered material was subjected to extraction with hexane (2.5 L X 3 days) at room temperature to remove the fat and oil. After removing the oil content, the residue was then subjected to extraction with DCM (2.5 L X 3 days) at room temperature. About 25 g of DCM extract was obtained after removing the solvent using rotary evaporator. The residue was then sequentially extracted with acetone, ethanol and water to afford 28 g of acetone, 20 g of ethanol and 12 g of water extract. A schematic representation of the entire isolation procedure is given in Fig. **3A.6**.

3A.3.3.1. Isolation and Characterization of Compounds from the Seeds

DCM (35 g) extract was fractionated over silica gel (100-200 mesh) column chromatography using hexane, hexane-EtOAc gradient and EtOAc afforded 21 fraction pools based on the similarities in the TLC. These fractions on repeated column chromatographic separation resulted in the isolation of following compounds **32-45**. Major phytoconstituents of the species such as malabaricones were also obtained in excellent yield from the acetone extract after column chromatographic separation and crystallization technique.

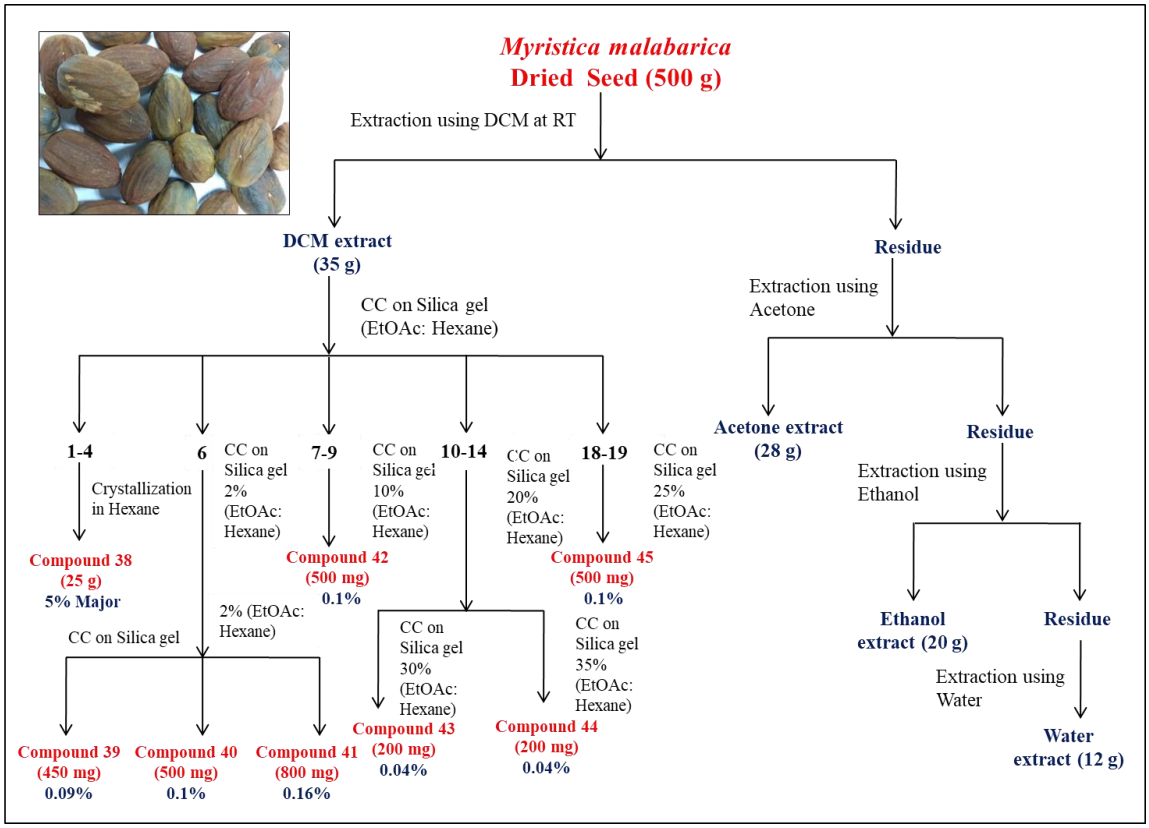


Figure 3A.6: Schematic representation of extraction and isolation procedure from the seeds of *M. malabarica*

Compounds **38-40** were identified as, **38** (trimyristin), **40** (1-(2,6-dihydroxyphenyl)tetradecan-1-one), **41** (malabaricone A), **42** (malabaricone D), **43** ((2R)-3(3,4-methylenedioxyphenyl)-1,2-propanediol), **44** (malabaricone B) and **45** (malabaricone C). These compounds were obtained after CC separation of the fractions of DCM extract using Hexane/EtOAc gradient. Structures of the compounds were confirmed on the basis of spectral data and in comparison with the literature. All these compounds were earlier reported from the species *M. beddomei* and their spectral details were already described in Chapter **2A.** Compound **39** was afforded as colourless liquid from fraction pool 6 after column chromatographic separation (Silica gel 100-200 mesh) using 2% ethylacetate-hexane. Spectral analysis of the compound and in comparison with literature confirmed the compound as bis-diethylhexylphthalate which is similar to the previously reported compound from the species *M. trobogarii* (Chapter **2B,** Compound **23**).

3A.3.4. Extraction of the Mace of *M. malabarica*

Around 270 g of air dried and powdered mace was extracted sequentially with acetone, ethanol and water (1.5 L x 3 days) at room temperature. The crude extract (50 g

acetone, 20 g ethanol & 15 g water) was obtained after the removal of solvent using a rotary evaporator. Schematic representation of entire extraction and isolation procedure are given in Fig. **3A.7**.

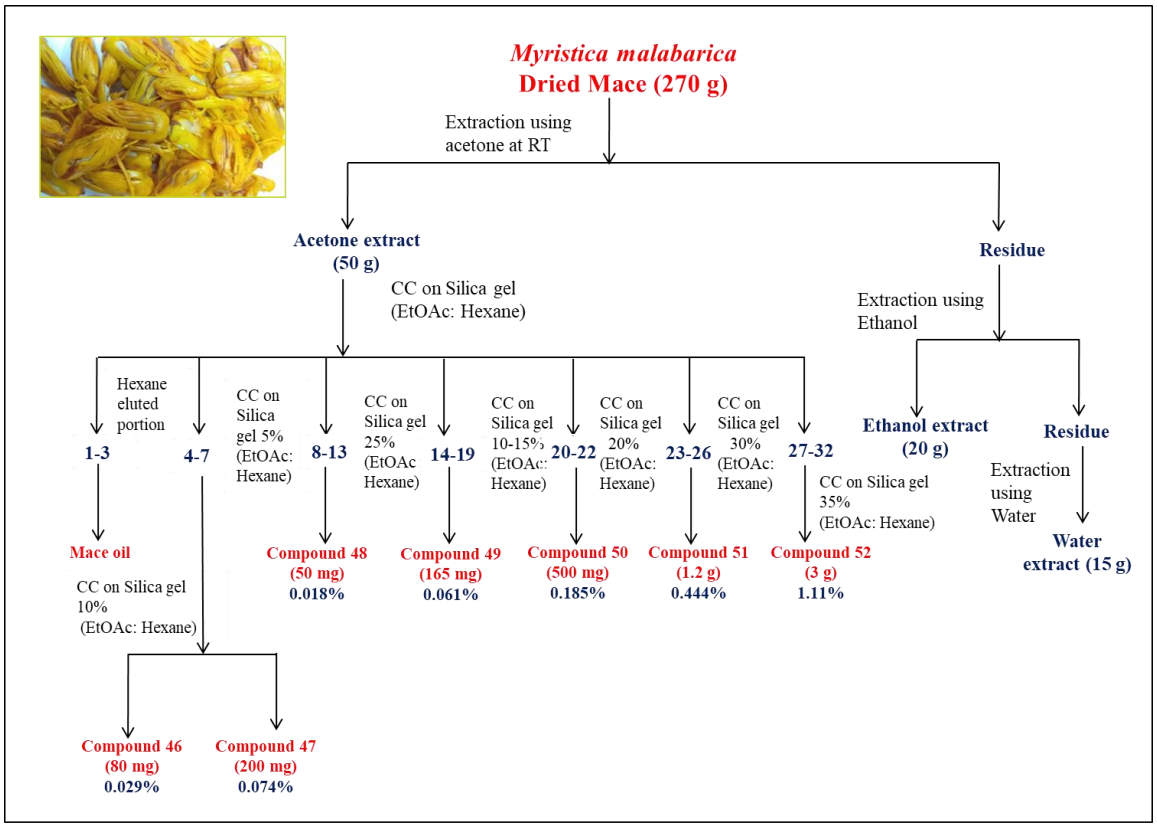


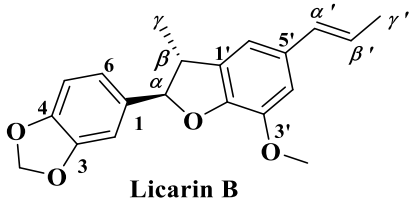
Figure 3A.7: Schematic representation of extraction and isolation procedure from the mace

3A.3.4.1. Isolation and Characterization of Compounds from the Mace

About 50 g of the acetone extract of the mace was subjected to silica gel (100-200 mesh) column chromatography using gradient elution of Hexane/ETOAc to obtain the fractions. Based on the similarities in TLC thirty two fraction pools were obtained. Hexane eluted fractions of the mace contained mainly fatty acid and oils.

Fraction pool 4-7 showed the presence of two UV active spot. On charring in Enholm yellow solution one spot on the TLC turned into purple colour and the other into red. Compound **46** was isolated as a white solid from the fraction pool 4-7 after CC separation using 5% ethylacetate-hexane as eluent. ^{1}H NMR spectrum of the compound displayed two sets of three proton doublets at δ 1.38 and 1.87 ppm corresponds to two methyl groups, a three proton singlet at δ 3.89 ppm for methoxy group and a two proton singlet at δ 5.95 ppm for methylenedioxy group. In addition presence of two sets of

doublet of doublet at δ [6.35 (dd, J_1=1.5 Hz, J_2=15.5 Hz, 1H) & 6.10 (dd, J_1=6.5 Hz, J_2=15.5 Hz, 1H) ppm] could be attributed to *trans olefinic* protons and a one proton doublet at δ 5.09 ppm along with a doublet of doublet at δ 3.39 ppm corresponds two -CH protons. Besides the peak corresponds to aromatic protons were also observed. ^{13}C NMR spectrum of the compound gave nineteen carbon resonances comprised of olefinic carbon, methoxy carbon, methyl carbon, methylenedioxy carbon along with aliphatic and aromatic carbons. The mass spectrum of the compound gave molecular ion peak at 347.1255 which is the $[M+Na]^+$ peak. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be licarin B [3].



Licarin B
Compound **46**

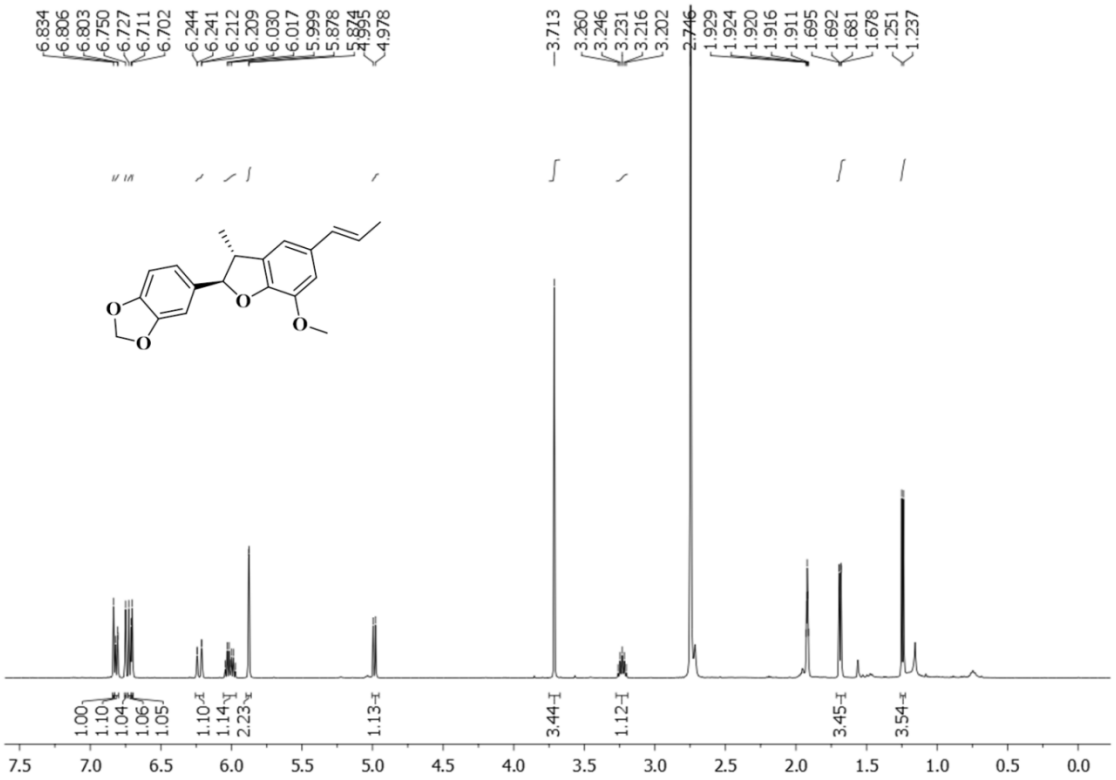


Figure 3A.8: ^{1}H NMR spectrum of licarin B

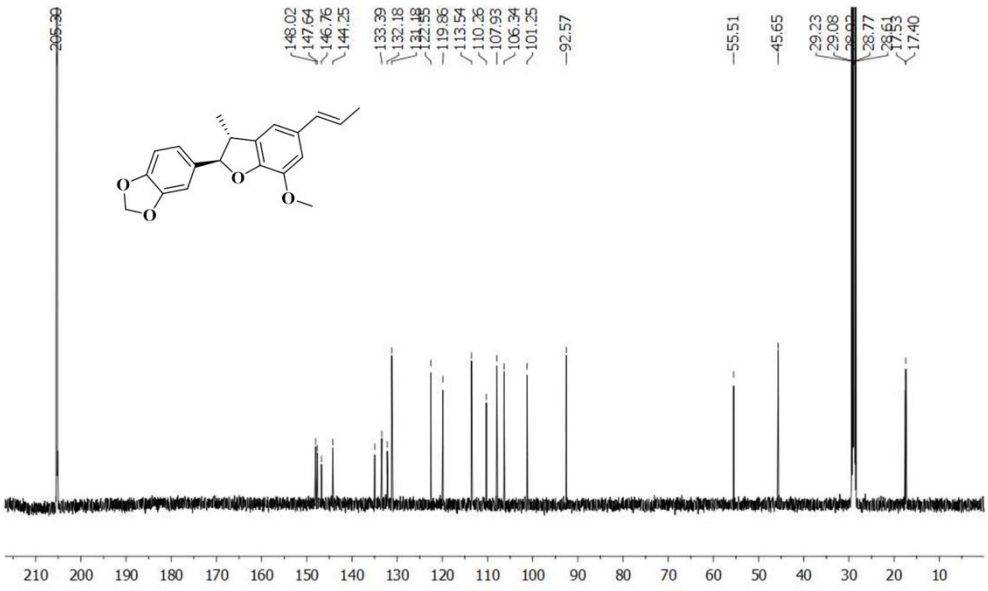
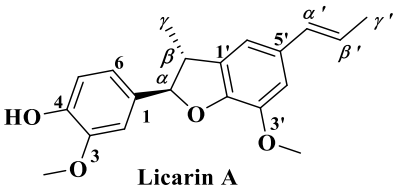


Figure 3A.9: ^{13}C NMR spectrum of licarin B

Compound **47** was also isolated from the same fraction pool as pale yellow crystals by eluting the column with 10% ethylacetate-hexane. By comparing the spectral details of the compound with literature the compound was confirmed as malabaricone A similar to the previously isolated compound **1** (Chapter **2A**). Compound **48** was afforded as pale yellow solid from the fraction pool 4-7 after CC separation using 5% ethylacetate-hexane as eluent and it was identified as 1-(2, 6-dihydroxyphenyl) tetradecan-1-one (Compound **2**, Chapter **2A**) based on the spectral data.

Fraction pool 14-19 on CC separation using 10-15% ethylacetate-hexane led to the isolation of compound **49** as white solid. Spectral data of the compound showed some similarities to that of compound **46** indicated the compound is a lignan with different substitution. ^{1}H NMR displayed two three proton singlets at δ 3.89 & 3.88 ppm indicated the presence of two methoxy groups. IR spectrum of the compound showed a broad absorption at 3426 cm^{-1} indicated the presence of a hydroxyl group. ^{13}C NMR of the compound gave nineteen carbon resonances. The mass spectrum of the compound gave molecular ion peak at 349.1414 which is the $[M+Na]^{+}$peak. By comparing the spectral data of the compound with the literature the compound was identified as licarn A [4].



Licarin A
Compound **49**

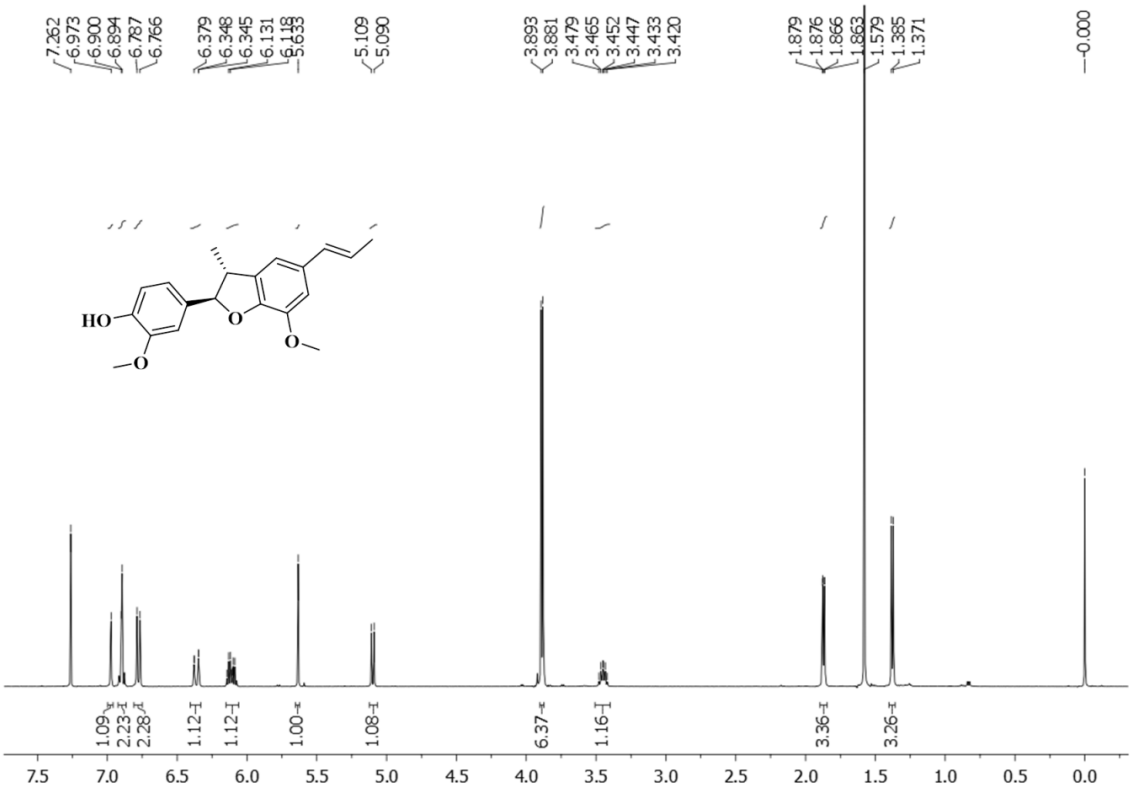


Figure 3A.10: ^{1}H NMR spectrum of licarin A

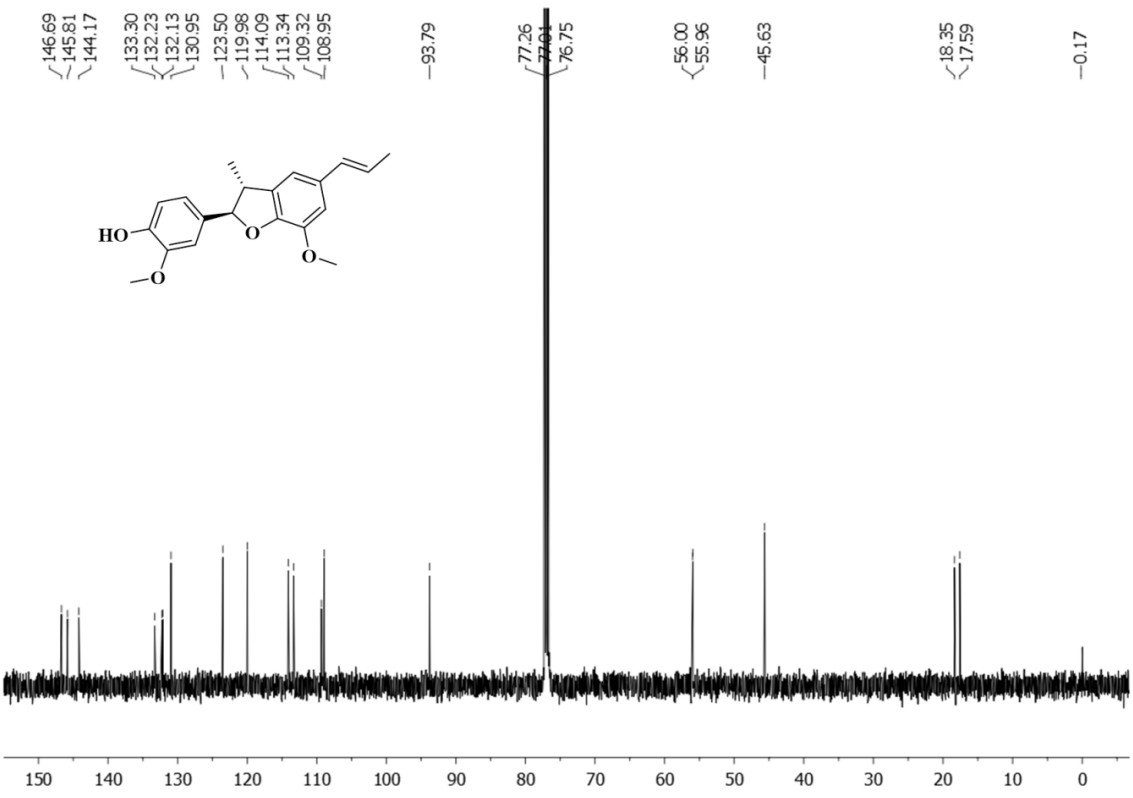


Figure 3A.11: ^{13}C NMR spectrum of licarin A

Compound **50** was identified as malabaricone D, **51** (malabaricone B) and **52** (malabaricone C) based on the spectral data. These compounds were also isolated earlier from the rind and seeds of *M. malabarica*.

3A.4. *In vitro* Antidiabetic Potential of Malabaricones

Type 2 diabetes mellitus (T2DM), a non-communicable chronic disorder characterized by hyperglycemia resulting from abnormal glucose metabolism and is expanding as a global health issue. Atherosclerotic cardiovascular disease, congestive heart failure, chronic kidney disease etc. are the high risk conditions associated with diabetes mellitus. Anti-diabetic medications include insulin injections and oral antihyperglycemic drugs such as glinides, sulfonylureas, α-glucosidase inhibitors, thiazolidinediones etc. are the active agents widely employed to control hyperglycemia. In addition healthy diet and lifestyle have a significant role in the management of T2DM. Increasing prevalence of T2DM and the severe side effects and limited efficacy associated with the existing antidiabetic drugs has created a significant medical need for new and effective therapeutic agents. Bioactive secondary metabolites from natural sources are highly preferred to overcome the challenges associated with diabetes treatment owing to their increased efficacy and safety [5-7].

Imbalanced glucose homeostasis affects the cellular balance of carbohydrate and lipid metabolism results in postprandial hyperglycemia which is the leading cause of diabetic complications associated with T2DM. The key enzymes involved in the digestion of carbohydrates are α-glucosidase and α-amylase. Most effective strategy adopted for the efficient management of hyperglycemia is the reduction of glucose production and absorption in stomach and intestine through the inhibition of carbohydrate digesting enzymes such as α-amylase and α-glucosidase along with the delaying of advanced glycation end products (AGEs) formation. Plants have long history of traditional use in treatment of T2DM. Plant secondary metabolites including flavonoids, phenolic compounds, alkaloids etc. were extensively studied for their antidiabetic potential [8-10]. Several studies demonstrated the potent inhibitory activity of phytochemicals from the plant species against digestive enzymes also [11]. In this regard we have evaluated the *in vitro* antidiabetic potential of phenylacylphenols isolated from *M. malabarica.* Acylphenols and phenylacylphenols including malabaricones are the major phytoconstituents of the wild nutmeg species *M. malabarica*. These molecules are responsible for the variety of biological activities displayed by the species. *In vitro* assays include α-glucosidase inhibition, α-amylase inhibition and antiglycation properties along

with molecular docking studies of the compounds were performed to evaluate the antidiabetic potential of the isolated compounds.

In vitro inhibitory potential of the compounds obtained from the fruits of the species such as 1-(2,6-dihydroxyphenyl)tetradecan-1-one (**1**), malabaricone A (**2**), malabaricone B (**3**), malabaricone C (**4**) and promalabaricone B (**5**) were assayed against carbohydrate hydrolyzing enzymes (α-amylase and α-glucosidase) and delaying of AGEs formation. *In vitro* assays were performed on the basis of reported procedure with slight modification in which acarbose and ascorbic acid are the positive control. IC_{50} values of the compounds were determined and are summarized in Table **3A. 1**.

Table 3A.1: IC_{50} values of isolated compounds against digestive enzyme inhibition and antiglycation properties

SL. NO	Compound Name	IC_{50} values (μM)		
		α-amylase	**α-glucosidase**	**Antiglycation**
1	1-(2,6-dihydroxy phenyl)tetradecan-1-one	74.12±1.278	171.90±0.89	120.84±0.547
2	Malabaricone A	**19.07±0.517**	91.444±1.245	**19.28±0.0454**
3	Malabaricone B	**12.89±0.068**	**63.70±0.0546**	**40.34±0.0948**
4	Malabaricone C	**10.63±0.171**	**43.61±0.620**	**14.99±0.114**
5	Promalabaricone B	81.97±1.233	**32.67±0.469**	227.26±0.773
Acarbose		8.93 ± 0.48	66.57 ± 0.982	-
Ascorbic acid		-	-	155.38 ± 0.547

Each value represents mean ± SD (standard deviation) from three individual measurements. Significance test between various groups was determined by using one-way analysis of variance, followed by Dunnett's t test. * $p < 0.01$ versus positive control.

3A.4.1. α-Amylase Inhibitory Activity

α-Amylase, the digestive enzyme present in saliva and pancreas is responsible for the hydrolysis of starch. Human pancreatic α-amylase catalyzes initial step in the hydrolysis of α-(1, 4)-D-glucosidic linkages in starch to maltose and glucose. Elevated levels of the enzyme in the blood are used as marker for the clinical identification of diabetes. Inhibition of the enzymatic activity can reduce the postprandial blood glucose level [12]. Since enzymatic inhibition is novel therapeutic target for the management of hyperglycemia we have evaluated the compounds for their α-amylase inhibitory potential

in human porcine pancreatic α-amylase in which acarbose as positive control. Acarbose is the first standard α-glucosidase inhibitor commercially available for diabetes treatment which can also inhibit the activities of α-amylase. The IC_{50} values of the tested compounds indicated its potential against α-amylase inhibition (Table **3A.1**). Among the compounds tested malabaricone A, B & C showed significant activity with IC_{50} values of 19.07±0.517, 12.89±0.068 & 10.63±0.171 μM respectively while the compounds 1-(2,6-dihydroxyphenyl)tetradecan-1-one and promalabaricone B showed lower activity. Inhibitory potential of malabaricone C was comparable to that of the standard drug acarbose indicating that it can act as an active lead molecule for α-amylase inhibition.

3A.4.2. α-Glucosidase Inhibitory Activity

α-Glucosidase is the membrane bound enzyme located in the epithelium of small intestine catalyzes the hydrolysis of oligosaccharides and disaccharides into simple glucose. Inhibition of α-glucosidase enzyme helps to retard the digestion of carbohydrates which in turn reduce the levels of glucose in blood and thereby suppress the postprandial hyperglycemia. α-Glucosidase inhibitors including acarbose, miglitol, voglibose etc. are one of the effective class of oral antidiabetic drugs capable of ameliorating hyperglycemia and are widely employed for the treatment of T2DM. Several plant extracts and secondary metabolites were proved to be effective α-glucosidase inhibitors [13-14]. Herein we evaluated the α-glucosidase inhibitory activity of the isolated compounds in which acarbose used as the positive control. All the compounds showed moderate to excellent inhibitory potential (Table **3A.1**). Amongst malabaricone B (IC_{50}=63.70±0.0546 μM), malabaricone C (IC_{50}=43.61±0.620 μM) and promalabaricone B (IC_{50}=32.67±0.469 μM) displayed significant inhibitory potential comparable to that of the standard. The inhibitory activity of the compounds were found to be higher than that of the standard acarbose (IC_{50}=66.57±0.982μM). Substantially higher activity was observed in promalabaricone B.

3A.4.3. Antiglycation Property

Advanced glycation end-products are complex and heterogeneous group of compounds results from the non-enzymatic reaction of reducing sugars with amino groups in proteins, lipids, and nucleic acids (Glycation). Diabetic complications including retinopathy, neuropathy and nephropathy are arises as consequence of formation and accumulation of advanced glycation end products (AGEs). Natural compounds isolated from plants such as flavonoids have shown promising antiglycation activity. Antiglycation agents can delay the process of AGEs formation and can be utilized for the

management of late diabetic complications [15-16]. Since the compounds isolated from *M. malabarica* showed excellent enzymatic inhibition activities we were eager to evaluate their potential against the formation of AGEs. Antiglyction property of the compounds was evaluated using reported methods in which ascorbic acid as the positive control. Interestingly the tested compounds, malabaricone A (IC_{50}=19.28±0.0454 μM), malabaricone B (IC_{50}=40.34±0.0948 μM) and malabaricone C (IC_{50}=14.99±0.114 μM) exhibited excellent antiglycation property than the reference standard ascorbic acid (IC_{50}=155.38±0.547 μM) whereas 1-(2,6-dihydroxyphenyl)tetradecan-1-one and promalabaricone B were found to have moderate activity. The compounds were proved to be effective in *in vitro* antidiabetic screening further detailed studies are needed for the exploring its potential against diabetes.

3A.5. Molecular Simulation Studies

Molecular simulation studies provide the data regarding the probable binding modes of interaction of active compounds with enzymes in the biological system which in turn supports the experimental results. Encouraged with the excellent experimental results from enzymatic inhibition potential of the isolated compounds, we were performed the molecular docking and simulation studies using a glide program of Schrodinger suite 2017-2 in which the digestive enzymes were selected as human pancreatic α-amylase (4GQQ), human maltase glucoamylase [C-terminal (2QMJ) and N-terminal (3TOP)] [17-18]. The binding efficiency expressed in terms of G-score/D-score of the compounds with the selected enzymes was evaluated and results are summarized in Table **3A.2**.

Table 3A.2: G-score/D-score of the compounds with enzymes

Compounds	**2QMJ**		**3TOP**		**4GQQ**	
	G-score	D-score	G-score	D-score	G-score	D-score
1-(2,6-dihydroxy phenyl)tetradecan-1-one	-2.06	-1.98	-3.75	-3.67	-4.67	-4.59
Malabaricone A	-3.34	-3.27	-3.94	-3.86	-5.16	-5.08
Malabaricone B	-4.07	-3.99	-7.81	-6.57	-6.51	-5.27
Malabaricone C	-5.48	-5.4	-8.8	-8.8	-6.94	-6.86
Promalabaricone B	-4.78	-4.78	-6.81	-6.81	-2.87	-2.87

The results revealed that malabaricone C binds the catalytic domain with a G-Score (−5.48 kcal/mol)/ D-Score (−5.4 kcal/mol) in human pancreatic α-amylase (4GQQ). Since the porcine pancreatic α-amylase shows 83% similarity with human pancreatic α-amylase the findings were in accordance with the obtained experimental

data. The compound was also binds effectively with 2QMJ. The amino catalytic domain of C-terminal human maltase glucoamylase (2QMJ) catalyzes the cleavage of short α-(1→4) oligosaccharide units. Malabaricone C formed a strong H-bond donor interaction with the Asp327 and Gln603 via the hydroxyl groups and carbonyls group in it. The carbonyl terminal catalytic domain of human maltase gluco amylase (3TOP) also shows better interaction with malabaricone C with good G-Score (−6.94)/D-Score (−6.86 kcal/mol). Promalabaricone B also exhibits strong interactions with 4GQQ, 2QMJ and 3TOP with good G-Score/D-Score. The 2D and 3D interaction diagram of malabaricone C with 4GQQ, 3TOP and 2QMJ are shown in Fig. **3A.12.**

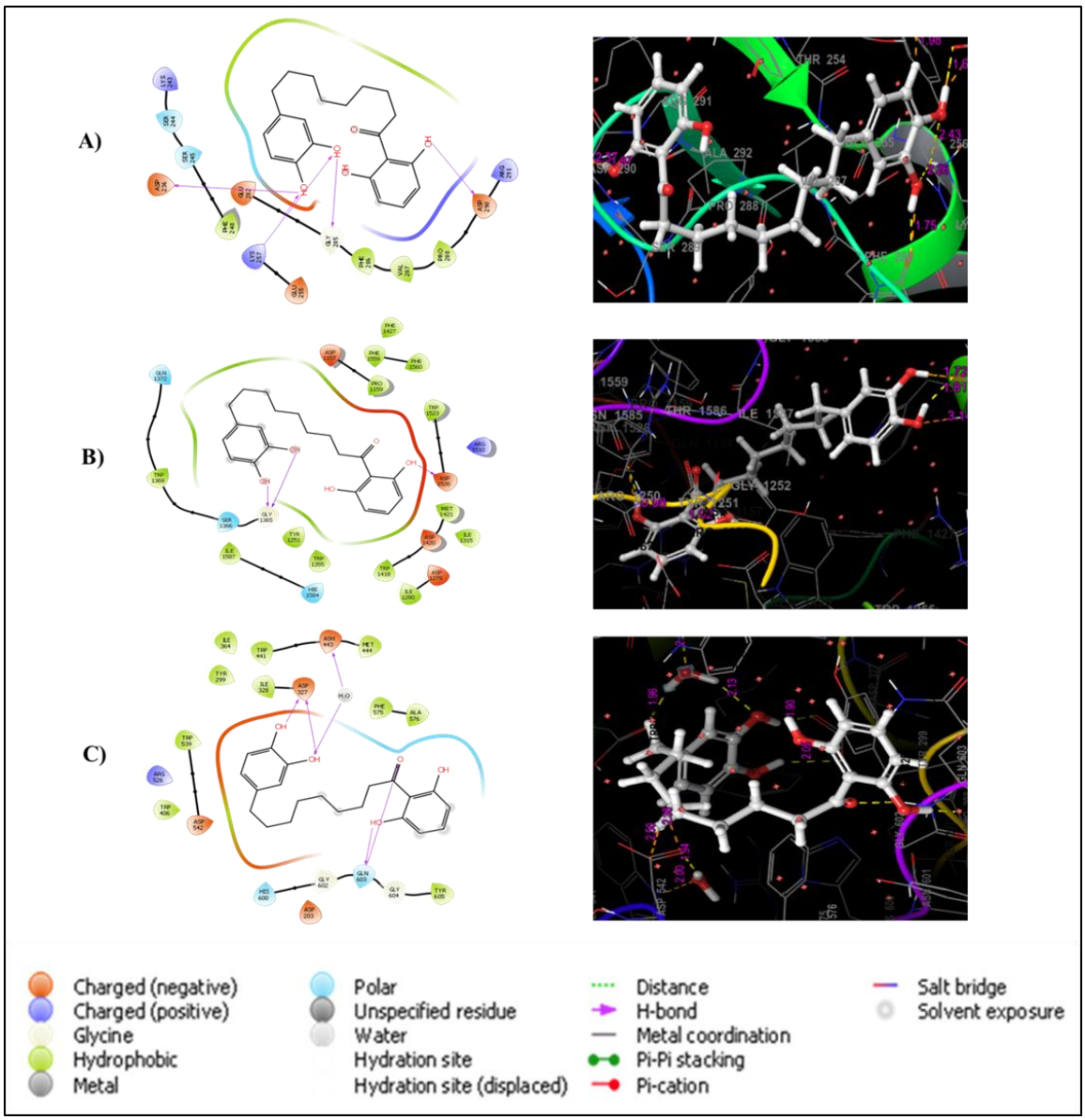


Figure 3A.12: The 2D and 3D interaction diagram of malabaricone C with A) 4GQQ, B) 3TOP and C) 2QMJ

Molecular simulation studies suggest that the compounds including malabaricones and promalabaricone B can act as efficient therapeutic agents for the treatment of T2DM. Further detailed studies are needed in order to understand their mechanism of action

In addition the pharmacokinetic parameters of the compound was analyzed by qikprop and the results (Table **3A.3**) revealed that all the tested compound satisfy the Lipinski Rule of Five and the adsorption, distribution, metabolism and excretion/toxicity (ADME/T) properties.

Table 3A.3: Pharmacokinetic parameters of the compounds

Compounds	**1**	**2**	**3**	**4**	**5**
M.W	320.471	326.435	342.434	358.433	360.449
#stars	3	3	0	0	0
#rotor	15	12	13	14	13
CNS	-2	-2	-2	-2	-2
SASA	729.068	697.858	701.324	715.598	725.605
FISA	115.108	114.984	169.717	216.82	209.734
HBA	1.5	1.5	2.25	3	6.2
HBD	0	0	1	2	2
QPlogKhsa	1.212	1.188	0.939	0.702	0.219
HOA	1	1	3	3	2
% HOA	100	100	86.654	87.297	81.422
QPlogPo/w	6.297	5.989	5.116	4.378	3.169
QPlogS	-6.806	-6.703	-6.132	5.717	5.624
ROf Five	1	0	1	1	0

M.W (Molecular Weight):130.0-725.0; #stars (few stars-more drug-like): 0-5; #rotor (Number of non-trivial rotatable bonds): 0 - 15; CNS (Central Nervous System activity): -2 to +2; SASA (Total solvent accessible surface area (SASA) in square angstroms using a probe with a 1.4 Å radius): 300.0-1000.0. FISA (Hydrophilic component of total solvent accessible area): 7.0-333.0; HBA (Hydrogen bond acceptor): 2.0-20.0; HBD (hydrogen bond donor): 0.0-6.0; Q Plog Khsa (Prediction of binding to human serum albumin): –1.5–1.5; Human Oral Absorption (HOA): Predicted qualitative human oral absorption: 1, 2, or 3 for low; Percent Human Oral Absorption (% HOA): Human oral absorption on 0 to 100% scale (>80% is high, <25% is poor); Q Plog Po/w (octanol/water partition coefficient): -2.0-6.5; Q PlogS (Aqueous solubility):-6.5-0.5; Ro5(Number of violations of Lipinski's rule of five): maximum is 4.

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3A.6. Conclusion

In summary, detailed phytochemical analysis of the different fruit parts (rind, seed and mace) of *M. malabarica* led to the isolation of thirteen compounds in which five compounds including licarin A, licarin B, trimyristin, bis-diethylhexylphthalate and (2R)-3(3, 4-methylenedioxyphenyl)-1,2-propanediol) were identified for the first time along with the known compounds including malabaricones (A-D), promalabaricone B, 1-(2,6-dihydroxyphenyl) tetradecan-1-one), biochanin A and prunetin. Malabaricones were isolated as the major phytoconstituents of the fruits of the species in excellent yield. In addition *in vitro* antibacterial screening of the compound was done in terms of their inhibition potential against digestive enzymes (α-amylase and α-glucosidase) along with antiglycation properties. All the tested compounds displayed moderate α-amylase and significant α-glucosidase inhibitory activity along with antiglycation properties. Compounds, malabaricone A, B & C showed significant α-amylase activity with IC_{50} values of 19.07±0.517, 12.89±0.068 & 10.63±0.171 μM respectively and malabaricone B (IC_{50}=63.70±0.0546 μM), malabaricone C (IC_{50}=43.61±0.620 μM) and promalabaricone B (IC_{50}=32.67±0.469 μM) displayed significant inhibitory potential against α-glucosidase. Malabaricone also showed significant antiglycation properties. Amongst malabaricone C was selected as excellent digestive enzyme inhibitor and was further supported by molecular docking studies. We established the antidiabetic potential of malabaricones from *M. malabarica*. These phytoconstituents can act as potential target for the development antidiabetic medication.

3A.7. Experimental Section

3A.7.1. General Methods

All the chemicals and solvents were of the best grade commercially available and were used without further purification. Different analytical techniques were used for the characterization of compounds. Merck precoated silica gel F_{254} plates were used for thin layer chromatography (TLC) and gravity column chromatography was performed using 100-200 & 230-400 mesh silica gel with gradient elution of Hexane/EtOAc. Melting point was determined on a Fisher Johns melting point apparatus and is uncorrected. IR spectra were recorded on Bruker Alpha FT-IR spectrometer and specific rotation was recorded using Jasco P-2000 polarimeter. Mass spectra were recorded under ESI/HRMS at 61800 resolution using Thermo Scientific Exactive Mass Spectrometer. The nuclear magnetic resonance spectra (NMR) were recorded on Bruker AMX 500 spectrometer ($CDCl_3$ & CD_3COCD_3 as solvents). Chemical shifts for ^1H NMR are reported as δ in

units of parts per million (ppm) downfield from $SiMe_4$ (δ 0.0). Multiplicities were given as: s (singlet); brs (broad singlet); d (doublet); t (triplet); m (multiplet); dd (doublet of doublet). Coupling constants are reported as *J* value in Hz. Commercially available porcine pancreatic α-amylase, rat intestinal α-glucosidase, acarbose, bovine serum albumin (BSA), α-D-glucose, ascorbic acid, 4-nitrophenyl α-D-glucopyranoside (p-NPG) were used to carry out antidiabetic assays.

3A.7.2. α-amylase inhibitory activity

The *α*-amylase inhibitory activity was determined by using the starch-iodine test based on the earlier reports [19]. Starch containing *α*-amylase solution (1 U mL^{-1}) was added to compounds of different concentration and was incubated for 30 minutes at 50 ^{o}C. After the incubation period, 1 M HCl was added to terminate the reaction subsequently 100 mL of iodine reagent was added to the reaction mixture. Finally *α*-amylase inhibition potential of the compound was quantified by measuring absorbance of the solution at 580 nm by using Synergy 4 Biotek multimode reader. Acarbose, the standard *α*-amylase inhibitor was used as the positive control and the percentage of inhibition of enzymatic activity was determined in accordance with the general equation,

$$\% \text{ of inhibition} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \text{ X } 100$$

The results were expressed in terms of IC_{50} value, which is defined as the concentration of *α*-amylase inhibitor required to reduce the enzymatic activity by 50% and is obtained from the plot of concentration along the x-axis and percentage inhibition along the y-axis.

3A.7.3. α-glucosidase inhibitory activity

The *α*-glucosidase inhibitory activity was assayed according to the previous method [20]. Rat intestinal α-glucosidase was used for the study.100 µL of the enzyme solution [1.0 U/mL prepared in 0.1 M phosphate buffer (pH 6.9)] were added to the solution of different concentrations of compounds and incubated for 10 min at 25 °C. After that, 50 µL of 5 µM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added and the mixture was again incubated at 25 °C for 5 min. The absorbance of the solution was determined at 405 nm using Synergy 4 Biotek multimode reader to quantify the enzymatic activity. The standard drug, acarbose was used as a positive control which gives the 100% enzymatic activity without the sample.

The percentage of inhibition of α-glucosidase enzyme was calculated using the above equation.

3A.7.4. Antiglycation property

Screening of antiglycation property of compounds was performed with some modifications in earlier methodology [21]. In brief, 1 mg/mL of bovine serum albumin (BSA) was incubated with 400 µL of α-D-glucose (500 mM) and different concentrations of the test compounds in 0.2 M potassium phosphate buffered saline at 60 °C for 24 h. The reaction was ended by adding 100% trichloroacetic acid (TCA) and kept at 4 °C for 10 minutes. Further, the samples were centrifuged (10,000 g) and the precipitate was dissolved in alkaline PBS. The fluorescence of the formed glycated end products (AGEs) was measured by excitation at 370 nm and emission at 440 nm using Synergy 4 Biotek multimode reader (USA). Ascorbic acid is used as the positive control. The AGEs formation was calculated using the following equation,

$$\text{AGEs formation} = \frac{\text{Fluorescence of control - Fluorescence of sample}}{\text{Fluorescence of control}} \text{ X } 100$$

3A.7.5. Molecular Docking Studies

The molecular docking simulation studies were performed by glide program of Schrodinger suite 2017-2. The crystal structures of human pancreatic α-amylase (PDB Code: 4GQQ) [22], human maltase glucoamylase C-terminal (PDB Code: 2QMJ) [23] and human maltase glucoamylase N-terminal protein (PDB Code: 3TOP) [24] were retrieved from the Protein Data Bank and the cleaned PDB files were further used for grid generation and simulation studies. The conformers of the ligands were prepared by ligprep and the pharmacokinetic parameters were analyzed using qikprop data. The molecular dynamics simulation studies were done by Schrodinger-Desmond programme for 3 ns using OPLS-2005 force field. The pharmacophore modeling was generated by pharmacophore hypothesis generation using seven point hypotheses.

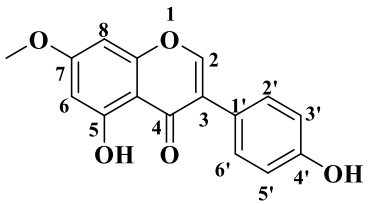
3A.7.6. Statistical Analysis

The experimental results were expressed as means±SD of triplicate measurements. The statistical analyses were performed by one-way analysis of variance, followed by Dunnett's t test. The difference was considered to be statistically significant when the p value was <0.01.

3A.8. Spectral Data

Compound 37 [Prunetin]

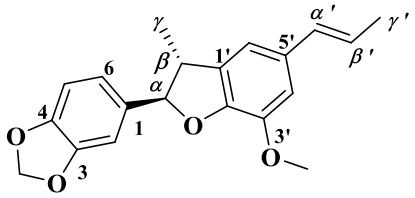
Fraction pool 12 after CC separation on silica gel using 15% ethyl acetate- hexane solution afforded the compound **37** as white powder. Based on the detailed analysis of spectral data and in comparison with the literature reports the compound was confirmed to be prunetin which is the structural isomer of the previously isolated compound biochanin A.



Molecular formula	:	$C_{16}H_{12}O_5$
Melting point	:	238-239 ^{o}C
FT-IR (neat, ν_{max}, cm^{-1})	:	3400, 2950, 1645
^{1}H NMR (500MHz, CD_3COCD_3)	:	δ 13.01 (s, 1H, OH-5), 8.68 (brs, 1H, OH-4'), 8.23 (s, 1H, H-2), 7.47 (d, *J*=8.5Hz, 2H, H-2', 6'), 6.91 (d, *J*=8.5Hz, 2H, H-3', 5'), 6.56(d, *J*=2.5Hz, 1H, H-8), 6.36 (d, *J*=2.5Hz, 1H, H-6), 3.93 (s, 3H, -OMe) ppm
^{13}C NMR (125MHz, CD_3COCD_3)	:	δ 181.7 (C=O), 166.6 (C-7), 163.5 (C-5), 158.9 (C-9), 158.5 (C-4'), 154.5 (C-2), 131.1 (C-2', 6'), 124.2 (C-1'), 122.8 (C-3), 115.9 (C-3',5'), 106.7 (C-10), 98.8 (C-6), 92.9 (C-8), 56.4 (-OMe) ppm
HR-ESIMS	:	285.07595 $[M+H]^+$

Compound 46 [Licarin B]

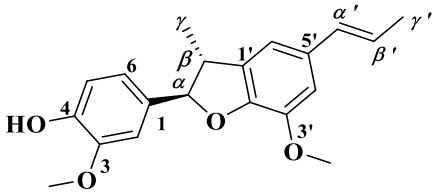
Compound **46** was afforded as a white solid from the fraction pool 4-7 after CC separation using 5% ethylacetate-hexane as eluent. By comparing the spectral data and literature values the compound was confirmed to be licarin B.



Molecular formula	:	$C_{20}H_{21}O_5$
Melting point	:	82-84 ^oC
FT-IR (neat, ν_{max}, cm^{-1})	:	2921, 1604, 1494, 1247, 1038, 963, 815
^{1}H NMR (500MHz, CD_3COCD_3)	:	δ 6.83(s, 1H), 6.81 (dd, J_1 =8 Hz, J_2=1.5 Hz, 1H), 6.75 (s, 1H), 6.72 (d, J = 8 Hz, 1H), 6.70 (s, 1H), 6.23 (dd, J_1= 16, J_2= 1.5 Hz, 1H), 6.04-5.97 (m, 1H), 5.88 (d, J = 2 Hz, 2H), 4.99 (d, J = 8.5 Hz, 1H), 3.71 (s, 3H), 3.27 – 3.19 (m, 1H), 1.69 (dd, J_1= 7, J_2= 1.5 Hz, 3H), 1.24 (d, J = 7 Hz, 3H) ppm
^{13}C NMR (125MHz, CD_3COCD_3)	:	δ 148.0, 147.6, 146.8, 144.2, 135.0, 133.4, 132.2, 131.1, 122.5, 119.9, 113.5, 110.3, 107.9, 106.3, 101.2, 92.6, 55.5, 45.6, 17.5, 17.4 ppm
HR-ESIMS	:	347.1255 $[M+Na]^+$

Compound 49 [Licarin A]

Fraction pool 14-19 on CC separation using 10-15% ethylacetate-hexane led to the isolation of compound **49** as white solid. IR spectrum of the compound showed a broad absorption at 3426 cm^{-1} indicated the presence of a hydroxyl group. ^{1}H NMR displayed two three proton singlets at δ 3.89 & 3.88 ppm indicated the presence of two methoxy groups. ^{13}C NMR of the compound gave nineteen carbon resonances. Finally by comparing the spectral data with the literature the compound was identified as licarin A.



Molecular formula	:	$C_{20}H_{22}O_4$
Melting point	:	119-120 ^{o}C
FT-IR (neat, ν_{max}, cm^{-1})	:	3426 (OH), 2924,1614, 1497, 1221, 1123, 956, 860
^{1}H NMR (500MHz, $CDCl_3$)	:	δ 6.97 (s, 1H), 6.90 (d, J = 3 Hz, 2H), 6.78 (d, J = 10.5 Hz, 2H), 6.36 (dd, J_1= 15.5, J_2= 1.5Hz, 1H), 6.15 – 6.06 (m, 1H), 5.63 (s, 1H), 5.10 (d, J = 9.5 Hz, 1H), 3.89 (s, 3H), 3.88 (s, 3H), 3.48-3.42 (m, 1H), 1.87 (dd, J_1= 6.5, J_2= 1.5 Hz, 3H), 1.38 (d, J = 7 Hz, 3H) ppm
^{13}C NMR (125MHz, $CDCl_3$)	:	δ 146.7, 145.8, 144.2, 133.3, 132.2, 132.1, 130.9, 123.5, 120.0, 114.1, 113.3, 109.3, 108.9, 93.8, 56.0, 55.9, 18.3, 17.6 ppm
HR-ESIMS	:	349.1414 $[M+Na]^+$

3A.9. References

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Chemoprofiling of the Mace of *Myristica fragrans* & *In Vitro* antidiabetic Potential of Lignans & Neolignans from the Mace

3B.1. Introduction

Kerala, recognized as "the land of spices or spice garden of the world" owing to its cultivation of variety of spices including nutmeg, cardamom, pepper, clove etc. These spices are tremendously exploited as the key flavoring agents in numerous food cuisines because of its unique aroma and flavor. Western Ghats are enriched with these species and flourished in the shaded slopes of the region. Agastyamala Biosphere Reserve is considered as the genetic reservoir of these spices. The nutmeg, *Myristica fragrans* is the flagship species of the renowned myristicaceae family commonly called as 'nutmeg family'. The species is native to Moluccas Island and is widely cultivated in India. The warm, humid climate, reasonable rainfall, special features of the soil of Kerala contributes towards its cultivation. Apart from the use as a spice, the species gained enormous interest in terms of its pharmacological activities and nutritional benefits and is well employed in Ayurvedic medicine to treat various ailments. Fruits of the nutmeg (Jathikka) including its rind, seed and mace were used as a foremost ingredient in several Ayurvedic formulations such as *Jatiphaladichurna*, *Jatiphaladivati*, *Jeerakarishta* etc. Due its wide range of medicinal applications the species is well studied and reported to have antimicrobial, antioxidant, anticancer, anti-inflammatory, hepatoprotective, memory enhancing activities etc. The species is reported to have phenolic compounds, lignans, neolignans, phenylpropanoids etc. as the major class of secondary metabolites. The essential oil and the fat (nutmeg butter) from the nutmeg have high medicinal value [1-3].

Figure 3B.1: *Myristica fragrans* fruit and mace

***Taxonomic description of the species*:** Tree, evergreen, 10-15 m tall, dioecious; main trunk 90–120 cm in diameter, bark rough, brown, exudate pale red coloured, watery, branching usually whorled. Leaves alternate, petiolate; petiole 1–2 cm long, cylindrical, glabrous; lamina 8–12 × 5–7 cm, broadly lanceolate, apex acute to acuminate, margin undulate, base cuneate, adaxially dark green, gland-dotted, abaxially glaucous, glabrous; lateral veins adaxially prominent, 12-14 paired. Male inflorescence 4-5 flowered, axillary umbel; peduncle erect, 5 mm long, cylindrical, glabrous. Flowers caducous, urceolate, 5.5–7.3 × 2.3–3.5 mm, yellow, pedicellate; pedicel 8-10 mm long, glabrous. Perianth fleshy, lobes, equal, acute, re-curved outward, yellow, scented, scabrid outside; staminal column 3–4 mm long, cylindrical, scabrid at base; anthers 5-6. Female inflorescence 1-2 flowered, axillary umbel; peduncle erect, 4-5 mm long, stout, cylindrical, glabrous. Flowers urceolate, 3.5-5 × 3–4 mm, yellow, persistent, pedicellate; pedicel up to 5 mm long, glabrous; perianth fleshy, lobes 3, equal, acute, scabrid outside; stigma short, bilobed; ovary ovate, 1.5–3 mm, scabrid with golden hairs. Fruits usually solitary, rarely in pairs, dehiscent, 4.5–6 × 4–5.5 cm, sub globose to ovoid, yellow, apex and base round, longitudinal suture on both sides, glabrous; rind fleshy, soft, 1–1.5 cm thick, yellow; seed 2.5–3 × 2–3 cm, sub globose to ellipsoid, black, arillate; aril bright red, lacerated.

A detailed description of the *M. fragrans* including phytochemistry and pharmacological activities was already mentioned in Chapter **1B.4**.

3B.2. Aim and Scope of the Present Work

The nutmeg generally being consumed as a spice in food offers various health benefits in addition to its flavor. The species is well explored for its pharmacological potential but the studies regarding its antidiabetic activity are very limited. The prevalence of Type 2 diabetes mellitus is primarily due to sedentary life style and unhealthy food habits. Healthy lifestyle and a balanced carbohydrate controlled diet can manage the blood glucose level in great extent. In view of this our efforts are directed towards the effective utilization of secondary metabolites from the mace of the species for their antidiabetic potential in terms of their activity against carbohydrate hydrolyzing enzyme inhibition along with antiglycation properties. Since the mace of the species is largely consumed as a spice we focused on the phytochemical analysis of the same in order to obtain the bioactive phytoconstituents. Besides, the present study also aims a comparative study on chemotaxonomy of the true nutmeg with the wild nutmeg species based on the phytoconstituent present in the species. So far no such comparative study in terms the secondary metabolites is done.

3B.3. Extraction, Isolation and Characterisation of Phytochemicals from the Mace of *Myristica fragrans*

3B.3.1. Collection of Plant Material

Mace of *M. frgrans* (1 kg) was purchased from a local market in Aluva, Ernakulum district, Kerala state, India during April 2017. A geographical map showing distribution and collection site of the species is given in Fig. **3B.2**.

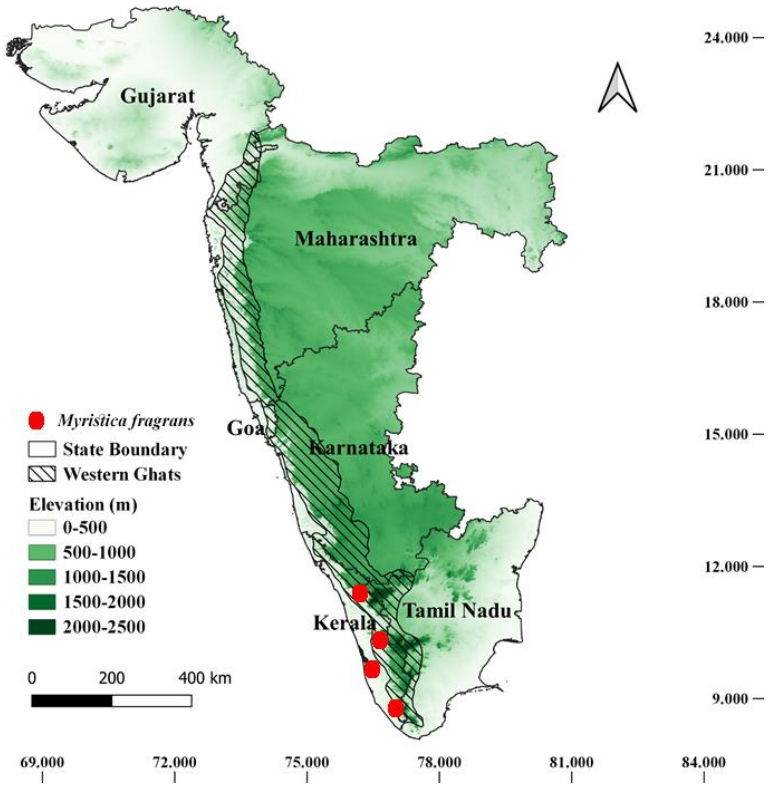


Figure 3B.2: Distribution and collection site of *M. fragrans*

3B.3.2. Extraction & Isolation

Purchased material was air dried and then powdered mechanically. About 800 g of powdered material was subjected to extraction with acetone (2 L x 4 days) at room temperature. Removal of the solvent under reduced pressure gave 90 g of the acetone extract. The residue was further extracted with ethanol (2 L x 4 days) and finally with water to afford 40 g of ethanol and 15 g of water extract.

Acetone extract (70 g) was fractionated over silica gel (100–200 mesh) column chromatography with the elution of hexane and Hexane/EtOAc gradient afforded 48 fractions. Hexane eluted fraction (oil from the mace) was removed initially and the remaining fractions on repeated CC separation and precipitation technique led to the isolation of following compounds. A schematic representation of entire extraction and isolation procedures are given in Fig. **3B.3**.

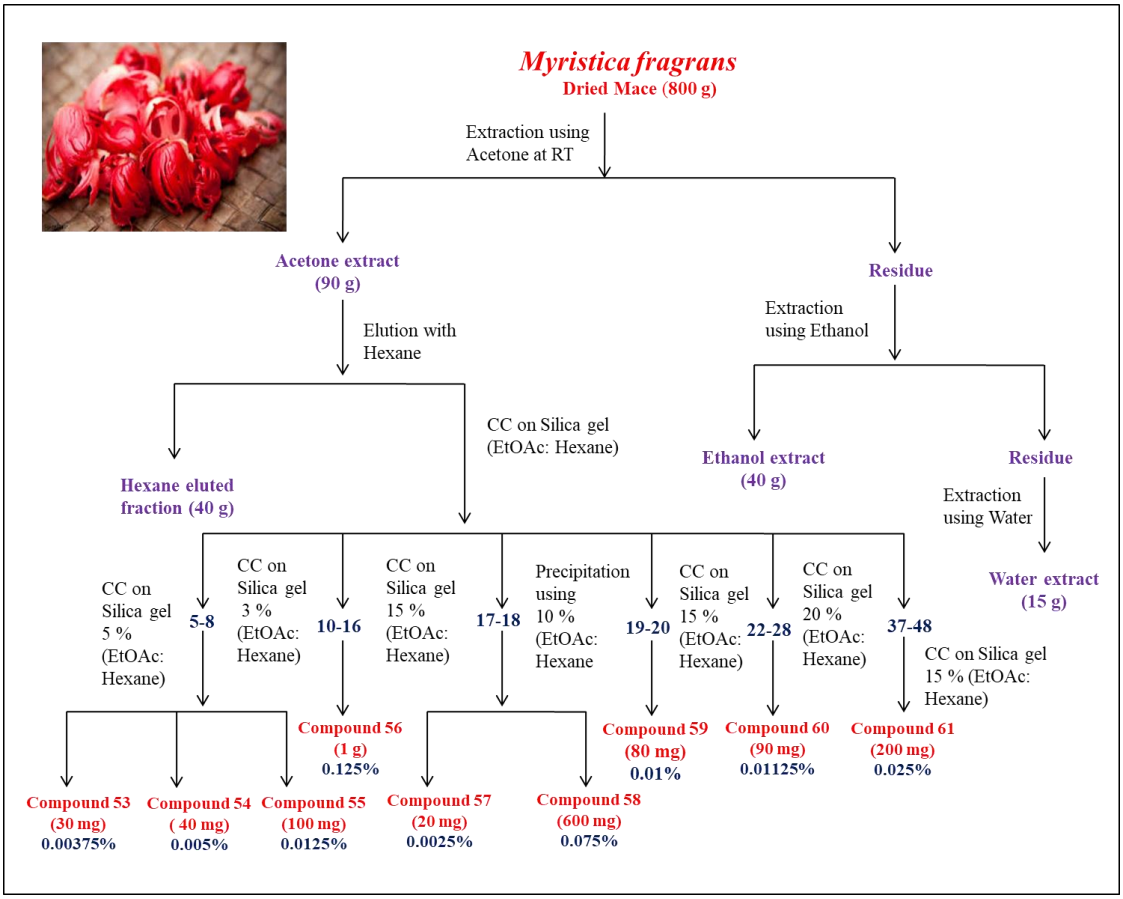
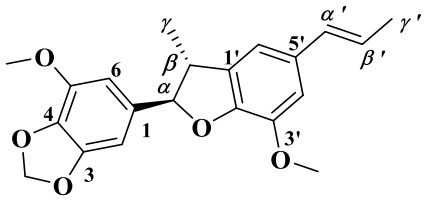


Figure 3B.3: Extraction and isolation procedure from the mace

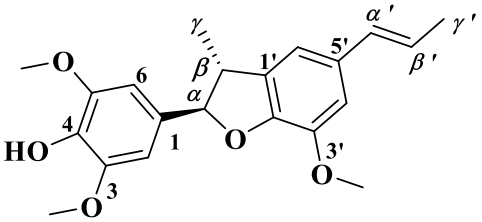
3B.3.3. Characterisation of Isolated Compounds from the Mace

Fraction pool 5-8 showed the presence of three UV active spots. Compound **53** was isolated as colourless viscous liquid from the fraction after CC separation using 5% ethyl acetate-hexane eluent. Based on the detailed analysis of spectral data and in comparison with the literature reports the compound was identified as (2S, 3R)-7-methoxy-3-methyl-5-((E)-prop-1-enyl)-2-(5-methoxy-3,4-methylenedioxyphenyl)-2,3-dihydrobenzofuran [4]. ^{1}H NMR spectrum of the compound showed a doublet of doublet at δ 6.35 (dd, J_1 =15.5 Hz, J_2 =1.5 Hz, 1H) ppm and a doublet of quartet at δ 6.10 (dq, J_1= 15.5 Hz, J_2= 6.5 Hz, 1H) ppm corresponds two *trans olefinic* protons along with a two proton singlet at δ 5.97 ppm for methylene dioxy group, a six proton singlet at δ 3.90 ppm for methoxy group and a set of doublet of doublet at δ 1.87 ppm and a doublet at δ 1.37 ppm for methyl groups. Besides, the aromatic protons were observed in the range of δ 6.78-6.61 ppm. ^{13}C NMR gave twenty one carbon resonances owing to methylenedioxy carbon, olefinic carbons, methoxy carbons, methyl carbons and aromatic carbons. The mass spectrum of the compound gave a molecular ion peak at 377.13644 which is the $[M+Na]^+$ peak [4].



(2S,3R)-7-methoxy-3-methyl-5-((E)-prop-1-enyl)-2-(5-methoxy-3,4-methylenedioxyphenyl)-2,3-dihydrobenzofuran
Compound **53**

Compound **54** was obtained from the same fraction as white powder and identified as licarin B, earlier isolated from the mace of *M. malabarica* (Compound **46,** Chapter **3A**). Compound **55** also isolated from the same fraction as colourless crystals. Spectral data of the compound indicated that it possess a lignan skeleton similar to that of the previous compounds. IR spectrum of the compound showed a broad absorption band at 3432 cm^{-1} indicates the presence of a hydroxyl group and the hydroxyl group resonated as a singlet δ 5.51 ppm in the ^{1}H NMR spectrum. ^{1}H NMR also displayed a doublet at δ 6.36 (d, J = 16 Hz, 1H) ppm and a multiplet at δ 6.13 – 6.08 (m, 1H) ppm for the *trans* olefinic protons, three sharp singlet at δ 3.92, 3.90 & 3.82 ppm for three methoxy group, two sets of doublet at δ 1.87 & 1.41 ppm for methyl groups along with signal corresponds to aromatic and -CH protons of benzofuran ring. ^{13}C NMR spectrum of the compound gave twenty one carbon resonances comprised of olefinic carbons, methoxy carbons, methyl carbons and aromatic carbons. The mass spectrum of the compound gave a molecular ion peak at 379.14664 which is the $[M+Na]^{+}$ peak. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be odoratisol A [5].



Odoratisol A
Compound **55**

Fraction pool 10-6 afforded the compound **56** as white solid after CC separation with 15% ethyl acetate-hexane eluent. Based on the spectral data and in comparison with the literature reports the compound was confirmed as licarin A, earlier reported from the species *M. malabarica* (Compound **49**, Chapter **3A**).

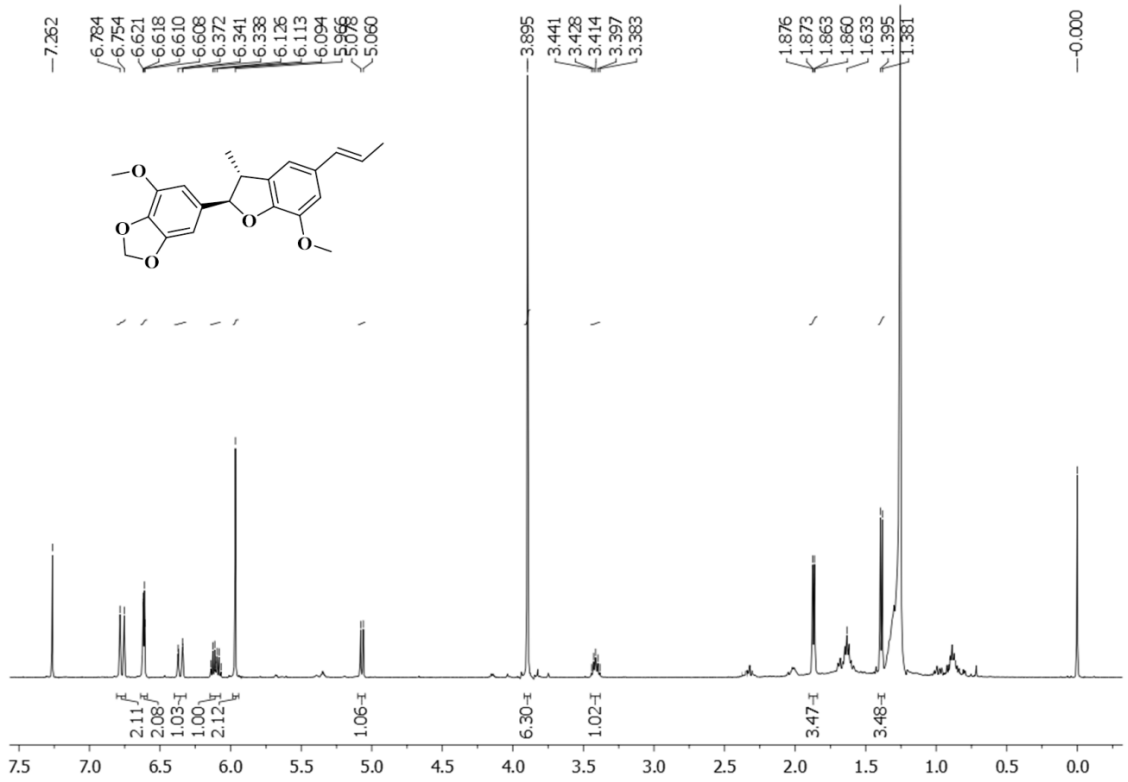


Figure 3B.4: [1]H NMR spectrum of (2S, 3R)-7-methoxy-3-methyl-5-((E)-prop-1-enyl)-2-(5-methoxy-3,4-methylenedioxyphenyl)-2,3-dihydrobenzofuran

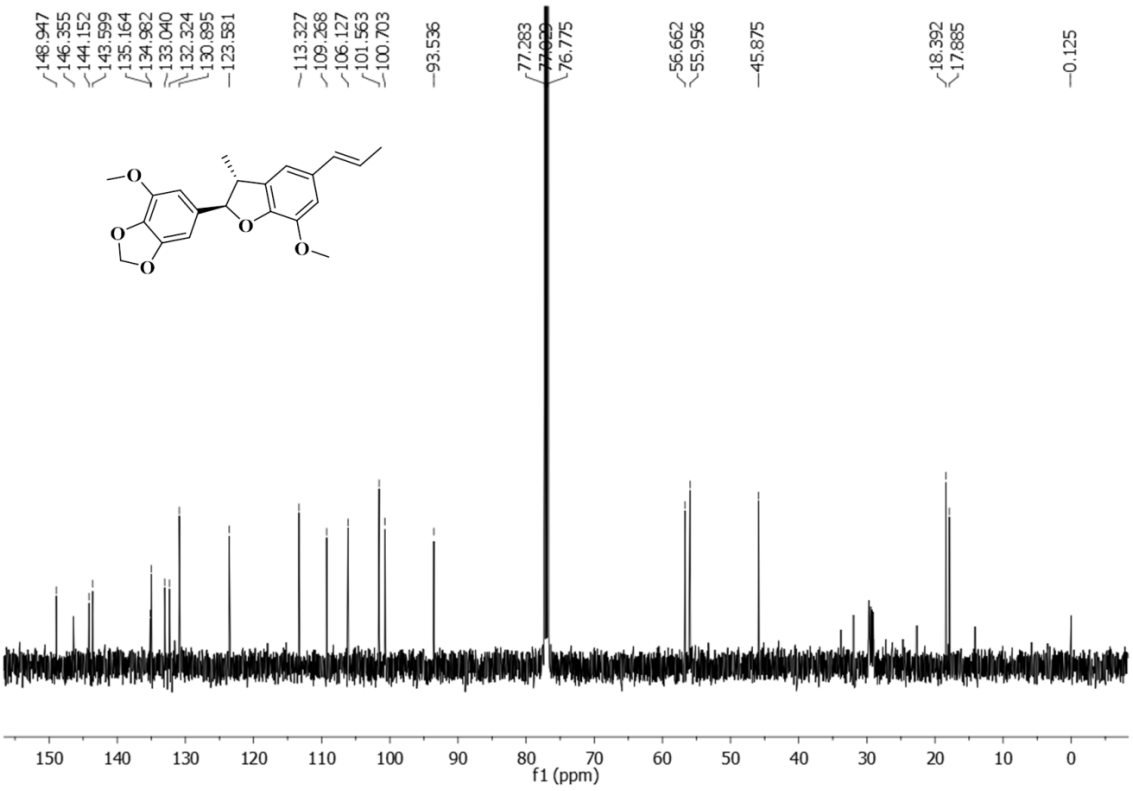


Figure 3B.5: (2S, 3R)-7-methoxy-3-methyl-5-((E)-prop-1-enyl)-2-(5-methoxy-3,4-methylenedioxyphenyl)-2,3-dihydrobenzofuran

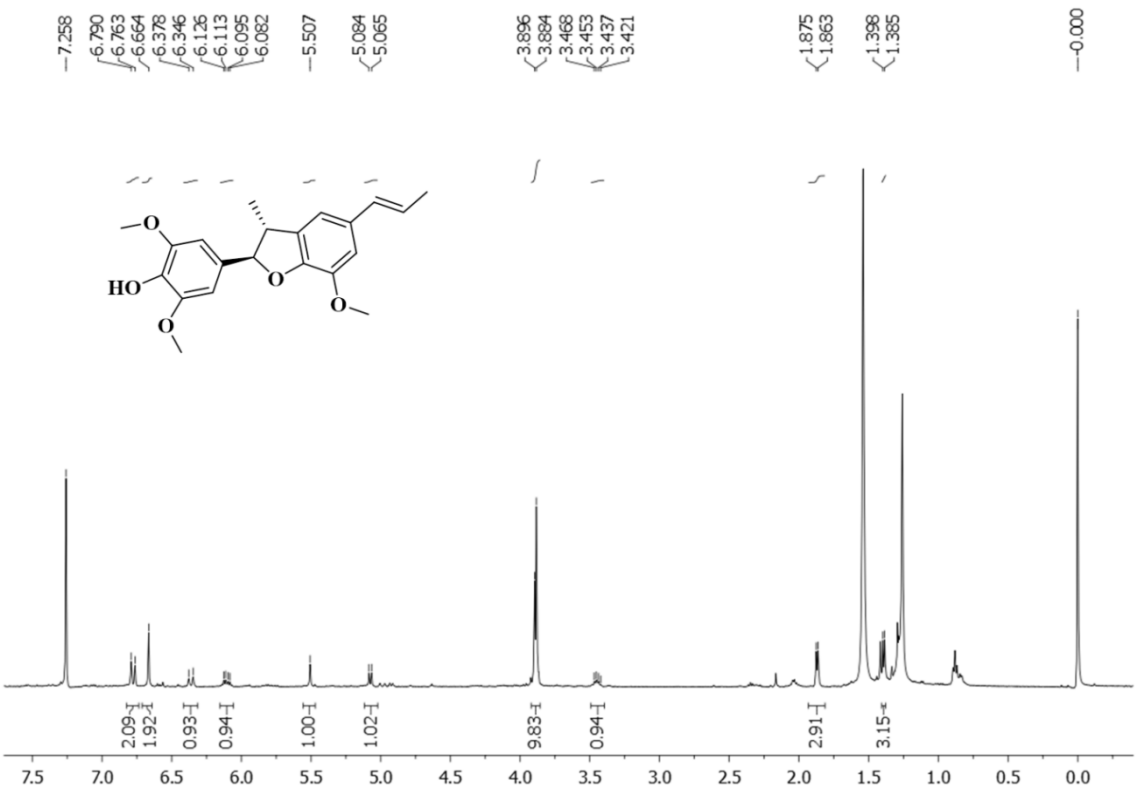


Figure 3B.6: ^{1}H NMR spectrum of odoratisol A

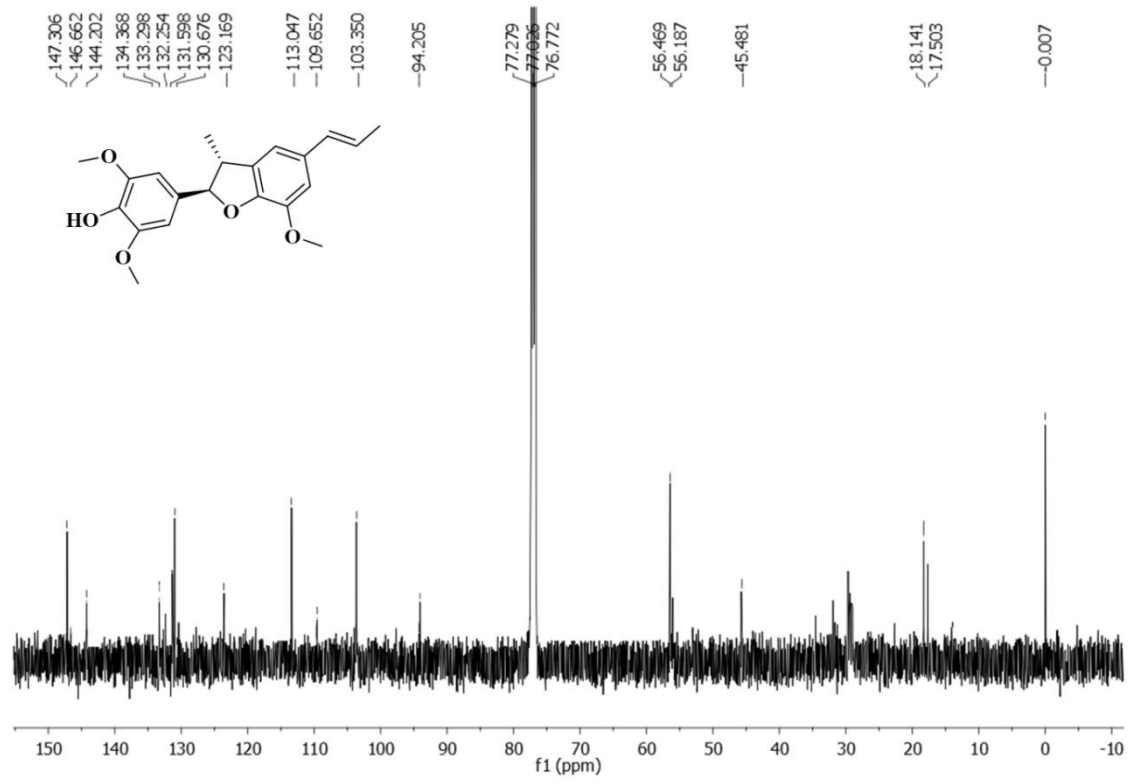
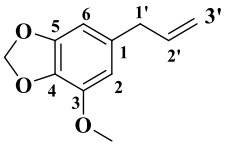


Figure 3B.7: ^{13}C NMR spectrum of odoratisol A

Fraction pool 17-18 showed the presence of two UV active spots. Compound **57** was isolated as colourless viscous oil with the elution hexane. ^{1}H NMR spectrum of the compound displayed a doublet of doublet at δ 5.07 (dd, J_1 = 16.5, J_2= 9.5 Hz, 2H) and δ 5.96 – 5.84 (m, 1H) ppm correspond to olefinic protons, a two proton singlet at δ 5.92 corresponds to methylenedioxy group, a doublet at δ 3.28 ppm for methylene group along with aromatic protons. ^{13}C NMR gave eleven carbon resonances comprises of olefinic carbons, methylene carbon, methylenedioxy carbon, methoxy carbon and aromatic carbons. The mass spectrum of the compound gave a molecular ion peak at 193.2140 which is the $[M+H]^+$ peak. By analyzing the spectral data and in comparison with literature value the compound was confirmed as myristicin [6].



Myristicin
Compound **57**

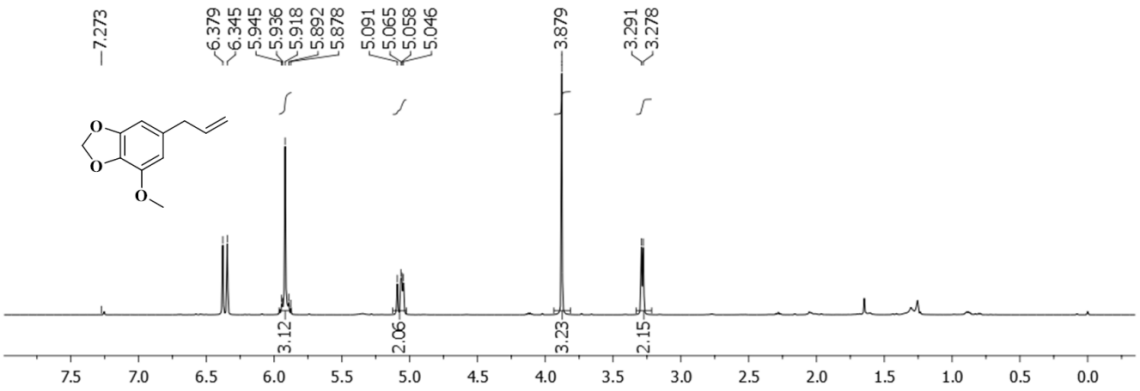


Figure 3B.8: ^{1}H NMR spectrum of myristicin

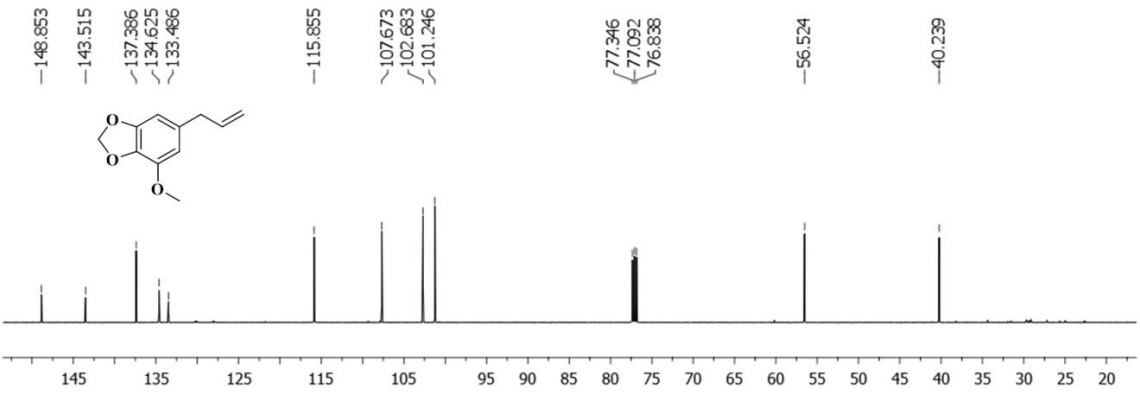


Figure 3B.9: ^{13}C NMR spectrum of myristicin

Compound **58** also isolated as white powder from the fraction after precipitation with 10% ethylacetate-hexane. Various spectral data analysis of this compound and in comparison to the literature values the compound was confirmed to be guaiacin [7]. ^{1}H NMR displayed a multiplet at δ 1.46 – 1.41 (m, 2H) ppm corresponds to two -CH protons of position 8 & 8'. Two diastereotopic protons of position 7 were obtained as two sets of doublet of doublet at δ [2.58 (dd, J_1 = 15.5 Hz, J_2= 3 Hz, 1H) & 2.44 (dd, J_1 = 15.5 Hz, J_2= 13 Hz)] ppm. In addition ^{1}H NMR showed two sets of three proton singlets at δ 3.65 & 3.64 ppm for methoxy group and two broad singlet at δ 7.22 & 6.99 for hydroxyl group along with aromatic and methyl protons. ^{13}C NMR spectrum displayed twenty carbon resonances including methoxy carbon, methyl carbon, carbon bearing hydroxyl group and aromatic and aliphatic carbons and finally mass spectrum of the compound gave a molecular ion peak at 351.15657 which is the $[M+Na]^{+}$ peak.

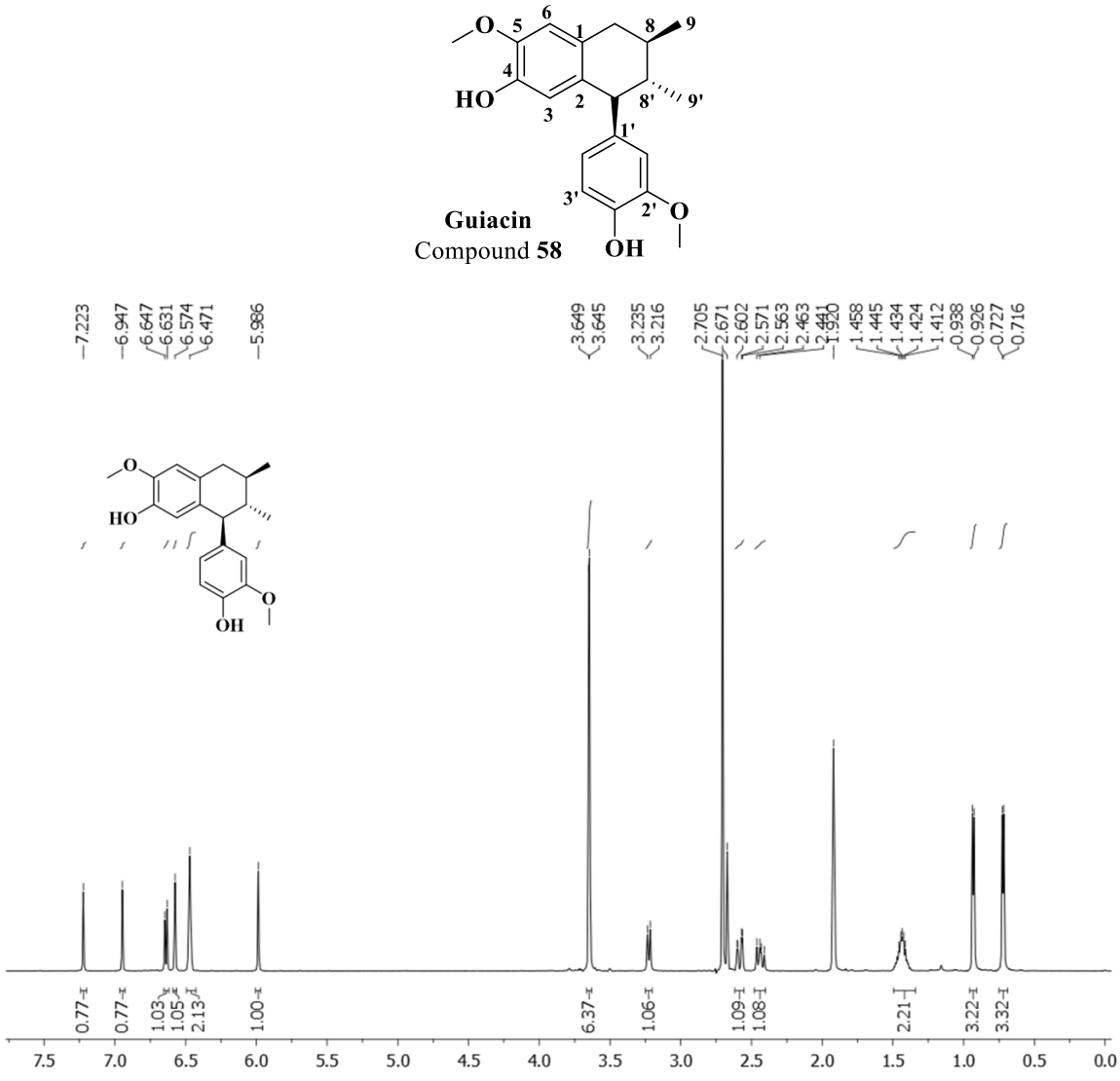


Figure 3B.10: ^{1}H NMR spectrum of guaiacin

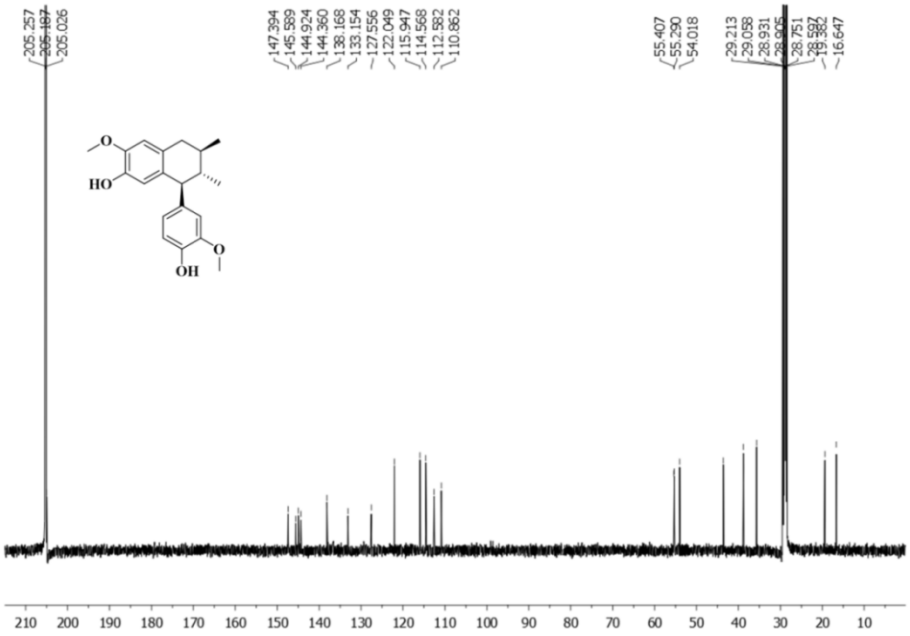
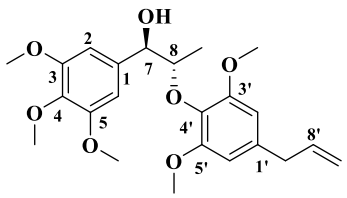


Figure 3B.11: ^{13}C NMR spectrum of guaiacin

Compound **59** was obtained as colourless viscous liquid from the fraction pool 19-20 after CC separation using 15% ethyl acetate-hexane eluent. Based on the comparative study of spectral data of the compounds with the literature the compound was identified as the neolignan, raphidecursinol B [8]. The IR absorption bands at 3506 cm^{-1} specifies the presence of hydroxyl functionality. The ^{1}H NMR spectrum displayed a broad singlet at δ 4.15 ppm confirmed the presence of hydroxyl group. The five, three proton singlet at δ (3.88, 3.86, 3.85, 3.82, 3.78) ppm indicated the presence of five methoxy group and a mutiplet, a doublet of doublet at δ [6.03-5.95 (m, 1H) & 5.14 (dd, J_1 = 16.5 Hz, J_1 =4.5 Hz, 2H)] ppm corresponds to olefinic protons. Besides, ^{1}H NMR spectrum exhibited signals corresponds to methyl protons at δ 1.13 (d, J = 6.5 Hz, 3H) ppm, methylene protons at δ 3.38 (d, J = 6.5 Hz, 2H) ppm, -CH protons at δ (4.80 & 4.36) ppm along with aromatic protons. DEPT 135 NMR confirmed the presence of two methylene group (δ 116.3, 40.6 ppm). ^{13}C NMR spectrum displayed thirty carbon resonances and mass spectrum of the compound gave a molecular ion peak at 441.18870 which is the [M+Na]$^{+}$ peak.



Raphidecursinol B
Compound **59**

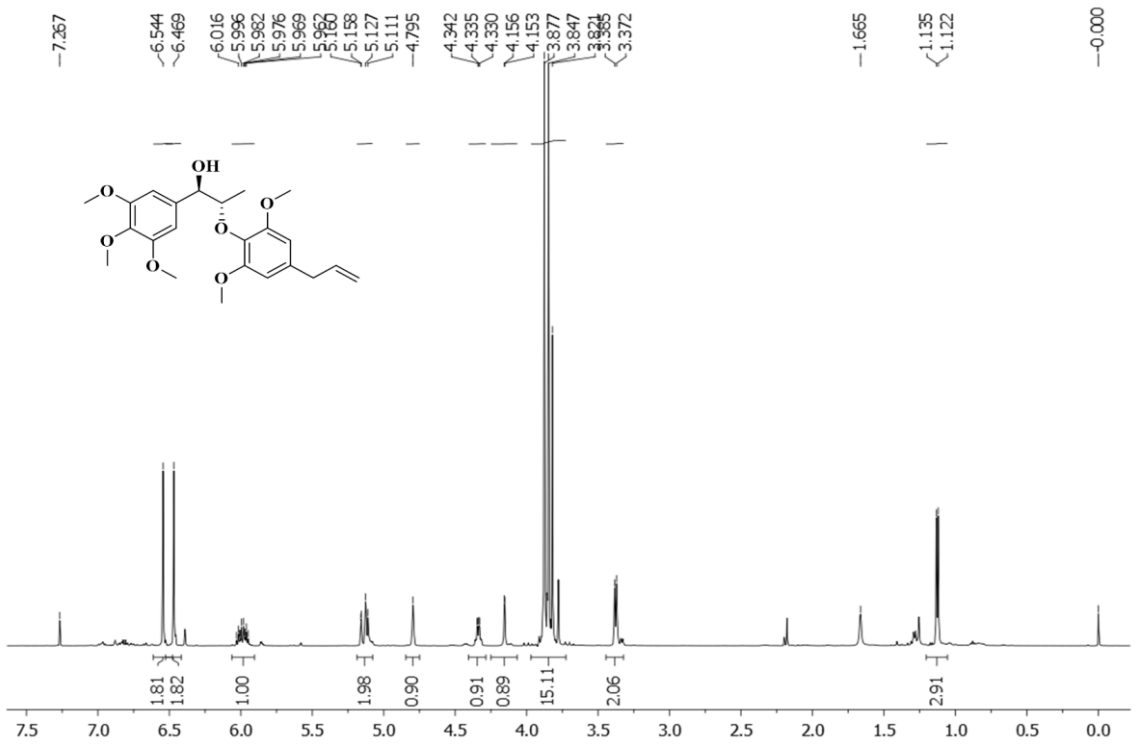


Figure 3B.12: ^{1}H NMR spectrum of raphidecursinol B

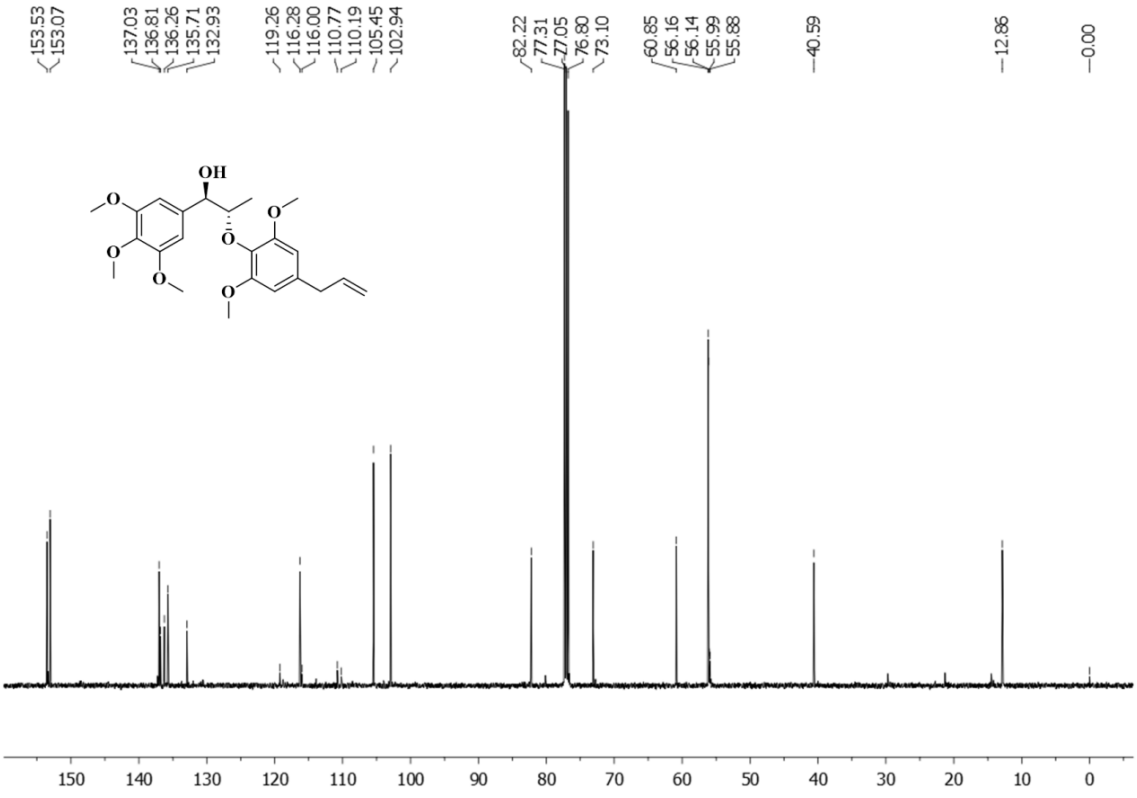
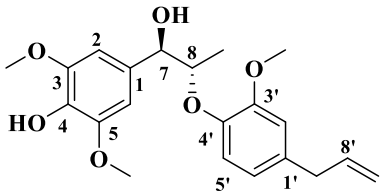


Figure 3B.13: ^{13}C NMR spectrum of raphidecursinol B

Fraction pool 20-22 led to the isolation of compound **60** as a colourless liquid after CC separation using 20% ethylacetate-hexane eluent. Spectral data of the compound was similar to that of previous compound indicated that the compound is a neolignan with different functionalities. The ^{1}H NMR spectrum displayed three proton singlets at δ 3.90, 3.89 & 3.87 ppm suggested the presence of three methoxy groups. Two sets of doublet at δ [6.83 (d, J = 8.5 Hz, 1H) & 6.68 (d, J = 8 Hz, 1H)] ppm corresponds to two *ortho* coupled protons at 5' & 6'. ^{13}C NMR spectrum displayed twenty six carbon resonances comprised of methoxy carbons, methylene carbon, olefinic carbons, carbon bearing hydroxyl group along with aliphatic and aromatic carbons and mass spectrum of the compound gave a molecular ion peak at 397.15738 which is the $[M+Na]^{+}$ peak. By comparing the spectral data with literature value the compound was confirmed as erythro-(7S, 8R)-$\Delta^{8'}$-4, 7-dihydroxy-3, 5, 3'trimethoxy-8-O-4'neolignan [8].



Erythro-(7S, 8R)-$\Delta^{8'}$-4, 7-dihydroxy-3, 5, 3'trimethoxy-8-O-4'neolignan

Compound **60**

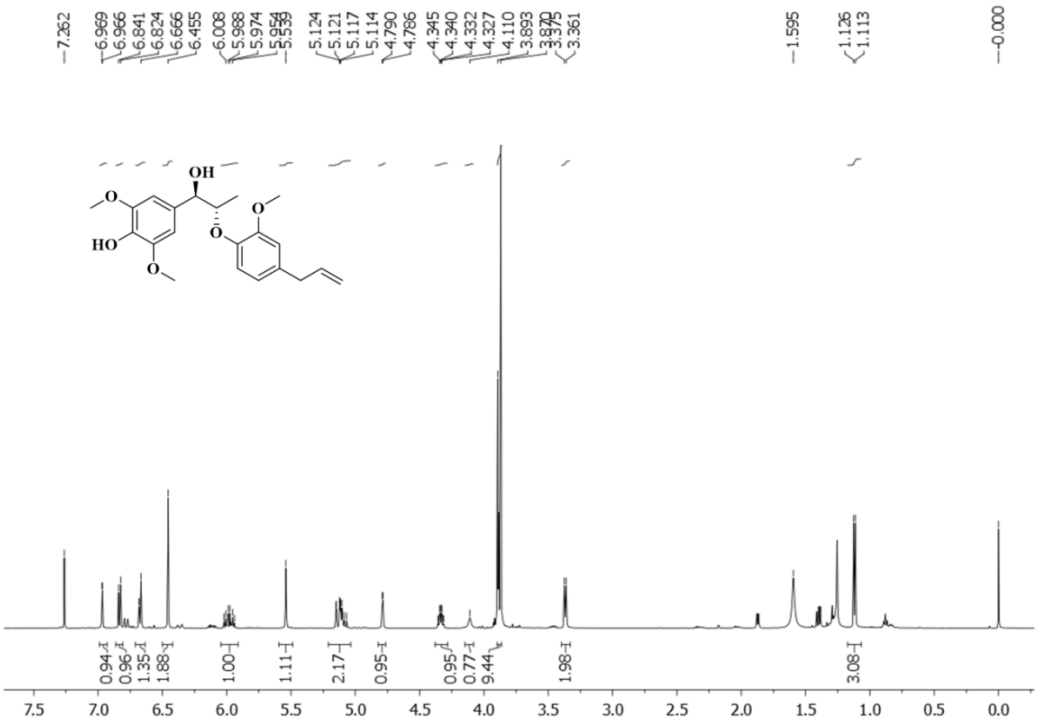


Figure 3B.14: ^{1}H NMR spectrum of erythro-(7S, 8R)-$\Delta^{8'}$-4, 7-dihydroxy-3, 5, 3'trimethoxy-8-O-4'neolignan

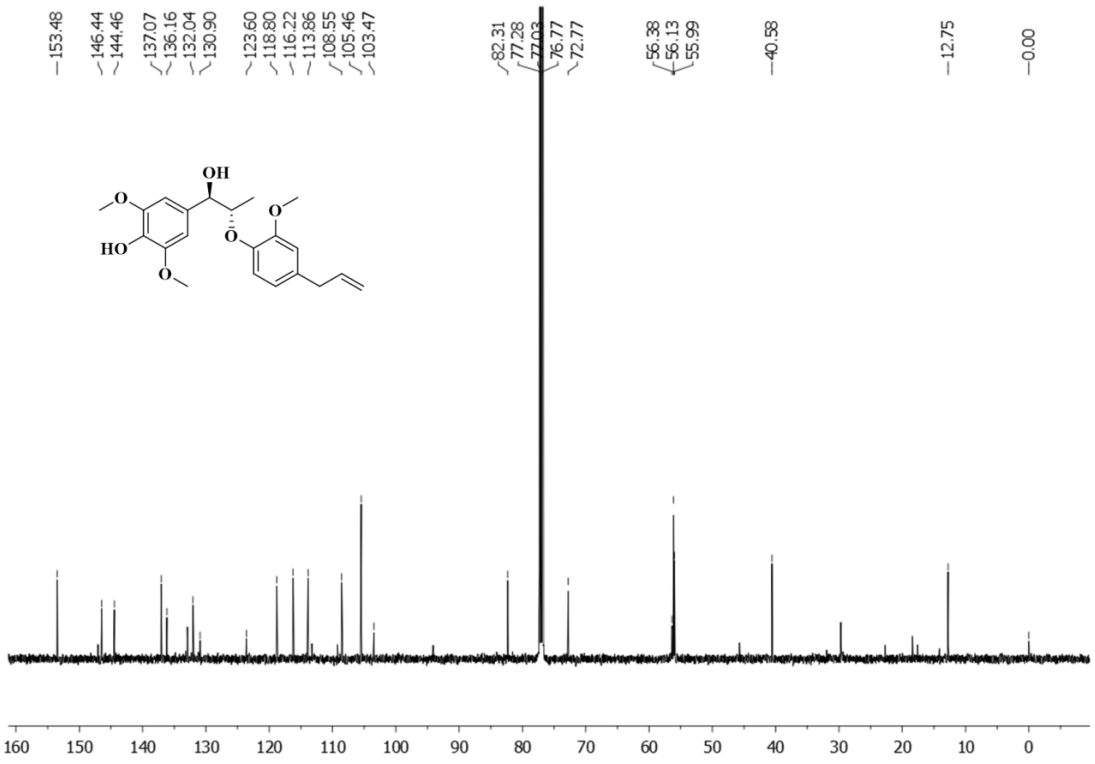


Figure 3B.15: ^{13}C NMR spectrum of erythro-(7S, 8R)-$\Delta^{8'}$-4, 7-dihydroxy-3, 5, 3'trimethoxy-8-O-4'neolignan

Compound **61** was afforded as pale yellow solid from the fraction pool 37-48 after CC separation using 15% ethylacetate hexane eluent. Based on the spectral data the compound was confirmed as malabaricone C which was earlier isolated from *M. beddomei* (Compound **4**, Chapter **2A**).

3B.4. *In vitro* Antidiabetic Screening of Lignans and Neolignans

Lignans and neolignans are a large group of secondary metabloties originated in plants as a result of shikimic acid biosynthetic pathway [9]. They were identified as the major phytoconstituents of the mace of the true nutmeg *Myristica fragrans*. Tetrahydrofuran lignans are important groups of natural products found in variety of plant species including *M. fragrans* [10]. Owing to their wide spectrum of the biological activities lignans and neolignans were well studied and reported to have antihypertensive, anti-inflammatory etc. activities. In addition antidiabetic potential of several lignan and neolignan obtained from plants were also reported [11]. The neolignan compound licarin B was reported to have the bioactive potential for insulin resistance and associated complications through its partial PPARγ activity [12]. The neolignan Licarin A was found to possess anti-parasitic activity [13-14].

Prevalence of T2DM is associated with the unhealthy food habits and drastic changes in life style. Since the mace of the nutmeg is extensively used as a spice in food cuisine we have evaluated the antidiabetic potential of phytoconstituents from the mace. Phytoconstituents of the mace were studied for their inhibitory potential against carbohydrate hydrolyzing enzymes (α-glucosidase and α-amylase) along with antiglycation properties.

Initially the isolated compounds from the mace of the nutmeg including (2S, 3R)-7-methoxy-3-methyl-5-((E)-prop-1-enyl)-2-(5-methoxy-3, 4-methylenedioxyphenyl)-2,3-dihydrobenzofuran (**1**), licarin A (**2**), licarin B (**3**), guaiacin (**4**), raphidecursinol B (**5**) were screened for their *in vitro* antidiabetic potential. The IC_{50} values of the compounds were determined against digestive enzymes (α-glucosidase and α-amylase) along with antiglycation properties and are summarized in Table **3B. 1**.

Table 3B.1: IC_{50} values of the compounds against digestive enzymes and antiglycation

SL. NO	Compound Name	IC_{50} values (µM)		
		α-amylase	**α-glucosidase**	**Antiglycation**
1	(2S,3R)-7-methoxy-3-methyl-5-((E)-prop-1-enyl)-2-(5-methoxy-3,4-methylenedioxyphenyl)-2,3-dihydrobenzofuran	238.2±0.543	**56.89 ± 0.711**	**122.33 ± 0.378**
2	Licarin A	96.02 ± 0.42	102.9 ± 1.49	312.32±0.823
3	Licarin B	**2.52±0.912**	**57.7 ± 0.715**	386.09± 0.472
4	Guaiacin	61.67 ± 0.982	**52.01 ± 1.01**	321.55 ±0.713
5	Raphidecursinol B	118.32±0.262	**38.36 ± 0.525**	234.52± 0..398
Acarbose		8.93 ± 0.48	52.28 ± 872	-
Ascorbic acid		-	-	155.38 ± 0.547

3B.4.1. α-Amylase Inhibitory Potential

Selected compounds were tested for their porcine pancreatic α-amylase inhibitory activity in which acarbose is used as the positive control. Amongst licarin B showed excellent α-amylase inhibition potential with an IC_{50} value of 2.52 ± 0.912 µM and was found to be higher than the reference standard acarbose (IC_{50}=8.93 ± 0.48 µM) whereas the remaining compounds, licarin A (IC_{50}=96.02 ± 0.42 µM), (2S, 3R)-7-methoxy-3-

methyl-5-((E)-prop-1-enyl)-2-(5-methoxy-3,4-methylenedioxyphenyl)-2,3-dihydro benzo furan (IC_{50}=238.2 ± 0.543 μM), guaiacin (IC_{50}=61.67 ± 0.982 μM), raphidecursinol B (IC_{50}=118.32 ± 0.262 μM) showed comparatively very lower activity.

3B.4.2. α-Glucosidase Inhibitory Potential

Inhibition of α-glucosidase enzymes (found in small intestine) and delay the release of glucose from polysaccharides can substantially decrease the amount of blood glucose level. *In vitro* α-glucosidase inhibition assay of the isolated compounds were performed using acarbose as the positive control. All the tested compounds showed moderate to good inhibition potential against α-glucosidase enzyme. Amongst raphidecursinol B showed significant activity with an IC_{50} value of 38.36 ± 0.525 μM which was found to be slightly higher than the positive control acarbose (52.28 ± 872 μM). In addition compounds, licarin B (IC_{50}=57.7 ± 0.715 μM), (2S, 3R)-7-methoxy-3-methyl-5-((E)-prop-1-enyl)-2-(5-methoxy-3, 4-methylenedioxyphenyl)-2,3-dihydro benzo furan (IC_{50}=56.89 ± 0.711 μM), guaiacin (IC_{50}=52.01 ± 1.01 μM) also showed good inhibition potential.

3B.4.3. Antiglycation Potential

Advanced glycation end products (AGEs) are the leading cause of diabetic complications. Antiglycation agents help to prevent the formation of AGEs thereby reduce the complications associated with T2DM. Several plant extracts and bioactive secondary metabolites reported to have antiglycation potential [15]. Selected compounds were screened for their antiglycation potential in which ascorbic acid is used as the positive control. Among the tested compounds, (2S, 3R)-7-methoxy-3-methyl-5-((E)-prop-1-enyl)-2-(5-methoxy-3,4-methylenedioxyphenyl)-2,3-dihydro benzo furan (IC_{50}=122.33 ± 0.378 μM) showed significant antiglycation potential and it was found to be slightly higher than the control ascorbic acid (IC_{50}=155.38 ± 0.547μM). Remaining compounds showed comparatively lower activity.

3B.4.4. Molecular Docking Studies

In vitro antidiabetic potential of the compounds were found to be effective so we further focused on the molecular docking studies in order to find out probable binding modes of the compounds with digestive enzymes. *In silico* studies were performed using glide program of Schrodinger suite 2017-2 [16]. The models of human pancreatic α-amylase (4GQQ), human maltase-glucoamylase [C-terminal (2QMJ) and N-terminal (3TOP)] were selected for the study and binding interaction of the selected compounds with the enzymes were expressed in terms of G score/D score (Table **3B.2**). Compounds

showed good binding efficiency with the digestive enzymes. Licarin A showed strong interaction with 2QMJ, 3TOP and 4GQQ with G score/D score of -5.15 kcal/mol, -7.33 kcal/mol & -2.63 kcal/mol respectively. The hydroxyl group of the compound formed a strong H-bond donor interaction with the Asp1279 (3TOP). Similarly guaiacin showed strong interaction with 2QMJ & 3TOP. Hydroxyl group of the compound showed hydrogen donor interaction with Asp1526. The 2D & 3D interaction diagram of the compounds, licarin A & guaiacin are given in Fig. **3B.16**.

The pharmacokinetic parameters of the compound were analyzed by qikprop [17]. The results (Table **3B.3**) showed that all the tested compound satisfy the adsorption, distribution, metabolism and excretion/toxicity (ADME/T) properties and Lipinski Rule of Five.

Table 3B.2: G-Score/D-Score of isolated compounds with digestive enzymes

Compounds	**2QMJ**		**3TOP**		**4GQQ**	
	G-score	D-score	G-score	D-score	G-score	D-score
(2S,3R)-7-methoxy-3-methyl-5-((E)-prop-1-enyl)-2-(5-methoxy-3,4-methylenedioxyphenyl)-2,3-dihydrobenzofuran	-3.34	-3.27	-3.94	-3.86	-5.16	-5.08
Licarin A	-5.15	-5.15	-7.33	-7.33	-2.63	-2.63
Licarin B	-4.07	-3.99	-7.81	-6.57	-6.51	-5.27
Guaiacin	-2.06	-1.98	-3.75	-3.67	-4.67	-4.59
Raphidecursinol B	-5.48	-5.4	-8.88	-8.8	-6.94	-6.86

Table 3B.3: Pharmacokinetic parameters of the compounds

Compounds	**1**	**2**	**3**	**4**	**5**
M.W	354.402	326.391	324.376	328.407	418.486
#stars	1	0	1	0	0
#rotor	3	4	2	4	12
CNS	0	0	0	0	0
SASA	605.835	617.65	569.65	595.858	721.227
FISA	0	54.956	0	106.126	36.932
HBA	3.75	3	3	3	6.2

HBD	0	1	0	2	1
QPlogKhsa	0.672	0.824	0.702	0.701	0.518
HOA	3	3	3	3	3
%HOA	100	100	100	100	100
QPlogPo/w	3.828	4.648	3.958	3.968	4.956
QPlogS	-6.014	-5.784	-5.894	-5.195	-5.142
ROf Five	0	0	0	0	0

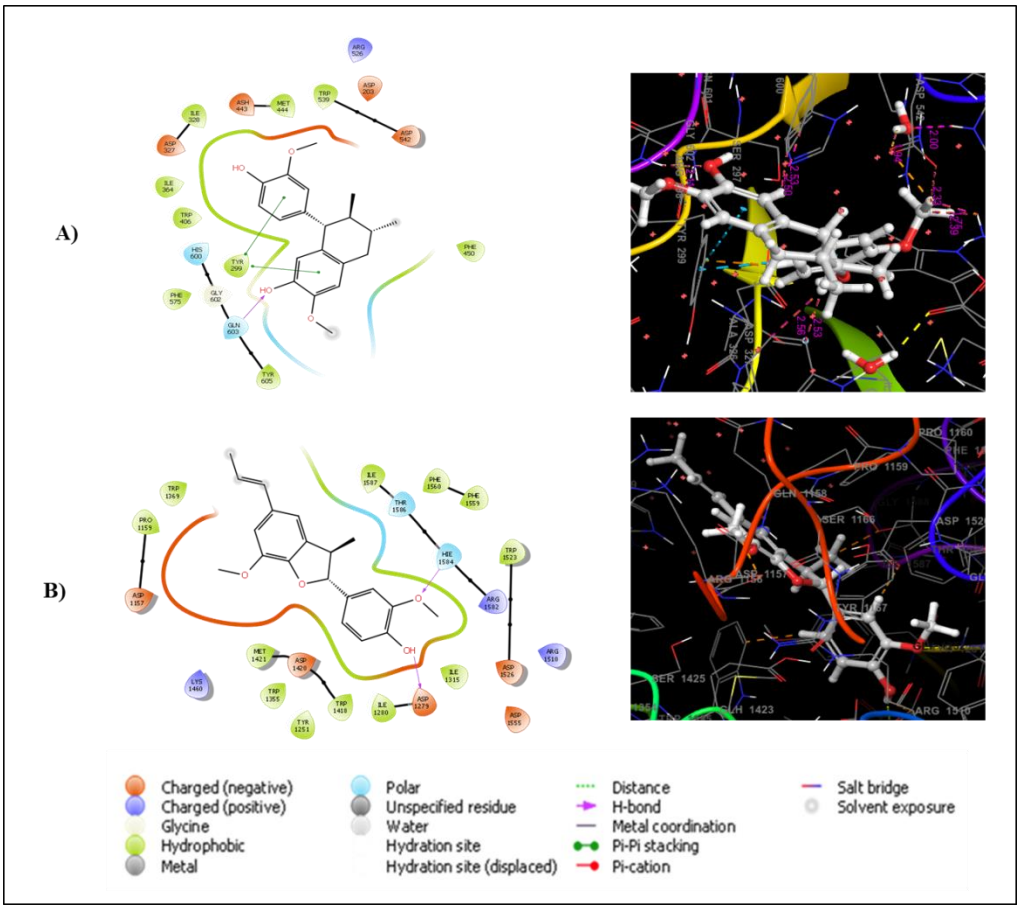


Figure 3B.16: The 2D and 3D interaction diagram of A) guaiacin with 2QMJ and B) licarin A with 3TOP

3B.5. Conclusion

In summary, phytochemical analysis of the mace of the common nutmeg *M. fragrans* was carried out in detail. Nine compounds including lignans, neolignans, phenylacyl phenols were isolated from the acetone extract of the mace. Among the compounds, odoratisol A, (2S, 3R)-7-methoxy-3-methyl-5-((E)-prop-1-enyl)-2-(5-methoxy-3, 4-methylenedioxyphenyl)-2,3-dihydrobenzofuran, myristicin & malabaricone C were identified for the first time from the mace of the species. Phytochemical analysis

showed that lignans and neolignans are the major phytoconstituents of the mace of the species. The study also revealed that there is a notable difference in the nature of the phytoconstituents present in true nutmeg and wild nutmeg species which in turn make difference in their pharmacological activities. In addition the isolated compounds exhibited potent activity against carbohydrate hydrolyzing enzymes and were found to be active antiglycation agents. Molecular docking study of the compounds well supported the experimental results. The phytoconstituents of the species can serve as promising candidates for the treatment of T2DM.

3B.6. Experimental Section

3B.6.1. General Methods

All the chemicals and solvents were of the best grade commercially available and were used without further purification. Analytical thin layer chromatography was performed on silica gel F_{254}, 0.25 mm, Merck. Gravity column chromatography was performed using 100-200 mesh silica gel with gradient elution of hexane-ethyl acetate used. Melting point was determined on a Fisher Johns melting point apparatus and is uncorrected. NMR spectra were recorded on Bruker AV 500 spectrometer. Chemical shifts was expressed δ in units of parts per million (ppm) downfield from TMS (δ 0.0). Multiplicities were given as: s (singlet); brs (broad singlet); d (doublet); t (triplet); m (multiplet). Coupling constants are reported as J value in Hz. Carbon nuclear magnetic spectra are reported as δ in units of parts per million (ppm) down field from $SiMe_4$ (δ 0.0). Mass spectra were recorded by using Thermo Scientific Exactive Mass Spectrometer under ESI/HRMS at 61800 resolution. IR spectra were recorded on Bruker Alpha FT-IR spectrometer and specific rotation was recorded using Jasco P-2000 polarimeter.

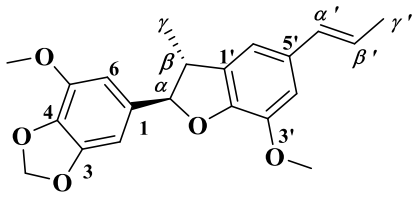
Commercially available porcine pancreatic α-amylase, rat intestinal α-glucosidase, acarbose, bovine serum albumin (BSA), α-D-glucose, ascorbic acid, 4-nitrophenyl α-D-glucopyranoside (p-NPG) were used to carry out antidiabetic assays. *In vitro* antidiabetic assays and molecular docking studies were performed on the basis of reported methods which are described in detail in Chapter **3A**, Section **3A.7**.

3B.7. Spectral Data

Compound 53 [(2S, 3R)-7-methoxy-3-methyl-5-((E)-prop-1-enyl)-2-(5-methoxy-3, 4-methylenedioxyphenyl)-2,3-dihydrobenzofuran]

Compound **53** was afforded as colourless viscous liquid from the fraction pool 5-8 after CC separation using 5% ethyl acetate-hexane eluent. ^{1}H NMR spectrum of the compound displayed the signals corresponds to the protons of methoxy groups,

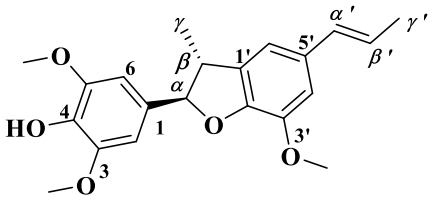
methelenedioxy group, *trans* olefinic bond and aromatic moiety. Based on the detailed analysis of spectral data and in comparison with the literature reports the compound was identified as (2S, 3R)-7-methoxy-3-methyl-5-((E)-prop-1-enyl)-2-(5-methoxy-3,4-methylenedioxyphenyl)-2, 3dihydrobenzofuran.



Molecular formula	:	$C_{21}H_{22}O_5$
FT-IR (neat, ν_{max}, cm^{-1})	:	2960, 1677, 1495,1325, 1204, 1138, 1044, 996, 931
^{1}H NMR (500MHz, $CDCl_3$)	:	δ 6.78 (s, 1H), 6.75 (s, 1H), 6.62 (d, J = 1.5 Hz, 1H), 6.61 (d, J = 1 Hz, 1H), 6.35 (dd, J_1 =15.5 Hz, J_2 =1.5 Hz, 1H), 6.10 (dq, J_1= 15.5 Hz, J_2= 6.5 Hz, 1H), 5.97 (s, 2H), 5.07 (d, J = 9 Hz, 1H), 3.90 (s, 6H), 3.46 – 3.36 (m, 1H), 1.87 (dd, J_1= 6.5 Hz, J_2 =1.5 Hz, 3H), 1.39 (d, J = 7 Hz, 3H) ppm
^{13}C NMR (125MHz, $CDCl_3$)	:	δ 148.9, 146.5, 144.1, 143.6, 135.2, 135.0, 133.0, 132.3, 130.9, 123.6, 113.3, 109.3, 106.2, 101.6, 100.7, 93.5, 55.7, 55.9, 45.9, 18.4, 17.9 ppm
HR-ESIMS	:	377.13644 $[M+Na]^+$

Compound 55 [Odoratisol A]

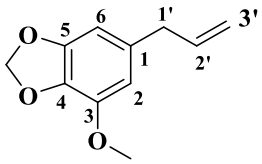
Fraction pool 5-8 led to the isolation of compound **55** as colorless crystals after the CC separation using 10% ethylacetate-hexane eluent. IR spectrum of the compound showed a broad absorption at 3432 cm^{-1} indicates the presence of a hydroxyl group and the hydroxyl group resonated as a singlet δ 5.51 ppm in the ^{1}H NMR spectrum. Spectral data of the compound was similar to that of previous one indicated the presence of lignan skeleton. The mass spectrum of the compound gave a molecular ion peak at 379.14664 which is the $[M+Na]^+$ peak. By comparing the spectral data with the literature report the compound was identified as odoratisol A.



Molecular formula	:	$C_{21}H_{24}O_5$
Melting point	:	116-118 ^oC
FT-IR (neat, ν_{max}, cm^{-1})	:	3432 (-OH), 2999, 2934, 1607, 1516, 1516, 1374, 1113, 961, 889
^{1}H NMR (500MHz, $CDCl_3$)	:	δ 6.79 (s, 1H), 6.76 (s, 1H), 6.66 (s, 2H), 6.36 (d, J = 16 Hz, 1H), 6.13 – 6.08 (m, 1H), 5.51 (s, 1H), 5.07 (d, J = 9.5 Hz, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.89 (s, 3H), 3.50– 3.41 (m, 1H), 1.87 (d, J = 6 Hz, 3H), 1.41 (d, J = 8.5 Hz, 3H) ppm
^{13}C NMR ($CDCl_3$)	:	δ 147.3, 146.7, 144.20, 134.4, 133.3, 132.2, 131.6, 130.7, 123.2, 113.0, 109.6, 103.3, 94.2, , 56.5, 56.2, 45.5, 18.1, 17.5 ppm
HR-ESIMS	:	379.14664 $[M+Na]^+$

Compound 57 [Myristicin]

Hexane eluted fractions of 17-18 led to the isolation of compound 57 as colourless viscous liquid. ^{1}H NMR spectrum of the compound displayed signals corresponds to olefinic protons, methylenedioxy protons, methylene protons along with aromatic protons. ^{13}C NMR exhibited eleven carbon resonances comprises of olefinic carbons, methylene carbon, methylenedioxy carbon, methoxy carbon and aromatic carbons. By analyzing the spectral data and in comparison with literature value the compound was identified as myristicin.

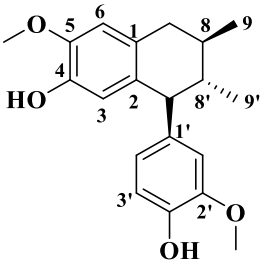


Molecular formula	:	$C_{11}H_{12}O_3$
FT-IR (neat, ν_{max}, cm^{-1})	:	3069, 2892, 1627, 1504, 1435, 1357,1127

^{1}H NMR (500MHz, $CDCl_3$)	:	δ 6.38 (s, 1H), 6.45 (s,1H), 5.92 (s, 2H), 5.96 – 5.84 (m, 1H), 5.07 (dd, J_1 = 16.5 Hz, J_2= 9.5 Hz, 2H), 3.88 (s, 3H), 3.28 (d, J = 6.5 Hz, 2H) ppm
^{13}C NMR (125MHz, $CDCl_3$)	:	δ 148.8, 143.5, 137.4, 134.6, 133.5, 115.8, 107., 102.7, 101.2, 56.5, 40.2 ppm ppm
HR-ESIMS	:	193.2140 $[M+H]^+$

Compound 58 [Guaiacin]

Compound 58 was afforded as white powder from the fraction pool 17-18 by eluting the column with 10% ethylacetate-hexane. IR spectrum of the compound showed the presence hydroxyl group. ^{1}H NMR spectrum indicated the presence of methoxy group, methyl group, methylene group and aromatic moiety. DEPT 135 NMR confirmed the presence of a methylene group. ^{1}H NMR, ^{13}C NMR and mass spectral studies of the compound and in comparison with literature value confirmed the compound as guaiacin.



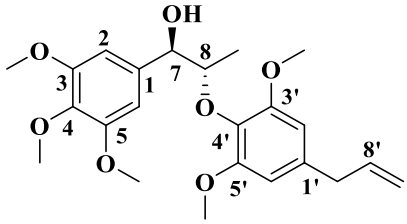
Molecular formula	:	$C_{20}H_{24}O_4$
Melting point	:	190-193 ^{o}C
FT-IR (neat, ν_{max}, cm^{-1})	:	3409, 2889, 1513, 1276, 1026, 770
^{1}H NMR ($500MHz$, CD_3COCD_3)	:	δ 7.22 (s, 1H), 6.95 (s, 1H), 6.64 (d, J = 8.0 Hz, 1H), 6.57 (s, 1H), 6.47 (s, 2H), 5.99 (s, 1H), 3.65 (s, 3H), 3.64 (s, 3H), 3.23 (d, J = 9.5 Hz, 1H), 2.58 (dd, J_1 = 15.5 Hz, J_2= 3 Hz, 1H), 2.44 (dd, J_1 = 15.5 Hz, J_2= 13 Hz), 1.46 – 1.41 (m, 2H), 0.93 (d, J = 6 Hz, 3H), 0.72 (d, J = 5.5 Hz, 3H) ppm
^{13}C NMR	:	δ 147.4, 145.6, 144.9, 144.4, 138.2, 133.1,

(125MHz, CD_3COCD_3) 127.6, 122.0, 115.9, 114.6, 112.6, 110.9, 55.4, 55.3, 54.0, 43.6, 38.8, 35.7, 19.4, 16.6 ppm

HR-ESIMS : 351.15657 $[M+Na]^+$

Compound 59 [Raphidecursinol B]

Compound **59** was obtained as colourless viscous liquid from the fraction pool 19-20 after CC separation using 15 % ethyl acetate-hexane eluent. The IR absorption bands at 3506 cm^{-1} specifies the presence of hydroxyl group. ^1H NMR of the compound displayed five three proton singlet for five methoxy group and signals corresponds to methyl group, olefinic proton, -CH protons, methylene proton and aromatic protons. ^{13}C NMR spectrum displayed thirty carbon resonances and mass spectrum of the compound gave a molecular ion peak at 441.18870 which is the $[M+Na]^+$ peak. Finally based on spectral data the compound was confirmed as raphidecursinol B.



Molecular formula : $C_{23}H_{30}O_7$

FT-IR (neat, ν_{max}, cm^{-1}) : 3506 (-OH), 2938, 2837, 1590,1503, 1228, 1127, 974, 823

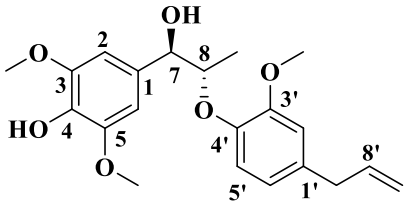
^{1}H NMR (500MHz, $CDCl_3$) : δ 6.54 (s, 2H), 6.47 (s, 2H), 6.03-5.95 (m, 1H), 5.14 (dd, J_1 = 16.5 Hz, J_1 =4.5 Hz, 2H), 4.80 (s, 1H), 4.36-4.32 (m, 1H), 4.15 (s,1H), 3.88 (s, 3H), 3.86 (s, 3H), 3.85 (s, 3H), 3.82 (s, 3H), 3.78 (s, 3H), 3.38 (d, J = 6.5 Hz, 2H), 1.13 (d, J = 6.5 Hz, 3H) ppm

^{13}C NMR (125MHz, $CDCl_3$) : δ 153.5, 153.1, 137.03, 136.8, 136.3, 135.7, 132.4, 119.3, 116.3, 116.0, 110.8, 110.2, 105.4, 102.9, 82.2, 73.1, 60.9, 56.2, 56.1, 56.0, 55.9, 40.6, 12.8 ppm

HR-ESIMS : 441.18870 $[M+Na]^+$

Compound 60 [Erythro-(7S, 8R)-$\Delta^{8'}$-4, 7-dihydroxy-3, 5, 3'trimethoxy-8-O-4'neolignan]

CC separation of fraction pool 22-28 using 20% ethylacetate-hexane eluent gave the compound **60** as colourless viscous liquid. Spectral data of the compound showed similarities with the previously isolated compound. IR spectrum indicated the presence of hydroxyl groups which was further confirmed from NMR spectrum. Mass spectrum of the compound gave a molecular ion peak at 397.15738 which is the $[M+Na]^+$ peak. Based on the spectral data and comparison with the literature reports the compound was identified as erythro-(7S, 8R)-$\Delta^{8'}$-4, 7-dihydroxy-3, 5, 3'trimethoxy-8-O-4'neolignan.



Molecular formula	:	$C_{21}H_{26}O_6$
FT-IR (neat, ν_{max}, cm^{-1})	:	3506 (-OH), 2937, 2840, 1587, 1497, 1273, 1121, 1034
^{1}H NMR (500MHz, $CDCl_3$)	:	δ 6.97 (d, *J* = 1.5 Hz, 1H), 6.83 (d, *J* = 8.5 Hz, 1H), 6.68 (d, *J* = 8 Hz, 1H), 6.46 (s, 2H), 6.02-5.94 (m, 1H), 5.54 (s, 1H), 5.15 – 5.06 (m, 2H), 4.79 (d, *J* = 2 Hz, 1H), 4.36-4.31 (m, 1H), 4.11 (s, 1H), 3.90 (s, 3H), 3.89 (s, 3H), 3.87 (s, 3H), 3.37 (d, *J* = 7Hz, 2H), 1.12 (d, *J* = 6.5 Hz, 3H) ppm
^{13}C NMR (125MHz, $CDCl_3$)	:	δ 153.5, 146.4, 144.5, 137.1, 136.2, 132.0, 130.9, 123.6, 118.8, 116.2, 113.9, 108.5, 105.5, 103.5, 82.3, 72.8, 56.4, 56.1, 56.0, 40.6, 12.7 ppm
HR-ESIMS	:	397.15738 $[M+Na]^+$

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CHAPTER 4

Antibacterial Agents from Natural Origin: Malabaricone B as a Novel Inhibitor against Multi drug resistant *Staphylococcus aureus* (MDR-SA) Infections

4.1. Introduction

Infectious diseases were recognized as the leading cause of morbidity and mortality in worldwide due to the inherent resistance of disease causing pathogens over the frontline therapeutic agents. Natural products (NPs) from various sources (plants, microbes etc.), offer chemically diverse, unique and bioactive secondary metabolites for the development of potent drug leads against numerous infectious diseases. The antimicrobial agents from natural origin were proved to be effective to target the microbial virulence. The diverse structural scaffolds from the NPs were always utilized in clinics to fight against the life threatening diseases. Unique chemodiversity and various modes of action of naturally derived compounds can fill the drug discovery pipeline of antibiotics in response to the drug resistance mechanisms. To date the 50% of the FDA antibacterial agents were derived from natural products primarily from microbial sources. Synergistic combination of natural derived compounds and conventional antibiotics along with the repurposing of the drugs to combat the drug resistant strains was also emerged as an efficient strategy in infection control [1-5]. Herein we briefly outline the history and development of antibiotics and antibiotic resistance.

4.1.1. History and Development of Antibiotics

Discovery of antibiotics; “the miracle discovery” considered as the paramount breakthrough in modern medicine. The “golden antibiotic era” started from the accidental discovery of penicillin by Alexander Fleming in 1928 from a culture of the fungus *Penicillium notatum* [6]. Penicillin, the “*β*-lactam antibiotic” was found to be very effective against *Staphyllococcus* infections and its first human use was reported in 1941. The term ‘antibiotic’ originates from two Greek words *anti* means 'against' and *bios* means 'life' and it was first introduced by Selman Waksman in 1941 to describe any small molecule, produced by a microbe, with antagonistic properties on the growth of other microbes [7]. Introduction of Salvarsan, the potent antibacterial agent against syphilis causing pathogen *Treponema pallidum* by Paul Ehrlich & Sahachiro Hata made a starting point in the discovery of antibiotics and which in turn provide the concept of ‘magic

bullet' that can specifically acts on pathogens without destroying the host. In earlier 1940s the development was excited with sulfa drugs, or sulfonamides, all of which are related to the compound sulfanilamide, offered the first successful remedy for many bacterial diseases. Arsenal compound were also extensively used to treat microbial infections during this time. In 1943 there happened the discovery of first aminoglycoside, streptomycin from a strain of soil bacteria *Streptomyces griseus*, which was the first useful drug for tuberculosis, tuberculosis meningitis and later for gram negative and positive pathogens outside penicillin's spectrum of activity. Cephalosporins were the second class of beta-lactam antibiotics discovered in 1945 after penicillin. Cephalexin, an oral antibiotic, introduced in 1967 and is still one of the major first line antibiotics used today. Later in 1947 chloramphenicol, the first 'broad-spectrum' antibiotic introduced by a Yale researcher from a complex soil bacteria *actinomycete Streptomyces venezuelae* against microorganisms with different cell walls and varied gram staining characteristics. It was the first FDA approved broad spectrum antibiotic having excellent tissue and fluid permeability. Chlortetracycline (Aureomycin), a new and potent broad spectrum antibacterial agent belongs to the class of tetracycline was introduced in 1948 against numerous infectious diseases. Erythromycin was the first macrolide antibiotic introduced in 1952 against several gam positive and gram negative bacterial infections. It was produced by a strain of *Saccharopolyspora erythraea*. Streptrogramin another class of antibiotics introduced in 1950s. Virginiamycin was the first representative of this class identified in 1952 from *Streptomyces virginiae.* Later glycopeptides (Vancomycin, 1956), quinolones (1961) and carbapenems (third class of beta-lactam antibiotic in 1976) were introduced. Most of the novel antibiotic classes deployed in clinics were discovered in golden antibiotic era (1950s-1970s). Oxazolidinones (linezolid), lipopeptides, macrocylic classes are the recently discovered antibiotics after a long interval [8-14].

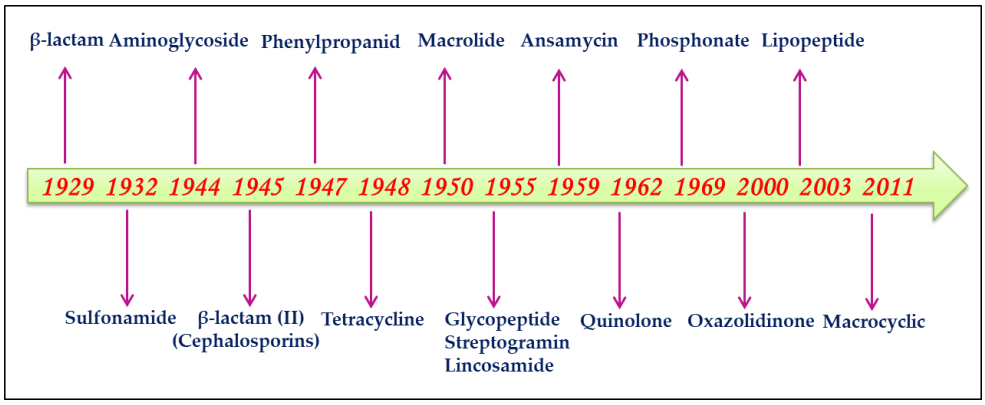


Figure 4.1: Time-line of the discovery of different classes of antibiotics in clinical use

4.1.2. Classification of Antibiotics

Antibiotics can be classified into numerous ways depends up on their spectrum of activity, chemical structure, mechanism of action etc. Broad spectrum antibiotics can act against a number of disease causing pathogens (gram positive and gram negative) while narrow spectrum antibiotics were used to get specific targeted action. Antibiotics are structurally diverse compounds derived naturally or synthetically and the basic structural features contribute towards its therapeutic action. Most of the antibiotics in clinical use are derived from natural products especially from microorganisms or their semisynthetic derivatives while limited numbers are chemically synthesized. Antibiotics possess various modes of pharmacological action by killing the bacteria (bactericidal antibodies) or preventing the bacterial growth (bacteriostatic antibodies) in hosts [15-16].

Table 4.1: Different classes of antibiotics based on structure, their action & origin

Class of Antibiotics	Examples	Mode of Action	Origin
β-lactams	Penicillin, Amoxicillin, Cephalosporin	Inhibit bacteria cell wall biosynthesis	Natural Product
Sulfonamides	Sulfanilamide Prontosil	Inhibit bacterial growth & multiplication	Synthetic
Aminoglycosides	Streptomycin, Neomycin	Inhibit protein synthesis by bacteria leading cell death	Natural Product
Tetracyclines	Tetracycline, Dexycycline	Inhibit protein synthesis & bacterial growth	Natural Product
Chloramphenicol	Chloramphenicol (not a first line drug)	Inhibit protein synthesis & bacterial growth	Natural Product
Macrolides	Erythromycin Azithromycin	Inhibit protein synthesis by bacteria	Natural Product
Glycopeptides	Vancomycin, Teicoplanin	Inhibit bacterial cell wall biosynthesis	Natural Product
Ansamycins	Rifamycin, Geldanamycin	Inhibit the synthesis of RNA by bacteria	Natural Product
Quinolones	Levofloxacin, Ciprofloxacin	Interfere with bacteria DNA replication & transcription	Natural Product
Streptogramins	Pristinamycin II A, Pristinamycin I A	Inhibit protein synthesis by bacteria	Natural Product
Oxazolidinones	Linezolid, Posizolid	Inhibit protein synthesis by bacteria, preventing growth	Synthetic
Lipopeptides	Daptomycin, Surfactin	Disrupt multiple cell membrane function	Natural Product

4.1.3. Antimicrobial Resistance (AMR)

With the advent of antibiotics and their persistent use against infectious diseases laterally created the problem of drug resistance. Antimicrobial resistance (AMR) in short is described as the ability of microbes to become resistant and grow in vicinity of antibacterial agent (drugs) employed to destroy it. AMR created the high resistant microorganisms known as "superbugs" which act as real threat for the treatment of infectious diseases. At present AMR has emerged as an unmet looming threat worldwide resulting increased rate of morbidity and mortality in developed and developing countries like India. It is estimated that AMR leads to ~700,000 deaths at present annually worldwide and if not controlled, could reach 10 million by 2050. Because of its prevalence WHO declared AMR is one among the top ten global threats to humanity. The foremost factors associated with alarming status of AMR include rampant consumption, unprescribed availability, misuse of antibiotics in agriculture and food industry, poultry etc. [17-19].

Probably the first report of AMR was in 1940s by Abraham & Chain towards the resistance of penicillin by *E. colli* bacteria. Microorganisms easily evolve intrinsic resistance mechanisms through gene mutation and adaptation. In initial stages *β*-lactams antibiotics were severely affected and compromised their activity due to resistance by the gram-positive pathogen *S. aures* [20]. In recent years the so called "ESKAPE" pathogens imparted prominent resistance over several classes of antibiotics (multidrug resistance) thereby created challenges in infection control. ESKAPE pathogens are a group of bacteria consisted of gram-negative and gram-positive bacteria such as *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa,* and *Enterobacter spp* are the WHO priority listed pathogens causing life threatening diseases worldwide. Amongst the ESKAPE pathogens carbapenem resistant *A. baumannii, P. aeruginosa, K. pneumoniae* and *Enterobacter* spp. are listed as critical whereas, vancomycin resistant *E. faecium (*VRE*)* and methicillin and vancomycin resistant *S. aureus* (MRSA and VRSA) are in high priority [21-23]. Majority of high risk nosocomial infections are associated with these pathogens and there is an urgent need of new antibacterial agents to specifically target them and stay ahead of infections [24]. The common therapeutic alternatives to combat with the ESKAPE pathogens include bacteriophage therapy, combination of antibiotics, synthetic chemicals, antimicrobial peptides, nano-materials, and photodynamic light therapy etc. [25-26].

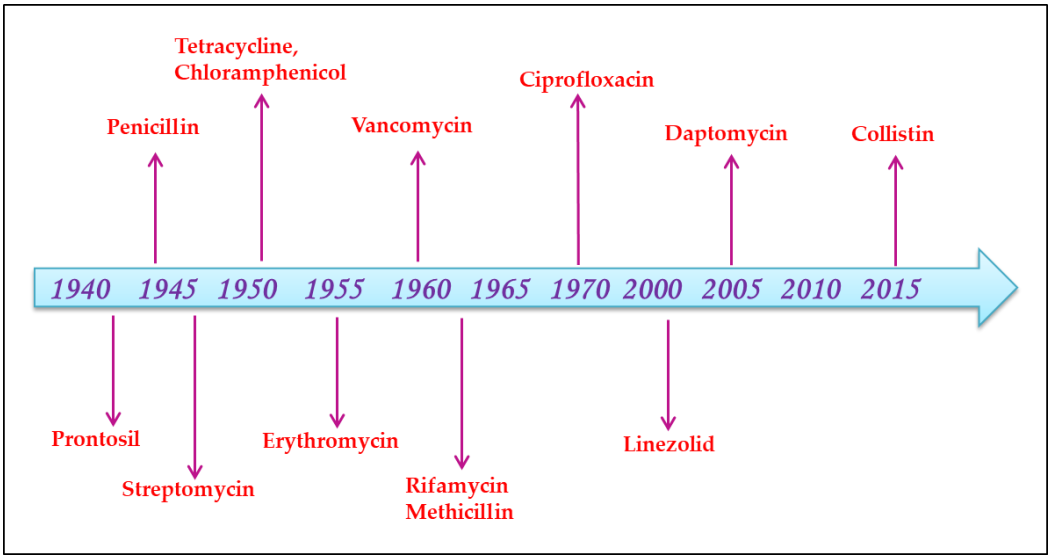


Figure 4.2: Time line of antimicrobial resistance (AMR)

4.1.4. Multi-Drug Resistant (MDR) *S. aureus*

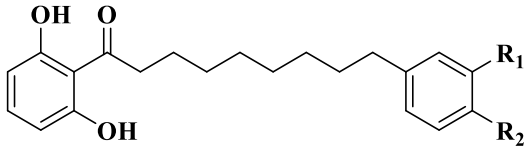
S. aureus is the opportunistic, highly drug resistant pathogen responsible for the prevalence of nosocomial infections. Antibiotic resistance of *S. aureus* was first reported against penicillin in 1940s. Penicillinase produced by the resistant strains of *S. aureus* can attack the *β*-lactam antibiotics leading to its inefficiency. To withstand the penicillin resistant *S. aureus* a new chemically modified agent methicillin was introduced in clinics in 1959. Unfortunately even after two years methicillin resistant strains of *S. aureus* (MRSA) were reported in Britain. MRSA were resulted from the methicillin sensitive *S. aureus* (MSSA) through the horizontal gene transfer. Soon after MRSA strains were spread across worldwide and acted as a global pandemic during the time, which in turn created clinical challenges. Both hospital and community acquired MRSA triggered the occurrence of high risk skin and skin structure infections, bacteremia, endocarditis, osteomyelitis and pneumonia. Vancomycin was introduced as first line treatment to MRSA isolates. But vancomycin resistant *S. aureus* (VRSA) was also reported in 2000. Infections caused by MRSA and VRSA are associated with increased morbidity, mortality, long term stay in hospital and higher financial burden on the healthcare system. Frontline antibiotics were failed to prevent MRSA infections due its inherent resistance. At present only limited number of FDA approved antibiotics such as daptomycin (lipopeptide) and linezolid (oxazolidinone), are available for the treatment of serious MRSA infections. But several strains were started resistance over daptomycin also. Since MRSA and VRSA are resistant to wide spectrum of existing antibiotics there is a growing concern to develop novel drugs to overcome the existing resistance mechanisms [27-30].

4.1.5. Drug Resistance in *Enterococci*

Unlike *S. aureus Enterococcus* species were also emerged as drug resistance pathogens imparting resistance over first line antibiotics. Among the species *E. faecalis* and *E. faecium* account for the majority of human *enterococcal* infections. Acquisition of virulence factor and MDR determinants lead to the drug resistance of these bacteria against *β*-lactam and cephalosporin antibiotics. However the first report of vancomycin resistant *enterococci* appeared in 1980s and the strains were developed resistant over the last resort of antibiotics very rapidly. The resistance genes of bacteria were very prominent in establishing wide range of infections and in very short time itself VRE infections become increasingly appeared worldwide. Priority rank of these pathogens reveals their challenging nature in healthcare [31-33]

4.1.6. Phenylacyl phenols/ Malabaricones

Malabaricones, the phenyl acyl phenols or diaryl nonanoids are an unusual class of bioactive secondary metabolites produced by wide variety of plant species belongs to the renowned "myristicaceae" family. Malabaricones named after their identification from the fruit rind of wild nutmeg species *M. malabarica* for the first time [34]. Malabaricones were also found to be the bioactive constituents of other species of *myristica* and *kenema* (limited) genus. Acylphenols are prominent class of natural products which include monomers and sometimes the uncommon dimers. Malabaricones mainly constitute 2, 6-dihydroxyphenyl type, acylphenols and their derivatives. The structure consists of 2, 6-dihydroxyacetophenone moiety is attached to a benzene ring through a C8 alkyl chain (Fig. **4.3**) and it was stabilized by various -O-HO hydrogen-bonding interactions.



1) R_1= H, R_2= H Malabaricone A
2) R_1= H, R_2= OH Malabaricone B
3) R_1= OH, R_2= OH Malabaricone C
4) -O-CH_2-O Malabaricone D

Figure 4.3: Basic skeleton of malabaricones

Generally phenolic compounds are produced as a result of shikimic acid biosynthetic pathway in plants. There is no detailed report available on the exact biosynthetic pathway involved in the synthesis of malabaricones. In 2000 Pham *et. al*

proposed a hypothetical biosynthetic pathway of phenylacyl phenols including malabaricones and promalabaricones. The biosynthesis of malabaricones started from the phenylalanine and tyrosine amino acid units. A cinnamoyl type compound is believed to be the biosynthetic precursor of malabaricones. The cinnamoyl CoA (derived from shikimic acid) precursor undergo chain elongation with six acetate units subsequently the reduction of first three acetate units along with cyclisation of the last three acetate units into a triketonic cyclohexane ring. Reduction and enolization of two carbonyl groups in the cyclohexane ring resulted promalabaricones which in turn afford malabaricones after reduction and enolization [35]. Schematic representation of hypothetical biosynthesis of malabaricones is given below (Fig. **4.4**).

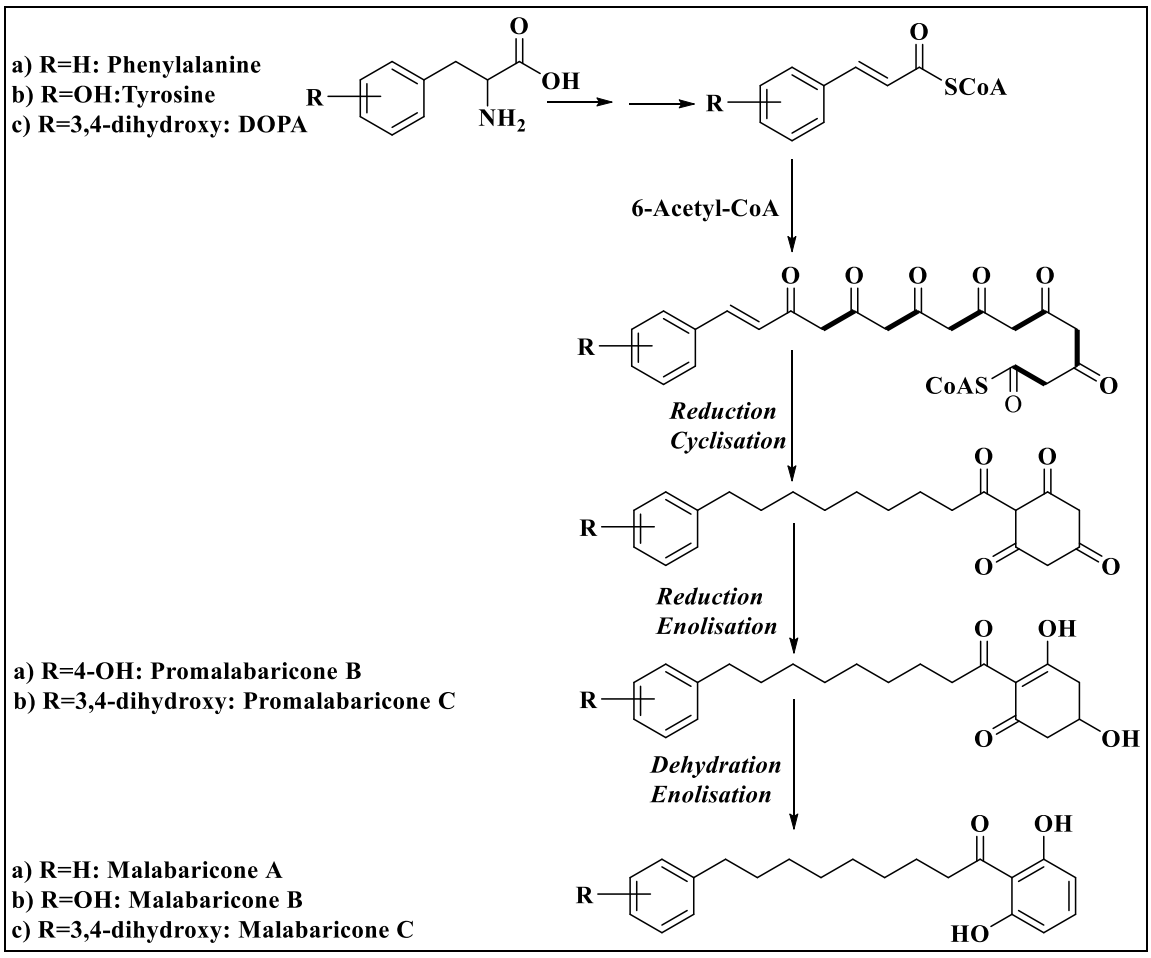


Figure 4.4: Hypothetical biosynthetic pathway of Malabaricones and Promalabaricones

Since malabaricones constitutes a significant class of natural products, they have gained considerable attention among researches owing to their structural characteristics and interesting biological activities. They were reported to possess wide spectrum of pharmacological activities including anticancer, anti-inflammatory, antihypertensive,

antioxidant, CNS etc. [36-40]. Antibacterial potential of the compounds were not well explored apart from the preliminary screening [41]. In this regard we have mainly focused on exploring the antibacterial potential of the compound. Isolation and characterization of the compounds were previously discussed in detail (Chapter **3A**).

4.2. Aim and Scope of the Present Study

Antibacterial agents that have potent activity against MDR strains are of enormous interest. Bacterial resistance over the existing drugs have become the most challenging issue in curing the infections caused by the pathogens including bacteria, fungus etc. Over consumption and rampant use of antibiotics resulted in developing bacterial resistance over it. Bacteria became more adaptable to antibiotics and they have improved their ability to defeat the drugs designed to kill them. Continuous exposures to the commonly existing antibiotics make them evolve resistance over it and which in turn reduces the effectiveness of the drug to manage the infectious diseases. In this context, limitations of the existing antibiotics highlight the critical need for novel antibacterial agents capable of competing against the bacterial resistance and to improve the effectiveness of the therapy. In this regard our approach is mainly focused on the effective utilization of naturally abundant secondary metabolites for the development of more potent antibacterial drug leads. Since various biological activities possessed by the polyphenolic compounds are impressive, we have utilized the phenyl acyl phenol class of compounds from the myristicaceae family for the detailed antibacterial screening. In the present study, we intend to investigate the antibacterial efficacy of malabaricones the unusual class of phenylacyl phenols against *S. aureus* including MDR strains and to study its mechanism of action in detail. One advantage of developing this natural product into a potential antibacterial lead is that it is extracted from the fruit rind which is locally available in plenty. In addition, the compound will be studied for its antibiotic effect in synergistic combination with standard drugs such as daptomycin and gentamycin. The present study aims the development of a highly active and cost effective antibacterial lead for the efficient management of diseases caused by MDR *S. aureus*.

4.3. Antibacterial Activity of Malabaricones/Phenyl acyl phenols

Validation and evaluation of antimicrobial potency of the natural products invole a series of *in vitro* studies along with their *in vivo* efficacy in animal model. Herein the antibacterial study of the phenyl acyl phenols (Fig **4.5**) include antibiotic suceptability testing against ESKAPE panel of pathogen, cytotoxicity assay, activity in MDR strains and enterococcus panel, time-kill kinetics, biofilm inhibition, intracellular killing assay,

induced resistant mutant generation studies, synergistic studies with the existing antibiotics, PMBN assay, post antibiotic effect and the *in vivo* neutropenic thigh infection model. These aspects are well explained in the following sections.

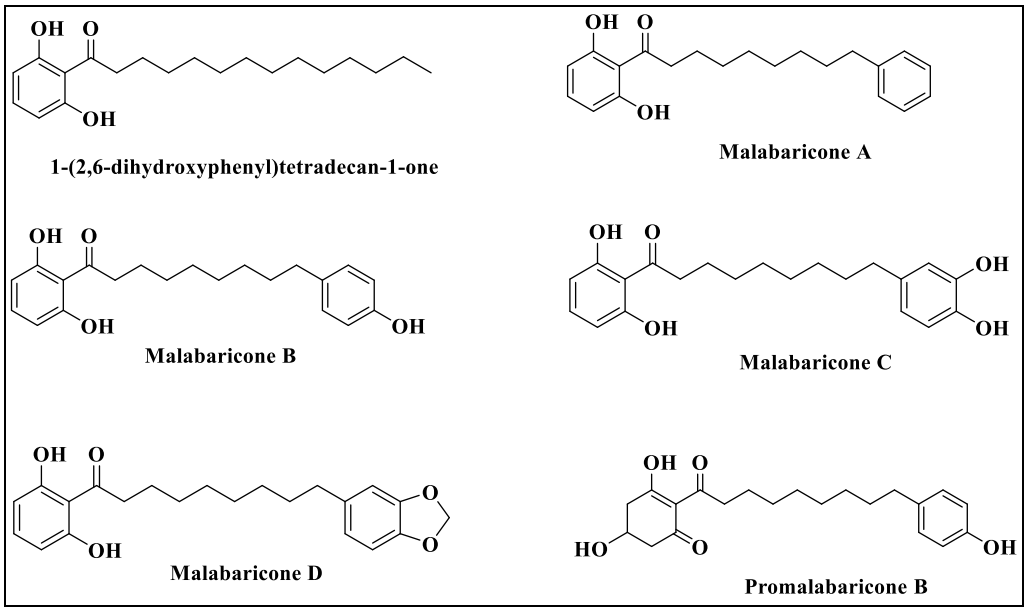


Figure 4.5: Structures of compounds subjected to antibacterial screening

4.3.1. *In vitro* Antibacterial Screening of the Compounds

4.3.1.1. Antibacterial Susceptibility Testing of the Compounds against ESKAP Panel

The isolated natural products [1-(2, 6-dihydroxyphenyl) tetradeacan-1-one (**NS-1**), malabaricone A (**NS-3**), promalabaricone B (**NS-5**), malabaricone B (**NS-7**), malabaricone C (**NS-9**) & malabaricone D (**NS-11**)] were screened for their antibacterial potential against ESKAP pathogen panel by broth micro dilution assay as per CLSI guidelines [42]. Antibiotic susceptibility testing was performed by determining MIC (Minimum inhibitory concentration) value of the compound against different strains of gram positive and negative bacteria. Levofloxacin was used as the reference compound and the results are given in Table **4.2**. Interestingly the compounds NS-5 to NS-11 exhibited potent and selective activity in gram positive bacterial strain of *S. aureus* ATCC 29213 and all the compounds are found to be inactive in other bacterial strains. Compounds NS-7, NS-9 & NS-11 showed excellent activity with an MIC value of 0.5, 4 & 0.5 µg/ml respectively. Since the compounds were active against gram positive bacteria further activity studies of the compounds were done in ATCC 29213 strain of *S. aureus*.

Table 4.2: MIC **(**µg/ml**)** value of the screened compounds against ESKAP pathogen panel

SL. NO	Code	Minimum Inhibitory Concentration (MIC) (µg/ml)				
		E. coli ATCC 25922	*S. aureus* ATCC 29213	*K. pneumonia* BAA 1705	*A. baumannii* BAA 1605	*P. aeruginosa* ATCC 27853
1	NS-1	>128	>128	>128	>128	>128
2	NS-3	>128	>128	>128	>128	>128
3	NS-5	>128	16	>128	>128	>128
4	**NS-7**	>128	**0.5**	>128	>128	>128
5	**NS-9**	>128	**4**	>128	>128	>128
6	**NS-11**	>128	**0.5**	>128	>128	>128
Levofloxacin			<0.03	0.125	64	8

4.3.1.2. Cytotoxicity Assay against Vero Cells

Cell cytotoxicity of the active compounds (NS-7, NS-9 & NS-11) was evaluated in Vero (African green monkey kidney cell line, ATCC CCL-81) cells using MTT assay [43] in which Doxorubicin was the positive control. The CC_{50} value of the compounds was determined and selectivity index is calculated as CC_{50}/MIC. The results are tabulated in Table **4.3**. The CC_{50} values of NS-7 (>40 µg/ml) & NS-11 (40 µg/ml) revealed that the compounds are non-toxic to Vero cells whereas NS-9 (>100 µg/ml) was found to be cytotoxic. Compounds NS-7 (>80 µg/ml) & NS-11 (80 µg/ml) showed a favourable selectivity index (>40). Based on the CC_{50} and selectivity index both the compounds (NS-7 & NS-11) were selected for further screening.

Table 4.3: Cytotoxicity profile of the compounds against Vero cells and selectivity index

SL. NO	Compound Code	MIC (µg/ml)	CC_{50} (µg/ml)	Selectivity Index
1	NS-7	0.5	>40	**>80**
2	NS-9	4	>100	>25
3	NS-11	0.5	40	**80**
Levofloxacin		0.125		

4.3.1.3. Activity against MDR (MSSA, MRSA & VRSA) Strains of *S. aureus*

Selected non-toxic compounds (NS-7 & NS-11) were screened against well-defined and characterized multiple clinical strains of MDR *S. aureus* to determine their spectrum of activity. MDR strains of *staphylococci* include methicillin sensitive *S. aureus* (MSSA), methicillin resistant *S. aureus* (MRSA) and vancomycin resistant *S. aureus* (VRSA). MIC value of the compounds in each strain (two replicate measurements I & II) was evaluated with levofloxacin, meropenem, methicilin and vancomycin as standard drugs and the results are summarized in Table **4.4** & **4.5**. Both the compounds NS-7 & NS-11 showed an MIC value of 1-2 μg/ml against MRSA & MSSA panel. MIC was slightly lower than that of the standard drug vancomycin reveals that compounds are very effective against MRSA & VRSA panel irrespective of its drug resistant status. In addition NS-7 & NS-11 exhibited excellent activity in all VRSA strains. Strains of MRSA were resistant to antibiotics such as methicillin, ceftriaxone, meropenem, gentamycin and linezolid while the VRSA strains are resistant to methicillin, ceftriaxone, meropenem, gentamycin, vancomycin and teicoplanin. The potent antibacterial activity displayed by NS-7 & NS-11 against MRSA and VRSA clearly indicate that they are capable of overcoming the currently existing drug-resistance mechanisms and can act as lead against MDR *S. aureus* infections.

Table 4.4: MIC of the compounds in multidrug-resistant strains of *staphylococci*, such as methicillin-sensitive *S. aureus* (MSSA) & methicillin-resistant *S. aureus* (MRSA)

			NS-7	NS-11	Levofloxacin	Meropenem	Methicillin	Vancomycin
MSSA	***S.aureus* ATCC 29213**	I	1	1	<0.5	0.5	2	1
		II	1	1	<0.5	<0.5	1	2
MRSA	***S.aureus* NRS 100**	I	1	1	<0.5	>64	>64	2
		II	1	1	<0.5	>64	>64	2
	***S.aureus* NRS 119**	I	1	1	16	>64	>64	2
		II	1	2	16	>64	>64	2
	***S.aureus* NRS 129**	I	1	1	<0.5	32	64	1
		II	1	1	<0.5	32	64	1
	***S.aureus* NRS 186**	I	2	2	8	32	64	2
		II	1	1	8	32	>64	1
	***S.aureus* NRS 191**	I	2	2	32	>64	>64	2
		II	1	2	32	>64	>64	2

	***S.aureus* NRS 192**	I	1	1	8	64	>64	1
		II	1	1	8	64	>64	2
	***S.aureus* NRS 193**	I	2	2	32	>64	>64	2
		II	2	1	32	>64	>64	2
	***S.aureus* NRS 194**	I	1	2	<0.5	8	16	1
		II	2	1	<0.5	16	32	1
	***S.aureus* NRS 198**	I	1	2	32	>64	>64	2
		II	1	1	32	>64	>64	2

Table 4.5: MIC of the compounds in vancomycin-resistant *S. aureus* (VRSA) panel

Minimum Inhibitory Concentration (MIC) (µg/ml)						
Compound code	**VRSA**					
	***S. aureus* VRS 1**		***S. aureus* VRS 4**		***S. aureus* VRS 12**	
	I	**II**	**I**	**II**	**I**	**II**
NS-7	1	2	2	1	2	1
NS-11	1	2	1	2	2	1
Levofloxacin	64	64	>64	>64	64	32
Meropenem	>64	>64	>64	>64	32	64
Methicillin	>64	>64	>64	>64	64	>64
Vancomycin	>64	>64	>64	>64	>64	>64

4.3.1.4. Time-Kill Kinetic Study

The bactericidal activity of NS-7 & NS-11 was explored by the time-kill method according to CLSI guidelines. Kill curves were constructed by plotting the colony forming unit (CFU/mL) surviving at each time point in presence of the compound. Fig. **4.6** illustrates the time kill kinetics of the compounds against *S. aureus* ATCC 29213. NS-11 at 1x MIC exhibited ~3 $\log_{10}$ reduction of CFU/mL after 2 h of exposure and remained the same as the starting log for the next 1 h showed bactericidal activity but as the time precedes activity was diminished and the bacterial growth was observed. Similar effects were observed for the compound when the concentration corresponds to

10x MIC. Initially the compound showed bactericidal activity but over the time activity is decreased.

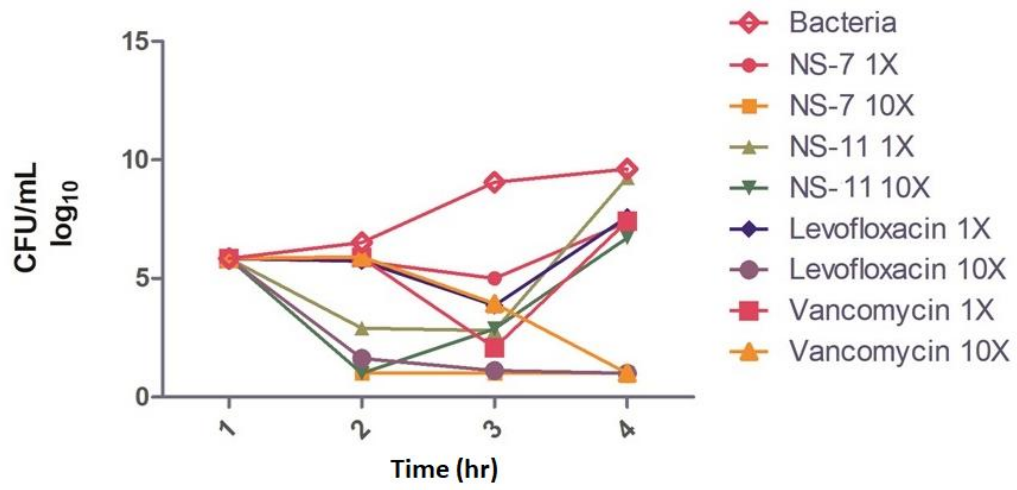


Figure 4.6: Time-kill kinetics of NS-7 & NS-11

While NS-7 exhibited concentration dependent bactericidal activity (Fig. **4.7**). It showed bacteriostatic effect by ~5 $\log_{10}$ reduction of CFU/mL (99.9 % killing) after 1h of exposure to *S. aureus* and the CFU/mL remained the same over time. Even after 1 h of exposure the bacterial growth is suppressed by the compound at 10x MIC and no visible growth was observed up to 24 h indicated its efficacy to inhibit the growth of *S. aureus*. The activity of the compound was found to be similar to that of the standard levofloxacin at 10x MIC. Since the bacterial strain, *S. aureus* ATCC 29213 inhibited very shortly after exposure to NS-7 we have selected the compound for further detailed analysis.

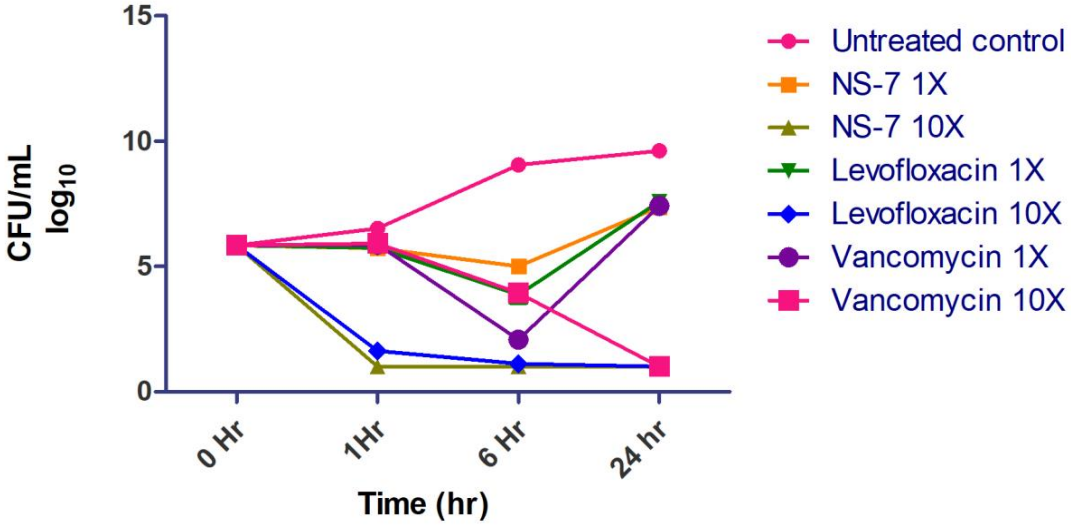


Figure 4.7: Time-kill kinetics of NS-7

4.3.1.5. Activity of NS-7 in MDR *Enterococcus* Panel

Enterococci, a group of gram positive bacteria are regarded as the leading cause of serious nosocomial infections. The human pathogenic bacteria found in gastro intestinal tract including *Enterococcus faecalis* and *Enterococcus faecium* is responsible for blood stream and urinary tract infections. Vancomycin was employed as the first line treatment for *Enterococcal* infections but the recent development of multidrug resistant genes (*Van A*) created the vancomycin resistant *E. faecium* (VRE) and it became life threatening pathogen. Natural resistance of VRE to distinct class of antimicrobials and its ability to acquire and transmit genetic resistance determinants created challenges in the treatment of VRE infections. Currently available FDA approved therapeutics for VRE infections include linezolid and a combination of quinupristin and dalfopristin. *Enterococcus faecalis* are sensitive to vancomycin also emerged resistance to other class of antibiotics. Vancomycin sensitive *E. faecalis* also developed infections related to hospitals [44-45]. The emerging risk associated with multidrug resistant *Enterococci* and limited number of effective drugs demanded the new antibacterial agents to defeat the drug resistance mechanisms.

Since NS-7 showed interesting activity against the gram positive bacteria *S. aureus* we have extended the study in *Enterococcus* panel. The well-defined clinical strains of VSE and VRE were used to screen the compounds. MIC of the compound was determined in the different strains of *E. faecalis* (NR 31884, NR 31885, NR 31886, NR 31887 & NR 31888) and *E. faecium* (NR 31903, NR 31909 & NR 31912) in which levofloxacin, methicillin, minocycline & vancomycin as the standard drugs. The results are summarized in Table **4.6** & Table **4.7**.

Table 4.6: MIC of NS-7 in MDR strains of vancomycin-sensitive *E. faecalis*

Compounds/ Drugs	**MIC (µg/ml) of NS-7 in *Enterococcus* panel (VSE)**				
	***E. faecalis* NR 31884**	***E. faecalis* NR 31885**	***E.faecalis* NR 31886**	***E.faecalis* NR 31887**	***E.faecalis* NR 31888**
NS-7	2	2	2	2	2
Levofloxacin	0.5	0.5	0.5	0.5	0.5
Methicillin	64	>64	>64	16	>64
Minocycline	0.125	0.125	0.125	16	0.125
Vancomycin	2	2	2	1	1

Table 4.7: MIC of NS-7 in multidrug-resistant strains of vancomycin-resistant *E. faecium*

Compounds/ Drugs	MIC (µg/ml) of NS-7 in *Enterococcus* panel (VRE)		
	***E. faecium* NR 31903**	***E. faecium* NR 31909**	***E. faecium* NR 31912**
NS-7	2	2	1
Levofloxacin	>64	>64	>64
Methicillin	>64	>64	>64
Minocycline	16	16	16
Vancomycin	>64	>64	>64

The compound showed excellent activity in both the strains of VRE & VSE with MIC value of 2 µg/ml. MIC value of the compound is found to be slightly higher than that of the standard vancomycin in VSE strains and in VRE strains NS-7 exhibited potent activity (MIC 1-2 µg/ml) than the drug minocycline which indicate that the compound can act as a new lead agent against multi drug resistant VRE infections.

4.3.1.6. Biofilm Inhibition Potential

Microbial biofilm formation has a primary role in the pathogenesis and is recognized as a serious issue in the treatment of infectious diseases. Microbial biofilms are formed as a result of the colonization and adherence of bacteria to a self-produced fouled surface. Inherent tolerance of bacteria in the biofilm towards the antibiotics and limited diffusion of the antibacterial agents through the polymeric matrix is responsible for antimicrobial resistance. The foremost risk associated with bacterial biofilm is the persistency and recurrence of infections. *S. aureus* is having the prominent virulence mechanism through the creation of biofilm especially in the case of wound infections (skin infections) which will cause difficulties in bacterial inhibition. In this regard, there is an urge of new strategies to prevent the bacterial biofilm formation and its dispersal. Significantly advanced antibacterial agents are required to penetrate into the biofilm matrix and overcome the antibiotic tolerance of bacteria. Naturally occurring peptides, secondary metabolites from plants and marine organisms were reported to have the efficacy to disrupt the biofilm formation [46-48].

Encouraged with the results obtained from the MDR activity and time killing kinetics of the naturally derived compound malabaricone B (NS-7), we have further evaluated its antibiofilm potential. The ability of the compound to inhibit the biofilm

formation was evaluated along with the conventional antibiotics through the biofilm inhibition assay. Vancomycin and levofloxacin was used as the standard drugs to reduce the biofilm.

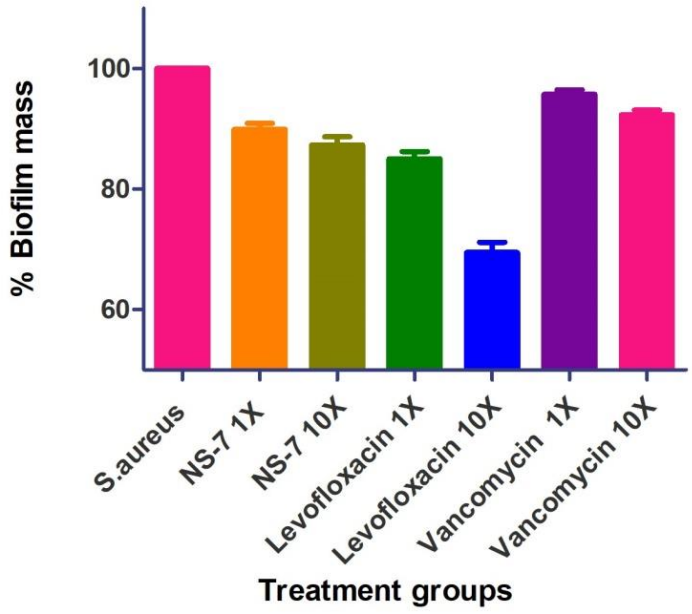


Figure 4.8: Biofilm inhibition of NS-7

The preformed biofilm by *S. aureus* ATCC 29213 was treated with malabaricone B (NS-7) and control antibiotics (vancomycin and levofloxacin) at 1x & 10x MIC and stained to enumerate the reduction in biofilm. The percentage reduction of biofilm is plotted against to treated groups to determine the efficacy of the compound (Fig. **4.8**). The result revealed that NS-7 at 1x MIC caused the 10% reduction of the preformed biofilm which is comparable to that of vancomycin at 10x MIC and the compound at 10x MIC caused ~15% reduction of preformed biofilm which is comparable to the activity of levofloxacin at 1x MIC. This suggests that the antibiofilm activity of the compound was comparable to that of vancomycin and levofloxacin at different concentrations. Compound was found to have potent activity in disrupting the preformed biofilm and can act as active agent to overcome the antibiotic tolerance of gram positive pathogen *S. aureus*. The excellent antibiofilm activity of the compound revealed that it can be used as a potent drug lead in topical antibiotic for skin infections.

4.3.1.7. Activity against Intracellular *S. aureus*

Intracellular *S. aureus* infections (blood stream infections) causes long term chronic inflammations. Persistence and recurrence of intracellular bacterial infections arises significantly due to the survival of bacteria inside the phagocytes and poor cellular

penetration of the conventional antibiotics. Generally monocytes and macrophages are involved in phagocytosis and intracellular killing by providing natural immunity against bacteria. Incomplete inhibition of intracellular bacteria will lead to its dispersal into new cells (the phenomenon of "Trojan horses") and evolve resistance mechanisms to withstand the antibiotics. Severe intracellular *S. aureus* infections and its antibiotic tolerance is a burden to infection control. Promising strategies for targeted elimination of intracellular pathogens with new and potent antibacterial agents having excellent penetrating power is urgently needed to prevent the infections and antibiotic tolerance. Several naturally derived compounds, synthetic compounds, nanoparticles etc. is proved to be efficient in intracellular killing of pathogens [49-51].

The potent inhibitory activity of NS-7 against *S. aureus* ATCC 29213 encouraged us to evaluate its ability in intracellular killing of bacteria. Intracellular killing assay of the compound was performed in J774.A1 mouse macrophage cell line in which vancomycin and levofloxacin as the positive control. Macrophages are placed in a culture with the bacteria. After 30 minutes the remaining bacteria are killed with a standard antibiotic and the phagocytes are stained and examined for number of bacteria they have killed. The bacterial colonies are enumerated before and after a killing period. Colony forming unit of the bacteria was determined on exposure with the active compound and conventional antibiotics (Fig. **4.9**).

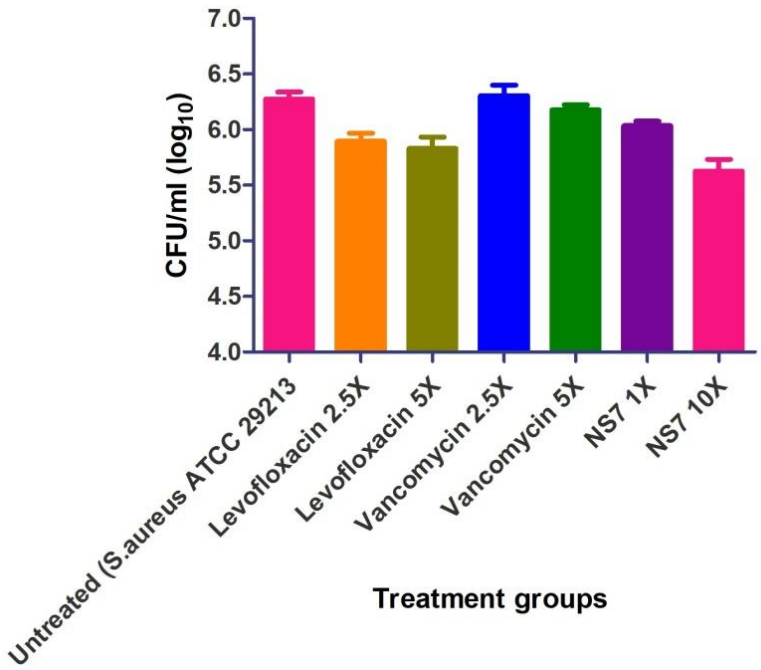


Figure 4.9: Intracellular killing of *S. aureus* ATCC 29213 by NS-7

Bactericidal activity of the NS-7 is evidenced by reduced CFU of the *S. aures*. NS-7 displayed a substantial reduction in CFU/ml at the concentration corresponds to 1x & 10x MIC which is found to be higher than levofloxacin and vancomycin. Compound was found to be effective against bacteria inside macrophages at 10x MIC and the study revealed that the compound can easily penetrate into the cell membrane and can overcome the intracellular mediated antibiotic tolerance of *S. aureus* ATCC 29213 strain.

4.3.1.8. Induced Resistant Mutant Generation Studies

Drug resistance of the *S. aureus* has a severe impact in the prevention of chronic infections ranging from skin to skin structure and nosocomial infections. Genetic mutation of the bacteria and over exposure of antibiotics are the leading cause of drug resistance. *S. aures* acquired specific resistance genes to combat the drugs and even created MDR strains. The bacterial resistance over the existing class of drugs necessitated the development of new alternatives [52-53].

We further moved to check the efficacy of compound in resistance studies with *S. aureus*. The tendency of bacterial resistance over NS-7 was evaluated using serial exposure of the compound against bacterial strain. Resistance profile of the compound is evaluated in *S. aureus* ATCC 29213 strain along with levofloxacin. MIC of the compound is determined after a number of serial passages up to 50 days (Fig. **4.10**).

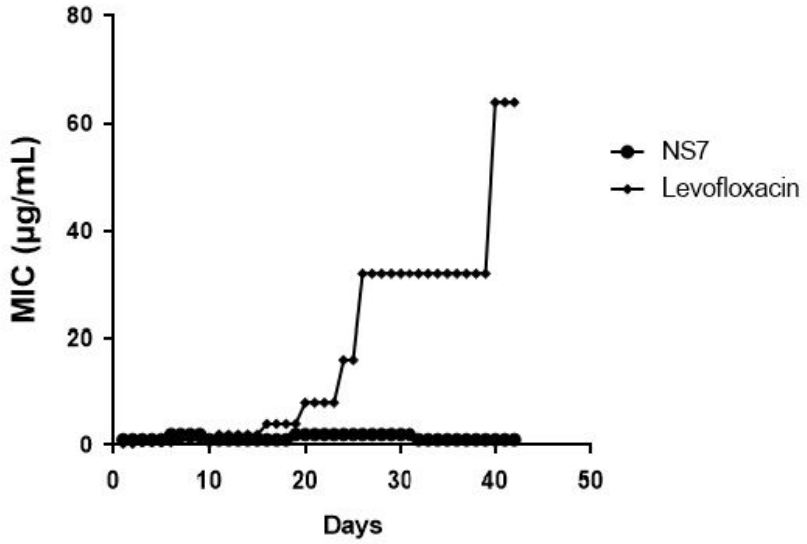


Figure 4.10: Induced resistant mutant generation of *S. aureus* ATCC 29213 with NS-7

The results indicated that MIC of the compound is not substantially altered after 50 passages and remain the same as the initial value. While the initial MIC value of the levofloxacin increased within 10 days and keep on increased after each passage finally

reached an MIC value of >64 µg/ml (64 fold) after 50 days indicating the complete resistance. The results revealed that bacteria have developed no cross resistance over the compound even after 40 days indicating the compound can withstand the bacteria. The propensity to generate resistance in compound is very lower as compared to levofloxacin and suggests that the compound can escape the drug resistant mechanism of *S. aureus*.

4.3.1.9. *In Vitro* Post Antibiotic Effect (PAE)

Post-antibiotic effect (PAE) is a well-established pharmacodynamics parameter that reflects an arrested bacterial growth following limited exposure to active antibacterial agent from the growth medium. Dosage regimens were designed in clinics for the patient on the basis of PAE values of the antibiotic. It allows determining the dosing schedule of antibiotics. Generally antibiotics needed prolonged PAE for less dosage. Administration of antibiotics in lower dosage to acquire better activity has the advantage of reducing the toxicity and side effects associated with antibiotic treatment [54-55].

Naturally derived compound malabaricone B (NS-7) displayed excellent activity against *S. aureus* in all *in vitro* antibacterial assays and suggest that it can act as an active antibacterial agent. As already mentioned dosage regimes of antibacterial agents depends up on its PAE we have evaluated the *in vitro* post antibiotic effect of the compound at different concentration against *S. aureus* 29213 strain. The compound is tested for its persistent suppression of bacterial growth (PAE) after a brief exposure (1 or 2 hours) of bacteria to an antibiotic. Briefly, bacteria were exposed to 1x and 10x MIC of the hit compound for 1 h, washed and plated on Mueller-Hinton media. The time taken for the test culture to demonstrate re-growth is compared to that of drug-free control and PAE is determined.

Table 4.8: *In vitro* post antibiotic effect of NS-7 at different concentration

Treatments	**Time for 1 log_{10} (h)**	**PAE (h)**
S. aureus 29213 (Untreated)	2	0
NS-7 1X	~3	~1
NS-7 10X	>24	>22
Levofloxacin 1X	~2.5	~0.5
Levofloxacin 10X	~3.5	~1.5
Vancomycin 1X	~3	~1
Vancomycin 10X	~3.5	~1.5

Compound possessed a prolonged PAE of 1 h and >22 h for the concentration corresponds to 1x and 10x MIC respectively. PAE value of the compound at 10x MIC which is very high compared to vancomycin (~1.5 h) and levofloxacin (~1.5 h) at the same concentration indicating compound suppress the bacterial growth in the administration of less dosage. Compound showed a concentration dependent PAE and short exposure is needed for better activity which is an added advantage.

4.3.1.10. Outer Membrane Susceptibility of NS-7 against Gram negative Pathogen

In vitro studies of the compound revealed that the compound is having specific and potent activity against gram positive pathogen and is inactive to gram negative pathogen. Membrane permeability of pathogens played a crucial role in antibacterial treatment. The outer membrane of gram negative pathogens has unique features which serve as a barrier for the penetration of antibacterial agents. This is the reason for failure of several antibiotics such as vancomycin, daptomycin etc. to target the gram negative pathogens [56-57].

In order to evaluate the outer membrane permeability of the compound (NS-7) against gram-negative pathogens, MIC of the compound against PMBN assay was carried out in gram negative bacterial strains. Combination of the compound with sub-lethal concentrations of Polymyxin B nonapeptide (PMBN) [to permeabilize the outer membrane] was tested against *E. coli* ATCC 25922 and *A. baumannii* BAA-1605.

Table 4.9: MIC of NS-7 in presence PMBN (Polymyxin B Nonapeptide Hydrochloride at 10 µg/ml) against gram negative bacterial strain

Gram negative Bacterial strain	**Minimum Inhibitory Concentration (MIC) (µg/ml)**								
	PMBN	**NS-7**		**Rifampicin**		**Vancomycin**		**Levofloxacin**	
		PMBN		**PMBN**		**PMBN**		**PMBN**	
		(−)	**(+)**	**(−)**	**(+)**	**(−)**	**(+)**	**(−)**	**(+)**
E. coli ATCC 25922	>64	>512	2	8	0.06	512	128	0.03	0.015
A. baumannii ATCCBAA-1605	>64	>512	4	2	0.03	256	64	8	8
PMBN-polymyxin B nonapeptide; **(−)**-No PMBN was added to the media; **(+)**-10 µg/ml of PMBN was added to the media									

The MIC value (Table **4.9**) of the compound in presence of PMBN against *E. coli* (2 µg/ml) and *A. baumannii* (4 µg/ml) clearly indicated that the compound is not completely inactive to the gram negative pathogen but it is having an entry issue not a target issue. Due to this the MIC is dropping in the presence of PMBN which slightly perturbs the outer membrane of gram-negative bacteria. In the case of gram positive bacteria the compound is very active which revealed the specificity of the compound towards *S. aureus*.

Table 4.10: MIC of NS-7 against gram positive bacteria

Gram positive Bacterial strain	**Minimum Inhibitory Concentration (MIC) (µg/ml)**			
	NS-7	**Rifampicin**	**Vancomycin**	**Levofloxacin**
E. coli ATCC 25922	2	0.0075	1	0.25

4.4. *In vivo* antibacterial Efficacy of the Compound

Malabaricone B exhibited potent antibacterial activity against drug resistance isolates. *In vitro* studies of the compound suggest that it can act as an active antibacterial agent against *S. aures* infections. So performing *in vivo* studies of the compound in animal model is appropriate for further development of the compound and its exploration for an antibacterial agent in clinical use. We have evaluated the *in vivo* efficacy of the compound in animal model.

4.4.1. Determination of Maximum Tolerance Dose (MTD) of NS-7

Prior to the testing of the efficacy of the compounds in animal model maximum tolerance dose of the compound was determined. Maximum tolerated dose (MTD) refers to the highest dose of a therapeutic agent to exhibit the desired effect without causing any side effects and it act as a basis for the selection of dose of active compound for phase I clinical trial. The MTD value of the active compound was evaluated in mice. A single 250 mg/kg dose of NS-7 was given to three Balb/c mice weighing between 20-21 g intraperitoneally (IP). No mortality was witnessed over the period of observation. The change in mice weight during the observation period is given in Table **4.11**. The study revealed that the MTD dose of compound is 250 mg/kg/day.

Table 4.11: MTD data of NS-7

Sample	Weight (g)			
	Day 0	Day 1	Day 5	Day 10
Mice 1	20	20	20	22.5
Mice 2	21	21	21.5	23
Mice 3	20	20	20.5	22.5

4.4.2. Murine Neutropenic Thigh Infection Model

The thigh infection model provides a sensitive system to evaluate the *in vivo* antimicrobial efficacy in a mammalian system. We have evaluated the *in vivo* efficacy of NS-7 in murine neutropenic thigh infection model. In brief, a group of neutropenic mice were infected at its right thigh with *S. aureus* followed by the intraperitoneally administration of NS-7/vancomycin. Control animals were treated with vancomycin (positive control) and saline (negative control). CFU count was determined and CFU/gm $\log_{10}$ is plotted against treatment dose to evaluate the animal efficacy of the compound (Fig. **4.11**).

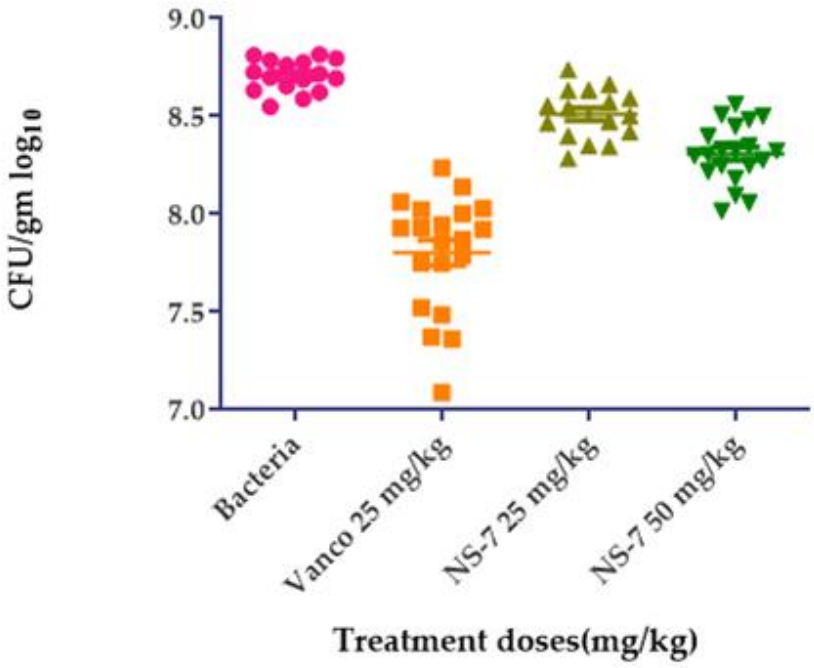


Figure 4.11: *In vivo* efficacy of NS-7 in murine neutropenic thigh infection model

A significant reduction of bacterial count (>1$\log_{10}$) is observed with mice treated with NS-7 (50 mg/kg) in the thigh model compared with the control group ($P<0.005$)

which is comparable to vancomycin at 25 mg/Kg (>1.5 $\log_{10}$) as compared to no drug control. Mice treated with NS-7 (25 mg/kg) showed 0.7 $\log_{10}$ reduction of bacterial count. The results showed the effectiveness of NS-7 in reducing the bacterial load in infected mice which was comparable to that of vancomycin. The *in vivo* efficacy data of NS-7 (Malabaricone B) shows good animal efficacy at 50 mg/kg as compared to vancomycin at 25 mg/kg.

Altogether *in vitro* and *in vivo* antibacterial studies of the plant derived secondary metabolite malabaricone B (NS-7) proposed that the compound is an effective antibacterial drug lead that can overcome the existing drug resistance mechanisms of the gram positive pathogen *S. aureus*.

4.5. Synergistic Activity Studies

Challenges associated with antimicrobial resistance enforced to develop new strategies to survive against disease causing pathogens. Recently there is an enormous interest in combination therapy to defeat life threatening infections. It is reported that synergistic combination of two active agents further enhances its potential rather than their individual usage and it is clinically proven. Synergistic combination of the conventional drugs was extensively employed in therapeutics especially in cancer chemotherapy and in infectious diseases due to the advent of prominent drug resistance mechanisms. In addition to the synergistic combination of conventional drugs, naturally derived bioactive compounds were also used to combine with the effective drug. Natural product served as a powerful therapeutic agent against infectious diseases and has long back history in the treatment of infectious diseases. In initial stages indigenous knowledge associated with plants made their application in traditional medicine. Later onwards the application of pure natural products were succeeded in targeting specific activity. Owing to the enormous bioactive potential and lower side effects of naturally derived secondary metabolites, they have gained adequate attention in combination therapy. Several plant extracts and secondary metabolites were found to possess efficient interaction with classical antibiotics which in turn provide augmented results. Synergistic combination therapy acts as a new paradigm in drug discovery approach [58-59].

Increasing frequency of antibiotic resistance and the development of superbugs threatened the health care system. Even the susceptible pathogens were become drug resistant by achieving spontaneous gene mutations. *S. aureus*, considered as the second most harmful pathogen (according to WHO) because of their intrinsic resistance over different class of antibiotics (methicillin, carbapenems, β-lactam etc.) and are the leading

cause of majority of high risk bacterial infections. Multidrug resistance (MDR) strains of *S. aureus* constitute a wide range of bacterial infections which are the serious health care problem worldwide and limited number of chemotherapeutic agents is currently available [28]. Since the compound malabaricone B (NS-7) proved as a potent inhibitor against MDR *S. aureus* we were eager to evaluate the synergistic activity of malabaricone B with classical antibiotics. Our approach involved the initial screening of the compound for synergistic activity followed by the study of active combination in MDR strains, time-kill kinetics and biofilm inhibition.

4.5.1. Synergistic Studies of NS-7 in Combination with FDA Approved Drugs

Compound was screened to determine the synergy between FDA approved antibiotics commonly used for the treatment of MDR *staphylococcus* infections which include ceftazidime, gentamycin, levofloxacin, linezolid, meropenem, minocycline, rifampicin and vancomycin. Checkerboard assay was used to determine the synergy between compound and the conventional antibiotics. According to the CLSI reference, before testing serial two-fold dilutions of NS-7 to at least 2x MIC were freshly prepared. MIC of the NS-7 alone and in combination with antibiotics was initially evaluated along with the fractional inhibitory concentration (FIC). Obtained results from the synergy studies were summarized in Table **4.12**.

Table 4.12: Synergistic combination studies of NS-7 with the FDA approved antibiotics

Drug	MIC (µg/ml)	MIC of NS-7 in presence of drug (A)	MIC of drug in presence of NS-7 (B)	FIC A	FIC B	FIC (A+B)	Inference
NS-7	**2**						
Ceftazidime	16	2	16	0.5	1	1.5	No interaction
Daptomycin	1	0.06	0.25	0.015	0.25	**0.265**	**Synergism**
Gentamycin	0.5	0.06	0.125	0.015	0.25	**0.265**	**Synergism**
Levofloxacin	0.25	2	0.25	0.5	1	1.5	No interaction
Linezolid	4	1	2	0.25	0.5	0.75	No interaction
Meropenem	1	2	1	0.5	1	1.5	No interaction
Minocycline	0.25	2	0.25	0.5	1	1.5	No interaction
Rifampicin	0.0078	2	0.0078	0.5	1	1.5	No interaction
Vancomycin	1	1	0.5	0.25	0.5	0.75	No interaction

NS-7 showed potent synergistic interaction only with daptomycin and gentamycin in *S. aureus* ATCC 29213 strain. Compound synergized with daptomycin and gentamycin with an FIC value of 0.265. The result was impressive and indicates antibacterial potential of the compound can be effectively utilized in combination with daptomycin and gentamycin to achieve enhanced activity for the treatment of MDR *staphylococcus* infections.

4.5.2. Activity of Synergistic Combination (NS-7+Gentamycin) against MDR-SA

Since compound exhibited excellent synergistic activity in combination with gentamycin, that particular combination was further screened against MDR strains of *S. aureus* including MSSA, MRSA and VRSA in which levofloxacin, meropenem, methicilin and vancomycin were used as reference standards. The MIC of compound alone and synergistic combination with gentamycin was determined in different strains. The results were summarized in Table **4.13** & **4.14**. The synergistic combination (NS-7+Gentamycin) showed enhanced activity in MSSA, MRSA and VRSA panel when compared to the compound/gentamycin alone. The combination was also found to be as effective as the standard drugs. So this particular combination can be an excellent alternative for the existing drugs in the treatment of various MDR *S. aureus* infections.

Table 4.13: MIC of synergistic combination (NS-7+Gentamycin) against MDR strains of *S. aureus* including MRSA and MSSA panel

		NS -7	Gentamycin	NS7+ Gentamycin	Levofloxacin	Meropenem	Vancomycin	Methicillin
MSSA	***S. aureus* ATCC 29213**	2	0.25	0.25	0.25	0.125	1	1
MRSA	***S .aureus* NRS 100**	2	0.125	0.125	0.25	32	2	>64
	***S. aureus* NRS 119**	2	>64	2	16	64	1	>64
	***S. aureus* NRS 129**	2	0.25	0.125	0.25	2	1	32
	***S. aureus* NRS 186**	2	0.25	0.25	4	4	1	64
	***S. aureus* NRS 191**	2	0.25	0.125	16	32	1	>64
	***S. aureus* NRS 192**	2	0.25	0.25	8	8	1	>64
	***S. aureus* NRS 193**	2	0.25	0.25	32	64	1	>64
	***S. aureus* NRS 194**	2	0.25	0.25	0.125	1	2	32
	***S. aureus* NRS 198**	2	0.25	0.25	32	32	1	>64

Table 4.14: MIC of synergistic combination (NS-7+Gentamycin) against VRSA panel

MIC (µg/ml) of combination against VRSA panel			
Compound / Drug	**VRSA**		
	***S. aureus* VRS 1**	***S. aureus* VRS 4**	***S. aureus* VRS 12**
NS-7	2	2	2
Gentamycin	32	0.5	8
NS7+ Gentamicin	2	0.25	2
Levofloxacin	32	>64	32
Meropenem	64	32	4
Vancomycin	>64	>64	>64
Methicillin	>64	>64	>64

4.5.3. Time-Kill Kinetic Studies of the Combination (NS-7+Gentamycin)

Compound was found to be effectively synergised with gentamycin. Furthermore the bactericidal activity of the combination was evaluated by the time-kill method according to CLSI. *S. aureus* ATCC 29213 cells were treated with 1x MIC of combination along with 1x MIC of Gentamycin, NS-7 alone and standard vancomycin. CFU/ml at various time points (0, 1, 6 and 24 h) was determined. Kill curves were constructed by plotting the CFU/mL surviving at each time point in the presence and absence of combination (NS-7+Gentamycin)/ Drugs alone (Fig. **4.12**). From the data it is evident that the combination effectively reduced the colony forming unit even after 1h of exposure and complete suppression of bacterial growth is achieved in 24 h which was found to be as effective as the individual drugs.

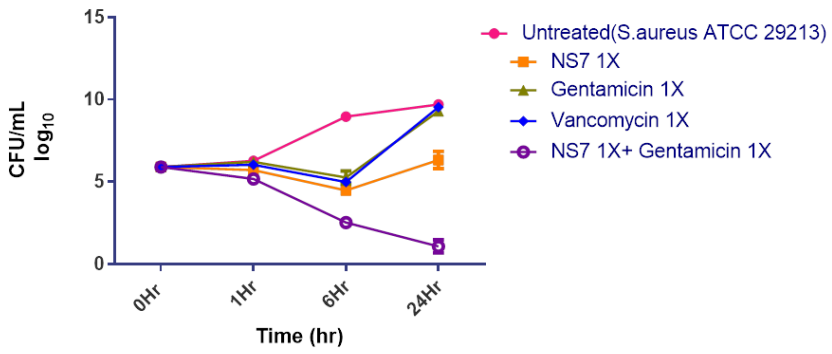


Figure 4.12: Time-kill kinetics of the synergistic combination

4.5.4. Effect of Synergistic Combination (NS-7+Gentamycin) on Biofilm Inhibition

Effect of synergistic combination of compound on the preformed biofilm by *S. aureus* ATCC 29213 was determined. The combination (NS-7+Gentamycin) at 1x MIC along with standard drugs (levofloxacin and vancomycin) at 1x and 10x MIC was evaluated and percentage inhibition of preformed biomass was determined.

The combination reduced 10% of the preformed biofilm at 1x MIC which is comparable to that of levofloxacin at 10x MIC. It proved that the combination in lower concentration is needed to reduce the biofilm than the individual drugs at higher concentration. The combination was found to be very effective than the standard drugs. So the conditions of severe *S. aureus* infections can be treated with this particular combination more efficiently.

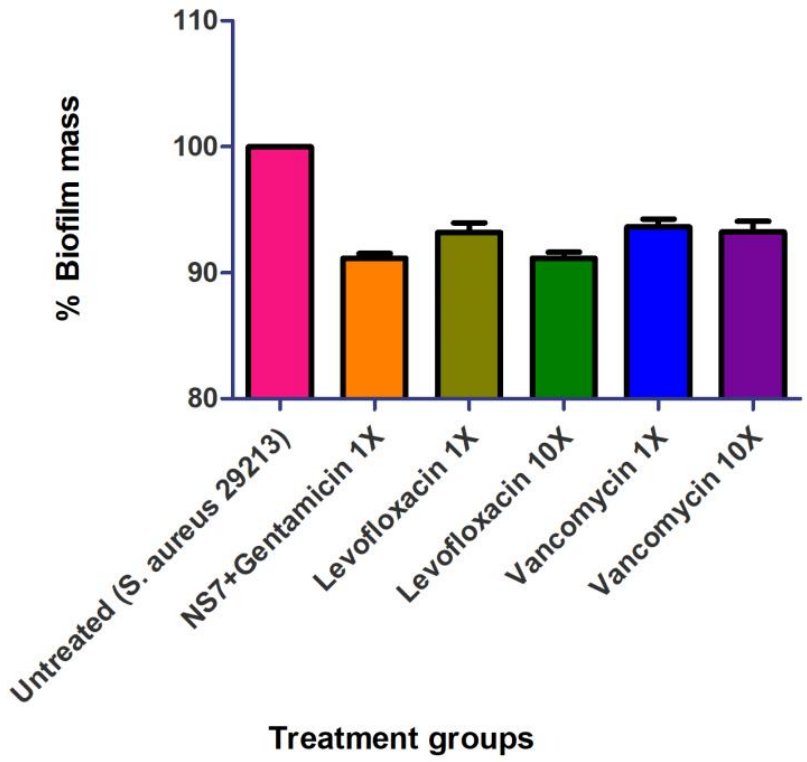


Figure 4.13: Biofilm inhibition of the combination (NS-7+Gentamycin)

Taken together the *in vitro* activity studies of the synergistic combination (NS-7+Gentamycin) suggest that the combination of the active compound and the conventional antibiotic, gentamycin is effective against *S. aureus*. Compound alone or in combination can be used to get better activity. While in combination the active ingredients are needed in lower concentration which is an advantage. *In vivo* efficacy studies are needed to understand its action in animal model.

4.6. Conclusion

In summary, a detailed *in vitro* and *in vivo* antibacterial screening of the compound, malabaricone B (NS-7) along with its synergistic activity against MDR *S. aureus* clinical isolates were demonstrated. We have effectively utilized the secondary metabolites from the Myristica species for the development of an alternative, safe and efficient therapeutic agent to address the issue related to antibiotic resistance. Clinical relevance and dosage regimens of the compound revealed that it requires less dosage administration and less exposure time for better activity. It is noteworthy that the malabaricone B exhibits a concentration-dependent bactericidal activity, effectively synergized with gentamycin and significantly reduced preformed biofilm and have good animal efficacy. These exhibited properties of the compound offers a new choice of a novel scaffold from natural origin for anti-staphylococcal therapy.

4.7. General Experimental Procedures

4.7.1. Reagents and Growth Media

Required bacterial media and supplements such as Mueller-Hinton agar (MHA), Mueller-Hinton cation supplemented broth II (MHBII) and Tryptic soy broth (TSB) were purchased from Becton-Dickinson (Franklin Lakes, NJ, USA). All the antibiotics and best graded chemicals were procured from Sigma-Aldrich (St. Louis, MO, USA). The medium including Roswell Park Memorial Institute Medium (RPMI) and Fetal Bovine Serum (FBS) were purchased from Lonza (Lonza, USA). All experimental procedures were performed in accordance with the standard guidelines and regulations.

4.7.2. Bacterial Strains

The strains of ESKAP pathogen panel consists five pathogens; *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Klebsiella pneumoniae* BAA-1705, *Acinetobacter baumannii* BAA-1605 and *Pseudomonas aeruginosa* ATCC 27853 were obtained from the American type culture collection (ATCC, Manassas, VA). The clinical MDR *S. aureus* strains including MRSA (NRS100, NRS119, NRS129, NRS186, NRS191, NRS192, NRS193, NRS194 & NRS198), VRSA (VRE1, VRE4 &VRE12) and enterococcus strains of *E. faecalis* (NR 31884, NR 31885, NR 31886, NR 31887 & NR 31888) and *E. faecium* (NR 31903, NR 31909 & NR 31912) were procured from Biodefense and Emerging Infectious disease/ Network on Antimicrobial Resistance in *Staphylococcus aureus* (BSI/NARSA). All the strains were routinely cultivated on MHA, MHBII and TSB. Before the starting of experiment, to get the starter culture single colony

was picked from MHA plate and was inoculated in liquid medium and incubated at 37 ^{0}C with shaking for overnight (18-24 h).

Table 4.15: Bacterial strains used in the study

Strains		Antibiotics Resistant to	Molecular Details of Strains
Gram negative Bacteria	*E. coli* ATCC 25922	None	Type strain
	K. pneumoniae BAA-1705	Carbapenem-resistant (Imipenem and Ertapenem)	Type strain
	A. baumannii BAA-1605	Ceftazidime, Gentamicin, Ticarcillin, Piperacillin, Aztreonam, Cefepime, Ciprofloxacin, Imipenem and Meropemem	Type strain
	P. aeruginosa ATCC 25923	None	Type strain
MSSA	SA 29213	None	
MRSA	NR 100	Methicillin, Ceftriaxone, Meropenem	✓ Resistant to tetracycline ✓ Positive for mec (subtype I) ✓ Large variety of virulence factors
	NR 119	Methicillin, Ceftriaxone, Meropenem, Gentamycin and Linezolid	✓ Positive for mec (subtype IV) ✓ G2576T mutation in domain V in one or more 23S rRNA genes
	NR 10129	Methicillin, Ceftriaxone, Meropenem	✓ Also known as TCH60
	NR 10186	Methicillin, Ceftriaxone, Meropenem	✓ Community-acquired MRSA ✓ Pulse-field gel electrophoresis (PFGE) typed as USA300 ✓ Positive for the Panton-Valentine leucocidin (PVL) virulence factor ✓ Contains staphylococcal chromosome cassette mec type IV
	NR 10191	Methicillin, Ceftriaxone, Meropenem	✓ Community-acquired MRSA ✓ Pulse-field gel electrophoresis (PFGE) typed as USA600 ✓ Negative for the Panton-Valentine leucocidin (PVL) virulence factor ✓ Contains staphylococcal

			chromosome cassette mec type II
	NR 10192	Methicillin, Ceftriaxone, Meropenem	✓ Community-acquired MRSA ✓ Pulse-field gel electrophoresis (PFGE) typed not as USA100-1100 ✓ Negative for the Panton-Valentine leucocidin (PVL) virulence factor ✓ Contains staphylococcal chromosome cassette mec type II
	NR 10193	Methicillin, Ceftriaxone, Meropenem	✓ Community-acquired MRSA ✓ Negative for the Panton-Valentine leucocidin (PVL) virulence factor ✓ Contains staphylococcal chromosome cassette mec type II
	NR 10194	Methicillin, Ceftriaxone	✓ Community-acquired MRSA ✓ Positive for the Panton-Valentine leucocidin (PVL) virulence factor ✓ Contains staphylococcal chromosome cassette mec type V
	NR 10198	Methicillin, Ceftriaxone, Meropenem	✓ Community-acquired MRSA ✓ Pulse-field gel electrophoresis (PFGE) typed as USA100 ✓ Negative for the Panton-Valentine leucocidin (PVL) virulence factor ✓ Contains staphylococcal chromosome cassette mec type II
VRSA	VRS 1	Methicillin, Ceftriaxone, Meropenem, Gentamycin, Vancomycin, Teicoplanin	✓ Positive for mec (subtype II) and vanA ✓ Negative for vanB, vanC1, vanC2, vanD, vanE, PVL and arginine catabolic mobile element (ACME) ✓ Pulsed-field type USA100
	VRS 4	Methicillin, Ceftriaxone, Meropenem, Vancomycin and Teicoplanin	✓ Positive for mec (subtype II) & vanA ✓ Negative for vanB, vanC1, vanC2, vanD, vanE, PVL & arginine catabolic mobile element (ACME) ✓ Pulsed-field type USA100

	VRS 12	Methicillin, Ceftriaxone, Meropenem, Vancomycin and Teicoplanin	NA (Data Not available)
E. faecalis	NR 31884	Gentamycin	✓ Hemolytic, cytolytic isolate ✓ Complete genome is sequenced (GenBank: AIRF00000000)
	NR 31885	Gentamycin	✓ Cytolytic isolate ✓ Complete genome is sequenced (GenBank: AIRH00000000)
E. faecium	NR 31903	Ampicillin	✓ Complete genome is sequenced (GenBank: AJDX00000000)
	NR 31912	Vancomycin	✓ Complete genome is sequenced (GenBank: AJDX00000000)

4.7.3. Antibacterial Susceptibility Testing

The compounds were screened for their antibacterial potential against ESKAP pathogen panel by broth microdilution assay utilizing CLSI guidelines. The panel consists of *E. coli, S. aureus, K. pneumonia, P. aeruginosa and A. baumannii*. Initially 10 mg/mL stock solutions of test compounds (malabaricones/extracts) were prepared in DMSO and were kept under −20 ^{0}C. The bacterial cultures were inoculated in Muller-Hinton supplemented broth and the optical density (OD) of the cultures were measured at 600 nm followed by dilution for ~10^{6} cfu/mL. Working stock solutions of concentration 2.56 mg/ml were prepared for different antibiotics using DMSO from stock solution of 10 mg/ml. Stock solutions were diluted further for differential dilutions of 64-0.5 µg/ml. Two controls *i.e.*, cells alone and media alone (without compound + cells) were studied. The plates are incubated at 37˚C for 24 h. Results were observed by the absence or presence of visible growth. The MIC is defined as the lowest concentration of the compound which inhibits visible growth in comparison to control. For each test compound MIC determination in three replicate measurements.

4.7.4. Cytotoxicity Assay

To test the effect of compounds on the growth of mammalian cells, a cytotoxicity assay was performed using MTT assay as per published protocols. In brief, 5000 Vero (African green monkey kidney) cells were seeded in a 96-well plate and grown for 24 hours at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in DMEM medium supplemented with 10% fetal bovine serum, 1.5 g Sodium bicarbonate/L, 100 µg/mL of

Penicillin and 10 µg/mL of Streptomycin. The next day different concentrations of compound were added in media in triplicate. Following 72 hours of incubation, MTT [(3-(4, 5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide)] was added to a final concentration of 1 mg/mL for 2 h. At the end of the incubation period, the media were removed by gently inverting the plate. 0.1 ml of DMSO was added to the wells to solubilize the formazan crystals and absorbance was read at 595 nm in a plate reader. The absorbance of treated vs. untreated cells was compared. % survival vs. concentration of compound was plotted to determine the CC_{50}. The selectivity index is calculated as CC_{50}/MIC and must be at least >10. Doxorubicin was used as positive control and each experiment was repeated in triplicate.

4.7.5. Activity against MDR Strains of *S. aureus* & Enterococcus Panel

MIC of the compound and synergistic combination (NS-7+Gentamycin) was determined in well-defined clinical strains MDR *S. aureus* such as MSSA, MRSA & VRSA and in Enterococcus panel (VRE & VSE) by broth microdilution assay as described earlier.

4.7.6. Time-Kill Kinetic Study

The bactericidal activity was explored by the time-kill method according to CLSI. *S. aureus* ATCC 29213 cells were diluted up to ~ 10^5 CFU/ml in MHBII. Bacteria were incubated with 1x and 10x MIC of compound and vancomycin and incubated at 37 ^{0}C with shaking for 24 h. Aliquots were withdrawn at various time points (0, 1, 6 and 24 h) serially diluted in PBS and CFU/mL was determined by plating on Mueller-hinton Agar. The kill curves were constructed by counting the colonies from plates and plotting the CFU/mL surviving at each time point in the presence and absence of compound. Experiment was repeated in three times induplicate and the mean data were plotted.Bactericidal activity was defined as a reduction of at least ≥1 $\log_{10}$ of the total count of CFU/mL in the original inoculum.

4.7.7. Biofilm Inhibition Assay

S. aureus ATCC 29213 strains was cultured overnight in TSB supplemented with 1% glucose on rotary shaker (180 rpm) at 37 ^{0}C. The overnight culture was diluted in fresh TSB broth (1:100) and the freshly diluted culture (0.2 mL/well) was transferred into 96 well flat bottom tissue culture plates. The plates were covered with adhesive lid to maintain low oxygen conditions to increase biofilm formation and incubated for 48 h at 37^{0}C. Subsequently, media was decanted, plates were rinsed gently thrice with 1x PBS (pH 7.2) to remove the planktonic bacteria. The plates were refilled with TSB containing

different drug concentrations and incubated for 24 h at 37 ^{0}C. Post drug treatment media was once again decanted and wells were rinsed with thrice with 1x PBS. The plates were incubated at 60 ^{0}C for 1 h for biofilm fixation and stained by 0.06% crystal violet for 10 min. Wells were rinsed with PBS and dried a room temperature. Biofilm bound crystal violet was eluted by 30% acetic acid (0.2 mL each) and quantified by measuring the absorbance at 600 nm.

4.7.8. Intracellular Killing Assay

The J774.A1 mouse macrophage cell line was seeded at 50,000 cells/well in 12 well tissue culture plates and was infected with *S. aureus* ATCC 29213 for 1 h at multiplicity of infection (MOI) of 1:100. After infection, cells were washed with 1x PBS (pH 7.4) to remove extracellular bacteria and wells are replaced by RPMI medium containing different concentration of drugs containing gentamycin (0.5 mg/L) to prevent extracellular bacterial growth. The plates incubated for 24 h at 37 °C in 5% CO_2 incubator. Following the incubation, the cells were washed 3 times with 1x PBS (pH 7.4) and lysed with RIPA buffer (#89901 Thermo). The cell lysate were serially diluted, plated on TSA and incubated for 24 h at 37 °C for enumeration of colony forming units (CFU).

4.7.9. Induced Resistant Mutant Generation Studies

After MIC was determined, serial passaging was initiated by harvesting bacterial cells growing at the highest concentration of the compound with one-half MIC of the MIC and inoculating into fresh media. This inoculum was subjected to another MIC assay. After 24 h incubation period, cells growing in the highest concentration of the compound from the previous passage were once again harvested and assayed for the MIC. The process was repeated for 35 passages. The MIC value of the compound was plotted against the number of passages and the fold increase in MIC was determined.

4.7.10. *In vitro* Post Antibiotic Effect (PAE)

Overnight culture of *S. aureus* ATCC 29213 was diluted in MHBII ~10^5 CFU/mL and exposed to 1x and 5x MIC of vancomycin, levofloxacin, test compound (NS-7) and incubated at 37 °C for 1 h. Following the incubation period, culture was centrifuged and washed 2 times with pre-warmed MHBII to remove any traces of antibiotics. Finally, cells were re-suspended in drug free MHBII and incubated further at 37 °C. Samples were taken after every 1h, serially diluted and plated on TSA for enumeration of CFU. The PAE was calculated as PAE = T – C; where, T is referred to the difference in time required for 1 $\log_{10}$ increase in CFU versus CFU observed immediately after the removal of drug and C in a similarly treated drug free control.

4.7.11. Polymyxin B nonapeptide (PMBN) Assay

The MIC of the compound and control antibiotics (Rifampicin, Vancomycin & levofloxacin), in presence of polymixin B nonapeptide (PMBN), against gram-negative bacteria (*E. colli* & *A. bumanni*) was measured as described in the antibacterial susceptibility testing (Broth microdilution assay). Sub lethal concentration of PMBN (10 μg/ml) was added to TSB to increase the outer membrane permeability and facilitate the entrance of the compound.

4.7.12. Maximum Tolerable Dose (MTD) Study

MTD studies were done using Balb/c mice. Three Balb/c mice of weight 20-21 kg were administrated a dose of 250 mg/kg of the active compound NS-7 with once daily (s.i.d.) intraperitoneally (IP) for 10 days. The dosed animals were monitored daily and clinical observations including weight loss (total for challenge group, time phased), morbidity (number of mice showing morbidity, type of morbidity, time-phased), and time to death for all mice (within 12 h window) were recorded.

4.7.13. Murine Neutropenic Thigh Infection Model / Skin infection model

Mice (BALB/c) of same age and same weight (~22–24 g as near as possible) will be grouped into 3 animals/per cage and will be marked on the tail. Mice will be injected intraperitoneally (IP) with 1 mL syringe with 26G needles containing 100 μL of Cyclophosphamide at a dose of 150 mg/Kg and 100 mg/kg, 24 h and 1 h respectively to induce neutropenia before the introduction of infection. Mid log phase bacteria (10^7–10^9) in PBS will be injected into the right thigh IM. After 3 & 6 hours post bacterial infection, the test compound ~200 μL (200 mg/kg) and Vancomycin at 50 mg/Kg of body weight per mice will be administered IP. The control animals were administered saline in the same volume and frequency as those receiving treatment. Untreated control animal were sacrificed after 2 h of the inoculation of bacteria to confirm establishment of infection. After 24 h the mice were sacrificed, thigh will be removed, homogenized individually in 5 ml of PBS, serially diluted and plated on the Mueller-Hinton agar plates for CFU count. After incubation for 18–24 h at 37 °C, the CFU were enumerated. Experiments were performed in triplicate and the mean data is plotted.

4.7.14. Synergy Screening

Checkerboard method was used to determine synergy between compound and the antibiotics. The antibiotics included in the study are Daptomycin, Gentamycin, Levofloxacin, Minocycline, Rifampicin, Linezolid, Meropenem and Vancomycin against a panel of MRSA and VRSA strains. According to the recommendations of CLSI, serial

two-fold dilutions of each drug to at least double the MIC were freshly prepared prior to testing. Compound was twofold diluted along the ordinate while the antibiotics were serially diluted along the abscissa in 96 well microtiter plates. An inoculum equal to 10^5 or 10^6 CFU/mL was prepared from each MRSA and VRSA isolate in MHB. Each microtiter well was inoculated with 100 µL of a bacterial inoculum of 10^5 or 10^6 CFU/mL, and plates were incubated at 37 °C for 24 h under aerobic conditions. After the incubation period was over, FIC was calculated. The ΣFICs (fractional inhibitory concentrations) were calculated as follows: ΣFIC=FIC A + FIC B, where FIC A is the MIC of drug A in the combination/MIC of drug A alone, and FIC B is the MIC of drug B in the combination/MIC of drug B alone. The combination is considered synergistic when the ΣFIC is ≤0.5, indifferent when the ΣFIC is > 0.5 to 4 and antagonistic when the ΣFIC is > 4.

4.7.15. Statistical Analyses

Statistical analysis was performed using Graph Pad Prism 7.0 software (Graph Pad Software, La Jolla, CA, USA). Comparison between three or more groups was analyzed using one-way ANOVA, with post-hoc Tukey's multiple comparisons test. P-values of <0.05 were considered to be significant.

4.8. References

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Phytochemical Investigation of the Leaves of *Hyptis capitata*

5A.1. Introduction

Aromatic and medicinal plants have been regarded as the foremost source of major bioactive phytoconstituents. From the time immemorial they have played a pivotal role in traditional system of medicine to cure various ailments. Lamiaceae family is considered as the versatile plant family known for their enormous medicinal and therapeutic values. Lamiaceae**,** formerly called 'Labiatae', 'the mint family' is the sixth largest family of flowering plants and the largest family of the order Lamiales. It consists of 236 genera and more than 7,000 species distributed worldwide, growing under wide variety of soil and climate, but more abundant in Mediterranean regions and in the hills. Around 64 genera and 380 species of plants were documented in India. *Salvia* L. is the largest genera of this family having 960 species followed by *Hyptis Jacquin* (400), *Scutellaria* L. (360) etc. along with some economically important genera including *Mentha* L., *Lavandula* L., *Ocimum* L., *Thymus* L. and *Melissa* L. Most of the members of this family are perennial or annual aromatic herbs [1]. The leaves are fragrant and contain volatile oils. The flowers are usually arranged in clusters and the fruit is commonly a dry nutlet. The high essential oil content of the species made this family useful for flavor, fragrance and medicinal application. Some of the well-known members of this family are a variety of aromatic spices like thyme, mint, oregano, basil, sage, savory, rosemary, etc. Most of the species of this family are used traditionally as antioxidant, anti-cancer, antipyretic, anti-inflammatory, anesthetic, hepatoprotective, anti-diabetic, smooth muscle relaxant, antitumor, antibacterial, antifungal, antiviral and for the treatment of wound healing, headache, pains, chronic rheumatism, sedative, gastrointestinal disorders owing to the presence of phenolic compounds, flavonoids or benzoic acids and terpenoids as well as steroids [2-4].

5A.2. Hyptis Genus

Hyptis is one of the largest and most widely distributed plant genera in the world belongs to lamiaceae family. It consists of 775 species widely distributed in tropical America and South America, as well as parts of West Africa. The generic name *Hyptis* is derived from the Greek word *Hyptios* meaning laid back or resupinate, referring to the limb of the corolla, which is turned on its back [5-6]. Species of the *Hyptis* genus are

characterized by their strong aromatic constituents and widely used to obtain the essential oil. Most of the species are commonly called bush mint. The genus mainly composed of herbs, shrubs, subshrubs, and more rarely small trees. The stems of the species of this genus are often square in cross section and the leaves are usually opposite, occasionally whorled, simple or rarely with slits, petiolate and aromatic, hairs gland-headed with essentials oils, simple, non-glandular, usually multicellular or both multicellular and unicellular [7]. Species of this genus are widely used in traditional folk medicine to treat various illnesses such as gastrointestinal infections, cramps, pains and skin infections and have been found to possess tumorigenic, anti-fertility and antimicrobial activities.

5A.3. *Hyptis capitata* Jacq

Hyptis capitata Jacq, commonly known as the "false ironwort, buttonweed or knobweed" (In Malayalam it is known as *karinthumba*) belongs to Lamiaceae family is native to tropical America. The species is naturalized in Western Ghats and ubiquitous in Kerala. It is an aromatic, annual to perennial weed with woody stems, pantropical distribution and also found in rain forests at elevations up to 750 m. It is a traditionally important medicinal plant extensively utilized in folk and traditional medicine to cure various ailments. The leaves are excitant, tonic and were widely used to treat skin infections. A cold water infusion of the crushed leaves is used to treat 'black diarrhoea' and also used as a sedative and calmative, and to reduce heart palpitations. A decoction of the leaves is used to clean wounds. The plant is well known for its wound healing property. The crushed leaves are applied to cuts and abrasions in order to prevent infections. The paste of the young leaves is employed to treat stomach ache [8-9].

Figure 5A.1: *H. capitata* plant and leaves

***Taxonomic description of the species*:** Stem profusely branched, quadrangular, shallowly furrowed on the sides. Leaves opposite petiolate, broadly ovate-oblong, serrate, cuneate and glabrous or minutely peberulous on the nerves beneath. Flowers in globose head; bracts ovatelanceolate, calyx glabrous, sub-equaly five toothed, teeth erect, sublote

corolla slightly two liped; upper lip four lobed. Stamns four, declinate; filaments free; anther cells confluent. Overy four partite; sryle bifed, snutlets four; basal scar small [10].

5A.3.1. Phytochemical Constituents

Most of the species of Hyptis species are aromatic and rich in essential oil content in all parts including leaves and flowers. Owing to the abundance of essential oil and its variety of biological activities they gained enormous interest. Essential oil of *Hyptis* species were well studied and demonstrated that they are composed mainly of mono and sesquiterpenes irrespective of their origin and significant differences were observed in the percentage compositions of the major components [11]. In 1995, Thoppil and Jose studied the essential oil of the leaves & inflorescence of Indian *Hyptis capitata* and reported that monoterpenoids (68%) were the major phytoconstituents in the oil along with minor amounts (4.6%) of sesquiterpene, *β*-caryophyllene. The monoterpenoids detected from the yellow coloured essential oil include borneol (16.7%), piperitone oxide (14.8%), terpinen-4-ol (11%), citronyl acetate (9.7%), fenchone (9.6%), geranyl acetate (4.3%) and citronellal (2.8%) [12]. GC-MS analysis of the leaves of Indonesian *H. capitata* was reported to contain terpenoid compounds including l-limonene, eugenol, farnesol isomers A, d-nerolidol, hexahydrofarnesol and neophytadiene and was assumed to serve as anti-infective agents [13]. Physicochemical and phytochemical evaluation of stem and leaves of *Hyptis capitata* was reported in various solvents. Majority of phytochemicals were detected in leaf extract of petroleum ether and methanolic extract of stem. Preliminary phytochemical screening revealed alkaloids, coumarins, glycosides, flavonoids, phenols, quinines, tannins, terpenoids, steroids, iridioids and saponins were present in leaf extract of ethyl acetate [14]. In 2019 Sumitha and Mini reported the pharmacognostic evaluation of root, stem and leaves of the medicinal herb *Hyptis capitata* as per WHO guidelines [9]. Recently preliminary phytochemical analysis of the leaves of Indonesian *H. capitata* was reported to contain alkaloids, flavonoids, tannins, carbohydrates, and coumarins [15].

Phytochemical investigations of the species of *Hyptis* genus revealed that diterpenoids, triterpenoids, flavonoids, lignanas are the major phytoconstituents present in this species (Fig. **5A.2**). Phytochemical investigation of the aerial parts of the medicinal plant *Hyptis capitata* reported to have 2,3-di(3’,4’-methylenedioxybenzyl)-2-buten-4-olide, 10-epiolguine, stigmasterol, 5-hydroxy-4’,7-dimethoxyflavone (apigenin-4’,7-dimethyl ether), oleanolic, ursolic and rosmarinic acids [16]. In 1988, Lee *et al*. reported the isolation of ursolic acid from the species [17]. Methanol extract of the aerial parts of

the species reported the presence of triterpene acids which include hyptatic acids-A and B along with 2α-hydroxyursolic acid, tormentic acid and maslinic acid [18].

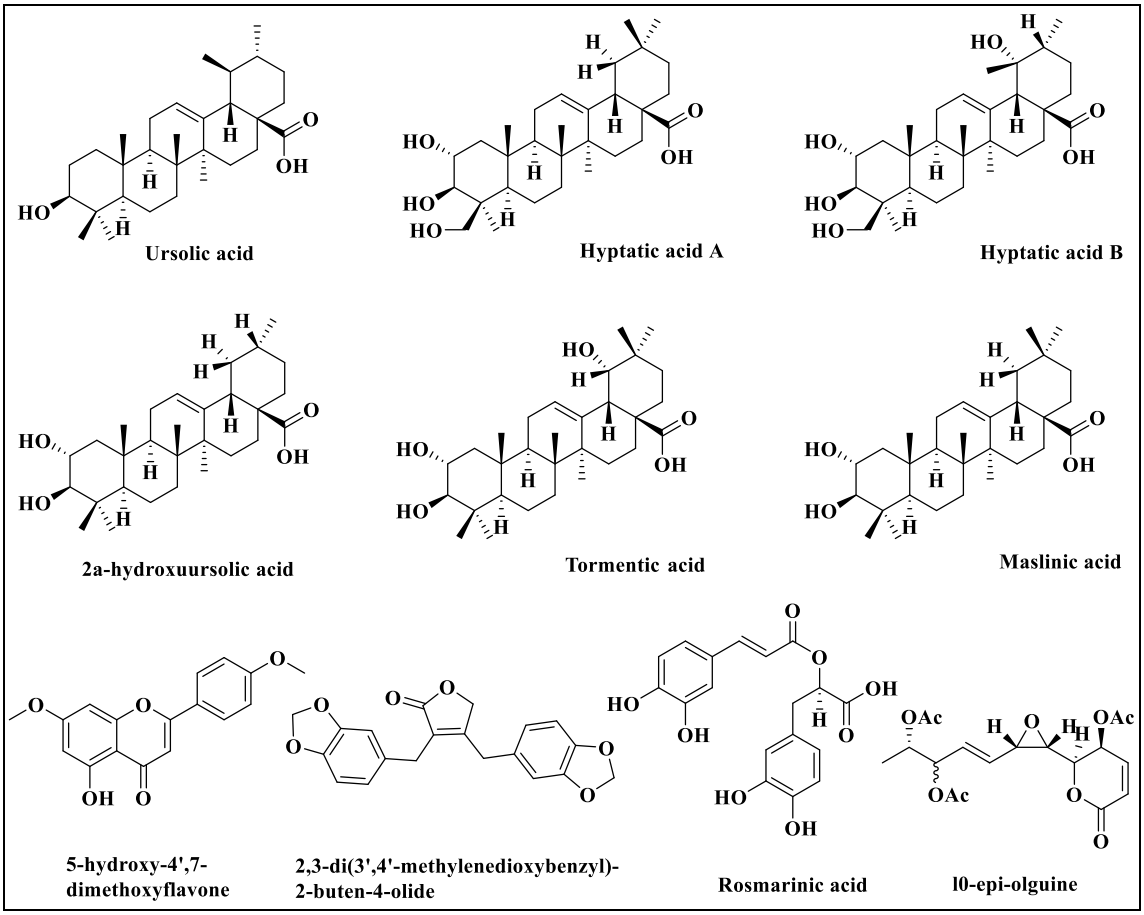


Figure 5A.2: Structures of the reported compounds from *H. capitata*

5A.3.2. Pharmacological Activities

Hyptis capitata is credited with various pharmacological activities. The plant has been used in traditional medicine for the treatment of gastro-intestinal disorders, hemorrhoids and asthma [17]. Essential oil from the *Hyptis* species was found to possess antimicrobial and antioxidant activities [19]. The species is reported to have inhibitory potential against *S. aureus* [20]. Lee *et al.* reported significant cytotoxicity of ursolic acid from the species in the lymphocytic leukemia cells P-388 and L-1210 as well as the human lung carcinoma cell A-549. It is also demonstrated marginal cytotoxicity in the KB and the human colon (HCT-8) and mammary (MCF-7) tumor cells [17]. Oleanolic acid isolated from the species is reported to have anti HIV activities [21]. Leaves, stem and branch extracts of *H. capitata* was reported to be active against snakebites [22].

5A.4. Aim and Scope of the Present Work

Medicinal plants are rich source of therapeutic agents and are well explored in traditional and folk medicine. The indigenous knowledge about the medicinal plants contributes towards the development of new medicinal lead from the plant. Scientific validation of traditional knowledge is also an important aspect. There are several medicinally important species used in traditional medicine which are not explored in terms of its phytoconstituents and pharmacological application. As already explained *Hyptis capita* is a traditionally important medicinal plant explored for its wound healing property. Several studies about this species grown in different places were reported. The species is ubiquitously distributed in Western Ghats especially in Kerala. Studies related to Indian *Hyptis capita* are limited. Detailed study regarding phytochemistry and pharmacology is not reported so far. Geographical and biological environment of growing locations of a wide spread plant species have an influence on the proportion and nature of secondary metabolites present in it. The factors including weather, temperature, latitude, soil, sunlight etc. plays a key role in the biosynthesis of metabolites. Plants have strong interaction with its environment throughout the growth process which in turn reflects in the production of secondary metabolites. Several studies demonstrated that medicinal plants that grow in various environments produce different active substance because of their wide distribution in different geological zones. In view of this our efforts are directed towards the detailed phytochemical analysis of the Indian *Hyptis capitata*. Since the leaves of the plant is having excellent wound healing property and extensively used to cure skin infections in the present work we aimed the phytochemical analysis of the leaves along with the evaluation of its antibacterial potential. As a promising medicinal plant, it is important to analyze the chemical contents and bioactive constituent of the leaves of *Hyptis capitata*.

5A.5. Extraction, Isolation and Characterization of Compounds from the Leaves of *Hyptis capitata*

5A.5.1. Collection of Plant Material

Leaves of *H. capitata* (5 kg) were collected in December 2015, from Kodungallur of Thrissur District, Kerala, India. Plant material was authenticated by the plant taxonomist. A geographical map showing the collection site is given in Fig. **5A.3**.

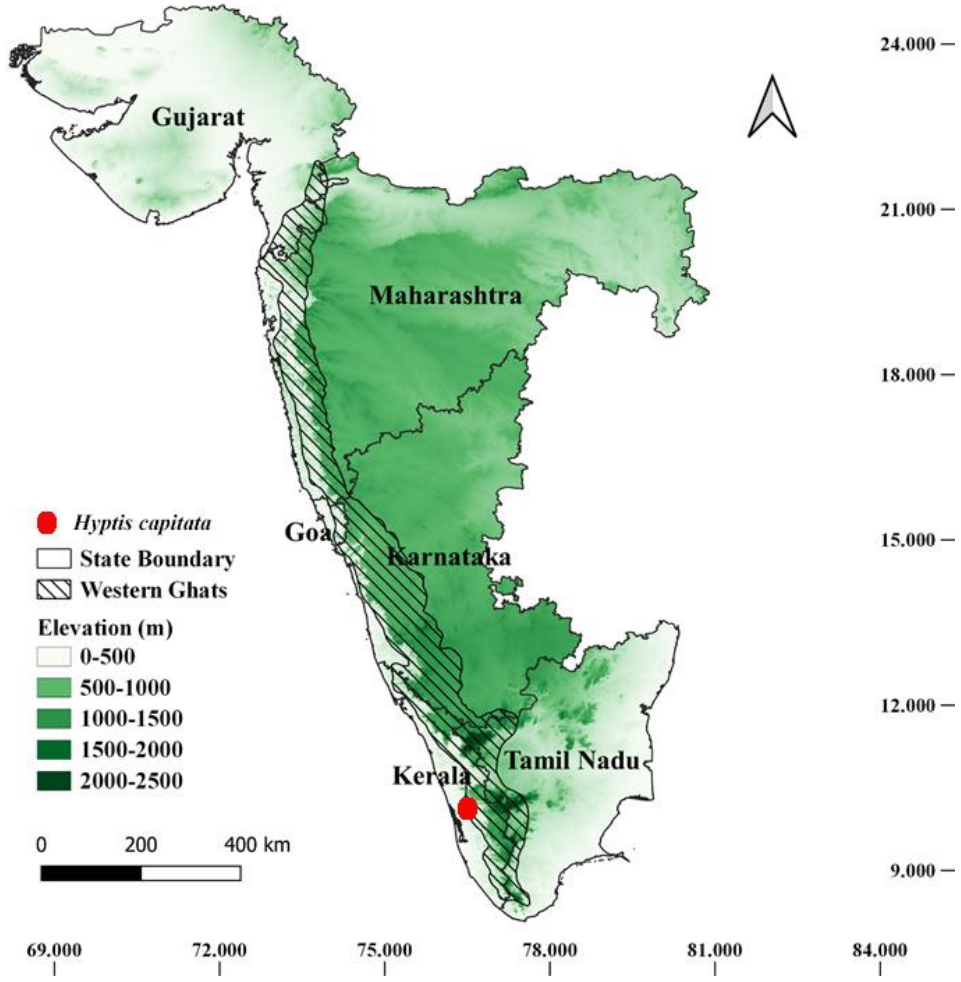


Figure 5A.3: Collection site of *H. capitata*

5A.5.2. Extraction of Essential Oil from the Leaves of *H. capitata*

The essential oils of the leaves were extracted by hydro-distillation using Clevenger's type apparatus. About 250 g of the fresh leaves were taken in a 3 L round bottomed flask and was filled with around 2 L of distilled water. Electrical heating mantle was used to heat the water. The flask was fitted with Clevenger and condenser. The essential oil remained floated in the oil collection area. After 4-5 hours of heating, when no visible essential oil was seen in the condenser area, essential oil was collected after draining the bottom water layer. Sodium sulphate was used to absorb any remaining moisture content in the essential oil. From this, we obtained around 1 ml of colorless and aromatic essential oil which was further subjected to GCMS analysis.

From the GCMS analysis (Table **5A.1**) it was found that the essential oil mainly consists of 1-isopropyl-4,7-dimethyl-1,2,3,5,6-8a-hexahydronaphthalene, γ-muurolene, 1-octen-3-ol, caryophyllene, β-bourbonene, α-murrolene are the major volatile components. The studies revealed that there are significant differences in terms of active

constituent of the essential oil depending upon its growing area. Structure of the major compounds from the GC-MS analysis is given in Fig. **5A.4**.

Table 5A.1: GCMS analysis of essential oil from the leaves of *H. capitata.*

SL. NO	Retention Time	Area %	Height %	Compound Name
1	21.512	6.47	5.37	1-isopropyl-4,7-dimethyl-1,2,3,5,6,8a-hexahydronaphthalene
2	20.429	6.33	5.36	γ-muurolene
3	6.879	5.67	4.06	1-octen-3-ol
4	19.037	5.51	5.16	Caryophyllene
5	18.100	5.23	5.33	β-bourbonene
6	21.002	5.21	4.09	α-murrolene
7	17.884	5.11	4.96	α-copaene
8	21.372	4.21	4.46	Naphthalene-1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1(1-methylethyl),(1α,4aβ,8aα)
9	20.520	3.73	3.66	Naphthalene-1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1(1-methylethyl)
10	20.085	3.45	3.41	4-isopropyl-β-methyl-benzene propanal
11	19.272	3.09	1.93	1H-cyclopenta[1,3]cyclopropa [1,2]benzene-octahydro-7-methyl-3-methylene-4(1-methylethyl),[3aS(3aα)]
12	34.929	2.69	2.25	Phytol
13	20.776	2.60	2.48	β-selinene
14	21.569	1.92	2.86	Naphthalene-1,2,3,4-tetrahydro-1,6-dimethyl-4(1-methylethyl),(1S-cis)
15	17.930	1.92	3.26	2-buten-1-one,1(2,6,6-trimethyl-1,3-cyclohexadiene-1-yl),(E)
16	19.651	1.91	2.06	5,9-undecadien-2-one-6,10-dimethyl(E)
17	22.024	1.70	1.97	1-isopropyl-4,7-dimethyl-1,2-dihydronaphthalene
18	20.850	1.61	1.88	1-isopropyl-7-methyl-4-methylene-1,2,3,4,4A,5,6,8a-octahydronaphthalene
19	18.409	1.60	1.56	2-propenal-3(2,6,6-trimethyl-1-cyclohexen-1-yl)

20	24.425	1.56	1.31	1-naphthaleneol-1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4(1-methylethyl),[1R(1α,4β,4 β,8aα)]
21	21.921	1.54	2.00	Naphthalene-1,2,4a,5,6,8a-hexahydro-4,7-dimethyl(1-methylethyl),[1R-(1α,4a α,8aα)]
22	17.705	1.49	1.79	Ylangene
23	19.754	1.49	1.03	1H-cycloprop(e)azulene-decahydro-1,1,7-trimethyl-4-methylene(1aR,4aS,7R,7aR,7bS)
24	24.857	1.47	1.91	2-pentadecanone-6,10,14-trimethyl
25	22.864	1.46	1.66	2-butanone-1(2,3,6-trimethylphenyl)
26	24.698	1.44	1.65	1-naphthaleneol-1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4(1-methylethyl),[1S-(1α,4α,4aα,8aα)]
27	24.857	1.34	1.47	1-farenesol
28	17.256	1.31	1.18	1,1,5-trimethyl-1,2-dihydronaphthalene
29	18.625	1.31	1.32	(E)-1(2,3,6-trimethylphenyl)buta-1,3-diene(TPB,1)
30	23.024	1.24	1.44	(-)-5-oxatricyclo[8.2.0.0(4,6)]dodecane-12-trimethyl-9-methylene[1R(1R*,4R*6R*,10S*)]

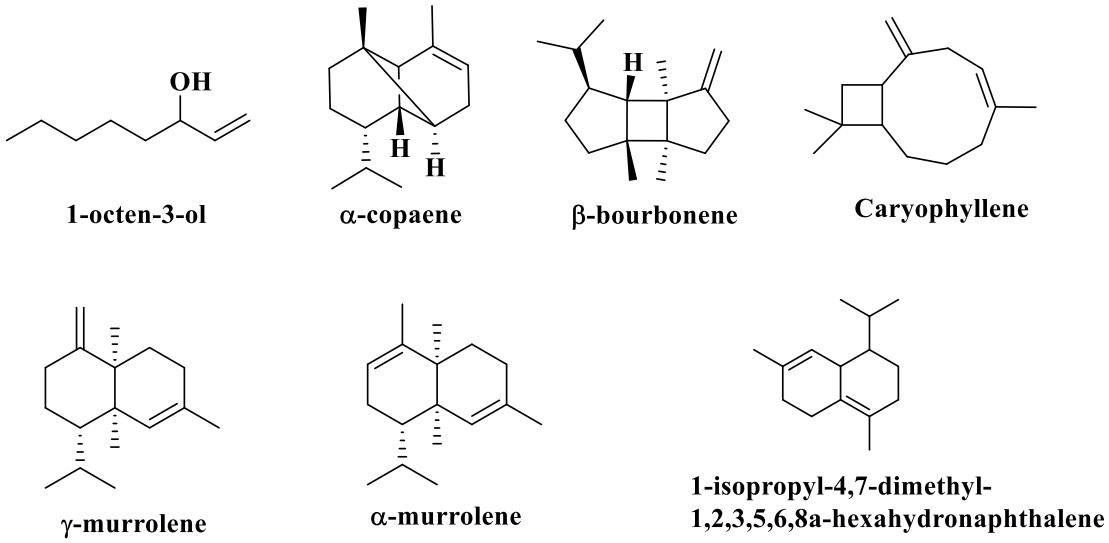


Figure 5A.4: Structures of the major components of essential oil of *H. capitata*

5A.5.3. Extraction and Isolation of Phytochemicals

Leaves of *H. capitata* (5 kg) were dried in an air oven maintained at 50 ^{o}C for two days, and then powdered mechanically. About 1 kg of powdered leaves was subjected to extraction with hexane (5 L x 4 days) at room temperature. Removal of the solvent under reduced pressure gave 5 g of crude hexane extract. The residue was then subjected to extraction using acetone (5 L X 4 days). About 60 g of acetone extract was obtained after removing the solvent using rotary evaporator. The above mentioned extraction procedures were repeated using ethanol and water to afford 20 g of ethanol and 18 g of water extract.

About 60 g of acetone extract was fractionated over silica gel (100-200 mesh) column chromatography using hexane, hexane-EtOAc gradient and EtOAc as eluent afforded 34 fraction pools based on the similarity in TLC analysis. The obtained fraction pools were subjected to repeated column chromatographic separation and purification led to the isolation of eight compounds. A schematic representation of the entire isolation procedure is given below (Fig. **5A.5**).

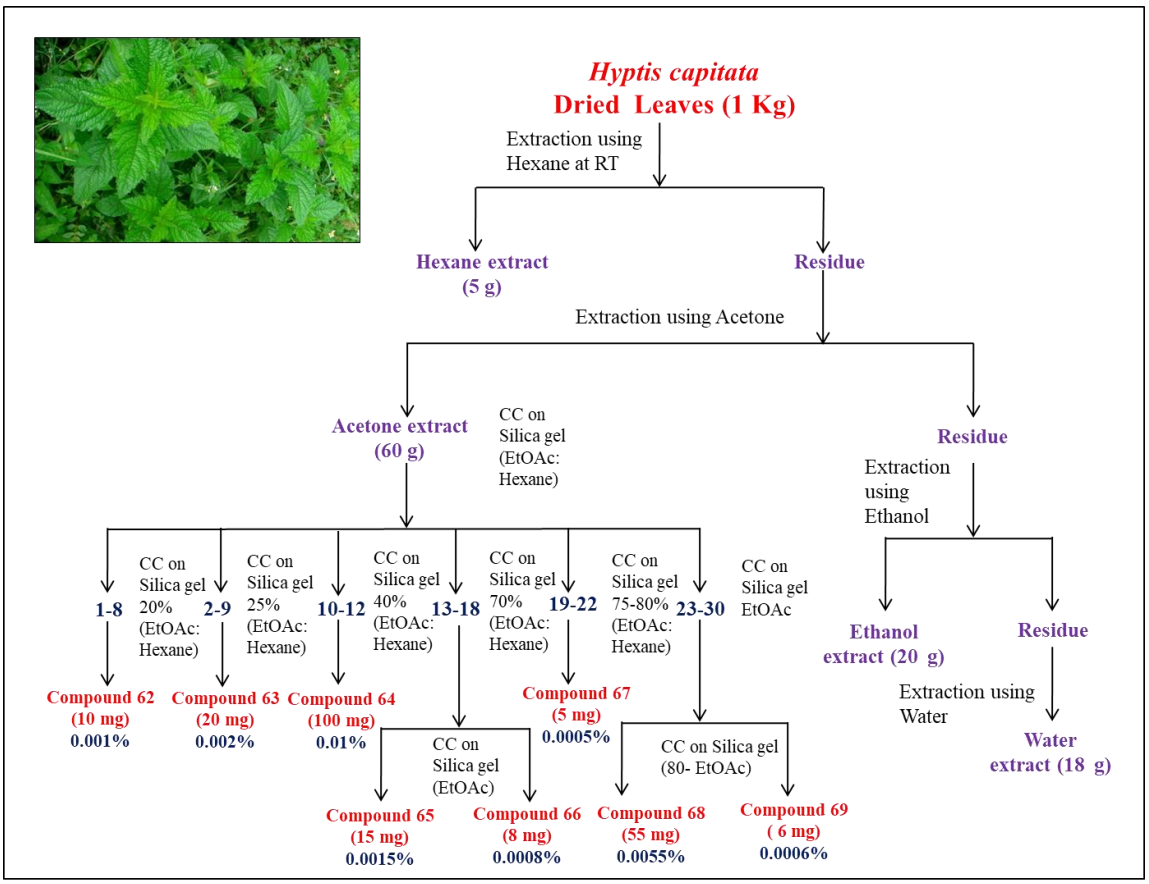
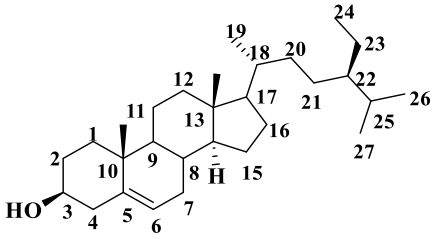


Figure 5A.5: Schematic representation of extraction and isolation procedure

Compound **62** was isolated as colourless needle shaped crystals from the fraction pool 1-4 after CC separation using 20% ethyl acetate-hexane. Based on the spectral data analysis the compound was confirmed to be the common phytosterol, *β*-sitosterol. The ^{1}H NMR displayed a doublet for *olefinic* proton at *δ* 5.35 (d, *J* = 5 Hz, 1H, H-6) ppm. The proton at position 3 resonated as a multiplet in the range *δ* 3.49-3.56 ppm indicating the stereo center. And it is one of the characteristic peaks. The ^{13}C NMR spectrum showed twenty nine carbon resonances including *olefinic* carbon, carbon bearing hydroxyl group and aliphatic carbons. DEPT 135 spectrum displayed eleven downward peaks corresponds to eleven methylene group. The mass spectrum of the compound gave a molecular ion peak at 415.1097 which is the $[M+H]^{+}$ peak [23].



***β*-sitosterol**
Compound **62**

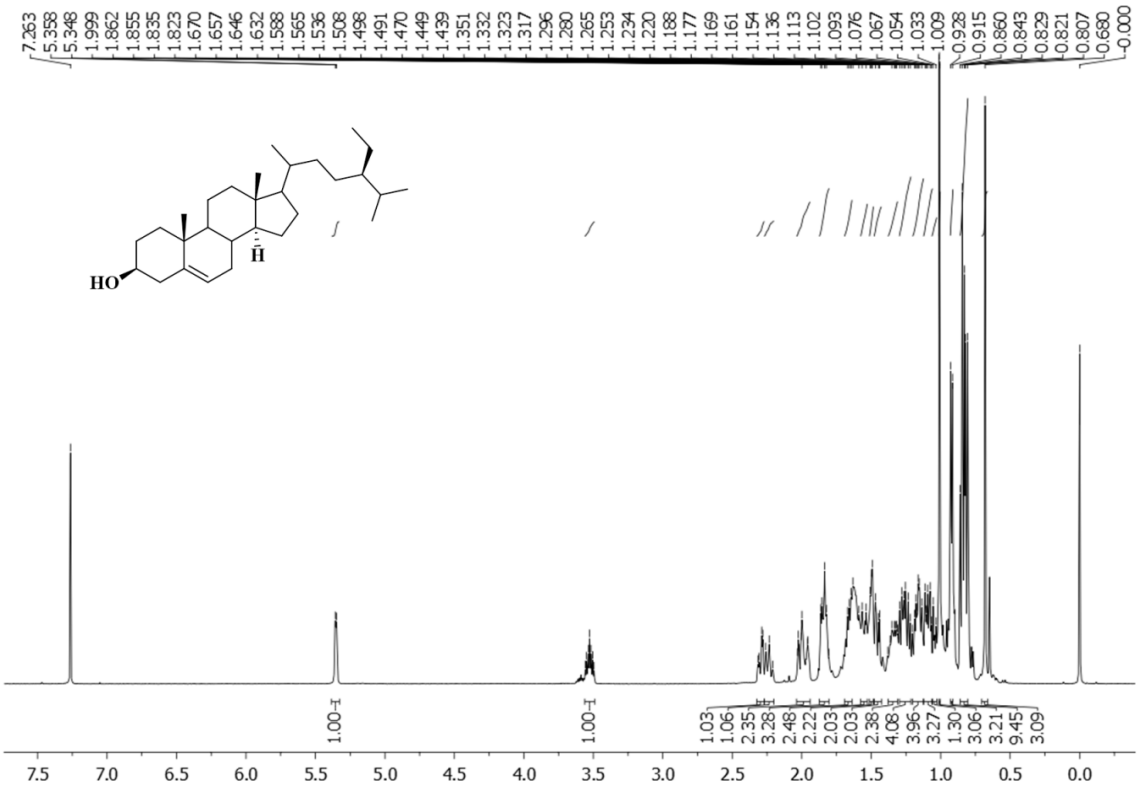


Figure 5A.6: ^{1}H NMR spectrum of *β*-sitosterol

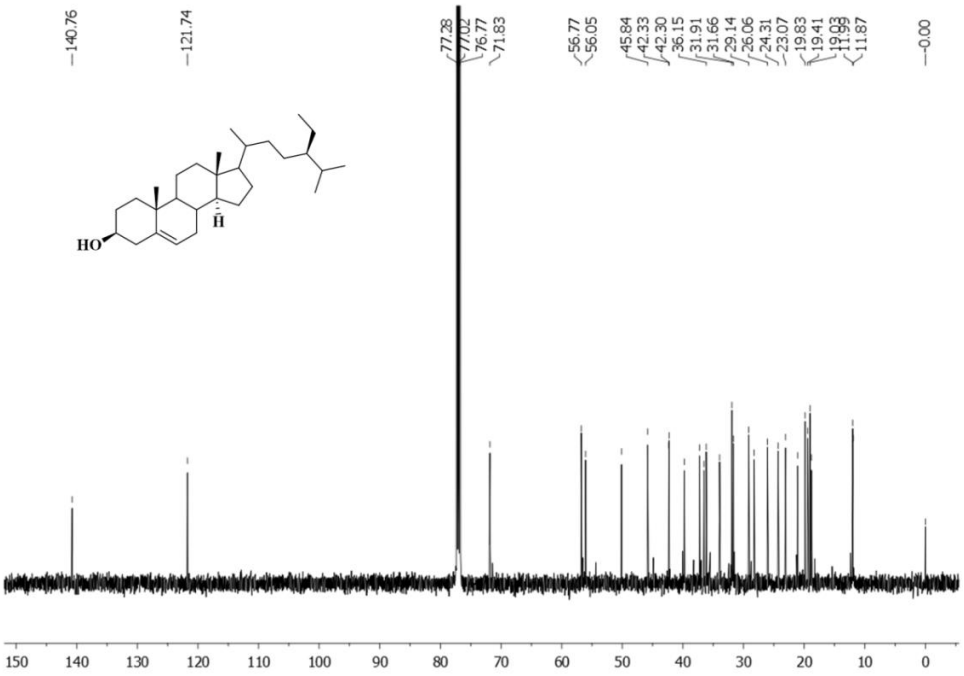
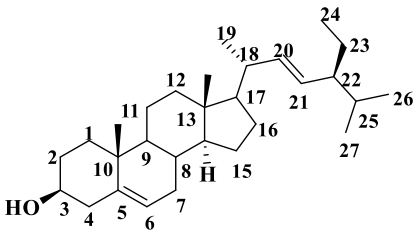


Figure 5A.7: ^{13}C NMR spectrum of *β*-sitosterol

Fractions 2-9 of acetone extract after column chromatographic separation, followed by crystallization yielded the compound **63**. Spectral data of the compound was similar to that of *β*-sitosterol the only difference is the presence of an additional *olefinic* bond in the structure. In the ^{1}H NMR spectrum two sets of doublet of doublet at [δ 5.15 (dd, J_1=15.5 Hz, J_2=9 Hz, 1H, H-22) & δ 5.02 (dd, J_1=15 Hz, J_2=8.5 Hz, 1H, H-23) ppm], indicating the presence of two *olefinic* protons. In the ^{1}H NMR spectrum proton at position 3 resonated as a multiplet in the range δ 3.57-3.49 ppm indicating the stereo center. And it is one of the characteristic peaks. ^{13}C NMR spectrum gave twenty one carbon resonances including *olefinic* carbons (δ 138.8 & 129.3 ppm), carbon bearing hydroxyl group and aliphatic carbons. DEPT 135 experiment reveals the compound contains ten methylene and six methyl groups. Finally ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be stigmasterol, The mass spectrum of the compound gave a molecular ion peak at 412.6197 which is the $[M+H]^+$ peak.



Stigmasterol
Compound **63**

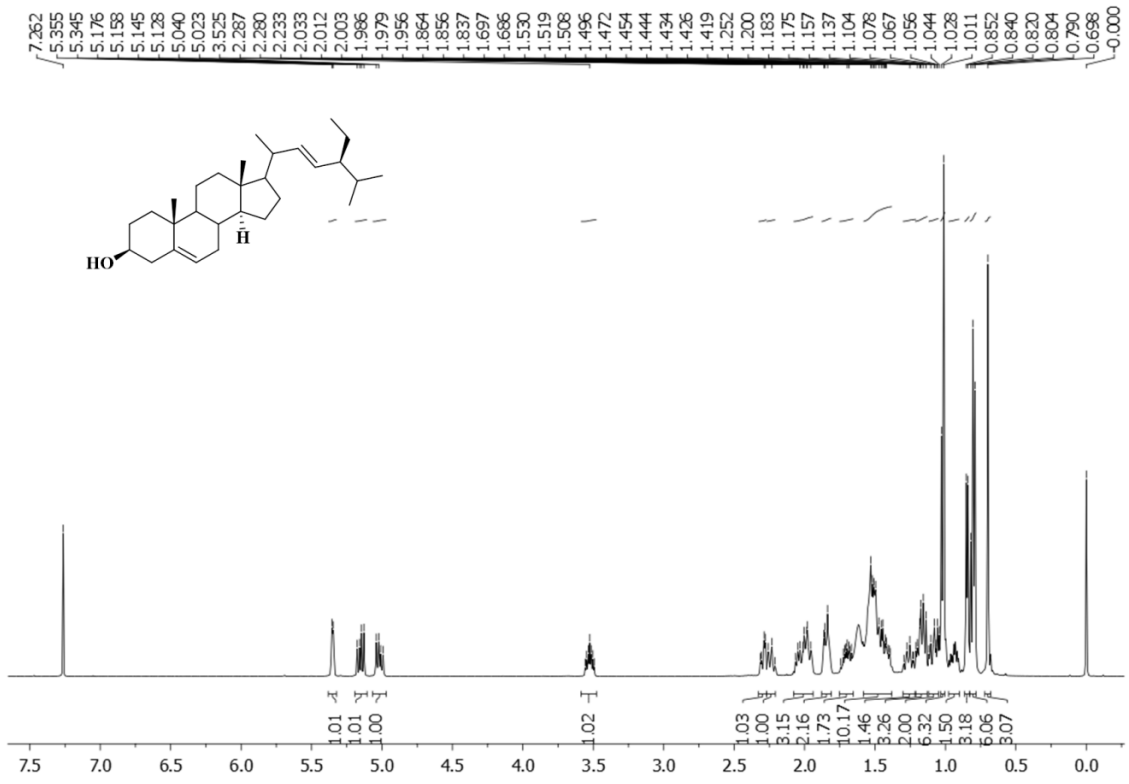


Figure 5A.8: ^{1}H NMR spectrum of stigmasterol

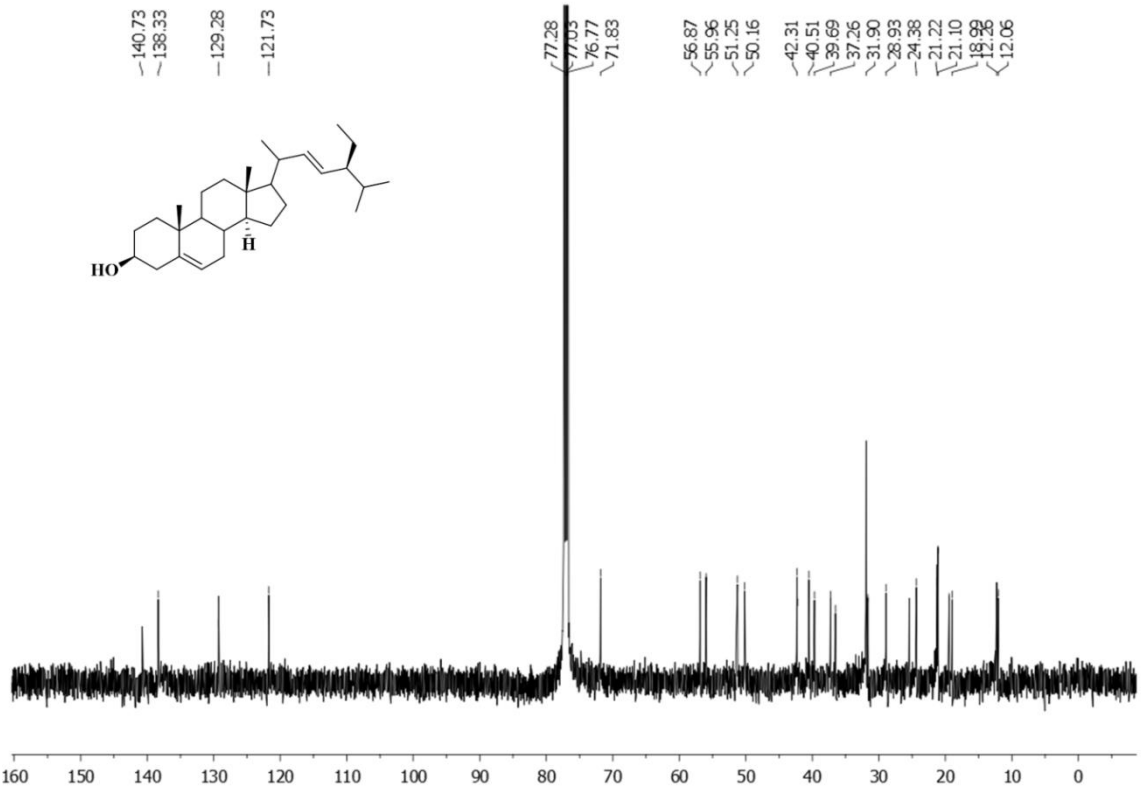
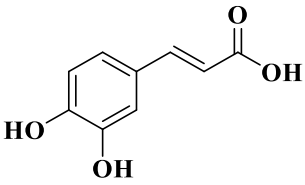


Figure 5A.9: ^{13}C NMR spectrum of stigmasterol

Fraction pool 10-12 showed the presence of an intense UV active spot. The TLC turned to violet colour on charring in McGill solution. These fractions on column CC separation using 40% ethyl acetate-hexane afforded the compound **64** as white solid. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be caffeic acid [14]. ^{1}H NMR spectrum showed two sets of doublets at δ 7.40 (d, *J*=16 Hz, 1H) & 6.13 (d, *J*=16 Hz, 1H) ppm indicates the presence of a *trans olefinic* bond. A broad two proton singlet at δ 8.20 ppm confirmed the presence of two hydroxyl groups. In addition ^{1}H NMR displayed two sets of doublets at δ 6.90 (d, *J*=8 Hz, 1H) & 6.74 (d, *J*=8Hz, 1H) ppm for *ortho* coupled aromatic protons and a singlet at δ 7.03 for the remaining aromatic proton. ^{13}C NMR gave nine carbon resonances including acid carbonyl carbon, *olefinic* carbons and aromatic carbons. The mass spectrum of the compound gave a molecular ion peak at 180.04226 which is the $[M+H]^{+}$ peak. To the best of our knowledge caffeic acid is reported for the first time from this species.



Caffeic acid
Compound **64**

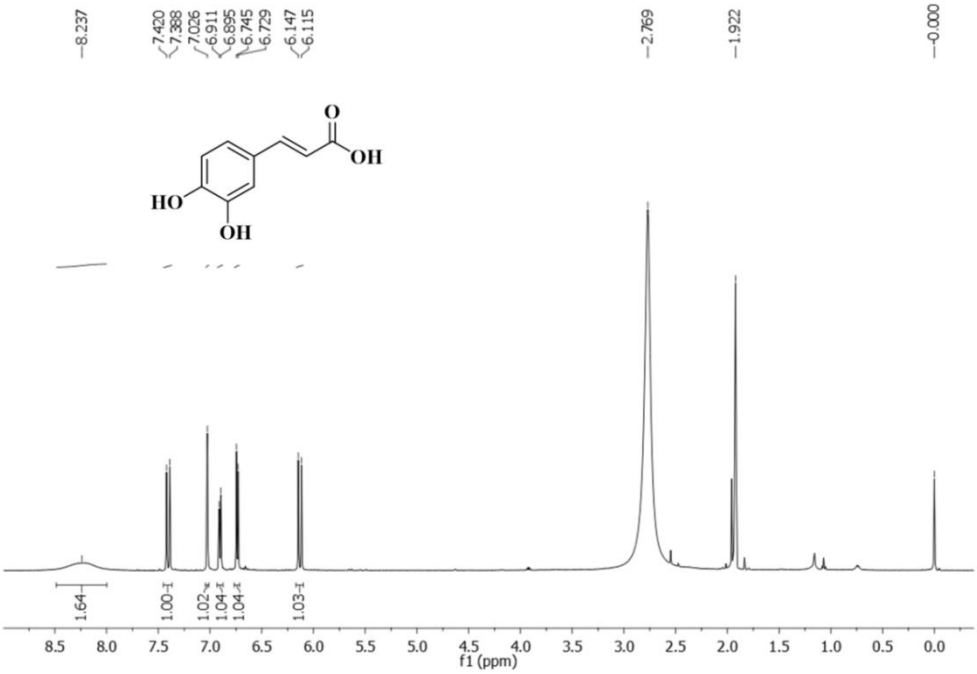


Figure 5A.10: ^{1}H NMR spectrum of Caffeic acid

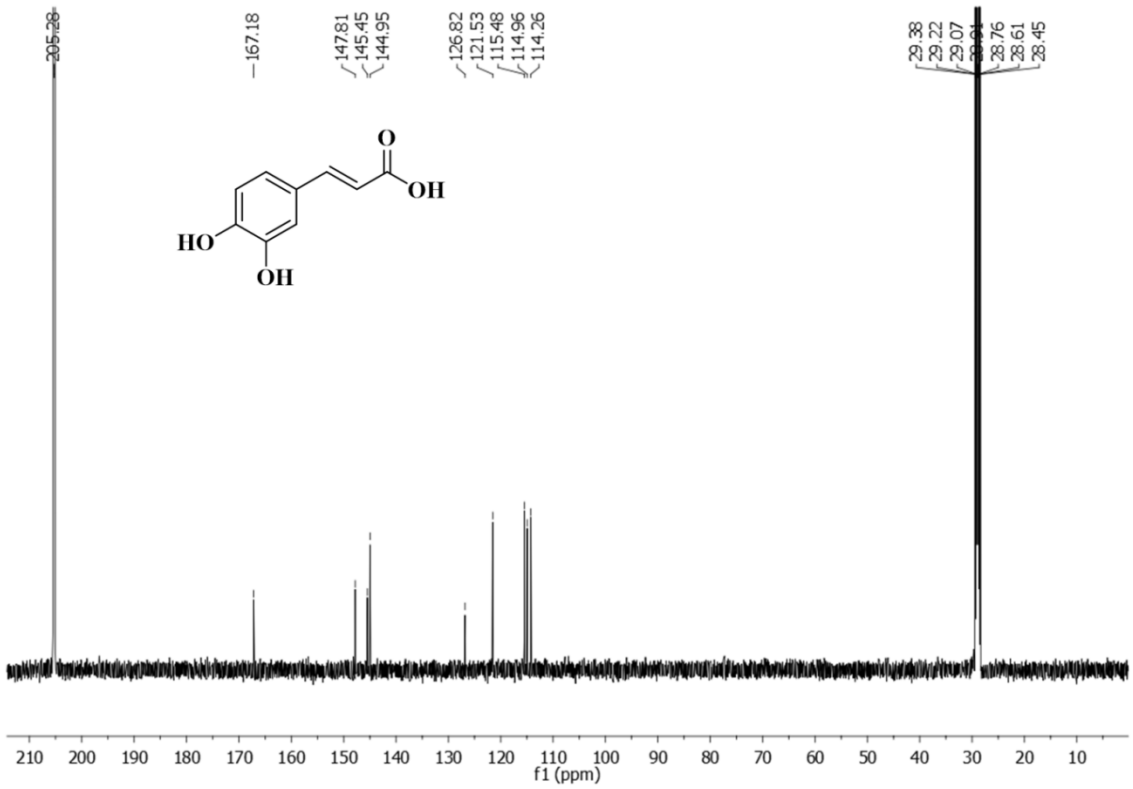
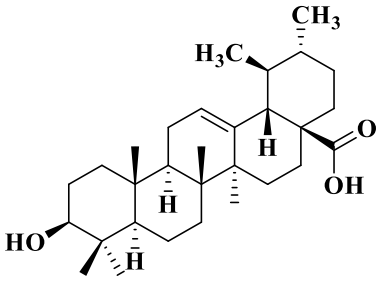


Figure 5A.11: ^{13}C NMR spectrum of Caffeic acid

Fraction pool 13-18 showed the presence of two intense spot on charring in McGill solution TLC turned to blue colour. CC separation using 70% ethyl acetate-hexane afforded the compound **65** as white solid. ^{1}H NMR spectrum showed a triplet at δ 5.28 ppm corresponding to an *olefinic* proton. *Olefinic* carbons resonated at δ 143.6 and 122.6 ppm in the ^{13}C NMR spectrum. DEPT 135 spectrum of the compound showed the presence of nine methylene and seven methyl groups. Presence of a carbonyl group was confirmed from the carbon resonance at δ 183.2 ppm in the ^{13}C NMR spectrum. Finally by incorporating all the spectral details of this compound and in comparison to the literature values the compound was confirmed to be Ursolic acid. The mass spectrum of the compound gave a molecular ion peak at 479.4397 which is the $[M+Na]^{+}$ peak [17].



Ursolic acid
Compound **65**

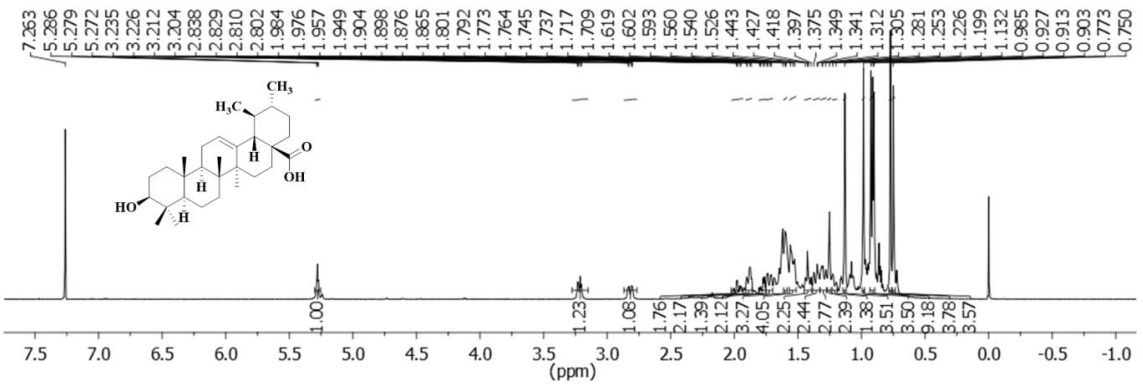


Figure 5A.12: ^{1}H NMR spectrum of ursolic acid

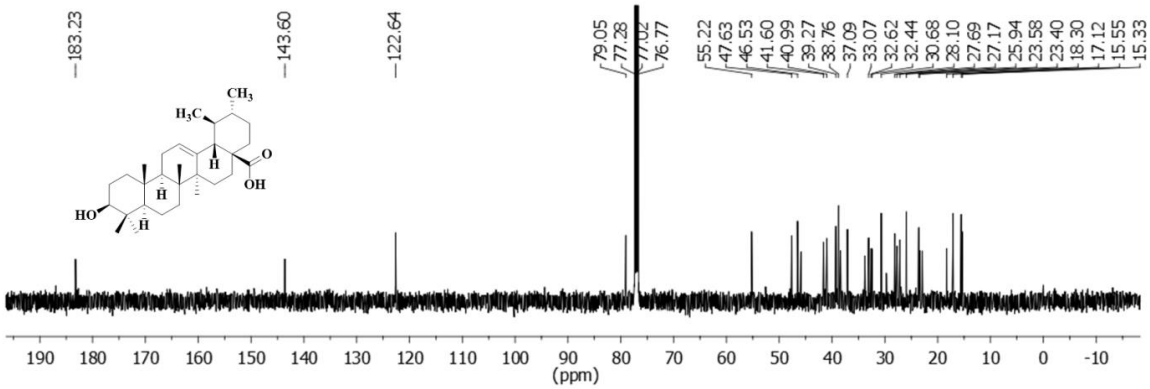


Figure 5A.13: ^{13}C NMR spectrum of ursolic acid

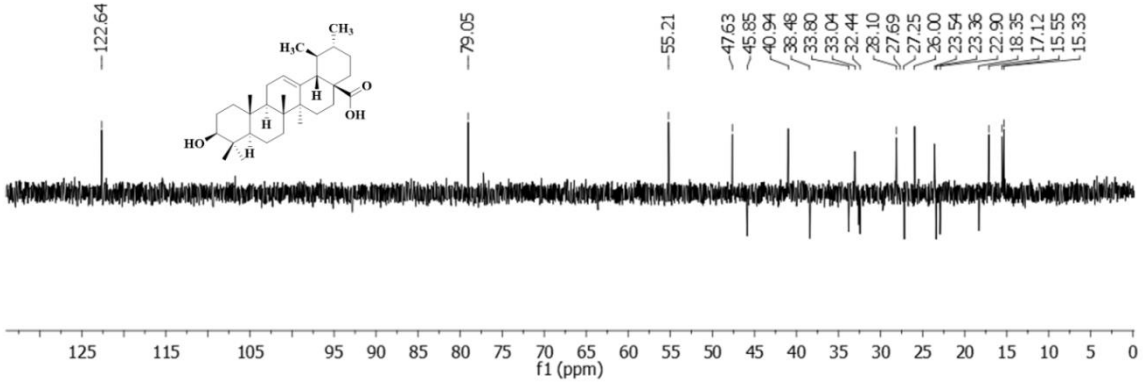
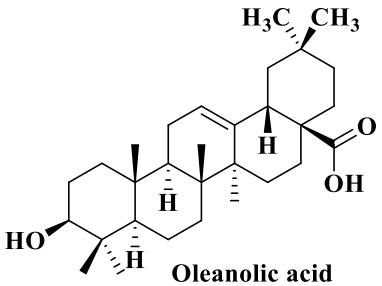


Figure 5A.14: DEPT 135 spectrum of ursolic acid

From the same fraction after column chromatographic separation afforded one more compound having almost same characteristics with that of ursolic acid. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound **66** was confirmed to be oleanolic acid. IR spectrum of the compound showed absorption at 1697 cm^{-1} suggesting the presence of an acid group.

^{1}H NMR displayed a triplet for *olefinic* proton at δ 5.28 (t, J = 3.5 Hz, 1H, H-12) ppm. Proton attached to the carbon bearing hydroxyl group resonated as a multiplet at δ 3.23-3.20 ppm. The signal obtained at δ 2.83-2.80 ppm corresponds to the proton adjacent to the acid group. All other aliphatic protons appeared in between δ 1.99 to 0.70 ppm. Comparison of the ^{13}C NMR spectral values, and DEPT 135 spectra suggested that there are seven methyl groups, ten methylene groups, four methine groups and six quaternary carbons in the molecule. ^{13}C NMR gave the carbon resonances corresponding to carbon bearing hydroxyl group (δ 79.0 ppm), acid carbonyl carbon (δ 183.1 ppm) and aliphatic carbons. The mass spectrum of the compound showed molecular ion peak at m/z 457.16516 which is the $[M+H]^{+}$ peak [18].



Oleanolic acid
Compound **66**

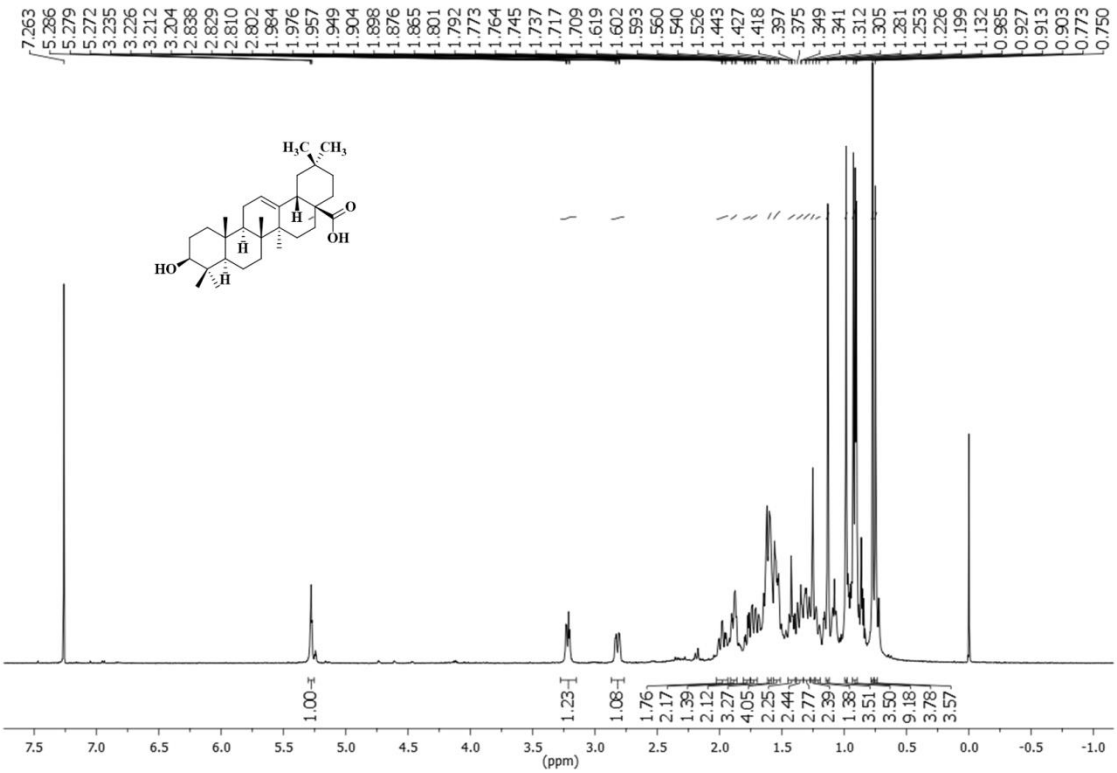


Figure 5A.15: ^{1}H NMR spectrum of oleanolic acid

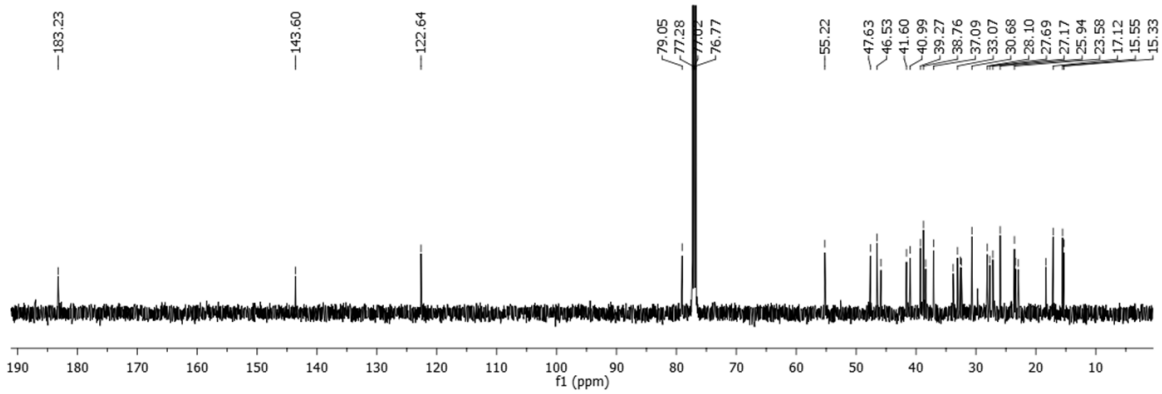


Figure 5A.16: ^{13}C NMR spectrum of oleanolic acid

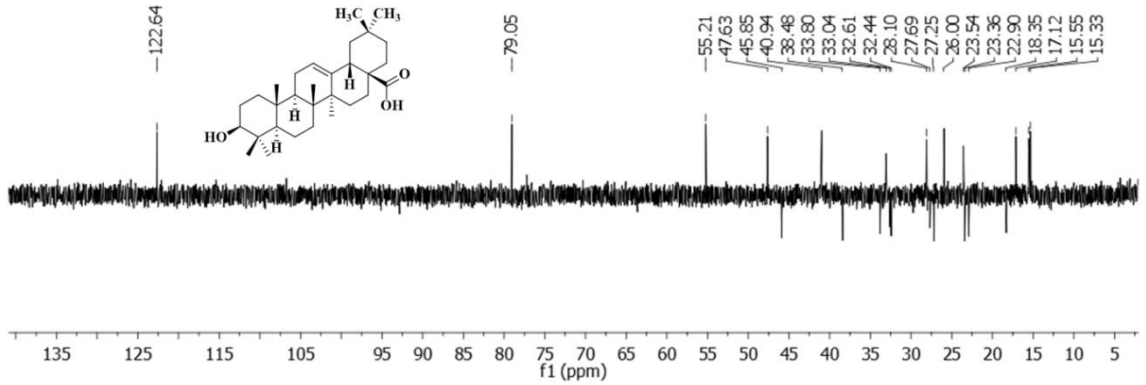
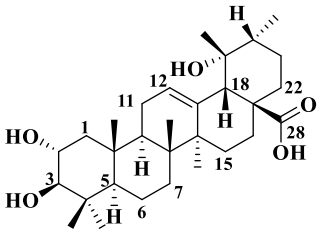


Figure 5A.17: DEPT 135 NMR spectrum of oleanolic acid

Column chromatographic separation of fraction pool 19-22 using 75-80% ethyl acetate-hexane afforded the compound **67** as a white solid. From spectral details of this compound and in comparison to the literature values the compound was confirmed to be tormentic acid. ^{1}H NMR spectrum showed a triplet at δ 5.49 ppm indicates the presence of an *olefinic* proton. Two sets of multiplet at δ 3.82 (m, 1H, H-2) & 3.11 (d, J=10 Hz, 1H, H-3) ppm corresponds to protons at position 2 & 3. Besides the aliphatic protons appeared at δ 2.32-1.6 ppm and methyl protons at δ 1.55-0.99 ppm.^{13}C NMR displayed twenty eight carbon resonances including *olefinic* carbons (δ 138.7 and 127.9 ppm), acid carbonyl carbon *(*δ 181.0 ppm), carbon bearing hydroxyl group and aliphatic carbons. DEPT 135 spectrum of the compound confirmed the presence of eight methylene and seven methyl groups. The mass spectrum of the compound gave a molecular ion peak at 511.33948 which is the $[M+Na]^{+}$ peak [18].



Tormentic acid
Compound **67**

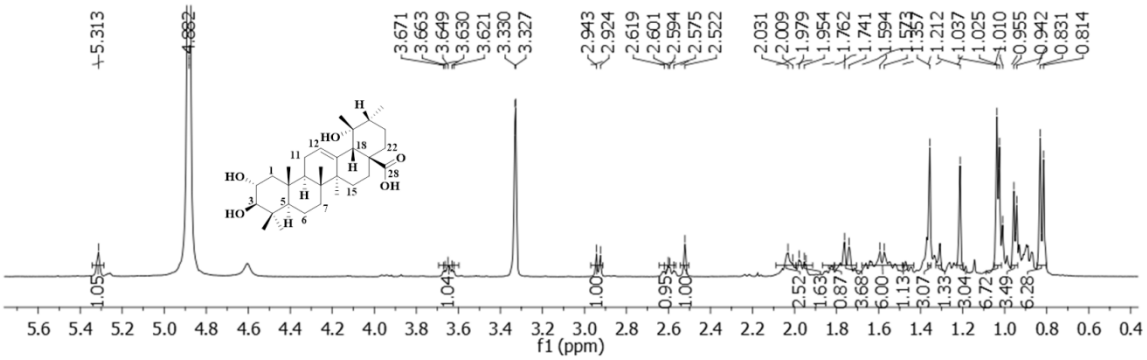


Figure 5A.18: ^{1}H NMR spectrum of tormentic acid

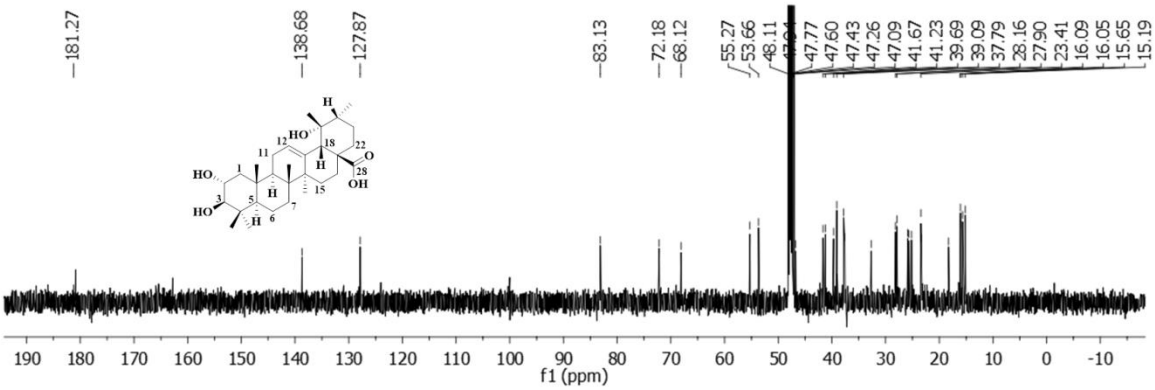


Figure 5A.19: ^{13}C NMR spectrum of tormentic acid

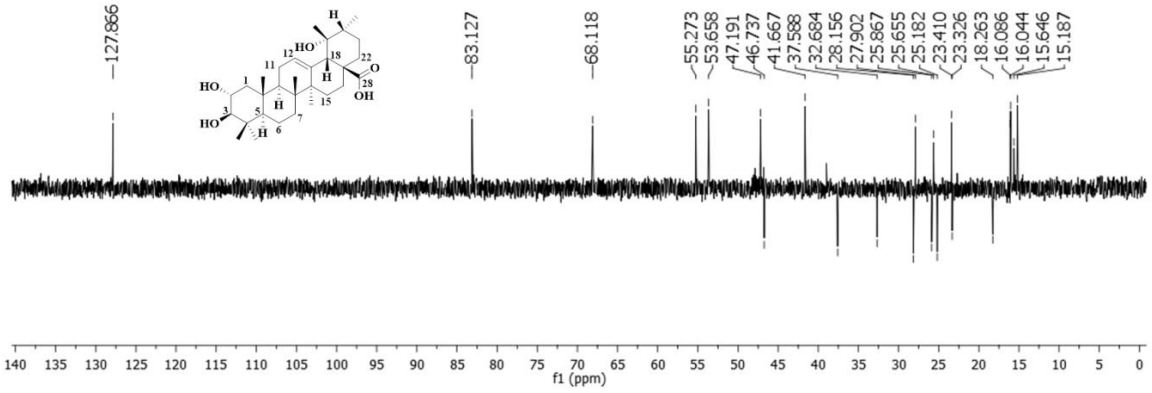
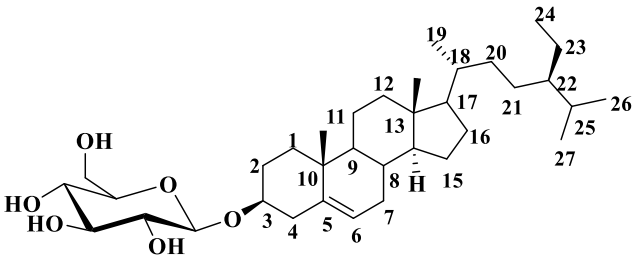


Figure 5A.20: DEPT 135 spectrum of tormentic acid

Compound **68** was isolated as white amorphous solid from the fraction pool 23-30 after CC separation using 80-90% ethyl acetate-hexane. The ^{1}H NMR spectrum of compound showed six signals at δ 0.58, 0.68, 0.71, 0.75, 0.83 and 0.90 ppm for methyl hydrogen (-CH_3) at C-18, C-29, C-27, C-26, C-21, C-19 respectively. The proton at C-3 appeared as a multiplet at δ 3.66 ppm. A doublet at δ 5.32 ppm was the characteristics of the double bond present in the ring in between quaternary carbon and methine carbon C-5 and C-6. ^{13}C NMR displayed twenty nine carbon resonances comprising of olefinic carbons, carbons of glucose moiety and aliphatic carbons. The glucose unit contained six carbons out of which oxygenated carbon C-1$^{'}$ appeared at δ 100.92 ppm and methylene carbon C-6$^{'}$ appeared at δ 61.66 ppm. The other four carbons of the glucose molecule appeared at δ 70.01, 75.60, 76.7 and 77.03 ppm. ^{1}H NMR and ^{13}C NMR data and in comparison to the literature the compound was confirmed to be β-sitosterol-β-D-glucopyranoside [25]. The compound displayed a molecular ion peak at 599.4284 which is the $[M+Na]^{+}$ peak. β-sitosterol-β-D-glucopyranoside is also identified for the first time from the species.



***β*-sitosterol-*β*-D-glucopyranoside**
Compound **68**

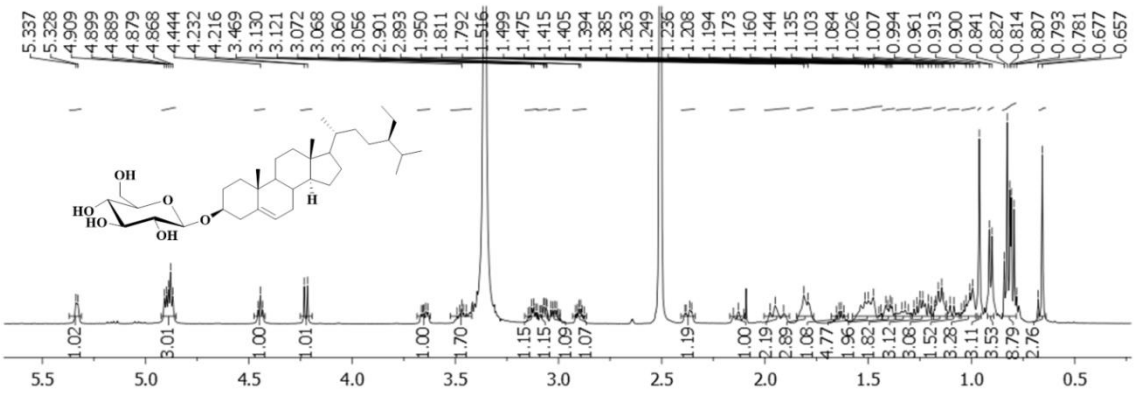


Figure 5A.21: ^{1}H NMR spectrum of β-sitosterol-β-D-glucopyranoside

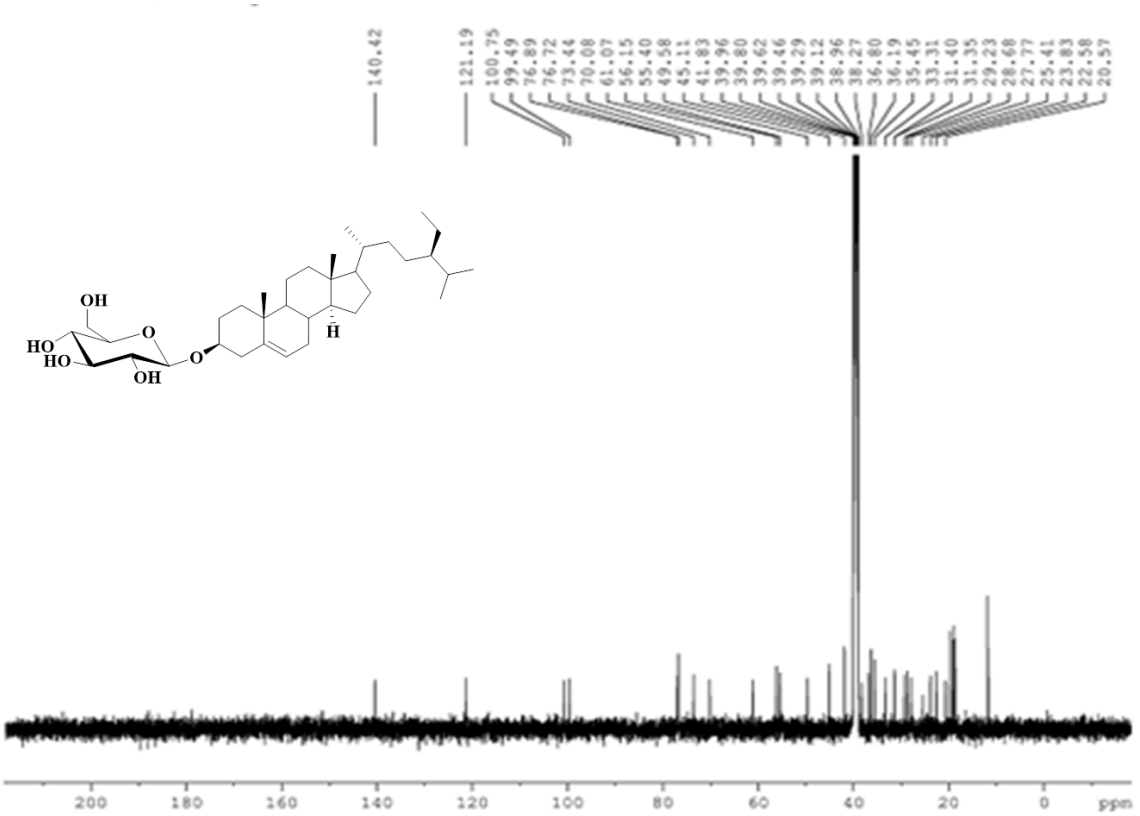
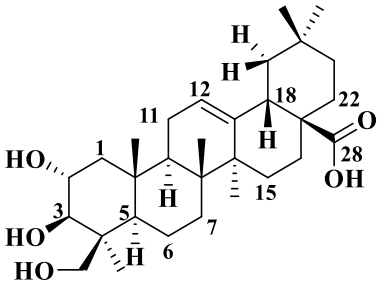


Figure 5A.22: ^{13}C NMR spectrum of β-sitosterol-β-D-glucopyranoside

Fraction pool 23-30 after CC separation using 80% ethyl acetate-hexane afforded the compound **69** as white solid. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be hyptatic acid A. ^{1}H NMR spectrum showed a triplet at δ 5.24 ppm indicates the presence of an olefinic proton. Signals ranging from δ 3-4 ppm confirmed the presence of four hydroxyl groups. DEPT 135 spectrum of the compound showed confirmed the presence of nine methylene groups. ^{13}C NMR gave the carbon resonances including acid carbonyl carbon, carbon bearing hydroxyl group and the aliphatic carbons. The mass spectrum of the compound gave a molecular ion peak at 511.33951 which is the $[M+Na]^{+}$ peak [26].



Hyptatic acid
Compound **69**

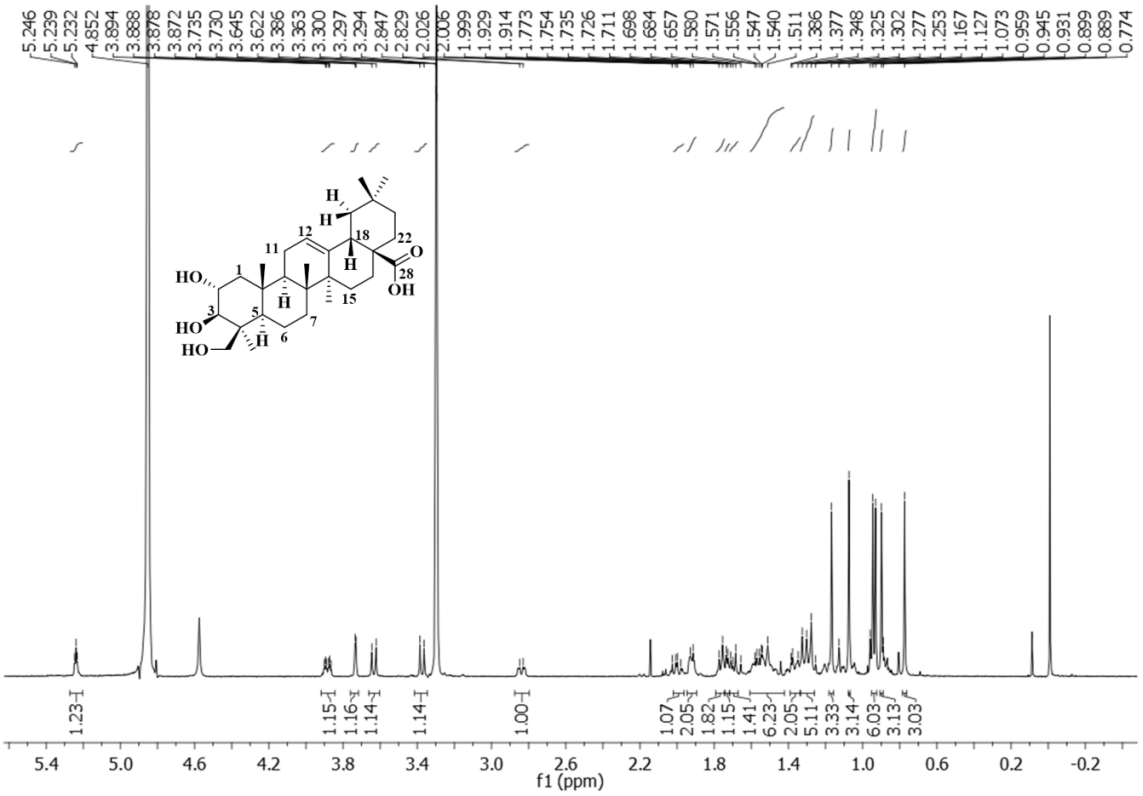


Figure 5A.23: ^{1}H NMR spectrum of hyptatic acid

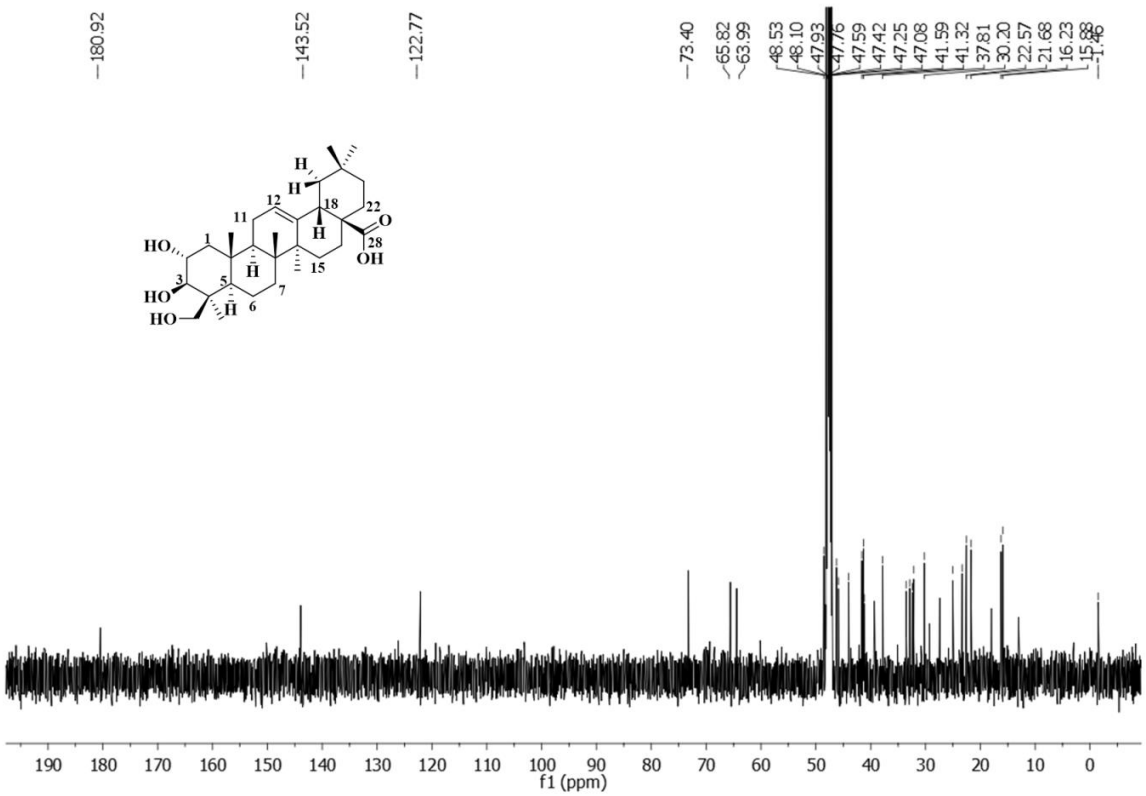


Figure 5A.24: ^{13}C NMR spectrum of hyptatic acid

5A.6. *In vitro* Antibacterial Screening of the Compounds from *H. capitata*

Plants are the promising source of active therapeutic agents. *Hyptis capitata*, the traditionally important plant is credited with various medicinal values. In traditional and folk medicine leaves of the species found extensive application against infectious diseases especially in skin diseases. Wound healing property, activity of the plant extracts against pathogens was reported. Hexane and ethanol extracts of the roots of the species reported to have significant potential to inhibit the *Candida albicans* [27]. The Indonesian species also reported to have antibacterial activity [15]. Apart from this no study on biological activity of phytoconstituents from the species is reported. We have evaluated *in vitro* antibacterial potential of the compounds against ESKAP pathogen panel consist of gram negative and positive pathogens.

5A.6.1. Antibacterial Susceptibility Testing against ESKAP Pathogen Panel

Antibiotic susceptibility testing of the isolated compounds was performed in accordance with standard CLSI guidelines [28]. Preliminary screening of the compound for their antibacterial potential against ESKAP pathogen panel was carried out by using Broth micro dilution assay. Minimum Inhibitory Concentration (MIC) of the compounds was determined against the pathogen panel (Table **5A.2**) in which levofloxacin as the positive control.

Table 5A.2: MIC values (µg/mL) of the tested compounds against ESKAP panel of bacteria

SL. NO	**Compound Name**	**Minimum Inhibitory Concentration (µg/mL)**				
		***E. coli* ATCC 25922**	***S. aureus* ATCC 29213**	***K. pneumoniae* BAA 1705**	***A. baumannii* BAA 1605**	***P. aeruginosa* ATCC 27853**
1	Ursolic acid	>128	**4**	>128	>128	>128
2	Tormentic acid	>128	128	>128	>128	>128
3	Caffeic acid	>128	128	>128	>128	>128
4	Hyptatic acid	>128	>128	>128	>128	>128
Levofloxacin		<0.03	0.125	64	8	0.5

Antibacterial susceptibility testing of the isolated compounds revealed that ursolic acid having excellent activity. Ursolic acid is an ursane-type pentacyclic triterpenoid

displayed selective and potent antibacterial activity against gram positive bacterial strain *Staphylococcus aureus* ATCC 29213 with an MIC value of 4 µg/mL. All other compounds are found to be inactive to both gram positive and negative bacterial strain. So it is evident that the antibacterial activity of *H. capitata* is mainly due to the presence of the active phytoconstituent ursolic acid.

5A.6.2. Cytotoxicity Assay against Vero Cells

The active compound ursolic acid was further tested for cytotoxicity against Vero cells using the MTT assay and its selectivity index was determined. CC_{50} is defined as the lowest concentration of compound which leads to a 50% reduction in cell viability. Doxorubicin was used as positive control and each experiment was repeated in triplicate.

Table 5A.3: Cytotoxicity profile against Vero cells and Selectivity index

SL. NO	Compound Code	MIC(µg/ml)	CC_{50} (µg/ml)	Selectivity Index
1	Ursolic acid	4	80	20
Selectivity Index=CC_{50}/MIC				

Unfortunately ursolic acid exhibited a selectivity index of 20 which indicated that the compound is slightly toxic to Vero cells (CC_{50} = 80 µg/mL).

5A.7. Conclusion

In conclusion, phytochemical analysis of the leaves of the medicinally important species *H. capitata* led to the isolation of seven phytoconstituents including sterols, glycosides and triterpenoids. We have isolated *β*-sitosterol, *β*-sitosterol-*β*-D-glucopyranoside, caffeic acid from the acetone extract of the leaves for the first time along with previously reported stigmasterol, ursolic acid, oleanolic acid, tormentic acid and hyptatic acid. In addition essential oil composition of the leaves of the species was also determined. The phytochemical analysis of this species grown in Kerala was studied for the first time and it showed some differences in phytoconstituents and its essential oil composition. From the findings it is evident that the habitat and environmental factors of the plants will effects the biosynthesis of secondary metabolites. Scientific validation of the traditional knowledge associated with the species was also achieved. Since the species used in wound healing and in skin infections in traditional medicine we have evaluated the constituents of the leaves for their antibacterial potential. Amongst ursolic acid exhibited potent and specific activity against gram positive bacteria *S. aureus* which are

the leading cause of skin and skin structure infections. So the antibacterial efficacy of the plant can be attributed to the presence of bioactive phytochemicals.

5A.8. Experimental Section

5A.8.1. General Experimental Details

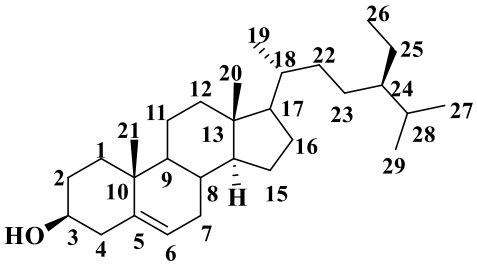
Melting points are uncorrected and were determined using Fisher-Jones melting point apparatus. IR spectra were recorded on a Bruker Alpha FT-IR spectrometer. ^{1}H NMR spectra were recorded at 500 MHz and ^{13}C NMR at 125 MHz using deuterated chloroform ($CDCl_3$) and deuterated methanol (CD_3OD) as the solvent on Bruker AMX 500 MHz spectrometer. Tetramethylsilane (TMS) was used as the internal standard and chemical shift values are expressed in δ-scale in units of parts per million (ppm) and coupling constants (*J*) in Hz. Abbreviations used in ^{1}H NMR are s-singlet, d-doublet, t-triplet, dd-doublet of doublet, brs-broad singlet and m-multiplet. Mass spectra were recorded using Thermo Scientific Exactive mass spectrometer under ESI/HRMS mode at 61800 resolutions. Specific rotations were recorded using Jasco P-2000 polarimeter. The plant materials were dried in convectional air drier at 40-45 °C. Solvents used for chromatography were distilled prior to use. Column chromatography was carried out using silica gel (100-200 mesh). The solvents were removed under reduced pressure using Büchi or Heidolph rotary evaporator. Analytical thin layer chromatography was performed on Merck silica gel 60 F_{254} aluminium sheets. Spots were first visualized under UV light (range 254-365 nm) and sprayed MC-gill solution followed by heating.

Antibacterial screening of the compound was carried out according to the previous methods as described in Chapter **4**.

5A.9. Spectral Data

Compound 62 [β-sitosterol]

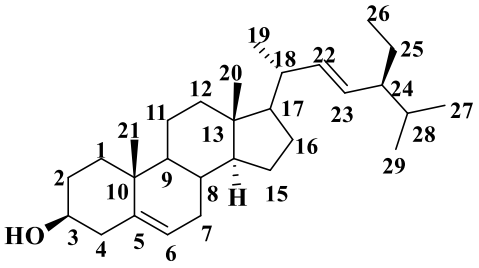
Fraction pool 1-2 obtained by eluting the column with 20% ethyl acetate in hexane followed by crystallization using the same solvent afforded colourless needle-like crystals of compound **62**, which was characterized as the common phytosterol, β-sitosterol, based on various spectral data (IR, ^{1}H NMR, ^{13}C NMR and HRMS) of the compound and in comparison with literature reports.



Molecular formula	:	$C_{29}H_{50}O$
Melting point	:	128-130 ^{o}C
FT-IR (neat, ν_{max}, cm^{-1})	:	3408, 2935, 2863, 1645, 1459, 1374, 1316, 1257, 1190, 1099, 1054, 1024, 958, 802
^{1}H NMR (500MHz, $CDCl_3$)	:	δ 5.35 (d, J = 5 Hz, 1H, H-6), 3.56-3.49 (m, 1H, H-3), [2.30 (dd, J_1=3.5 Hz, J_2=12.5 Hz, 1H),2.23 (t, J=13Hz, 1H), 2.02-1.96 (m, 2H),1.86-1.80 (m, 3H), 1.67-1.63 (m, 2H), 1.59-1.54 (m, 2H), 1.51-1.49 (m, 2H), 1.47-1.44 (m, 2H), 1.38-1.31 (m, 2H), 1.29-1.21 (m, 4H), 1.19-1.14 (m, 4H), 1.11-1.07 (m,3H), 1.05-1.03 (m, 1H), (other aliphatic hydrogens)], 1.01 (s, 3H, Me), 0.92 (d, J = 6.5 Hz, 3H, Me), 0.86-0.81 (m 9H, Me), 0.68 (s, 3H, Me) ppm
^{13}C NMR (125MHz, $CDCl_3$)	:	δ 140.8 (C-5), 121.7 (C-6), 71.8 (C-3), [56.7, 56.0, 50.1, 45.8, 42.3, 42.3, 39.8, 37.2, 36.5, 36.1, 33.9, 31.9, 31.7, 29.1, 28.3, 26.1, 24.3, 23.1, 21.1, 19.8, 19.4, 19.0, 18.8, 12.0, 11.9 (other aliphatic carbons)] ppm
HR-ESIMS	:	415.3928 $[M+H]^+$

Compound 63 [Stigma sterol]

Compound **63** was a UV inactive compound, isolated from fraction pools 2-9 after coloumn chromatographic separation using 25 % ethylacetate in hexane. The spectral data of the compound is found to be similar with β-sitosterol. After the detailed spectroscopic analysis and in comparison with literature reports the structure of the compound was confirmed to be the phytosterol, stigma sterol.

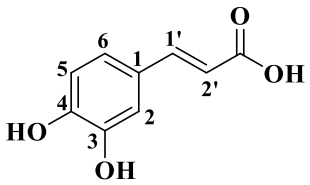


Molecular formula	:	$C_{29}H_{48}O$
Melting point	:	162-165 ^{o}C
FT-IR (neat, ν_{max}, cm^{-1})	:	3410, 3272, 2935, 2863, 1645, 1459, 1374, 1316, 1257, 1190, 1099, 1054, 1024
^{1}H NMR (500MHz, $CDCl_3$)	:	δ 5.35 (d, J = 5 Hz, 1H, H-6), 5.15 (dd, J_1=15.5 Hz, J_2=9 Hz, 1H,H-22), 5.02 (dd, J_1=15 Hz, J_2=8.5 Hz, 1H,H-23), 3.57-3.49 (m, 1H, H-3), [2.30 (dd, J_1=13 Hz, J_2=4 Hz, 1H), 2.23 (t, J=13Hz, 1H), 2.06-1.95 (m, 3H), 1.86-1.84 (m, 2H), 1.74-1.67 (m, 2H), 1.53-1.39 (m, 10H), 1.29-1.23 (m, 1H), 1.21-1.12 (m, 3H), 1.08-1.04 (m,2H), (other aliphatic hydrogens)], 1.02 (d, J = 8.5 Hz, 6H, Me), 0.85 (d, J = 6 Hz, 3H, Me), 0.80 (t, J=8Hz, 6H, Me), 0.78 (s, 3H, Me) ppm
^{13}C NMR (125MHz, $CDCl_3$)	:	δ 140.7 (C-5), 138.3 (C-22), 129.3 (C-23), 121.7 (C-6), 71.8 (C-3), [56.9, 56.0, 51.2, 50.2, 42.3, 42.2, 40.5, 39.6, 37.2, 36.5, 36.2, 31.9, 31.7, 28.9, 25.4, 24.4, 21.2, 21.0, 19.4, 19.0, 12.3, 12.1 (other aliphatic carbons)] ppm.
HR-ESIMS	:	412.6197 $[M+H]^+$

Compound 64 [Caffeic acid]

Compound **64** was obtained as colourless powder (100 mg) from the fraction pool 10-12 of the acetone extract after coloumn chromatographic separation using 40% ethylacetate in hexane. In the ^{1}H NMR spectra two doublets appeared at δ 7.40 and 6.13 ppm with a coupling constant of 16 Hz indicating the presence of *trans* olefinic bond. A signal appeared at δ 167.2 ppm in the ^{13}C NMR spectra suggested the presence of a

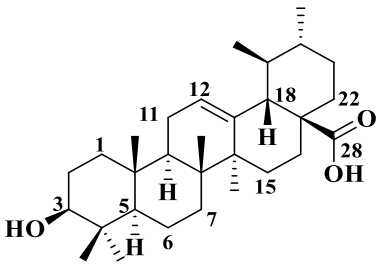
carbonyl group of acid. Finally the compound was identified as caffeic acid based on the various spectral analyses and literature reports.



Molecular formula	:	$C_9H_8O_4$
Melting point	:	185°C
FT-IR (neat, ν_{max}, cm^{-1})	:	3431, 3230, 3048,2989,2925, 1645,1524
^{1}H NMR (500MHz, CD_3COCD_3)	:	δ 8.23 (s, 2H, -OH), 7.40 (d, *J*=16 Hz, 1H), 7.03 (s, 1H), 6.90 (d, *J*=8 Hz, 1H), 6.74 (d, *J*=8Hz, 1H), 6.13 (d, *J* = 16 Hz, 1H) ppm
^{13}CNMR (125MHz, CD_3COCD_3)	:	δ 167.2 (C=O), 147.8 (C-4), 145.4 (C-3), 144.9 (C-1'), 126.8 (C-1), 121.5 (C-6), 115.5 (C-5), 114.9 (C-2'), 114.2 (C-2) ppm.
HR-ESIMS	:	181.03941 $[M+H]^+$

Compound 65 [Ursolic acid]

Compound **65** was isolated as colourless solid from the fraction 13-18 by eluting the coloumn with 70% ethylacetate in hexane. ^{1}H NMR spectrum of the compound showed a triplet at δ 5.28 ppm indicates the presence of an olefinic bond. IR spectrum of the compound showed absorption at 1646 cm^{-1} indicating the presence of carbonyl group. DEPT 135 experiment indicates the presence of nine methylene group. Finally the compound was confirmed to be ursolic acid by analyzing the spectral details and literature reports.

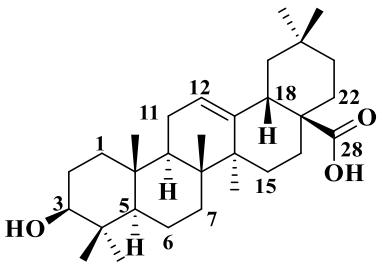


Molecular formula	:	$C_{30}H_{48}O_3$
Melting point	:	286-287 °C
FT-IR (neat, ν_{max}, cm^{-1})	:	3437, 3240, 3021, 2555, 1646, 1623

^{1}H NMR (500MHz, $CDCl_3$)	:	δ 5.28 (t, J = 3.5 Hz, 1H, H-12), 3.22 (dd, J_1= 11.5 Hz, J_2= 4.5 Hz, 1H, H-3), 2.82 (dd, J_1= 13, J_2= 4 Hz, 1H, H-18), [2.0-1.95 (m, 2H), 1.90-1.86 (m, 3H), 1.78 (dd, J_1=14 Hz, J_2=4.5 Hz, 1H), 1.73 (dd, J_1=14 Hz, J_2=4 Hz, 2H), 1.60 (d, J=4.5 Hz, 3H), 1.56–1.52 (m, 4H), 1.43–1.39 (m, 2H), 1.37–1.34 (m, 2H), 1.31–1.28 (m, 3H), 1.25 (m, 2H), 1.22–1.19 (m, 2H) (aliphatic hydrogens)], 1.13 (s, 3H, Me), 0.99 (s, 3H, Me), 0.93 (s, 3H, Me), 0.91 (s, 3H, Me), 0.90 (s, 3H, Me), 0.77 (s, 3H, Me), 0.75 (s, 3H, Me) ppm
^{13}C NMR (125MHz, $CDCl_3$)	:	δ 183.2 (C-28), 143.6 (C-13), 122.6 (C-12), 79.1 (C-3), 55.2, 47.6, 46.5, 45.9, 41.6, 40.9, 39.3, 38.7, 38.4, 37.1, 33.8, 33.1, 32.6, 32.4, 30.7, 28.1, 27.7, 27.2, 25.9, (aliphatic carbons)], 23.6 (Me), 23.4 (Me), 22.9 (Me), 18.3 (Me), 17.1 (Me), 15.5 (Me), 15.3 (Me) ppm
HR-ESIMS	:	479.3497 $[M+Na]^+$

Compound 66 [Oleanolic acid]

Compound **66** was obtained as colourless powder from the fraction pool 13-18 after eluting the column with 70% ethyl acetate in hexane. IR spectrum of the compound showed absorption at 1697 cm^{-1} suggesting the presence of an acid group and it was further confirmed from the peak obtained at δ 183.1 ppm in the ^{13}C NMR spectrum. DEPT 135 spectra confirm the presence of ten methylene group in the molecule. Finally by incorporating all the spectral data and in comparison with literature reports the compound was identified as oleanolic acid.

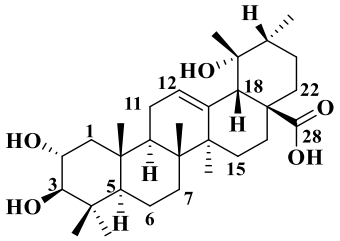


Molecular formula	:	$C_{30}H_{48}O_3$
Melting point	:	305-310 ^{o}C
FT-IR (neat, ν_{max}, cm^{-1})	:	3449, 2945, 2866, 1701, 1460, 1385, 1363, 1267, 1161, 1009
^{1}H NMR (500MHz, $CDCl_3$)	:	δ 5.28 (t, J = 3.5 Hz, 1H, H-12), 3.22 (dd, J_1= 11 Hz, J_2= 4 Hz, 1H, H-3), 2.82 (dd, J_1= 13.5, J_2= 4.5 Hz, 1H, H-18), [2.01-1.95 (m, 2H), 1.90-1.86 (m, 3H), 1.78 (dd, J_1=14 Hz, J_2=4.5 Hz, 1H), 1.73 (dd, J_1=14.5 Hz, J_2=4.5 Hz, 2H), 1.62–1.59 (m, 4H), 1.56–1.53 (m, 4H), 1.44–1.35 (m, 3H), 1.34–1.21 (m, 3H), 1.25 (s, 2H), (aliphatic hydrogens)], 1.13 (s, 3H, Me), 0.98 (s, 3H, Me), 0.93 (s, 3H, Me), 0.91 (s, 3H, Me), 0.90 (s, 3H, Me), 0.77 (s, 3H, Me), 0.75 (s, 3H, Me) ppm
^{13}C NMR (125MHz, $CDCl_3$)	:	δ 183.2 (C-28), 143.6 (C-13), 122.6 (C-12), 79.0 (C-3), 55.2, 47..56, 46, 45.9, 41.6, 40.9, 39.3, 38.8, 38.4, 37.1, 33.8, 33.1, 32.6, 32.4, 30.7, 28.1, 27.7, 27.2, 25.9, (aliphatic carbons)], 23.6 (Me), 23.4 (Me), 22.9 (Me), 18.3 (Me), 17.1 (Me), 15.5 (Me), 15.3 (Me) ppm
HR-ESIMS	:	457.1652 $[M+H]^+$

Compound 67 [Tormentic acid]

Compound **67** was obtained as colourless powder from the fractions of 19-22 after column chromatographic separation. NMR spectrum of the compound is found to be similar to that of the compound previously isolated from the plant and indicated that the

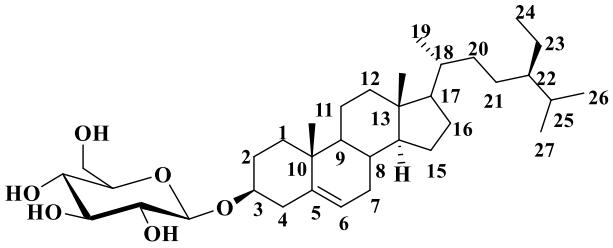
compound is a triterpenoid. In the ^{13}C NMR spectrum signal appeared at δ 181.3 ppm indicating the presence of a carbonyl group of acid. DEPT 135 spectrum of the compound suggested the presence of eight methylene and seven methyl group in the molecule. Finally ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be tormentic acid.



Molecular formula	:	$C_{30}H_{48}O_5$
Melting point	:	265-268^{o}C
FT-IR (neat, ν_{max}, cm^{-1})	:	3400, 2910, 1675, 1440, 1035, 950
^{1}H NMR (500MHz, CD_3OD)	:	δ 5.49 (t, *J*=3 Hz, 1H, H-12), 3.82 (m, 1H, H-2), 3.11 (d, *J*=10 Hz, 1H, H-3), 2.78 (m, 1H), 2.70 (s, 1H), [2.32-2.17 (m, 3H), 2.15-2.12 (m, 2H), 2.03–2.02 (m, 1H), 1.96–1.89 (m, 4H), 1.83–1.81 (m, 2H), 1.77-1.75 (m, 3H), 1.72-1.69 (m, 2H), 1.65-1.62 (m, 1H), (aliphatic hydrogens)], 1.55 (s, 3H, Me), 1.39 (s, 3H, Me), 1.21 (s, 3H, Me), 1.20 (s, 3H, Me), 1.13 (s, 3H, Me), 1.01 (s, 3H, Me), 0.99 (s, 3H, Me) ppm
^{13}C NMR (125MHz, CD_3OD)	:	δ 181.3 (C=O), 138.7 (C-13), 127.9 (C-12), 83.1 (C-3), 72.2, 68.1, 55.3, 53.6, 46.7, 41.7, 41.2, 39.7, 39.1, 37.8, 37.6, 32.7, 28.2, 27.9, 25.9, 25.6, 25.2 (aliphatic carbons)], 23.4 (Me), 23.3 (Me), 18.3 (Me), 16.1 (Me), 16.0 (Me), 15.6 (Me), 15.2 (Me) ppm
HR-ESIMS	:	511.3395 $[M+Na]^+$

Compound 68 [*β*-sitosterol-3-O-*β*-D-glucopyranoside]

Compound **68** was obtained as an amorphous solid from the fractions 23-30 after precipitation using acetone. IR spectrum of the compound showed a broad absorption at 3395 cm^{-1}, suggesting the presence of a hydroxyl group. In the ^{1}H NMR spectrum a doublet at δ 4.22 ppm integrating for one proton with a coupling constant of 8 Hz could be attributed to the anomeric proton of glucose, which is in *β*-configuration and the anomeric carbon resonated at δ 100.7 ppm in the ^{13}C NMR spectrum. Finally by incorporating all the spectral data and in comparison to the literature reports the compound is identified as *β*-sitosterol-3-O-*β*-D-glucopyranoside.

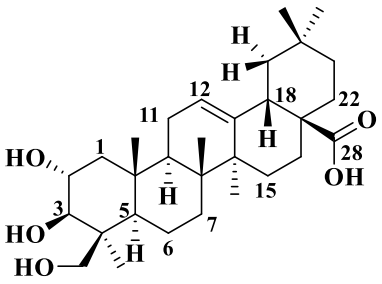


Molecular formula	:	$C_{35}H_{60}O_6$
Melting point	:	128-130^{o}C
FT-IR (neat, ν_{max}, cm^{-1})	:	3409, 3266, 2995, 2863, 1257, 1190, 1090, 1054, 1029, 968, 844
^{1}H NMR (500MHz, CD_3SOCD_3)	:	δ 5.33 (d, *J*=4.5 Hz, 1H, H-6), 4.91-4-87 (m, 3H, 2', 3', 4'-OH), 4.44 (t, *J*=6 Hz, 1H, 6'-OH), 4.22 (d, *J*=8 Hz, 1H, H-1'), 3.67-3.66 (m, 1H, H-2'), 3.49-3.47 (m, 2H, H-6'), 3.15-3.14 (m, 1H, H-5'), 3.09-3.06 (m, 1H, H-4'), 3.04-2.99 (m, 1H, H-3'), 2.92-2.88 (m, 1H, H3), 2.39-2.35 (m, 1H, H-4a), 2.15-2.10 (m, 1H, H-4b), 1.98-1.91 (m, 2H, H-2), [1.81-1.79 (m, 3H), 1.65-1.62 (m, 1H), 1.52-1.49 (m, 5H), 1.41-1.39 (m, 2H), 1.37-1.31 (m, 2H), 1.28-1.19 (m, 3H), 1.18-1.12 (m, 3H), 1.10-1.08 (m, 1H),1.05-0.99 (m, 3H), (aliphatic hydrogens)] 0.96 (s, 3H, Me), 0.91 (s, 3H, Me), 0.81 (m, 9H, Me), 0.66 (s, 3H, Me) ppm

^{13}C NMR (125MHz, CD_3SOCD_3)	:	δ 140.4 (C-5), 121.2 (C-6), 100.7 (C-1'), [99.5, 76.9, 76.7, 73.4, 70.1, 61.1, (Glucose ring carbons)], [56.1, 55.4, 49.6, 45.1, 41.8, 38.3, 36.8, 36.2, 35.4, 33.3, 31.4, 31.3, 29.2, 28.7, 27.8, 25.4, 23.8, 22.6, 20.6, 19.7, 19.1, 18.9, 18.6, 11.8, 11.6, aliphatic carbons)] ppm.
HR-ESIMS	:	599.4284 $[M+Na]^+$

Compound 69 [Hyptatic acid A]

Column chromatographic separation of the fraction 23-30 by using 80% ethyl acetate in hexane afforded the compound **69** as amorphous solid. ^{1}H NMR spectrum confirmed the presence characteristic peaks of a triterpenoid system. Presence of carbonyl group was confirmed from ^{13}C NMR spectrum. DEPT 135 spectrum indicated the presence of ten methylene and six methyl group. Finally by incorporating all the spectral data of the compound and in comparison with literature values the compound was identified as Hyptatic acid A.



Molecular formula	:	$C_{30}H_{48}O_5$
Melting point	:	298-304^{o}C
FT-IR (neat, ν_{max}, cm^{-1})	:	3410, 2920, 1675, 1440, 1370, 1040, 1015
^{1}H NMR (500MHz, CD_3OD)	:	δ 5.24 (t, J=3.5 Hz, 1H, H-12), 3.88 (m, 1H, H-2), 3.73 (d, J=2.5 Hz, 1H, H-3), 3.63 (d, J=11.5 Hz, 1H, H-23a), 3.37 (d, J=11.5 Hz, 1H, H-23b), 2.84 (d, J=9 Hz, 1H, H-18), [2.03-1.98 (m, 1H), 1.93-1.91 (m, 2H), 1.77-1.75 (m, 2H), 1.74-1.73 (m, 1H), 1.71-1.68 (m, 1H), 1.58-1.51 (m, 6H), 1.39-1.35 (m,

		2H), 1.33-1.25 (m, 5H), (aliphatic hydrogens)], 1.17 (s, 3H, Me), 1.07 (s, 3H, Me), 0.94 (s, 3H, Me), 0.93 (s, 3H, Me), 0.90 (s, 3H, Me), 0.77 (s, 3H, Me) ppm
^{13}C NMR (125MHz, CD_3OD)	:	δ 180.9 (C=O), 143.5 (C-13), 128.8 (C-12), 73.4 (C-3), 65.8 (C-2), 63.9 (C-23), 48.5, 46.2, 45.9, 44.0, 41.6, 41.3, 41.2, 37.8, 33.5, 32.9, 32.4, 32.1, 30.2, 25.0, 23.3, 22.6, 21.7, 16.2, 15.9, 13.1, 12.7, (aliphatic carbons)] ppm.
HR-ESIMS	:	511.3395 $[M+Na]^+$

5A.9. References

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Scientific Validation of '*Nayopayam Kwatha'* a Polyherbal Ayurvedic Formulation & Its Standardisation Protocol Development

5B.1. Introduction

Ayurveda is one of the ancient traditional systems of medicine in India. The word Ayurveda "the science of life" derived from the combination of two Sanskrit words *ayur* means life and *veda* means science or knowledge. Ayurveda has rich indigenous knowledge of herbal drug combinations for almost all ailments. Ancient Ayurvedic script *Atharva-veda* describes the healing property of more than a hundred medicinal plants against various diseases. According to Ayurveda diseases are originated as the result of the imbalance of three *doshas* (three principle energies of the body) such as *Vata* (responsible for body movement), *Pitta* (responsible for metabolic reaction of the body) and *Kapha* (responsible for growth, protection, lubrication and sustenance). So Ayurvedic treatment relies on marinating these *dhoshas* for the prevention of diseases. It is a holistic approach to maintain a healthy body and mind. Herbal products form the basis of Ayurvedic medicine. The extensive use of plant derived drugs to cure ailments is greatly encouraged by WHO by providing guidelines and standards to ensure its purity and quality [1-6].

5B.1.1. Polyherbal Ayurvedic Formulations

Ayurvedic medicines were prepared from the different parts of the plants including roots, seeds, flowers etc. in various forms such as decoctions (*Kwatha*/*kashayam*), hot and cold infusions, powders (*Churna*), medicated oil (*Taila*) etc. Apart from the pure herbal formulations Ayurveda also employed herbo-bio-mineral combinations for therapeutic efficacy. Herbal formulations were primarily derived from single herbs or the combination of herbs (polyherbal formulations) according to the principle of Ayurveda. Polyherbal formulations were recognized as an Ayurvedic therapeutic strategy for augmented efficacy at lower dosage. Ayurvedic dosage forms are mostly poly herbal, which is more effective compared to the single herb therapies. The Ayurvedic script "*Sarangdhar Samhita*" emphasized the concept of polyherbal formulations in Ayurvedic treatment. The synergistic (*sarvakarmaja)* activity of bioactive phytoconstituents of the individual herbs of such formulation is responsible for the increased efficacy which is clinically and pharmacologically proven. As a general

principle, most of the poly herbal formulations are combinations of ingredients in equal proportions. But there are several formulations in which the ingredients are mixed in particular proportions to achieve the specific effect. Drug ratios of individual herbs in such Ayurvedic formulation are of utmost importance in order to attain the desired curative effect. Classical drug ratios were employed for the preparation of polyherbal formulations rather than their equal combination. Every formulation has its unique indications and contraindications which help to ensure its quality and safety and make them an ideal treatment of choice for excellent therapeutic effect. It is amply documented that *Ayurvedic* medicines have adverse effects when formulated or used inappropriately so it is important to follow the standard protocols regarding the formulation and its uses [7-10].

Table 5B.1: Examples of polyherbal formulations

Polyherbal Formulation	Ingredient Herbs	Plant parts Used	Therapeutic Use
Nayopayam Kwatha	✓ *Sida cordifolia* ✓ *Cuminum cyminum* ✓ *Zingiber officinale*	✓ Roots ✓ Seeds ✓ Rhizomes	✓ Respiratory ailments ✓ Heart problems ✓ Postnatal care
Amrtottara kwatha	✓ *Tinospora cordifolia* ✓ *Terminalia chebula* ✓ *Zingiber officinale*	✓ Stem ✓ Fruit rind ✓ Rhizomes	✓ Inflammatory conditions ✓ Hyperpyrexia
Sahacharadi kashayam	✓ *Barleria prionitis* ✓ *Cedrus deodara* ✓ *Zingiber officnale*	✓ Leaves ✓ Heartwood ✓ Rhizomes	✓ Joint Pain ✓ Bodyache
Guluchyadi kashayam	✓ *Tinospora cordifolia* ✓ *Coriandrum sativum* ✓ *Pterocrpus Santalinus* ✓ *Azadiracta indica* ✓ *Prunus cerasoides*	✓ Stem ✓ Leaves ✓ Heartwood ✓ Leaves ✓ Fruit	✓ Fever ✓ Vomiting ✓ Burning sensation ✓ Thirstiness
Drakshadi Kashayam	✓ *Vitis vinifera* ✓ *Madhuca Indica* ✓ *Glycyrrhiza glabra* ✓ *Symplocos racemosa* ✓ *Gmelina arborea* ✓ *Hemidesmus indicus* ✓ *Cyperus rotundus* ✓ *Emblica officinalis* ✓ *Coleus zeylanicum* ✓ *Nelumbium speciosum* ✓ *Prunus cerasoides* ✓ *Santalum album* ✓ *Vetiveria zizanioides* ✓ *Jasminum sambac* ✓ *Phoenix famifera*	✓ Dried fruits ✓ Flowers ✓ Roots ✓ Roots ✓ Fruits ✓ Roots ✓ Rhizomes ✓ Fruits ✓ Roots ✓ Flowers ✓ Heartwood ✓ Heartwood ✓ Roots ✓ Flowers ✓ fruits	✓ Chronic fever ✓ Nausea & Vomiting ✓ Excessive thirst ✓ Giddiness ✓ Jaundice ✓ Internal bleeding ✓ Headache ✓ Vertigo ✓ Burning sensation

5B.1.2. *Nayopayam Kwatha* (NK)

Nayopayam kwatha is the well-known poly herbal formulation extensively used for respiratory ailments (bronchial asthma), cardiac diseases, gas trouble and postnatal care. It is a very good bronchodilator and carminative in actions. Several Ayurvedic textbooks describes the importance of NK include *Vaidya Manorama* [11] in the chapter *Kasa chiktsa* (treatment of cough) and *Arogyaraksha Kalpadruma* [12] in the chapter *Vata roga chiktsa* (diseases of *Vata* origin). *Bala* (*Sida cordifolia*), *Jeeraka* (*Cuminum cyminum*) *and Nagara* (*Zingiber officinale*) are the major ingredients of this decoction. The roots of *Sida cordifolia Linn* is taken as *Bala* in AFI [13] and *Sida rhombifolia ssp.Sida retusa Linn* in Kerala for the same [14]. Ancient Ayurvedic scripts explains different drug ratio of the individual herbs for this formulation to obtain specific curative effects. For the diseases related to '*vata*' the ingredients *Bala* (*S. cordifolia*), *Jeeraka* (*C. cyminum*), *Nagara* (*Z. officinale*) having the ratio 3:2:1 as per *Vaidya manorama* and for the treatment of cough the ingredients are in the ratio 10:1:1 as per *Arogya raksha kalpadrum*.

5B.1.3. Source Plants of *Nayopayam Kwatha*

Nayopayam Kwatha was formulated from the three medicinal plant species include *S. cordifolia*, *C. cyminum* and *Z. officinale*. These ingredient herbs were found to possess various pharmacological activities. A brief description of these plant species were given below.

5B.1.3.1. *Sida cordifolia* & *Sida retusa*

S. cordifolia, the country mallow (In Malayalam 'kurunthotti') belongs to malvaceae family is an annual to perennial herb grown in tropical and subtropical regions of India. The root (known as "*bala"*) of the species is a valuable herb widely used as an Ayurvedic medicine. The species constitutes alkaloids, ecdysteroids, flavonoids, terpenoids and fatty acids as the major phytoconstituents. According to Ayurveda the plant is a tonic, astringent and emollient and is used to cure respiratory ailments (bronchial asthma, nasal congestion & asthma), headache, cough, rheumatism, gastrointestinal and urinary infections, debility, and skin infections. Decoction of the plant is used to reduce bodyweight. Most of the Ayurvedic formulations signify the presence of *bala* as the major ingredient owing to their wide spectrum of therapeutic efficacy. The plant is well studied for their pharmacological activity and reported to possess analgesic, anti-inflammatory, antibacterial, hypoglycemic, hepatoprotective, antioxidant, CNS activity etc. [15-18].

Figure 5B.1: Plant, flower and root of *S. cordifolia*

S. retusa is considered as the sub species of *Sida rhobifolia* ubiquitously found in Kerala. It is also a well-established medicinal plant in Ayurveda found to have anti-rheumatic, antiasthma and CNS activities. Most of the time, the species is used as an adulterant to *Sida cordifolia* [19].

5B.1.3.2. *Cuminum cyminum*

Cuminum cyminum, the cumin ('Jeerakam' in Malayalam) is a popular spice used in various food cuisines. The species is native to East Mediterranean to South Asia and one of the old cultivated medicinal food herbs in Asia, belongs to apiaceae family. Cumin is a valuable neutraceutical and in Ayurveda it is employed for gastrointestinal disorders, respiratory disorders, toothache, diarrhea etc. Several Ayurvedic decoctions and fermented products (*Jeerakarishta)* for digestive and respiratory disorders contain cumin seeds as the major ingredients. Essential oil from cumin also has excellent therapeutic potential including antimicrobial, antioxidant and anti-osteoporotic activities. Flavonoids and its glycosides, sesquiterpenoid glycosides, steroids, alkaloids, tannins were the phytoconstituents of cumin seeds. Pharmacological activities of cumin include antioxidant, antidiabetic, antimicrobial, anti-inflammatory, analgesic, anticancer etc. [20-22].

Figure 5B.2: Plant and seeds of *Cuminum cyminum*

5B.1.3.3. *Zingiber officinale*

Zingiber officinale, the ginger belongs to the well- known zingiberaeae family is an exotic spice used to flavor the food and an active constituent in traditional medicine. The rhizome of the ginger is an excellent Ayurvedic drug endowed with wide array of medicinal properties. The potential of ginger is well exploited for respiratory ailments including asthma, hiccups, bronchitis along with digestive and appetite ailments. The essential oil of the rhizomes is considered as an efficient bronchodilator and also possesses antioxidant, anti-inflammatory, analgesic, anticancer and immune-modulatory activities. Gingerols are the major phytoconstituent of ginger responsible for its therapeutic potential. Dried rhizome of ginger (*Shunti*) is a versatile ingredient of Ayurvedic preparations. The pharmacological activity of the ginger include antioxidant, anti-inflammatory, anticancer, neuroprotective, antihyperglycemic, antihypertensive, antimicrobial etc. [23-25].

Figure 5B.3: Plant and rhizomes of *Zingiber officinale*

5B.1.4. Standardization and Quality Control of Herbal Formulations

Herbal based medicines have long back history in therapeutics and still plays potential role for the well-being of mankind. Herbal formulations were prepared from the crude extracts of the specific plant part by means of various processes. So the quality of ingredient herbs to the final product is a matter of concern. The ancient scripts of Ayurveda itself underlined the importance of studying the characteristic including nature, collection, method of preservation, method of preparation, purity, quality, therapeutic dosage and specific action of herbal formulations prior to the administration. According to WHO around 80% of the global population depends on herbal medicine for the health care needs. The increased use of herbal medicine necessitates the development of quality standard for herbal products. Most of the marketed herbal products were found to be not up to the quality standard. In this scenario quality control of the herbal products to safeguard the quality of the final products is significant and it will help to trace out the

impurities or contaminants in the formulations. Several studies on marketed Ayurveda products revealed the presence of adulterant drugs, heavy metal contents and other chemical substances which will adversely affects the health of human [26-28].

The reliability and acceptability on such products can be achieved by executing proper standardizations. In view of this WHO put forward stringent guidelines and regulations for the standardization of herbal drugs. The guidelines emphasized on several aspects including authenticity of the plant species, physicochemical parameters of the plants as well as final products, phytochemical evaluation of herbs and toxicity testing with the aid of advanced technique including HPLC, HPTLC, LC-MS/GC-MS etc. In addition Ayurveda Pharmacopeia of India (API) also provided reference and testing protocols of more than eighty medicinal plants employed in Ayurvedic preparation in terms of organoleptic characters, phamacognostic properties etc. So by the careful evaluation of the herbal drugs and their standardization in terms of predefined characteristics will serves scientific validation and which in turn promotes its acceptability [29-30].

5B.2. Aim and Scope of the Present Study

Ayurveda and its approaches were succeeded in curing various ailments. The herbal based formulations of Ayurveda always proved to be effective in therapeutics owing to the tremendous potential of medicinal plants. The acceptability of Ayurvedic drugs in modern medicine and its quality is always a matter of concern due to the lack proper validation. Commercialization of the herbal products resulted in improper formulation techniques and the increased use of adulterant drugs which in turn affected the purity and efficacy. In order to ensure the efficacy, stability and safety of polyherbal formulations standardization in terms of physicochemical properties, phytochemical screening of the individual herbs and physical properties of final formulation is important. There is an urgent need to validate basic principles as well as drugs used in the Ayurvedic system of medicine with the help of advanced techniques. In this regard our efforts are directed towards the development of standardization protocols for the well-known polyherbal formulation *Nayopayam Kwatha* based on the marker phytoconstituents of the individual herbs. So far no scientific validation protocol is developed for NK. Initially we aimed the detailed phytochemical analysis of the individual herbs and their pharmacognostic evaluation along with the development of separate profiles of NK with standard markers isolated from the individual plant ingredient to provide leads for clinical

research using ingredients in the needed ratios *i.e*. 3:2:1, 10:1:1 & 1:1:1. Our study also aims the comparison of classical drug ratios of this formulation with the general principle.

5B.3. Phytochemical Analysis of the Source Plants

5B.3.1. Collection and Identification of Plant materials

The herbal ingredients of NK were collected and authenticated for the study purpose. Roots of *S. cordifolia* Linn & *S. retusa* Linn were collected from Kanyakumari and Tamilnadu region respectively. *C. cyminum* Linn seeds were directly purchased from a cultivator in Gujarat and rhizomes of *Z. officinale* obtained from its natural habitat, Thiruvananthapuram. The source plant materials were authenticated from Pharmacognosy Unit of Govt. Ayurveda College, Thiruvananthapuram Poojappura and a voucher specimen was deposited in the herbarium of the institute. These plant materials were also utilized in the formulation of NK with different ratios.

5B.3.2. Extraction of the Source Plants

The collected plant materials were cleaned and air dried. Coarsely grounded, dried materials *viz*. roots of *S. cordifolia* (3 kg) and *S. retusa* (2 kg), seeds of *C. cyminum* (1.5 kg) and rhizomes of *Z. officinale* (618 g) were extracted with ethanol for three days at room temperature for three days. The crude extract of the plant materials (50 g of *S. cordifoila*, 20 g of *S. retusa*, 40 g of *C. cyminum* and 16 g of *Z. officinale*) were afforded after removal the solvent under reduced pressure using a rotary evaporator.

5B.3.3. Preliminary Phytochemical Analysis

In order to identify the presence various secondary metabolites, the alcoholic extract of the plant species was subjected to qualitative analysis. All the plant species gave positive detection of alkaloids and steroids; flavonoids and saponins were detected in the extracts of *C. cyminum* and *Z. officinale* while *S. cordifolia*, *S. retusa* & *Z. officinale* showed the presence of tannins. Presence of phenolic compounds was observed in *C. cyminum* only all these findings are in accordance with the API standards.

Table 5B.2: Preliminary phytochemical evaluation of the individual plants

Metabolites	***S. cordifolia***	***S. retusa***	***C. cyminum***	***Z. officinale***
Alkaloids	+	+	+	+
Flavonoids	-	-	+	+
Saponins	-	-	+	+
Tannins	+	+	-	+
Phenols	-	-	+	-
Steroids	+	+	+	+

5B.3.4. Isolation and Characterization of Phytochemicals from the Source Plants

The ingredient herbs were subjected to detailed phytochemical analysis to afford the major secondary metabolites with the aid of column chromatography.

5B.3.4.1. *Sida cordifolia*

About 50 g of the ethanol extract of the roots were fractionated over silica gel (100-200 mesh) column chromatography using hexane, hexane-EtOAc gradient and EtOAc eluent afforded 65 fraction pools. Based on the similarity in TLC they were combined into 18 fraction pools. These fraction pools were subjected to repeated column chromatographic separation using EtOAc-hexane gradient solution resulted in the isolation of the following compounds. The detailed schematic representation of extraction and isolation procedure is given in Fig. **5B.4**.

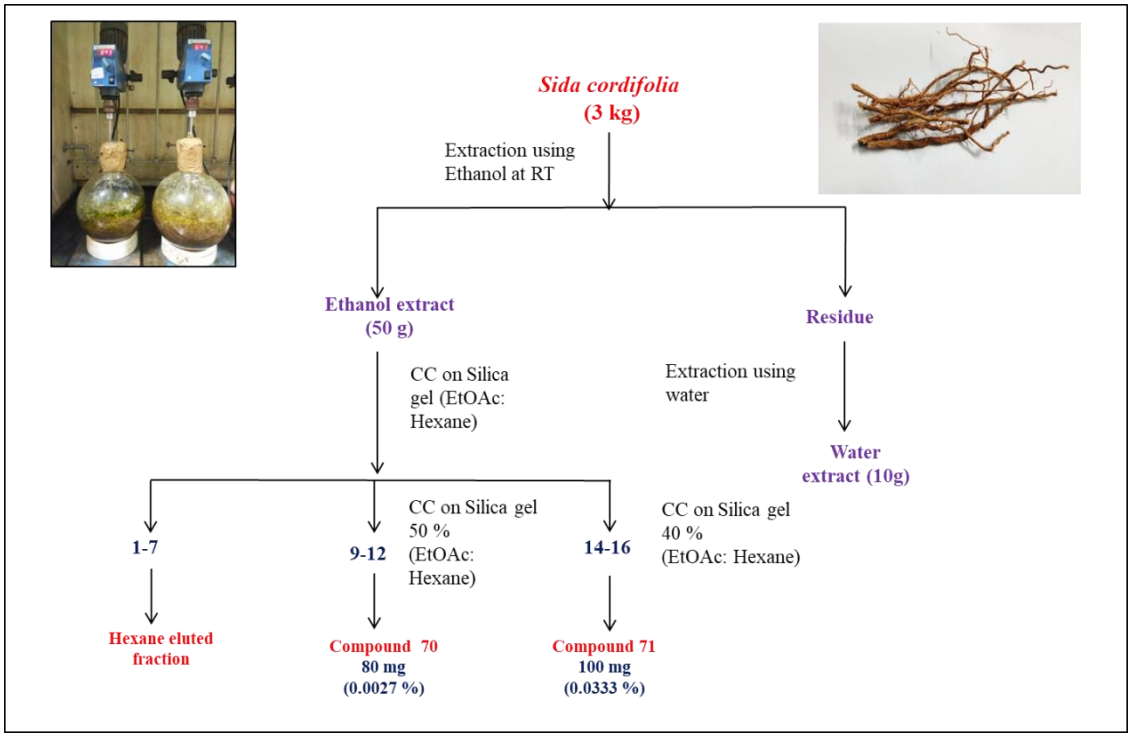
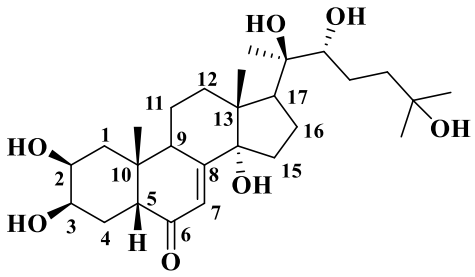


Figure 5B.4: Schematic representation of extraction & isolation procedure from the roots

Compound **70** afforded as white amorphous solid from the fraction pool 9-12 after CC separation using 50% ethylacetate-hexane eluent. Based on the spectral data analysis and in comparison with the literature reports the compound was confirmed to be *β*-sitosterol*β*-D-glucoside which is previously isolated from the *Hyptis capitata* species (Compound **68**). Spectral data of the compound was described in Chapter **5A**.

Fraction pool 14-16 after CC separation using 40% ethyl acetate-hexane afforded the compound **71** as white solid. ^{1}H NMR spectrum of the compound displayed a doublet

at δ 5.83 (d, *J*=2.5 Hz, 1H) ppm corresponds to the *olefinic* proton (H-7), a doublet at δ 3.97 (d, *J*=1.5 Hz, 1H) ppm for H-3, a multiplet at δ 3.88-3.84 (m,1H) ppm for H-2, a multiplet at δ 3.34-3.32 (m,1H) ppm for H-22 and a multiplet at δ 3.24-3.16 (m,1H) ppm for H-9. The remaining aliphatic protons were observed in the range of 3-0.91 ppm.^{13}C NMR spectrum of the compound gave twenty five carbon resonances comprised of carbonyl carbon (209.0 ppm), *olefinic* carbons (170.5 & 124.7 ppm) carbon bearing hydroxyl group (87.8, 80.9, 80.5, 73.8, 71.2, 71.1 ppm) and aliphatic carbons. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be ecdysterone. The mass spectrum of the compound gave molecular ion peak at 503.29892 which is the $[M+Na]^+$ peak [31].



Ecdysterone
Compound **71**

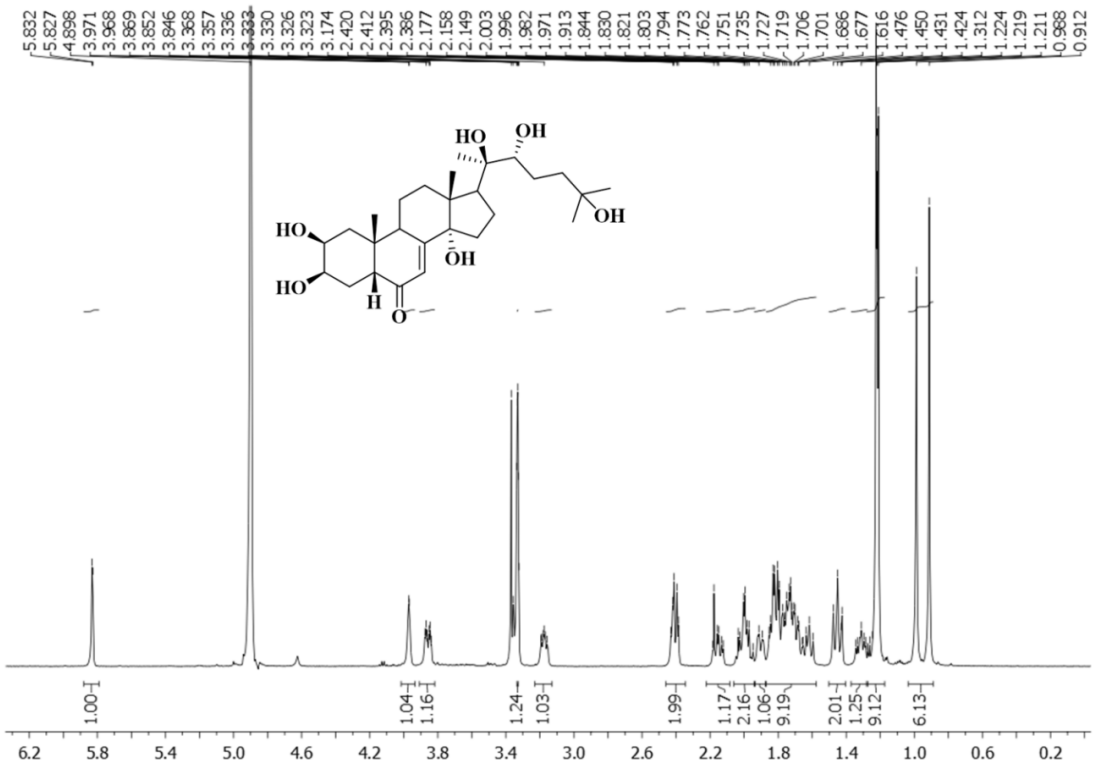


Figure 5B.5: ^{1}H NMR spectrum of Ecdysterone

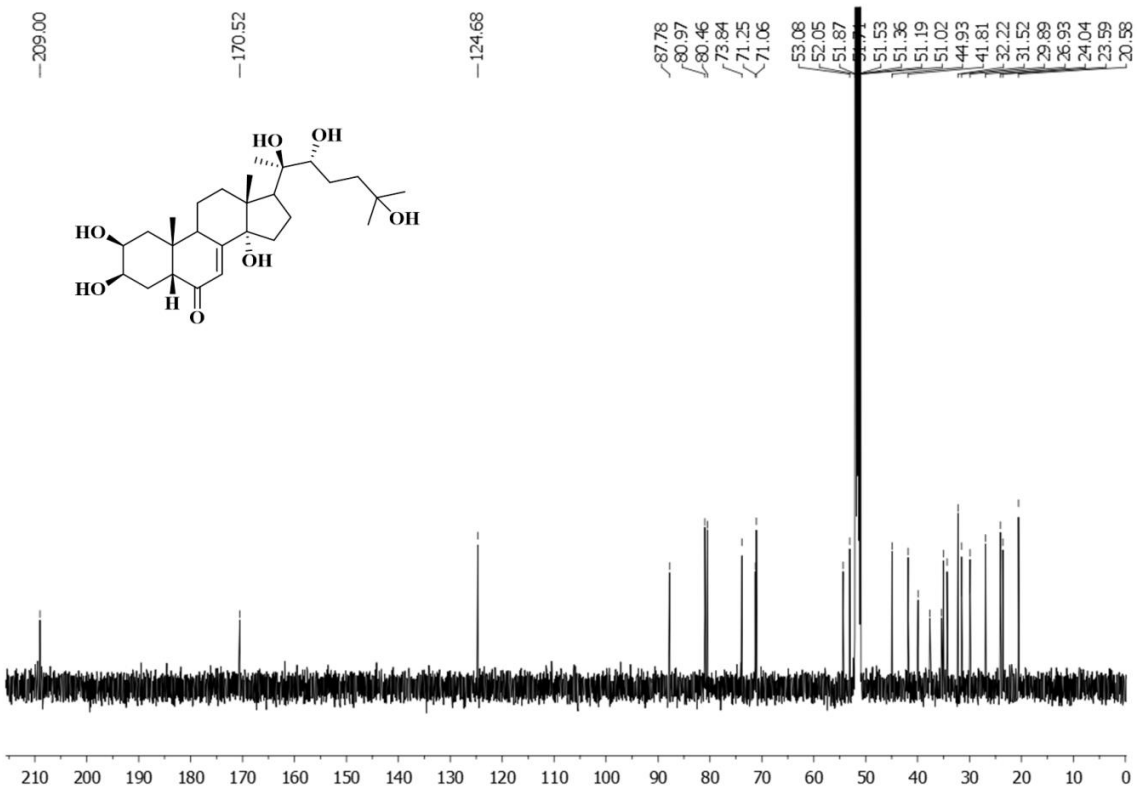


Figure 5B.6: ^{13}C NMR spectrum of Ecdysterone

5B.3.4.2. *Sida retusa*

Ethanol extract (20 g) of the roots was fractionated over silica gel (100-200 mesh) column chromatography by gradient elution of EtOAc/Hexane eluent afforded 18 fractions. Based on the TLC analysis similar fractions were combined and subjected to further purification using column chromatography in order to obtain the pure compounds. A schematic representation of extraction and isolation procedures is given in Fig. **5B.7**.

Compound **72** was isolated as colour less crystals from the fraction pool 9-12 after cc separation using 15% ethylacetate-hexane. Compound **73** was also obtained as colour less crystals from the fraction pool 13-16 after CC separation with 25% ethylacetate-hexane eluent. Spectral details of the two compounds showed several similarities and suggested the presence of phytosterol structure. By analyzing the various spectroscopic data and in comparison with the literature reports Compound **72** was identified as *β*-sitosterol while the compound **73** as stigmasterol. Both the compounds were previously isolated from *hyptis capitata*. The spectral details of the compounds were discussed in detail in the previous part (Chapter **5A**, Compound **62** & **63**).

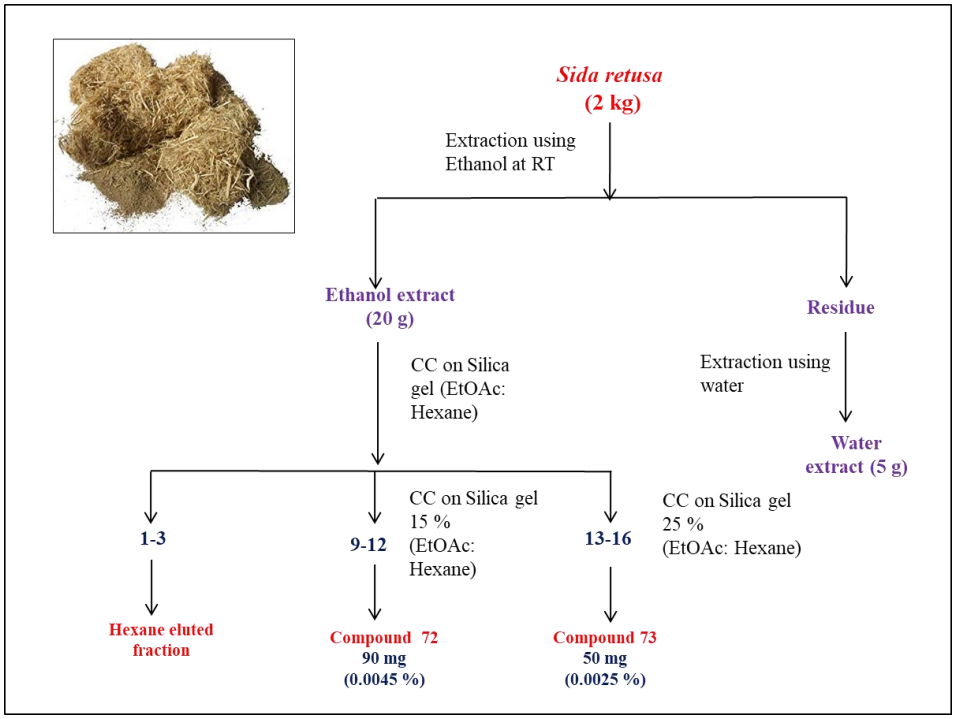


Figure 5B.7: Schematic representation of extraction & isolation procedure from the roots

5B.3.4.3. *Cuminum cyminum*

Around 40 g of the ethanol extract of the seeds of *C. cyminum* was fractionated over silica gel (100-200 mesh) column chromatography by the gradient elution of EtOAc/Hexane afforded twenty five fractions. These fractions were combined and purified by repeated CC separation based on the TLC analysis to obtain the isolates. Extraction and isolation procedure from the seeds is given in Fig. **5B.8**.

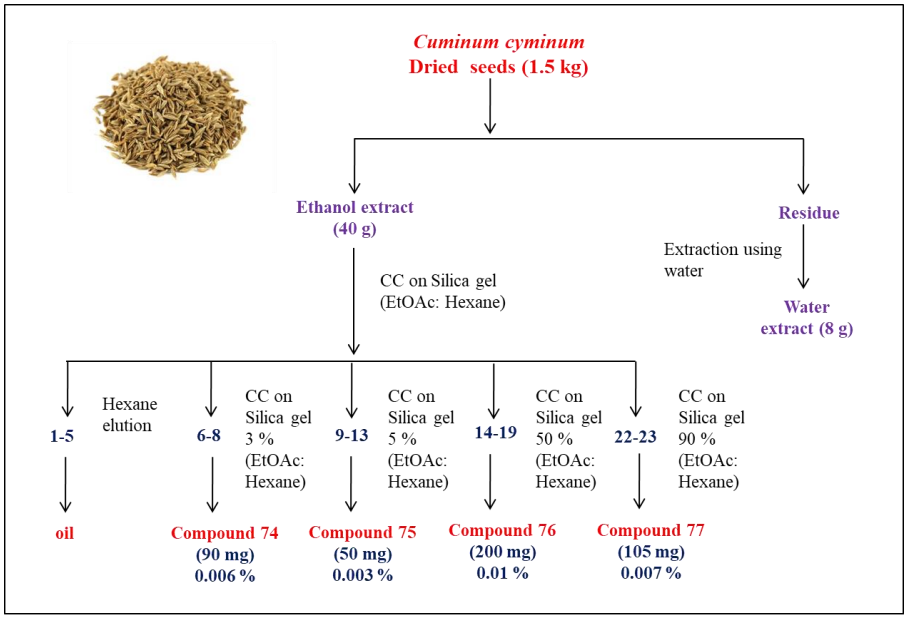
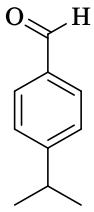


Figure 5B.8: Schematic representation of extraction isolation procedure from the seeds

Fraction pool 6-8 showed the presence of an intense UV active spot. It was then subjected to CC on silica gel using 3% ethyl acetate-hexane afforded the compound **74** as colourless viscous liquid having the smell of cumin. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be cuminaldehyde [32]. ^{1}H NMR displayed a sharp singlet at δ 9.97 (s, 1H) ppm corresponds to aldehyde proton, two sets of sharp singlets at δ 1.29 (s, 3H) & 1.28 (s, 3H) ppm for protons of two methyl group and a multiplet at δ 3.00 (m, 1H) ppm for the -CH proton. The 1, 4-disubstitution of the aromatic ring was evident from two sets of doublets at δ 7.82 (d, J=8 Hz, 2H) & 7.35 (d, J=8Hz, 2H) ppm. ^{13}C NMR of the compound was comprised of seven carbon resonances of carbonyl carbon of aldehyde (δ 192 ppm), -CH carbon (δ 34.5 ppm) and methyl carbons (δ 23.6 ppm) along with aromatic carbons. The mass spectrum of the compound gave a molecular ion peak of 149.2050 which is $[M+H]^{+}$ peak.



Cuminaldehyde
Compound **74**

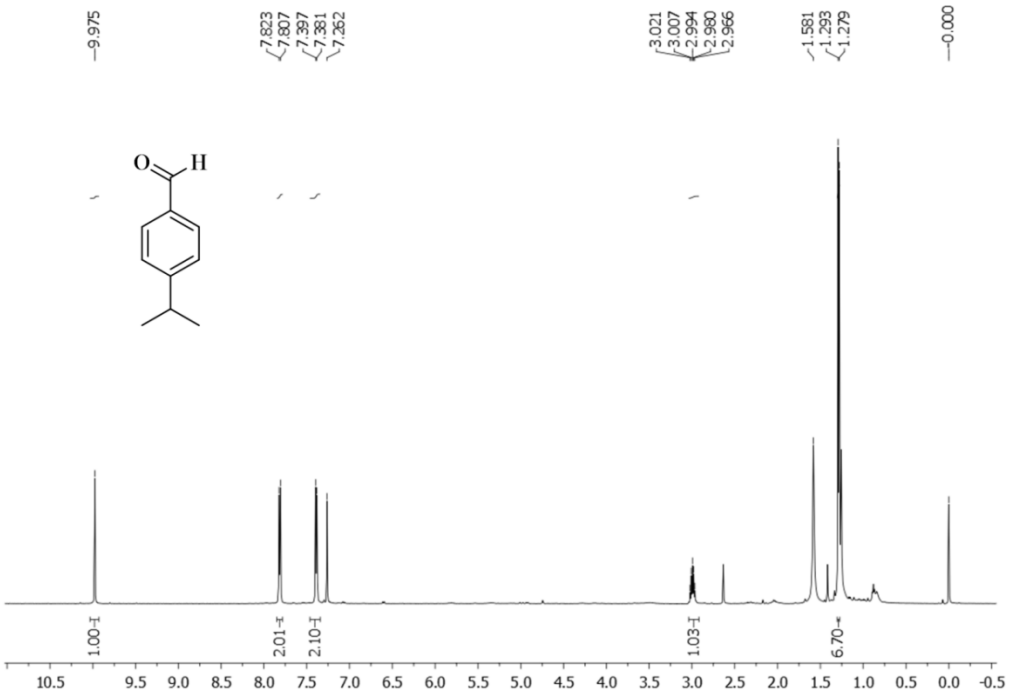


Figure 5B.9: ^{1}H NMR spectrum of cuminaldehyde

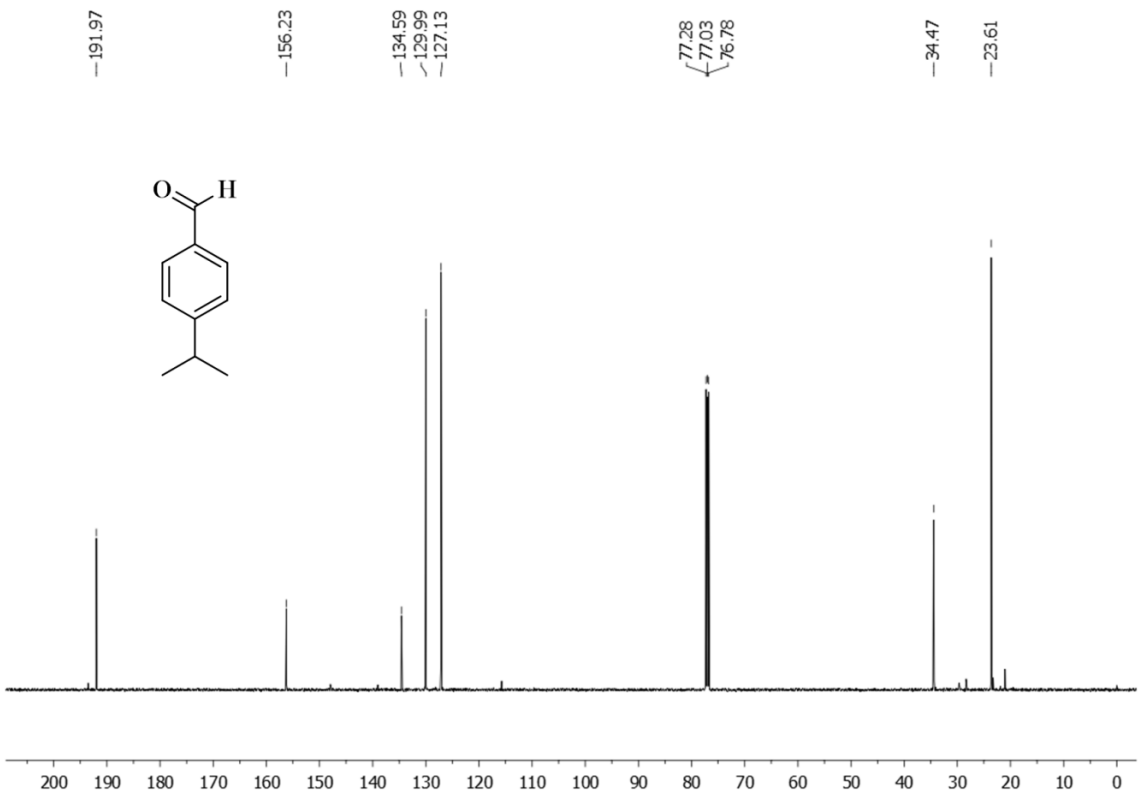
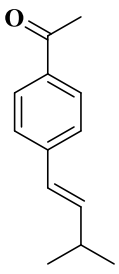


Figure 5B.10: ^{13}C NMR spectrum of cuminaldehyde

Fraction pool 9-13 after CC separation on silica gel using 5% ethyl acetate-hexane eluent afforded compound **75** as colourless liquid. ^{1}H NMR spectrum of the compound exhibited signals for trans *olefinic* protons [δ 6.68 (d, J = 16 Hz, 1H) & 7.51 (s, 1H) ppm], 1,4-disubsubstituted aromatic ring [δ 7.47 (d, J = 7.5 Hz, 2H) & 7.26 (d, J = 7.5 Hz, 2H) ppm], methyl protons [δ 1.26 (d, J = 6.9 Hz, 6H) ppm], -CH proton [δ 2.93 (m, 1H)] along with methyl group attached to carbonyl carbon [δ 2.37 (s, 3H) ppm]. ^{13}C NMR displayed the corresponding carbon resonances. The mass spectrum of the compound gave a molecular ion peak of 189.2700 which is $[M+H]^{+}$ peak. By the detailed analysis of various spectral data the compound was confirmed as (E)-1-(4-(3-methylbut-1-en-1-yl) phenyl) ethan-1-one.



(*E*)-1-(4-(3-methylbut-1-en-1-yl)phenyl)ethan-1-one
Compound **75**

Figure 5B.11: [1]H NMR spectrum of (E)-1-(4-(3-methylbut-1-en-1-yl) phenyl) ethan-1-one

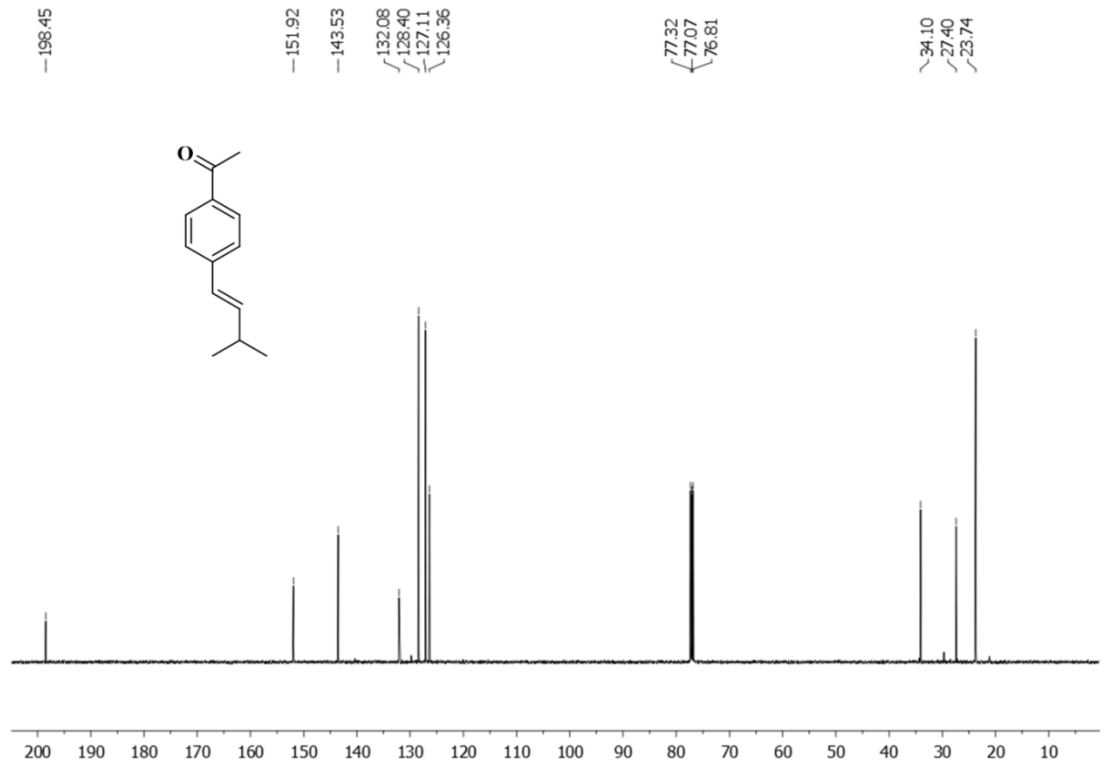
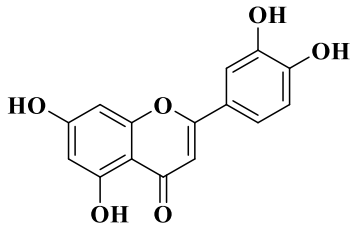


Figure 5B.12: [13]C NMR spectrum of (E)-1-(4-(3-methylbut-1-en-1-yl) phenyl) ethan-1-one

Compound **76** was isolated as yellow powder from the fraction pool 14-19 after CC on silica gel using 50% ethyl acetate-hexane eluent. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be flavone luteolin [33]. ^{1}H NMR spectrum of the compound showed a sharp singlet at δ 6.60 (s, 1H) ppm corresponds to *olefinic* proton of the flavone moiety which is a characteristic peak. A sharp singlet at δ 13.04 (s, 1H) ppm corresponds to a hydrogen bonded phenolic proton and two sets of singlets at δ 9.60 & 8.64 for other phenolic hydroxyl groups. Two sets of singlets observed at δ 6.54 (s, 1H, H-8) & 6.27 (s, 1H, H-6) ppm for aromatic protons of the first ring and aromatic protons of the second ring appeared at δ 7.01-7.52 ppm. ^{13}C NMR gave fifteen signals corresponding to carbonyl carbon (δ 182.2 ppm), *olefinic* carbons (δ 104, 164 ppm), carbon bearing hydroxyl groups and aromatic carbons. The mass spectrum of the compound gave a molecular ion peak of 287.05635 which is $[M+H]^{+}$ peak.



Luteolin
Compound **76**

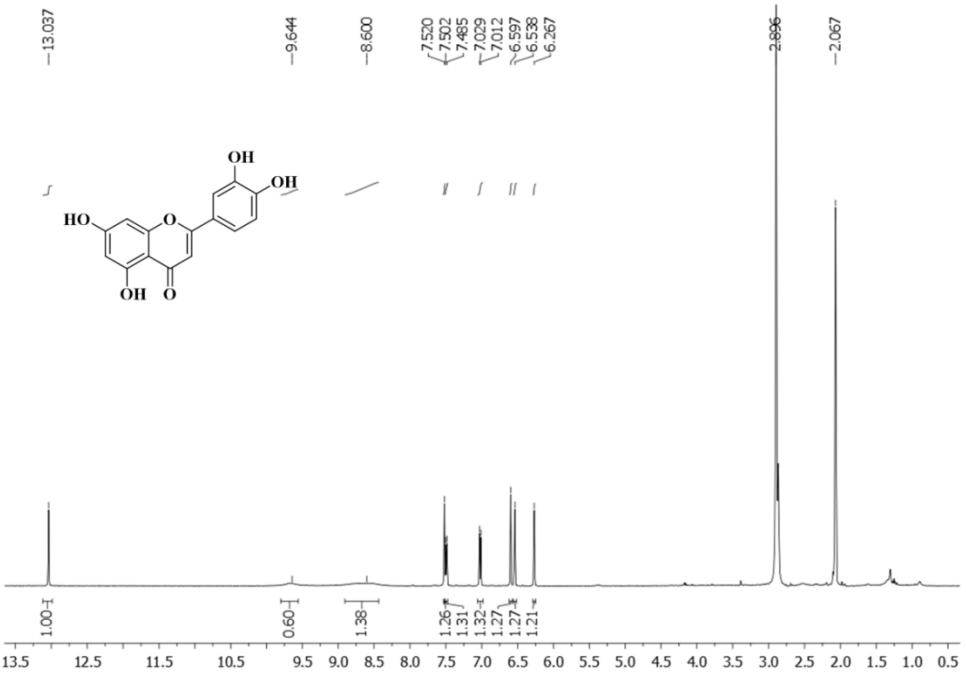


Figure 5B.13: ^{1}H NMR spectrum of luteolin

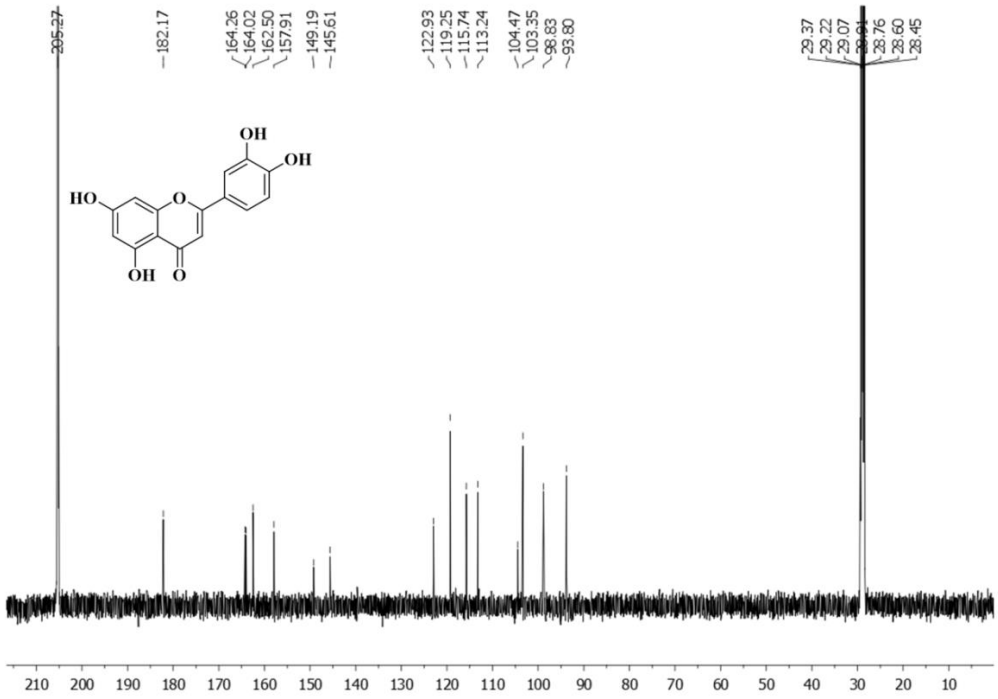
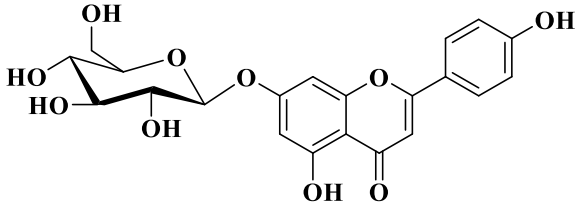


Figure 5B.14: ^{13}C NMR spectrum of luteolin

Fraction pool 20-23 after CC separation using 90% ethylacetate-hexane led to the isolation of compound **77** as yellow powder. By comparing all the spectral data with the literature the compound was identified as apigenin-7-O-*β*-D-glucopyranoside [34]. ^{1}H NMR spectrum of the compound displayed signals corresponds to flavone and glucose moiety. The aglycone (flavone) was identified as apigenin. ^{1}H NMR displayed signals for *olefinic* proton [δ 6.87 (s, 1H) ppm], protons of first aromatic ring [δ 6.84 (s, 1H) & 6.45 (s, 1H) ppm] and the *ortho* coupled protons of second aromatic ring [δ 7.95 (d, J = 8.5 Hz, 2H) & 6.95 (d, J = 8.5 Hz, 2H) ppm]. Besides, the protons of glucose moiety observed at δ 3.72-3.18 ppm and the anomeric proton appeared at δ 5.07 (d, J = 7 Hz, 1H). ^{13}C NMR gave twenty one carbon resonances comprised of olefinic carbon (δ 164.7, 103.6 ppm), aromatic ring carbons, and carbons of glucose ring (100.4, 77.7, 76.9, 73.9, 70.1 & 61.1 ppm). The mass spectrum of the compound gave a molecular ion peak of 433.12711 which is $[M+H]^{+}$ peak.



Apigenin-7-O-*β*–D-glucopyranoside
Compound **77**

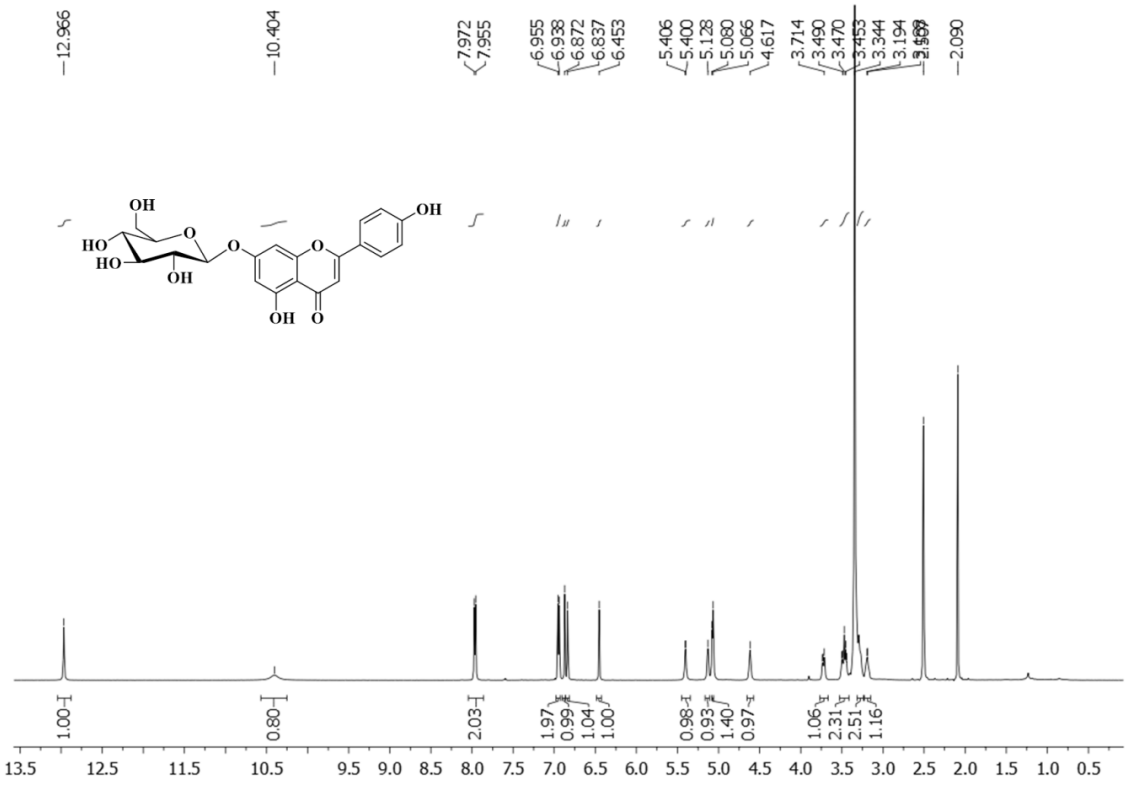


Figure 5B.15: ^{1}H NMR spectrum of apigenin-7-O-*β*-D-glucopyranoside

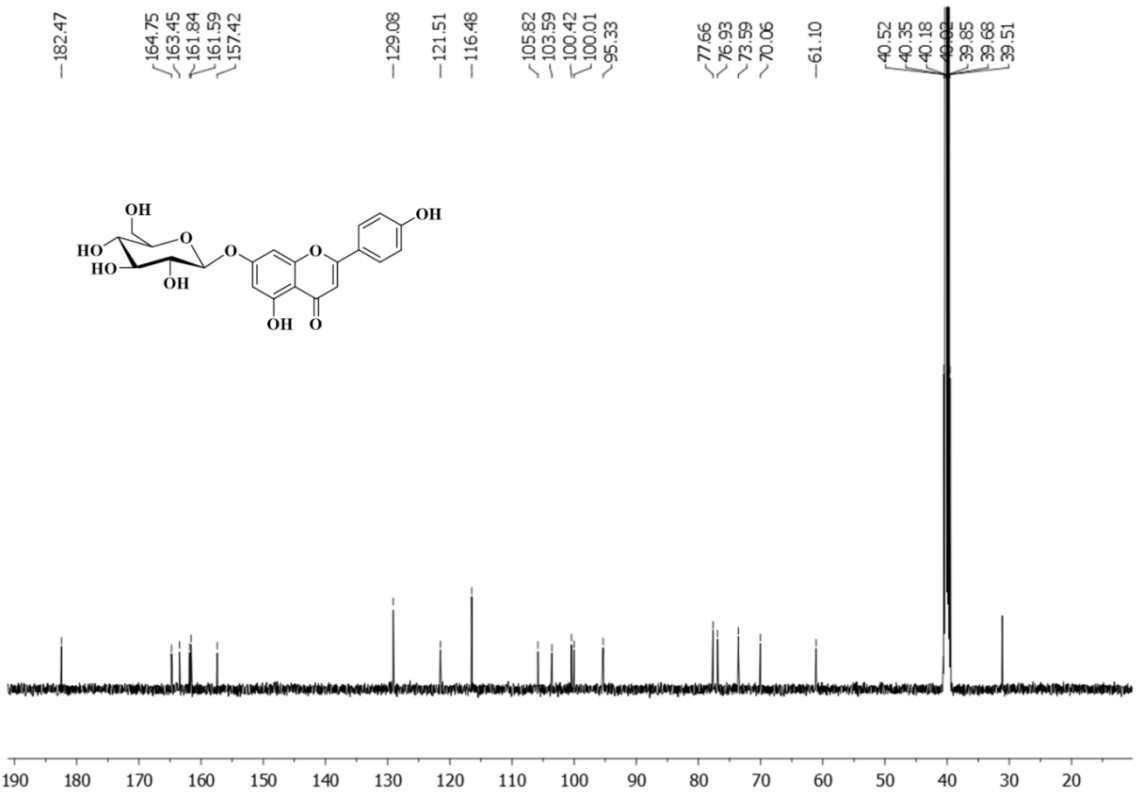


Figure 5B.16: ^{13}C NMR spectrum of apigenin-7-O-*β*-D-glucopyranoside

5B.3.4.4. *Zingiber officinale*

The ethanol extract (16 g) of the rhizome was chromatographed on silica gel column by gradient elution with EtOAc /Hexane afforded fifteen fractions. Based on TLC analysis similar fractions were purified to isolate the compounds. A schematic representation of extraction and isolation procedure is given in Fig. **5B.17**.

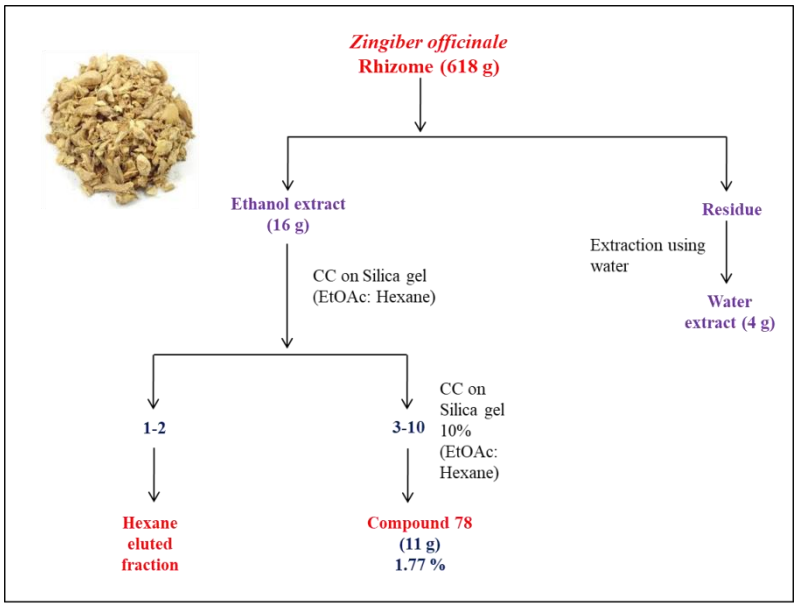
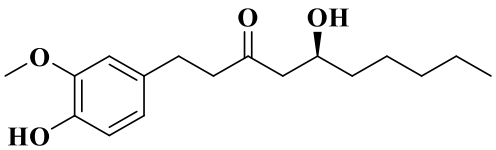


Figure 5B.17: Schematic representation of extraction & isolation procedure

Compound **78** was afforded as brown liquid from the fraction pool 3-4 after CC on silica gel using 10-20% ethyl acetate-hexane. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be 6-gingerol [35]. ^{1}H NMR spectrum of the compound displayed signals corresponds aromatic protons [δ 6.80 (dd, J_1 =8Hz, J_2 =2Hz, 1H, H-2'), 6.67 (s, 1H, H-6'), 6.63 (d, J= 8Hz, 1H, H-3') ppm], methoxy proton [δ 3.84 (s, 3H) ppm], -CH proton [δ 4.05-4.01 (m, 1H, H-5) ppm] and aliphatic protons [δ 2.83-0.8 ppm]. ^{13}C NMR of the compound gave seventeen carbon signals consist of carbonyl carbon, carbon bearing hydroxyl group, methoxy carbon, aromatic carbons and aliphatic carbons. The mass spectrum of the compound gave a molecular ion peak of 295.1831which is $[M+H]^+$ peak.



6-gingerol
Compound **78**

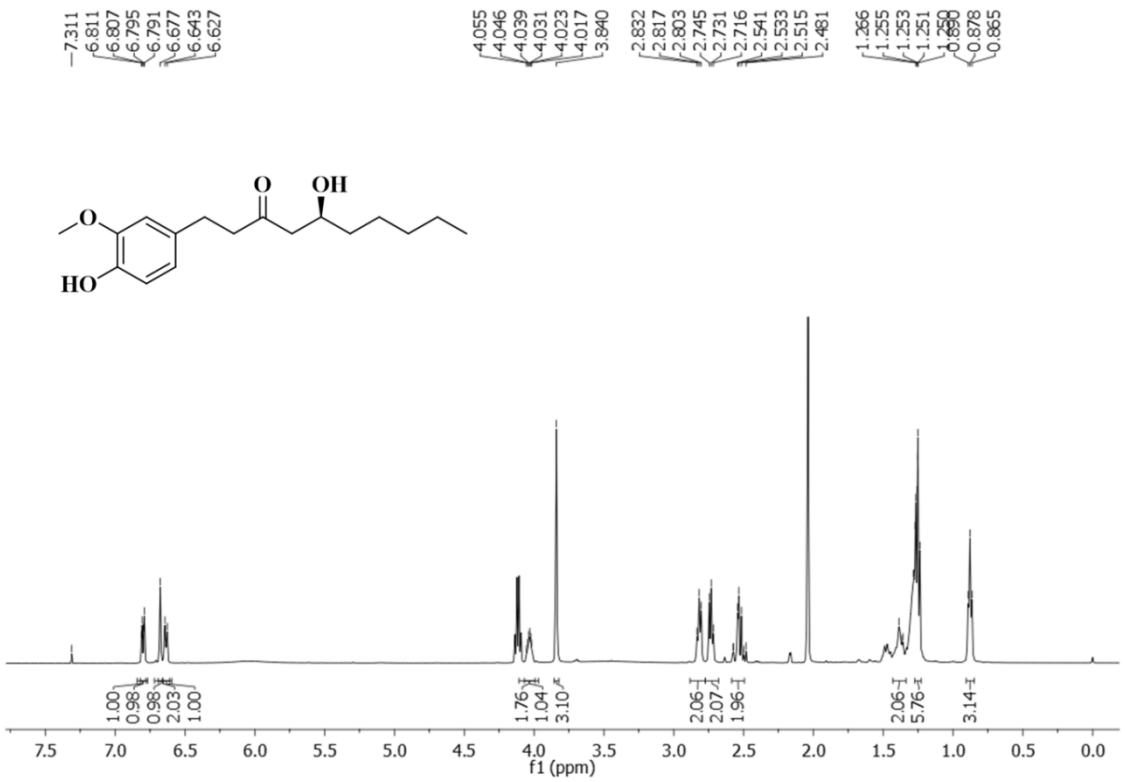


Figure 5B.18: ^{1}H NMR spectrum of 6-gingerol

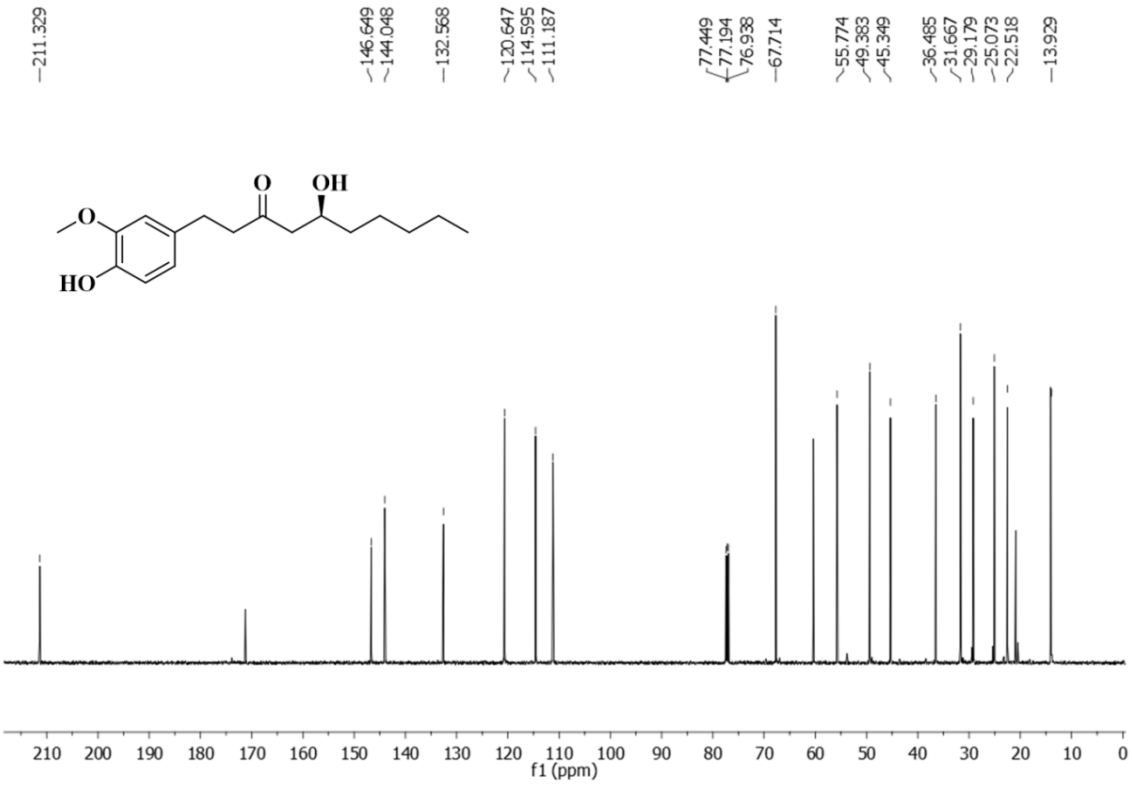


Figure 5B.19: ^{13}C NMR spectrum of 6-gingerol

5B.4. Pharmacognostic Evaluation of the Source Plants

Pharmacognostic evaluation is considered as the foremost step in quality control which will ensure the quality and purity of the raw materials used. WHO provided guidelines for the pharmacognostic evaluation with respect to several quality control parameters including the organoleptic character, physico-chemical parameters and microscopic features. The authenticity of the herbal ingredient is of utmost importance in herbal formulation. In view of this initially we have subjected the source plants of NK for pharmacognostic evaluation.

5B.4.1. Organoleptic Evaluation

The herbal products are manufactured primarily from crude material. So the quality and purity of crude herb is an important factor that determines the therapeutic efficacy of the final product. Adulterant species will contribute adverse effects so the authenticity of the herbal ingredients should be evaluated. Collected plant materials were analyzed in terms of sensory characters (Table **5B.3**). Organoleptic evaluation of the individual plant species exhibited characteristics features in terms of dimensions, external surface, fracture, shape, color, odor and taste. Scent of cumin seed, ginger rhizome is the main features observed and the data obtained was in good agreement with the features described in Pharmacognosy textbook [36]. Thus the collected raw drugs were proved to be authentic. In addition these results can be used to identify the right variety and the adulterants used in formulations.

Table 5B.3: Organoleptic evaluation of the individual herbs

Characters	***S. cordifolia* (B1) Root**	***S. retusa* (B2) Root**	***C. cyminum* Seed**	***Z. officinale* Rhizome**
Dimensions	Length varying from 7-10 cm	Length varying from 7-14 cm	5 mm long	5-7cm long
Shape	Long, cylindrical tortuous	Cylindrical, branched tortuous	Elongated, thick in the middle and laterally compressed	Fairly, smooth, laterally compressed bearing many branches
External surface	Smooth, with some thin rootlets	Numerous lateral rootlets	5 lines are present	Longitudinally striated
Fracture	Fibrous	Fibrous	None	Short
Colour	Pale yellow	Pale yellow	Yellowish brown	Yellowish brown to greyish brown

Odour	No characteristic odour	No characteristic odour	Aromatic	Characteristic
Taste	Slightly bitter	Slightly bitter	Slightly pungent	Pungent

5B.4.2. Microscopic Evaluation

Histological features of the collected plant parts (roots, seeds and rhizomes) and their powder characteristics were identified. Transverse sections of the plant part is stained and analyzed. Both the plant species *S. cordifolia* and *S. retusa* were identical in many aspects. Histological features were also found in good agreement with reference standards.

Table 5B.7: Histological features of the of the individual plants

Plant material	**Microscopic characters of transverse section**	**Characters of Powder microscopy**
S. cordifolia (Roots)	✓ Cork-thin walled tangentially elongated cells ✓ Phellogen-a single layer of narrow, thin walled tangentially elongated cells ✓ Cortex-made up of parenchymatous cells. Calcium oxalate crystals and starch grains are present. ✓ Secondary phloem-occurs in conical strands ✓ Medullary rays-many, long, uniseriate, extending and reaching up to the cortex in a straight course	Lignified fibers with parenchyma. Calcium oxalate crystals & Starch grains were present in the parenchyma cells.
S. retusa (Roots)	✓ Cork - thin walled, rectangular tangentially elongated cells ✓ Features of phellogen and cortex are similar to *S. cordifolia* ✓ Secondary phloem – appears in cortical strands and are much narrower and linear than in *Sida cordifolia*	Similar to *S. cordifolia*
C. cyminum (Seeds)	✓ Epicarp - composed of a layer of colorless cells, with thin walls and a faintly and irregularly striated cuticle ✓ Underlying the epicarp the thin-walled cells of the palisade are seen ✓ Endocarp- composed of a layer of fairly large, thin-walled cells. ✓ The endosperm - composed of moderately thick-walled cells containing aleuronic microrosette crystals of calcium oxalate	Presence of aleurone grains, surface cells and some calcium oxalate crystals

Z. officinale (Rhizome)	✓ Cork-consists of irregularly arranged, tangentially elongated, slightly brown-colored cells ✓ Cortex-contains thin-walled polygonal parenchymatous tissue ✓ Contains suberised oil cells, which holds yellowish-brown oleoresin ✓ The inner cortex contains closed, collateral vascular bundles ✓ Endodermis - characterized by a single layered pericycle ✓ Vascular bundles of stele resemble that of cortex with the exception of a ring of small scattered bundle within the pericycle ✓ Yellow polygonal oleo-resin cell are also present in the cortical region.	Presence of thin walled non lignified fibres, simple starch grains, oleoresin cells and reticulately thickened vessels

5B.4.3. Preliminary Physicochemical Evaluation

Physicochemical parameters of the medicinal herbs played a major role in therapeutical efficacy. Preliminary physicochemical evaluation based on the parameters such as foreign matter content, moisture content, fiber content, sugar content, total ash, acid insoluble ash, alcohol/water soluble extractive etc. of the collected plant material were evaluated according to standard procedures in API [36].

Table 5B.8: Preliminary physicochemical evaluation of the individual plants

Parameters	***S. cordifolia***	***S. retusa***	***C. cyminum***	***Z. officinale***
Foreign matter	0.5%	0.3%	2.0%	1.0%
Moisture	10.0%	9.03%	8.4%	9.0%
Total ash	1.45%	1.34%	8.0%	6.0%
Acid insoluble ash	0.64%	0.54%	1.0%	1.5%
Alcohol soluble extractive	2.01%	1.08%	7.0%	3.0%
Water soluble extractive	4.73%	4.0%	15%	10%
Fiber content	44.0%	46.02%	5.06%	8.0%
Total sugar	1.24%	1.78%	0.08%	1.17%
Reducing sugar	0.54%	0.57%	_	0.09%

The features of the plant species obtained were in compliance with the standard values in Ayurvedic Pharmacopoeia of India (API). In our analysis, total fiber content of

Sida cordifolia, *Sida retusa*, *Cuminum cyminum* and *Zingiber officinale* are 44%, 46.02%, 5.06%, and 8% respectively. The total sugar content of these drugs is 1.24%, 1.78%, 0.08% and 1.17% respectively. *S. retusa* was found to be the drug with maximum fiber content and sugar content. *C. cyminum* and *Z. officinale* possessed the maximum ash value which indicates their identity and purity. Foreign matter content of the sample drugs were also lower and in acceptable range. The water soluble extractive value of all plants is higher than its alcohol soluble value indicates the drug is highly soluble in water. Water soluble extractive value is more significant because the decoction were prepared in water. The quality and authenticity of the plant species in an Ayurvedic formulation contributes much towards the safety, effectiveness and acceptability. These basic data obtained can be used for future reference to ensure the authenticity of the plants and to trace out the presence of adulterants.

5B.5. Preparation and Evaluation of *Nayopayam Kwtha* (NK)

The decoction was formulated from the collected ingredient herbs according to the standard procedures as per *Sarngadhara samhita* [37]. As already mentioned classical drug ratios of polyherbal formulations plays a crucial role in achieving the specific healing action. Herein we have formulated the decoction according to the classical ratio 3:2:1 as per *Vaidya Manorama* and 10:1:1 as per *Arogya raksha Kalpadruma* along with 1:1:1 as per the general way of Ayurvedic poly herbal formulation. Nowadays most of the polyherbal decoctions were prepared by using the ingredients in equal proportions rather than the classical concept.

Crushed roots of [*Sida cordifolia* (B1) and *Sida retusa* (B2)], powdered seeds of *Cuminum cyminum* and rhizomes of *Zingiber officinale* were utilized for the preparation of NK in water. Since there are two source plants for *bala*, *ie Sida cordifolia* (B1) and *Sida retusa* (B2) two sets of NK (NKB1 & NKB2) were prepared in three different drug ratios. The different sets of prepared formulation were initially subjected to pharmacognostic evaluation.

5B.5.1. Organoleptic Evaluation of NK

Organoleptic evaluation will help to determine the preliminary quality evaluation. Prepared formulations were assesses in terms of the sensory organs. According to organoleptic evaluation brownish yellow colour, characteristic odour, liquid consistency and astringent taste were observed as the characteristic features of *Nayopayam kwatha*. Apart from the slight colour variation (not predominant), alteration of drug ratios not

affected the organoleptic characters of the formulation. Organoleptic characters of the prepared samples satisfy reference features described in API.

Table 5B.9: Organoleptic characters of NK

NK Formulations	
State	Liquid
Appearance	Brownish yellow
Nature	Liquid consistency
Odour	Characterestic
Tate	Astingent

5B.5.2. Physicochemical analysis of NK

Decoction samples were subjected to physicochemical analysis based on its parameters such as specific gravity, p^H and total solids. For the decoctions these parameters should be in an acceptable range as per WHO recommendation to avoid toxicity.

Table 5B.10: Physicochemical analysis of *Nayopayam kwatha*

Drug ratio/ Parameters	3:2:1		10:1:1		1:1:1	
	NKB1	NKB2	NKB1	NKB1	NKB1	NKB1
Specific gravity	1.02	1.05	1.04	1.08	1	1.03
p^H	6.56	6.52	6.8	6.65	6.3	6.2
Total solids (g/ml)	0.04	0.04	0.06	0.09	0.03	0.05

Specific gravity of the decoction prepared in three ratios was found to be around 1. The formulation NKB2 in the ratio 10:1:1 showed highest specific gravity and NKB1-1:1:1 having lowest specific gravity. p^H of the decoction was in an acceptable range for all formulations and was found to be around 6.2-6.8. Altering the drug ratio has no effect on its p^H and specific gravity. The sample NKB2 in the ratio 10:1:1 was found to contain more amounts of total solids whereas NKB1 in the ratio1:1:1 having least amount of total solids. The total solids represent the amount of organic and inorganic matter which is not considered as a primary contaminant and will not contribute health effects. It is found that, altering the ratio of drugs has a slight effect on the total solid contents of NK. Analysis of physicochemical parameters of the decoction revealed that altering the drug

ratio has no effect on its p^H and specific gravity while it will affect the presence of total solids.

5B.5.3. GC-MS Analysis of NK formulations

NK formulations were subjected to GC-MS analysis in order to obtain the volatile components present the in the different set of formulation. Decoctions (NKB1-3:2:1, NKB1-10:1:1, NKB1-1:1:1 & NKB2-3:2:1, NKB2-10:1:1, NKB2-1:1:1) were evaporated and the dry residues were dissolved in methanol for the analysis. The volatile chemical constituents were identified by matching mass spectra with spectra of reference compounds in mass spectral library of NIST and WILEY. Major compounds (more area %) obtained from the each decoction samples were given in the Table **5B.11A-F**.

Table 5B.11A: GC-MS analysis of NKB1-3:2:1

Retention time	Area %	Compound Name
39.266	11.46	Gingerol
23.643	11.13	4-(1-methylethyl)-benzamide
48.715	9.36	(3β,22e)-tigmasta-5,22-dien-3-ol,
22.387	9.29	(1-methoxy-1-methylethyl)-benzene
19.287	6.92	4-(1-methylethyl)-benzoic acid
38.97	6.68	Tricyclo[5.1.0.0(3,5)]octane-2,6-dione, 1,3,4,5,7,8-hexamethyl-, stereoisomer
24.224	6.35	4-(4-hydroxy-3-methoxyphenyl)- 2-butanone
30.065	5.44	5-(7a-isopropenyl-4,5-dimethyl-octahydroinden-4-yl)-3-methyl-pent-2-en-1-ol
37.371	3.60	1-(2,4-dihydroxyphenyl)-2-(4-methoxy-3-nitrophenyl)ethanone
18.858	2.64	Caryophyllene
41.744	2.43	3-decanone, 5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-, (s)-
44.712	2.37	Bis(2-ethylhexyl)-phthalate
21.474	2.20	5-(1-bromo-1-methyl-ethyl)-2-methyl-cyclohexanol
22.867	2.20	[1R-(1R*,4R*,6R*,10S*)]- (-)-5-oxatricyclo[8.2.0.0(4,6)]dodecane,,12-trimethyl-9-methylene
30.169	1.66	Hexadecanoic acid, methyl ester

Table 5B.11B: GC-MS analysis of NKB1-10:1:1

Retention time	Area %	Compound Name
23.645	14.04	Benzamide, 4-(1-methylethyl)-
19.298	11.15	Benzoic acid, 4-(1-methylethyl)-
39.283	10.31	Gingerol
18.872	7.28	Caryophyllene

38.979	5.01	Tricyclo[5.1.0.0(3,5)]octane-2,6-dione, 1,3,4,5,7,8-hexamethyl-, stereoisomer
24.238	4.84	2-Butanone, 4-(4-hydroxy-3-methoxyphenyl)-
22.409	4.39	Benzene, (1-methoxy-1-methylethyl)-
22.873	3.97	(-)-5-Oxatricyclo[8.2.0.0(4,6)]dodecane,,12-trimethyl-9-methylene-, [1R-(1R*,4R*,6R*,10S*)]-
30.069	3.22	5-(7a-Isopropenyl-4,5-dimethyl-octahydroinden-4-yl)-3-methyl-pent-2-en-1-ol
19.495	2.92	3-Oxabicyclo[3.3.0]octan-2-one, 7-isopropylidene-, E-
18.068	2.63	2,4-diisopropenyl-1-methyl-1-vinylcyclohexane
44.540	2.62	3,5,7-Tris(trimethylsiloxy)-2-[3,4-di(trimethylsiloxy)phenyl]-4H-1-benzopyran-4-one
37.387	2.60	(-)-Nortrachelogenin
44.718	2.51	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester
16.031	2.37	2-Methoxy-4-vinylphenol
30.179	2.31	Hexadecanoic acid, methyl ester
34.612	1.91	Phytol
28.381	2.30	Neophytadiene
19.634	1.47	(E)-β-famesene
19.772	1.36	α-humulene

Table 5B.11C: GC-MS analysis of NKB1-1:1:1

Retention time	**Area %**	**Compound Name**
22.425	12.25	Benzene, (1-methoxy-1-methylethyl)-
39.283	10.82	Gingerol
24.235	9.08	2-Butanone, 4-(4-hydroxy-3-methoxyphenyl)-
30.082	6.48	5-(7a-Isopropenyl-4,5-dimethyl-octahydroinden-4-yl)-3-methyl-pent-2-en-1-ol
38.986	6.35	1H-Indene, 2,3,3a,4,7,7a-hexahydro-2,2,4,4,7,7-hexamethyl-
23.720	5.81	Benzamide, 4-(1-methylethyl)-
44.720	4.86	2-([(2-ethylhexyl)oxy]carbonyl)benzoic acid
16.333	4.55	2-Oxabicyclo[2.2.2]octan-6-ol, 1,3,3-trimethyl-
16.156	3.44	Octadecanoic acid, 9,10-dihydroxy-, methyl ester, bis(trifluoroacetate)
15.036	3.39	Cinnamaldehyde, (E)-
16.046	2.80	2-Methoxy-4-vinylphenol
44.550	2.69	3,5,7-Tris(trimethylsiloxy)-2-[3,4-di(trimethylsiloxy)phenyl]-4H-1-benzopyran-4-one
19.482	2.07	Acetic acid, cinnamyl ester
25.448	2.03	limonene dioxide 1
20.865	1.67	2(3H)-Benzofuranone, 3a, 4, 5, 7a-tetrahydro-3a, 6-dimethyl-, cis-(. +-.)-

Table 5B.11D: GC-MS analysis of NKB2-3:2:1

Retention time	Area %	Compound Name
39.320	18.01	9-Octadecenoic acid, 12-hydroxy-, methyl ester, (Z)-
44.723	13.30	1,2-Benzenedicarboxylic acid, 1,2-bis(2-ethylhexyl) ester
34.381	12.00	6-Octadecenoic acid, methyl ester, (Z)-
34.161	5.96	9,12-Octadecadienoic acid (Z,Z)-, methyl ester
21.374	4.97	Dodecanoic acid, methyl ester
29.866	4.33	(1-methyldodecyl)benzene
25.987	3.93	Methyl tetradecanoate
41.759	3.69	Gingerol
30.174	3.65	Hexadecanoic acid, methyl ester
43.646	3.61	Phenol, 2,4-bis(1-phenylethyl)-
15.044	3.14	2-Propenal, 3-phenyl-
30.063	2.40	2(1H)-Naphthalenone, 3,4,4a,5,6,7,8,8a.alpha.-octahydro-5.alpha.-hydroxy-4a.alpha.,7,7-trimethyl-, acetate
24.266	1.95	2-Butanone, 4-(4-hydroxy-3-methoxyphenyl)-
18.862	1.60	Caryophyllene
14.155	1.52	Propanal, 2-methyl-3-phenyl-
29.043	1.39	(1-ethylundecyl)benzene
23.324	1.29	2-Tetradecanol
28.132	1.07	Benzene, (1-pentyloctyl)-

Table 5B.11E: GC-MS analysis of NKB2-10:1:1

Retention time	Area %	Compound Name
16.041	11.95	2-Mmethoxy-4-vinylphenol
22.480	11.09	Dodecanoic acid
39.319	9.64	9-octadecenoic acid, 12-hydroxy-, methyl ester, [R-(Z)]-
22.413	9.61	p-cymen-7-ol
15.875	5.81	5-(2',3',5',6'-Tetradeuterioocta-2',5'-dien-1'-yl)-4,5-dideuteriotetrahydrofuran-2-one
23.690	5.01	Benzamide, 4-(1-methylethyl)-
50.228	4.59	Glycerol tricaprylate
44.720	4.44	1,2-benzenedicarboxylic acid, diisooctyl ester
44.536	3.34	3,5,7-tris(trimethylsiloxy)-2-[3,4-di(trimethylsiloxy)phenyl]-4H-1-benzopyran-4-one
47.729	2.96	bis[1-methyl-1-silafluoren-1-yl]- Oxide
22.875	2.64	(-)-5-oxatricyclo[8.2.0.0(4,6)]dodecane,,12-trimethyl-9-methylene-, [1R-(1R*,4R*,6R*,10S*)]-
15.595	2.38	1,4-dihydroxy-p-menth-2-ene
34.296	2.33	Dodecanoic acid, 2,3-dihydroxypropyl ester

38.981	2.33	2,2,4,4,7,7-hexamethyl-2,3,3a,4,7,7a-hexahydro-1h-indene
20.081	2.09	Ethanone, 1-(2-hydroxytricyclo[3.3.1.13,7]dec-1-yl)-
16.330	1.68	2-oxabicyclo[2.2.2]octan-6-ol, 1,3,3-trimethyl-
30.070	1.66	5-(7a-Isopropenyl-4,5-dimethyl-octahydroinden-4-yl)-3-methyl-pent-2-en-1-ol
21.377	1.62	Dodecanoic acid, methyl ester

Table 5B.11F: GC-MS analysis of NKB2-1:1:1

Retention time	Area %	Compound Name
16.016	15.21	2-Methoxy-4-vinylphenol
23.643	14.73	Benzamide, 4-(1-methylethyl)-
22.395	11.20	Benzene, (1-methoxy-1-methylethyl)-
38.986	8.49	1H-Indene, 2,3,3a,4,7,7a-hexahydro-2,2,4,4,7,7-hexamethyl-
39.288	6.00	Gingerol
22.877	4.86	(-)-5-Oxatricyclo[8.2.0.0(4,6)]dodecane,,12-trimethyl-9-methylene-, [1R-(1R*,4R*,6R*,10S*)]-
9.799	4.16	Benzene, 1-methyl-2-(2-propenyl)-
18.874	3.43	Caryophyllene
30.072	2.91	5-(7a-Isopropenyl-4,5-dimethyl-octahydroinden-4-yl)-3-methyl-pent-2-en-1-ol
15.770	2.61	Phenol, 5-methyl-2-(1-methylethyl)-
30.181	1.88	Hexadecanoic acid, methyl ester
34.613	1.85	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-
48.697	1.72	2-Butenoic acid, 2-methyl-, dodecahydro-8-hydroxy-8a-methyl-3,5-bis(methylene)-2-oxonaphtho[2,3-b]furan-4-yl ester, [3ar-[3a.al
35.635	1.65	(3-Methoxymethoxy-2-nitrocyclohexyl)benzene

5B.6. Quantification of NK formulations with Marker Phytoconstituents

Standardization of herbal formulations is inevitable in order to ensure its safety and efficacy. Herbal based formulations are widely employed to cure various ailments on account of their higher efficacy, safety and cost effectiveness. One of the major challenges associated with herbal based formulation is its acceptability in modern medicine due to the lack of standardization. Most of the formulation available today in the market having no standardization protocols in terms of marker constituents of the individual herbs used in it. Quantification of Ayurvedic polyherbal formulations in terms of chemical markers of its ingredient herbs by HPLC is a reliable technique in quality control. In this regard we have developed a simple, optimized and validated HPLC

method for the standardization of NK with the isolated chemical markers from the individual herbs for the first time.

Chemical makers such as ecdysterone from *Sida cordifolia*, *β*-sitosterol from *Sida retusa*, luteolin from *Cuminum cyminum* and 6-gingerol from *Zingiber officinale* were identified and selected for the standardisation of NK formulations in three different drug ratios. Methanol extract of the decoction was chosen for the analysis because it has a polarity similar to that of water and the results were statistically analyzed and plotted in the Fig. **5B.20**.

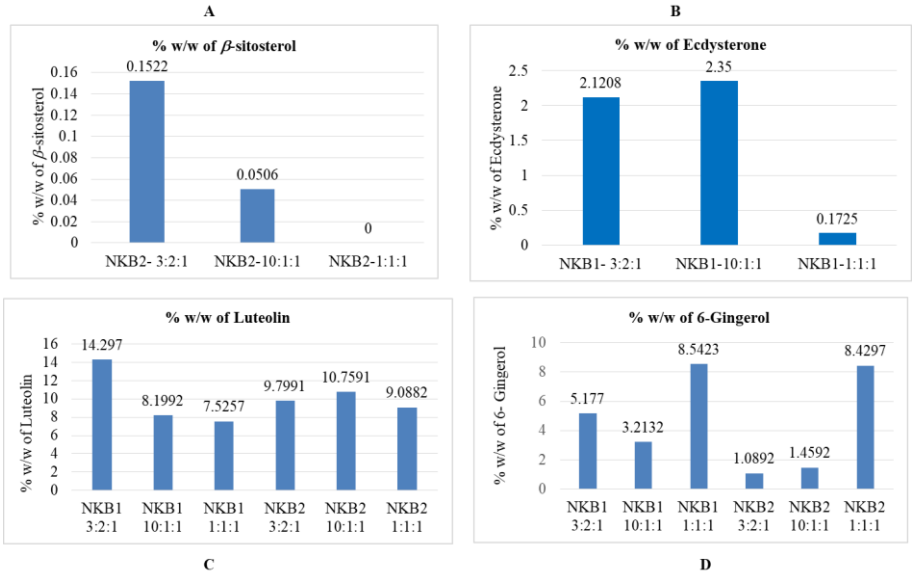


Figure 5B.20: Mean quantities of the major compounds present in NK

Three NK samples were taken for the quantification of ecdysterone (NKB1-3:2:1, NKB1-10:1:1, NKB1-1:1:1) and *β*-sitosterol (NKB2-3:2:1, NKB2-10:1:1, NKB2-1:1:1). The amount of ecdysterone was found to be more in NKB1-10:1:1. This may be due to the more %w/w of *bala* in this proportion. Amount of *β*-sitosterol was found to be more in NKB2-3:2:1 which was unexpected. No peaks were detected for NKB2-1:1:1. Among the decoctions made using B2 (*S. retusa*) as the source plant of *bala*, NKB2-10:1:1contain more amount of luteolin while the decoctions made using B1 (*S.cordifolia*) as the source plant of bala, NKB1-3:2:1contain more amount of luteolin. Among the six decoctions, the % w/w of 6- gingerol was more in NKB1-1:1:1. From the area percentage of standard 6-gingerol was 823525.47 and area percentages of NKB1-1:1:1, NKB2-3:2:1and NKB2-1:1:1were more than the standard. This suggests that the amount of 6-gingerol got increased in the *Nayopayam kwatha* than the extract. NKB2-1:1:1& NKB2-1:1:1contain more amount of gingerol than in other samples. Statistically analyzing these

four compounds, all were found to be significant at a P value <0.0001. By analyzing the chromatogram precise peaks were obtained for luteolin and 6-gingerol.

Pharmacological/therapeutic action of NK can be attributed to the bioactive phytoconstituents present in the individual herbs of the formulation. The constituent ginger (*Z. officinale*) and its active component 6-gingerol are known to possess potential activity against respiratory disorders and cardiovascular diseases [38]. In our study it is evident that NK in all drug ratios contains 6-gingerol in appreciable amount and substantially higher in the drug ratio 1:1:1 and there is a notable variation when the ingredient bala chosen as *S. cordifolia* and *S. retusa*. Literature suggests that flavonoids including luteolin are beneficial in controlling various respiratory diseases and having anti-allergy potential [39]. Luteolin is also found to present in all drug ratio of NK in significant amount. Thus these two compounds contribute much towards the therapeutic potential of NK against respiratory problems. In addition ecdysterone was present in higher amount in the drug ratio of 10:1:1 of NK when bala used as *sida cordifolia* in accordance with its weight percent in the ratio. But in the case of *Sida retusa* the significant amount of *β*-sitosterol was observed in the drug ratio 3:2:1 and not detected in the ratio 1:1:1 which is unusual. Both ecdysterone and *β*-sitosterol are pharmacologically important and from this study we can conclude that *S. cordifolia* can be a better choice when compared to *Sida retusa* for the NK formulation. Classical drug ratios are very important rather than the ingredients in equal proportion.

5B.7. Conclusion

In summary, we have developed standardization protocol for the well-known Ayurvedic polyherbal formulation *Nayopayam kwatha* in terms of the marker phytoconsituents of ingredient herbs. NK formulations with different drug ratios were scientifically validated and analyzed in detail with respect to its phytochemistry. Characteristic features of the formulations and its individual drugs along with the authenticity of the ingredient herbs were well established through the pharmacognostic evaluation. Detailed phytochemical analysis of the plants was carried out to afford the major phytoconstituents. HPLC quantification of NK formulations based on the marker compounds resulted in the detection marker compounds in appreciable amounts thus contributing to the therapeutic efficacy of the formulation. *Nayopayam kwatha* with different drug ratio are having different profiles. Also there is a notable change was observed in the phytochemical profile of *kwatha* (decoction) by using two source plants for bala *i.e. Sida cordifolia* and *Sida retusa*. Present study also revealed that the classical

drug ratios of polyherbal formulation are of utmost important rather than the ingredients in equal proportion. The present study is the first report of standardization of NK based on the marker constituents in the individual herbs and the characterization parameters of individual herbs and decoction described in this study may serve as a standard reference for quality control analysis of NK. This may prove to be a remarkable contribution to the existing knowledge, especially in the field of quality control & standardization. The method developed for HPLC and GC-MS analysis in this study can be used as a reliable technique for standardization of *Nayopayam kwatha*.

5B.8. Experimental Section

5B.8.1. General Methods

All the chemicals, reagents were commercial pure and the best grade available, used as such for the study. Detailed description of the experimental methods for the phytochemical analysis and GCMS analysis were already described in previous chapters. Preliminary phytochemical evaluation, organoleptic evaluation, microscopic evaluation and physicochemical analysis were carried out according the previously reported methods & standard procedures in API [36]. HPLC quantification was performed in Agilent 1260 series HPLC system (Agilent Technologies, USA) comprising a quaternary pump, a vacuum degasser, a variable wavelength detector, a 20 µl sample injector and a column thermostat. The data were analyzed using open lab software.

5B.8.2. Preparation of NK formulations

Crushed roots of *bala (S. cordifolia* (B1)), coarsely powdered seeds of *jeeraka* (*C. cyminum*) and rhizomes of *nagara* (*Z. officinale*) were mixed together to make 48 g. 768 ml (16 times) of water was added to it. Temperature was maintained at 80-90 ^{0}C with the aid of a thermometer, on a gas stove and reduced the total volume into 96 ml (1/8th). Finally the decoction obtained was filtered through a 4 layered clean cloth. The same procedure was repeated with *bala* as *S. retusa* (B2) to afford two sets (NKB1 and NKB2) *kwatha* (decoction) in 3:2:1 (24g: 16g: 8g), 10:1:1(40g: 4g: 4g) and 1:1:1 (16g: 16g: 16g) drug ratios.

5B.8.3. Estimation of Marker Compounds

10 ml each of the decoction prepared as per the drug ratio was taken in a beaker and kept on water bath maintained at 80 ºC for 1 ½ - 2 ½ hours until a dry residue was obtained. The residue was dissolved in 10 ml methanol by continuously stirring with a glass rod for 20 minutes. The undissolved portion was filtered through Whatman filter paper no 1 and the clear solution used for the analysis. A stock solution was prepared by

dissolving 10 mg of the compound in 1ml methanol and further making it up to a 0.1 mg/mL solution. The detection wavelength, mobile phase and flow rate was selected according to the compounds (Table **5B.12**). The column thermostat was maintained at 25±1 ºC. 10 µl of stock solution was made up to 1 mL using mobile phase and 20 µl was injected using sampler injector. In case of sample solution of decoction, 20 µl was directly injected after filtration through 0.2 µm millipore membrane filter. The graph obtained with each of the sample was compared with that of the standard and the peak area was measured, which was plotted against concentration to estimate the weight percent of the compound.

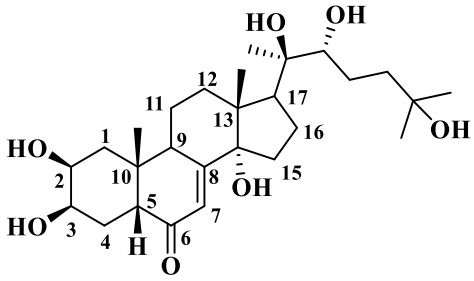
Table 5B.12: Different chromatographic conditions of marker compounds

Chromatographic conditions	Detection Wavelength (nm)	Mobile phase	Flow rate (mL/min)
Ecdysterone	254	Methanol : Water (1:1 v/v)	1.2
β-sitosterol	202	Acetonitrile : Water (95:5v/v)	2
Luteolin	260	Water : Methanol : Acetic acid (700:300:10 v/v)	1
6- gingerol	280	Methanol : 0.05% Ortho phosphoric acid in water (3:2 v/v)	1

5B.9. Spectral Data

Compound 71 [Ecdysterone]

Compound **71** was obtained from as a white solid from the fraction pool 14-16 after CC separation using 40% ethyl acetate-hexane. By comparing the spectral data of the compound with literature the compound was identified as naturally occurring steroid molecule ecdysterone.



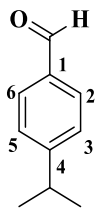
Molecular formula : $C_{27}H_{44}O_7$

Melting point : 160-164 ^{o}C

FT-IR (neat, ν_{max}, cm^{-1})	:	3450, 2950, 1660, 1450, 1340, 1030, 1015
^{1}H NMR (500MHz, CD_3OD)	:	δ 5.83 (d, *J*=2.5 Hz, 1H, H-7), 3.97 (d, *J*=1.54 1H, H-3), 3.88-3.84 (m, 1H, H-2), 3.34-3.32 (m, 1H, H-22), 3.24-3.16 (m, 1H, H-9), 2.43-2.38 (m, 2H), 2.18-2.12 (m, 1H), 2.03-1.97 (m, 2H), 1.91-1.89 (m, 1H), 1.85-1.79 (m, 3H), 1.77-1.70 (m, 3H), 1.67-1.56 (m, 3H), 1.47-1.42 (m, 2H), 1.234-1.25 (m, 1H), 1.23 (s,3H), 1.22 (s, 3H), 1.22 (s, 3H)0.99 (s, 3H), 0.91 (s, 3H) ppm
^{13}C NMR (125MHz, CD_3OD)	:	δ 209.0, 170.5, 124.7, 87.8, 81.0, 80.5, 73.8, 71.2, 71.1, 54.3, 53.1, , 44.9, 41.8, 39.9, 37.6, 35.4, 35.0, 34.3, 32.2, 31.5, 29.9, 26.9, 24.0, 23.6, 20.6 ppm
HR-ESIMS	:	503.2989 $[M+Na]^+$

Compound 74 [Cuminaldehyde]

Fraction pool 6-8 after CC on silica gel using 3% ethyl acetate-hexane led to the isolation of the compound **74** as colourless viscous liquid.^{1}H NMR displayed signals corresponds to aldehyde proton, methyl proton, -CH proton, 1, 4-disubstituted aromatic ring ^{13}C NMR of the compound was comprised of seven carbon resonances of carbonyl carbon of aldehyde (δ 192 ppm), -CH carbon (δ 34.5 ppm) and methyl carbons (δ 23.6 ppm) along with aromatic carbons. The mass spectrum of the compound gave a molecular ion peak of 149.2050 which is $[M+H]^+$ peak. Finally by analyzing the spectral data the compound was identified as cuminaldehyde.

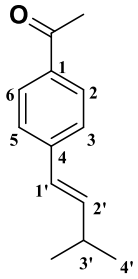


Molecular formula	:	$C_{10}H_{12}O$
^{1}H NMR (500MHz, $CDCl_3$)	:	δ 9.97 (s, 1H), 7.82 (d, *J*=8 Hz, 2H,H-2,6), 7.35 (d, *J*=8 Hz, 2H,H-3,5), 3.02-2.97 (m, 1H), 1.29 (s, 3H), 1.28 (s, 3H) ppm

^{13}C NMR (125MHz, $CDCl_3$)	:	δ 192.0, 156.2, 134.6, 130.0, 127.13, 34.5, 23.6 ppm
HR-ESIMS	:	149.2050 $[M+H]^+$

Compound 75 [(E)-1-(4-(3-methylbut-1-en-1-yl) phenyl) ethan-1-one]

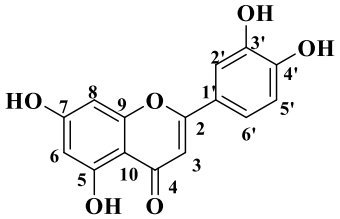
Compound **75** was afforded as colourless liquid from the fraction pool Fraction pool 9-13 after CC separation on silica gel using 5% ethyl acetate- hexane. Based on the analysis of spectral data the compound was identified as [(E)-1-(4-(3-methylbut-1-en-1-yl) phenyl) ethan-1-one.



Molecular formula	:	$C_{13}H_{16}O$
^{1}H NMR (500MHz, $CDCl_3$)	:	δ 7.51 (s, 1H), 7.47 (d, J = 7.5 Hz, 2H, H-2,6), 7.26 (d, J = 7.5 Hz, 2H, H-3,5), 6.68 (d, J = 16 Hz, 1H, H-1'), 2.95-2.91 (m, 1H, H-3') ppm
^{13}C NMR ($CDCl_3$)	:	δ 198.4, 151.9, 143.5, 132.1, 128.4, 127.1, 126.4, 34.1, 27.4, 23.7ppm
HR-ESIMS	:	189.2700 $[M+H]^+$

Compound 76 [Luteolin]

Column chromatographic separation of fraction pool 14-19 using 50% ethyl acetate-hexane eluent afforded compound as a yellow powder. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be flavone luteolin.

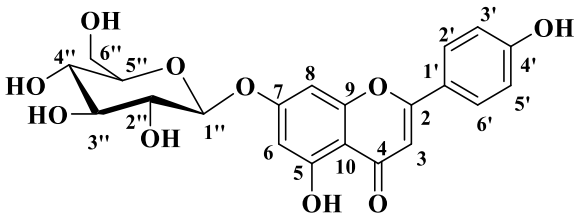


Molecular formula	:	$C_{15}H_{10}O_6$

Melting Point	:	273-278 ^{o}C
FT-IR (neat, ν_{max}, cm^{-1})	:	3420, 2919, 1618, 1508, 1454
^{1}H NMR (500MHz, CD_3COCD_3)	:	δ 13.04 (s, 1H, -OH-5), 9.64 (brs, 1H), 8.60 (brs, 1H), 7.52 (s, 1H), 7.49 (d, *J*=8.5 Hz, 1H), 7.02 (d, *J*=8.5 Hz, 1H), 6.60 (s, 1H, H-3), 6.54 (s, 1H), 6.27 (s, 1H) ppm
^{13}C NMR (125MHz, CD_3COCD_3)	:	δ 182.2, 164.3, 164.0, 162.5, 157.9, 149.2, 145.6, 122.9, 119.3, 115.7, 113.2, 104.5, 103.3, 98.8, 93.8 ppm
HR-ESIMS	:	287.05635 $[M+H]^+$

Compound 77 [apigenin-7-O-β-D-glucopyranoside]

Fraction pool 20-23 after CC separation using 90% ethylacetate-hexane led to the isolation of compound as yellow powder. Spectral data of the compound suggested the presence of a flavonoid glucoside. ^{1}H NMR displayed the peak corresponds to glucose moiety and flavone moiety. By analyzing the spectral data and in comparison with the literature reports the compound was confirmed as apigenin-7-O-β-D-glucopyranoside.

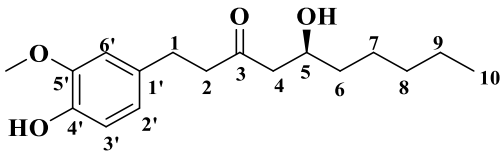


Molecular formula	:	$C_{21}H_{20}O_{10}$
Melting Point	:	230-237 ^{o}C
FT-IR (neat, ν_{max}, cm^{-1})	:	3450, 3445, 1645, 1640, 1600, 1495
^{1}H NMR (500MHz, DMSO-d_6)	:	δ 12.96 (s, 1H, -OH-5), 10.40 (brs, 1H), 7.95 (d, *J*=8.5 Hz, 2H), 6.95 (d, *J*=8.5 Hz, 2H), 6.87 (s, 1H, H-3), 6.84 (s, 1H), 6.45 (s, 1H), 5.40 (s, 1H), 5.13 (s, 1H), 5.07 (d, *J*=7 Hz, 1H), 4.62 (s,1H), 3.72-3.70 (m,1H), 3.50-3.44 (m,2H), 3.25-3.31 (m,2H), 3.19-3.18 (m,1H) ppm
^{13}C NMR	:	δ 182.5, 164.7, 163.4, 161.8, 161.6, 157.4,

(125MHz, DMSO-d_6)		129.1, 121.5, 116.5, 105.8, 103.6, 100.4, 95.3, 77.7, 76.9, 73.6, 70.1, 61.1 ppm
HR-ESIMS	:	433. 12711 $[M+H]^+$

Compound 78 [6-gingerol]

Compound **78** was afforded as brown coloured viscous liquid from the fraction pool 3-4 after CC on silica gel using 10-20% ethyl acetate-hexane. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and in comparison to the literature values the compound was confirmed to be 6-gingerol.



Molecular formula	:	$C_{17}H_{26}O_4$
^{1}H NMR (500MHz, $CDCl_3$)	:	δ 6.80 (dd, J_1 =8Hz, J_2 =2Hz, 1H, H-2'), 6.67 (s, 1H, H-6'), 6.63 (d, J= 8Hz, 1H, H-3'), 4.05-4.01 (m, 1H, H-5), 3.84 (s, 3H, -OCH_3), 2.83-2.80 (m, 2H, H-1), 2.74-2.71 (m, 2H, H-2), 2.57-2.48 (m, 2H, H-4), 1.38-1.35 (m, 2H, H-6), 1.28-1.23 (m, 6H), 0.87 (t, 3H, H-10) ppm
^{13}C NMR (125MHz, $CDCl_3$)	:	δ 211.3, 146.7, 144.0, 130.6, 120.6, 114.6, 111.2, 67.7, 55.8, 49.4, 45.4, 36.5, 31.7, 29.2, 25.1, 22.5, 13.9 ppm
HR-ESIMS	:	295.1831 $[M+H]^+$

5B.10. References

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ABSTRACT

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Faculty of Study	: **Chemical Sciences**	Year of Submission	: **2021**
AcSIR Academic Centre/CSIR Lab	: **CSIR-National Institute for Interdisciplinary Science & Technology (CSIR-NIIST), TVM, Kerala**	Name of Supervisor	: **Dr. K. V. Radhakrishnan**
Title of the thesis	: **Exploration of Bioactives from Rare and Endemic Species of Western Ghats for the Development of Potential Drug Lead against Infectious and Lifestyle Diseases**		

Natural products (NPs) offer great variety of bioactive secondary metabolites beneficial to mankind. Owing to their enormous bioactive potential secondary metabolites from natural resources were highly exploited in therapeutics against chronic diseases and still continue their legacy. In this scenario our efforts are directed towards the isolation and biological screening of secondary metabolites from rare and endemic wild nutmeg species of Agasthyamala Biosphere Reserve of Western Ghats.

Chapter 1 outlined a brief introduction about the major role of natural products in drug discovery and development with special emphasis on the treatment of lifestyle and infectious diseases. In addition the chapter also highlighted the phytochemical & pharmacological aspects of myristicaceae species of Western Ghats. In **Chapter 2** we have discussed the bioprospecting of two unexplored wild nutmeg species. In Part A we described the phytochemical evaluation & anti-proliferative potential of bioactives from *M. beddomei* wherein Part B discussed about the newly identified species *M. trobogarii* and its biological potential. In **Chapter 3,** Part A provided the chemoprofiling of *M. malabarica* & antidiabetic potential of malabaricones while Part B covered the phytochemical analysis of *M. fragrans* and study of antidiabetic potential of the lignans and neolignans. Exploration and utilization of a natural product Malabaricone B from myristicaceae species as a potent drug lead against bacterial infections was described in **Chapter 4**. In **Chapter 5**, part A outlined the chemoprofiling and antibacterial screening of traditional medicinal plant *H. capitata*. In part B we have developed standardization protocol for the well-known poly herbal Ayurvedic formulation *Nayopayam Kwatha* along with its scientific validation based on the marker phytoconstituents of the individual herbs was also achieved.

Details of the Publications Emanating from the Thesis Work

Published

1. **Neethu S**, Veena S. K, V. C. Indulekha, Jollykutty Eapen and K. V. Radhakrishnan, Phytoconstituents Assessment and Development of Standardization Protocol for 'Nayopayam Kwatha', a Polyherbal Ayurvedic Formulation (***Journal of Ayurveda and Integrative Medicine*, 2021*, 12* (3), 489-499**).
2. **Neethu S**, M. G. Govind, Vimalkumar P. S, Biji M, Sherin D. R, Mathew Dan and K. V. Radhakrishnan, Novel Flavonoids from the aerial parts of Unexplored and Endangered Wild nutmeg Species *Myristica beddomei* subsp. *spherocarpa* W.J. de Wilde (***Phytochemistry Letters*, 2021*, 45*, 72-76**).

Manuscript under Preparation

1. **Neethu S**, M. G. Govind, Anuja G. Joseph, Mathew Dan and K. V. Radhakrishnan, Chemotaxonomy of the newly identified species *Myristica trobogarii* Govind & M. Dan and its antiproliferative and antimicrobial Potential (**Manuscript Under Preparation**).
2. **Sivadas Neethu**, Grace Kaul, Manjulika Shukla, Murugan Govindakurup Govind, Mathew Dan, Sidharth Chopra and Kokkuvayil Vasu Radhakrishnan, Malabaricone B as a potent drug lead against Multi drug resistant *S. aureus* infections and its Synergistic Combination Studies with Conventional Antibiotics (**Manuscript Under Preparation**).
3. **Neethu S**, Vimalkumar P. S, M. G. Govind, Ajaikumar B. Kunnumakkara and K. V. Radhakrishnan, Malabaricones; natural phenylacylphenols from myristicaceae family as a potent therapeutic candidates against chronic diseases (**Review Under Preparation**).

List of Patents

1. Synergistic Combination of a Natural Product Malabaricone B and Conventional Antibiotics against Multidrug Resistant *Staphylococcus aureus* Infections, **Sivadas Neethu**, Kokkuvayil Vasu Radhakrishnan, Sidharth Chopra, Grace Kaul, Manjulika Shukla, Murugan Govindakurup Govind, Mathew Dan **(Patent filed on 1/10/2021**, **Patent No: 202111044825)**.

Papers Published from Other Related Works

1. B. Prabha, **S. Neethu**, S. Lekshmy Krishnan, D.R. Sherin, M. Madhukrishnan, R. Ananthakrishnan, K.B. Rameshkumar, T.K. Manojkumar, P. Jayamurthy, K.V. Radhakrishnan, Antidiabetic potential of phytochemicals isolated from the stem bark of *Myristica fatua* Houtt. var. magnifica (Bedd.) Sinclair, ***Bioorganic & Medicinal Chemistry***, 2018, 26, 3461–3467.
2. B. P. Dhanya, Greeshma Gopalan, Parameswaran Sasikumar, **Sivadas Neethu,** Murugan Thulasi Meenu, Puthiyaparambath Sharathna, Jubi John, Sunil Varughese, Mamiyil Sabu, Mathew Dan and Kokkuvayil Vasu Radhakrishnan, "Lewis Acid Catalysed Activation of Zerumbone towards Sesquiterpenoid Derivatives: Sustainable Utilisation of Natural Resources towards Chemically Diverse Architectures", ***Asian.J.Org.Chem.***, 2018, 7, 471–476. DOI: 10.1002/ajoc.201800006.
3. B. Prabha, S. Sini, D. R. Sherin, **S. Neethu**, K. B Rameshkumar, T. K Manojkumar, P. Jayamurthy, K. V. Radhakrishnan, Promalabaricone B from *Myristica fatua* Houtt. seeds demonstrate antidiabetic potential by modulating glucose uptake *via* the upregulation of AMPK in L6 myotubes, ***Nat Prod Res***., 2021, 35(5), 867-872. DOI: 10.1080/14786419.2019.1607852.
4. B. Prabha, S. Sini, D. R. Sherin, **S. Neethu**, M. G. Govind, Mathew Dan, T. K. Manojkumar, P. Jayamurthy and K. V. Radhakrishnan, Dactyloidin from *Myristica fatua* Houtt. seeds enhances glucose uptake through translocation of GLUT4 via upregulation of AMPK in L6 myotubes (***Accepted in Journal of Medicinal and Aromatic Plant Sciences***, 2021).

List of Conference Presentations

1. Isolation and characterization of triterpenoid type compounds from *Hyptis capitata*, **S. Neethu**, K. V. Radhakrishnan, Kerala Science Congress (29th KSC) organized by KSCSTE and KFRI at Mar Thoma College, Thiruvalla, Pathanamthitta, held on 28th to 30th January 2017 [**Poster presentation**].
2. *In vitro* Anitdiabetic Potential of Compounds from *Myristica fragrans*, *Myristica malabarica* and *Myristica fatua*, **S. Neethu**, B. Prabha, K. B. Rameshkumar and K. V. Radhakrishnan, 2nd International Conference on "Nutraceuticals and Chronic Diseases"(INCD 2017) organized by Indian Institute of Technology Guwahati

(IITG), Assam, India at Bogmallo, Goa, India from September 1-3, 2017 [**Poster presentation**].

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Original Research Article (Experimental)

Phytoconstituents assessment and development of standardization protocol for '*Nayopayam Kwatha*', a polyherbal Ayurvedic formulation

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ABSTRACT

Background: *Nayopayam kwatha* (NK) is a well-known polyherbal formulation widely used to cure respiratory ailments, heart problems, and postnatal difficulties. Literature suggests that so far no standardization protocol was developed for NK to validate its quality and purity.
Objective(s): To develop a standardization protocol for NK based on the marker phytoconstituents present in the individual herbs of the formulation.
Materials and methods: The roots of *bala* [*Sida cordifolia* (B1) and *Sida retusa* (B2)], seeds of *jeeraka* (*Cuminum cyminum*), and rhizomes of *nagara* (*Zingiber officinale*) were the ingredients of NK. Since there were two source plants for *bala*, two sets of NK (NKB1 and NKB2) were prepared in the ratio 3:2:1 as per *Vaidya Manorama* and 10:1:1 as per *Arogyaraksha Kalpadruma* along with 1:1:1 as per the general way of Ayurvedic polyherbal decoctions. Both the individual herbs and the *kwatha* (decoction) prepared were analyzed in terms of pharmacognostical, organoleptic, and physicochemical parameters as per the standard methods. Phytochemical analysis of the individual herbs resulted in the isolation of major phytoconstituents and the *kwatha* was quantified in terms of marker compounds with the aid of HPLC.
Results: HPLC quantification suggests that appreciable amount of marker phytoconstituents of individual herbs are present in the *kwatha*. Thus, the isolated compounds luteolin (*C. cyminum*), 6-gingerol (*Z. officinale*), β-sitosterol (*S. retusa*), and ecdysterone (*S. cordifolia*) can be used as markers to standardize NK.
Conclusion: Characteristics of NK, as well as its individual drugs, were well-established. The present study of NK with respect to its phytochemistry revealed that the classical drug ratios of the polyherbal formulation are of utmost importance rather than the ingredients in equal proportion. The characterization parameters of individual herbs and *kwatha* described in this study may serve as a standard reference for quality control analysis of NK and the method developed in this study can be used as a reliable technique for standardization of NK to ensure the purity and quality of raw drugs used.

1. Introduction

The science of Ayurveda has a rich traditional knowledge of drug combinations for almost all ailments [1–3]. Ayurvedic dosage forms are mostly polyherbal, which is more effective as compared to the single herb therapies. It is a therapeutic strategy to achieve the augmented therapeutic efficacy at a lower dosage [4,5]. Drug ratios of the individual herbs in an Ayurvedic formulation are of utmost importance in order to attain the desired curative effect [6]. Every formulation has its unique indications and contraindications which help to ensure its quality and safety and make them an ideal treatment of choice for excellent therapeutic effect. Most of these formulations exert their increased efficacy through the phytoconstituents present in the individual herbs. The synergistic (*sarvakarmaja*) activity of individual herbs in such formulations contributes much towards its efficacy [7]. It is amply documented

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that Ayurvedic medicines have adverse effects when formulated or used inappropriately. Hence, it is important to follow the standard protocols regarding the formulation and its uses. As a general principle, most dosage forms are polyherbal combinations of ingredients in equal proportions. However, there are only a few combinations in which the ingredients are mixed in particular proportions to achieve the specific curative effect.

Nayopayam kwatha (NK) is a renowned polyherbal formulation extensively used for all respiratory ailments (*swasa vikara*) especially for bronchial asthma (*thamaka swasa*), cardiac diseases, gas trouble, and postnatal care. It is a very good bronchodilator and carminative in actions. Several Ayurvedic textbooks describe the importance of NK including *Vaidya Manorama* [8] in the chapter *Kasa chiktsa* (treatment of cough) and *Arogyaraksha Kalpadruma* [9] in the chapter *Vata roga chiktsa* (diseases of *vata* origin). *Bala* (*Sida cordifolia*), *jeeraka* (*Cuminum cyminum*) and *nagara* (*Zingiber officinale*) constitute the ingredients of this *kwatha* (decoction). The roots of *S. cordifolia* Linn (B1) are taken as *bala* in Ayurveda Formulary of India (AFI) [10] as are those of *S. rhombifolia* ssp., and *S. retusa* Linn (B2) in Kerala [11]. Ancient Ayurvedic scripts explains different drug ratios of the individual herbs for this formulation to obtain specific therapeutic effects.

For the diseases related to '*vata*' the ingredients *bala* (*S. cordifolia*), *jeeraka* (*C. cyminum*), and *nagara* (*Z. officinale*) are in the ratio 3:2:1 as per *Vaidya Manorama,* and for the treatment of cough the ingredients are in the ratio 10:1:1 as per *Arogyaraksha Kalpadrum*. The reference *slokas* (verses) are cited as; "*Nayopaayayana mithe balajeerakanagarei kwatha peetha pramadhnaty sameerana balam balath*" (*Vaidya Manorama*); and "*Balayaam dasabhir bhage dwabhyaam jeeraka vishwayoo siddhakwatho nayopaya swasa hidma haram param*" (*Arogyaraksha Kalpadruma*). NK is indicated as *vatasamana* (pacification of *vata*) as per *Vaidya Manorama*, and for *swasa* (breathlessness) and *hikka* (hiccough) as per *Arogyaraksha Kalpadruma*.

It is important to ensure the efficacy, stability, and safety of a polyherbal formulation, standardization in terms of physicochemical properties, phytochemical screening of the individual herbs, and physical properties of the final formulation [12,13]. So far, no scientific validation protocol has been developed for NK-based on the marker compound present in the ingredient herbs. Hence, the present study was planned to assess the same. We attempted to develop separate profiles of these *kwathas* with standard markers isolated from the individual plant ingredients to provide leads for clinical research using ingredients in the needed ratios i.e., 3:2:1, 10:1:1, and 1:1:1.

2. Materials and methods

2.1. Collection and identification of plant materials

The ingredients of NK viz., *bala* (B1 - *S. cordifolia* and B2 - *S. retusa*), *jeeraka* (*C. cyminum*), and *nagara* (*Z. officinale*) were procured and authenticated for the study purpose (See Fig. 1). B1 was collected from Kanyakumari region during January-February and its root was cut off, cleaned, washed, and shade-dried. B2 was collected from Tamil Nadu region in the month of December and January, its root was cut off, cleaned, washed, and shade-dried. *Nagara* rhizomes (cultivated locally), were collected in the month of January, peeled, and dried in the sun for a period of 2 weeks. *Jeeraka* seeds were directly purchased from a cultivator in Gujarat state in the month of January. The source plants were authenticated by a botanist from Pharmacognosy unit, Govt. Ayurveda College, Poojappura and a voucher specimen was deposited in the herbarium of Govt. Ayurveda College, Poojappura, Thiruvananthapuram.

2.2. Methods

2.2.1. Macroscopic evaluation

The roots of B1 and B2, fruit of *jeeraka*, and rhizomes of *nagara* were subjected to organoleptic (including sensory) evaluation. The characters evaluated were dimensions, shape of pieces, outer surface, fracture, and odour.

2.2.2. Microscopic evaluation

The histological features of roots of B1 and B2, seeds of *jeeraka,* and rhizomes of *nagara* were analyzed in detail.

2.2.2.1. Microscopy of whole drug. Enough number of sections (T.S) of pre-soaked drug were taken and carefully transferred to a petri dish containing water using a fine paint brush. A few thin sections that floated in water were selected and transferred to a watch glass containing safranin stain. After 1 min, the sections were immersed in pure water to remove the excess satin and were thus ready, for mounting on a slide. A stained section was carefully transferred on a clean glass slide using thin paint brush. Two drops of glycerin was added on the section using a dropper and a clean coverslip was placed gently over the section. This slide was then placed on a microscope for examination and direct images were taken at 4x, 10x, and 40x magnifications.

2.2.2.2. Powder microscopy. Sufficient amount of coarsely powdered drug was mounted on a glass slide after mixing with glycerin. Ocular 10x and 40x objectives were used for all observations and diagnostic features were photographed.

2.2.3. Preliminary physicochemical evaluation

Preliminary physicochemical parameters included foreign matter, moisture content, ash value, volatile oil content, different extractive values, fiber content, and sugar content of roots of B1 and B2, *jeeraka* seeds, and *nagara* rhizomes were evaluated according to standard procedures in Ayurvedic Pharmacopoeia of India (API) [14].

2.2.4. Preliminary phytochemical evaluation

The alcoholic (100% ethanol) extract of the individual herbs (B1 and B2, *jeeraka, and nagara*) were subjected to qualitative analysis for the identification of various phytoconstituents including phenols, steroids, flavonoids, alkaloids, etc.

Fig. 1. Collected samples of **a)** *Sida cordifolia*, **b)** *Sida retusa*, **c)** *Cuminum cyminum* and **d)** *Zingiber officinale*.

2.2.5. Isolation and characterisation of major phytoconstituents

Major phytochemicals were isolated from the roots of B1 and B2, *jeeraka* seeds, and *nagara* rhizomes with the aid of gravitational column chromatography. Initially the coarsely powdered materials were subjected to extraction using ethanol (2.5 L x 3days). The extract was then filtered and concentrated under reduced pressure in a rotary evaporator to afford the crude ethanol extract. The crude ethanol extract was then subjected to repeated column chromatographic separation using silica gel (100–200 mesh) with hexane, hexane-ethyl acetate mixtures of varying polarity as eluent in order to obtain the compounds (Supporting information Figure S1, S6, S11 & S16). The isolated compounds were characterised on the basis of various spectroscopic technique.

2.2.6. Preparation of NK

Kwatha was prepared according to the standard procedures as per *Sarngadhara samhita* [15]. The *sloka* (verse) is sited below.

"*Paneeyam shodasa gunam kshunne dravyapalekshipeth*

Mrut patre kwathayeth grahyam astamamsha avasheshitam"

"One pala of coarsely powdered drug is boiled with 16 parts of water in an earthen pot, on mild fire till the required liquid is reduced to 1/8 of the original quantity. This liquid is known as shrta, qwatha, kasaya or niryuha (decoction)".

Crushed root of B1, coarsely powdered seeds of *jeeraka*, and rhizomes of *nagara* were mixed together to achieve a total of 48 g and 768 ml (16 times) of water was added to it. Temperature was maintained at 80–90 °C with the aid of a thermometer, on a gas stove and the total volume was reduced to 96 ml (1/8th). Finally, the decoction obtained was filtered through a 4-layered clean cloth. The same procedure was repeated with B2 to form two sets (NKB1 and NKB2) of *kwatha* in 3:2:1 (24g: 16g: 8g), 10:1:1 (40g: 4g: 4g), and 1:1:1 (16g: 16g: 16g) drug ratios.

The prepared sets of *kwatha* samples were analyzed with respect to the major compounds (β-sitosterol, ecdysterone, luteolin, and 6-gingerol) by analytical technique using HPLC [16–18].

2.2.7. Physicochemical analysis of NK

Different physical parameters of *kwatha* such as total solids, specific gravity, and pH were evaluated using standard pharmacopoeial methods.

2.2.8. Quantitative analysis using HPLC

HPLC analysis was carried out to quantify the major compounds β-sitosterol, ecdysterone, luteolin, and 6-gingerol in the *kwatha*. The analysis was carried out with Agilent 1260 series HPLC system (Agilent Technologies, USA) comprising a quaternary pump, a vacuum degasser, a variable wavelength detector, a 20 μl sample injector, and a column thermostat. The data were analyzed using open lab software.

2.2.8.1. Estimation of β-sitosterol. Ten millilitres each decoction prepared as per the drug ratio was taken in a beaker and kept on water bath maintained at 80 °C for 1.5 – 2.5 hours until a dry residue was obtained. The residue was dissolved in 10 ml methanol by continuously stirring with a glass rod for 20 min. The undissolved portion was filtered through Whatman filter paper no 1 and the clear solution was used for the analysis. A stock solution of β-sitosterol was prepared by dissolving 10 mg of standard β-sitosterol (isolated from the plant extract) in 1 ml methanol and further making it up to a 0.1 mg/mL solution. The detection wavelength was set at 202 nm. The mobile phase consisted of acetonitrile water (in a ratio of 95:5v/v) at a flow rate of 2.0 mL/min. The column thermostat was maintained at 25±1 °C. A stock solution of 10 μl was made up to 1 mL using mobile phase and 20 μl was injected using sampler injector. In case of sample solution of decoction, 20 μl was directly injected after filtration through 0.2 μm millipore membrane filter. The graph obtained with each of the sample was compared with that of the standard and the peak area was measured, which was plotted against concentration. The concentration of β-sitosterol in samples was estimated based on this.

The same procedures were followed for the estimation of ecdysterone, luteolin and 6-gingerol. Different chromatographic conditions of each are given in Table 1.

2.2.9. GC Chromatography-Mass Spectroscopy (GC-MS) analysis of kwatha

Ten millilitres of each *kwatha* were taken in a beaker and kept on a water bath maintained at 80 °C for 1.5–2.5 hours until a dry residue was obtained. The residue was dissolved in 10 ml methanol by continuously stirring with a glass rod for 20 min. The undissolved portion was filtered off through Whatman no.1 filter paper, and the clear solution was used for analysis.

The GC-MS phytochemical profiling was performed using GCMS-TQ8030 Shimadzu instrument. One microlitre of sample was injected to a GC, equipped with a MS and a medium polar capillary column Rxi-5Sil MS (30 m × 0.25 mm I. D, 0.25 μm). The oven program had an initial temperature of 60 °C for 2 min, which was then increased to 200 °C for 2 min at the rate of 5 °C per minute, which then increased to 220 °C for 1 min at the rate of 3 °C/min. Finally, the temperature was increased to 250 °C at the rate of 6 °C/min for 7 min. The total run time was 50 min. The detector temperature and the injection temperature were 250 °C, and helium was the carrier gas with purity 99.999% at a flow rate of 1 mL/min. The sample was injected in the split-less mode. The ion energy used for the electron impact ionization (EI) mode was 70 eV. The mass *m*/*z* was scanned for a range of 100–1000. The essential chemical constituents were identified by matching mass spectra with spectra of reference compounds in mass spectral library of NIST and WILEY. The relative amounts of individual components were expressed as percentage peak areas relative to total peak area.

3. Results

3.1. Macroscopic evaluation

Macroscopic evaluation of the individual herbs of NK is summarized in Table 2. The data obtained was in good agreement with

Table 1
Different chromatographic conditions of marker compounds.

Chromatographic Conditions	Ecdysterone	β-sitosterol	Luteolin	6- gingerol
Detection Wavelength (nm)	254	202	260	280
Mobile phase	Methanol: Water (1:1 v/v)	Acetonitrile: Water (95:5 v/v)	Water: Methanol: Acetic acid (700:300:10 v/v)	Methanol: 0.05% Ortho phosphoric acid in water (3:2 v/v)
Flow rate (mL/min)	1.2	2	1	1

Table 2
Organoleptic evaluation of the individual herbs.

Characters	*S. cordifolia* (B1) root	*S.retusa* (B2) root	*C. cyminum* seeds	*Z. officinale* rhizome
Dimensions	Length varying from 7 to 10 cm	Length varying from 7 to 14 cm	5 mm long	5–7 cm long
Shape	Long, cylindrical tortuous	Cylindrical, branched tortuous	Elongated, thick in the middle and laterally compressed	Fairly, smooth, laterally compressed bearing many branches
External surface	Smooth, with some thin rootlets	Numerous lateral rootlets are present	5 lines are present	Longitudinally striated
Fracture	Fibrous	Fibrous		Short
Colour	Pale yellow	Pale yellow	Yellowish brown	Yellowish brown to greyish brown colour
Odour	No characteristic odour	No characteristic odour	Aromatic	Characteristic
Taste	Slightly bitter	Slightly bitter	Slightly pungent	Pungent

Table 3
Histological features of the individual herbs.

Plant material	Microscopical characters of transverse section	Characters of powder microscopy
Bala (S. cordifolia) (B1)	Cork-thin walled tangentially elongated cells. Phellogen-a single layer of narrow, thin walled tangentially elongated cells. Cortex-made up of parenchymatous cells. Calcium oxalate crystals and starch grains are present. Secondary phloem-occurs in conical strands. Medullary rays-many, long, uniseriate, extending and reaching up to the cortex in a straight course	Lignified fibers with parenchyma, Calcium oxalate crystals in the parenchyma cells. Starch grains are also seen in the parenchyma
Bala (S. retusa) (B2)	Cork - thin walled, rectangular tangentially elongated cells. Features of phellogen and cortex are similar to *S.cordifolia*. Secondary phloem – appears in cortical strands and are much narrower and linear than in *S. cordifolia*	Similar to *Sida cordifolia*
Jeeraka (C. cyminum)	Epicarp - composed of a layer of colorless cells, with thin walls and a faintly and irregularly striated cuticle. Underlying the epicarp the thin-walled cells of the palisade are seen Endocarp - composed of a layer of fairly large, thin-walled cells. The endosperm - composed of moderately thick-walled cells containing aleuronic microrosette crystals of calcium oxalate	Presence of aleurone grains, surface cells and some calcium oxalate crystals
Nagara (Z.officinale)	Cork-consists of irregularly arranged, tangentially elongated, slightly brown-colored cells. Cortex-contains thin-walled polygonal parenchymatous tissue. Contains suberised oil cells, which holds yellowish-brown oleoresin. The inner cortex contains closed, collateral vascular bundles. Endodermis - characterized by a single layered pericycle. Vascular bundles of stele resemble that of cortex with the exception of a ring of small scattered bundle within the pericycle. Yellow polygonal oleo-resin cell are also present in the cortical region.	Presence of thin walled non lignified fibres, simple starch grains, oleoresin cells and reticulately thickened vessels

the literature which in turn proves the genuinity of the plant material chosen for the study.

3.2. Microscopic evaluation

Histological features of roots of B1 and B2, seeds of *jeeraka*, and rhizomes of *nagara* and their powder characters are given in Table 3. Trans- section of each herb and its powder microscopy images are given in Figs. 2 and 3.

3.3. Preliminary physicochemical evaluation

Preliminary physicochemical evaluations of the individual plants are tabulated in Table 4.

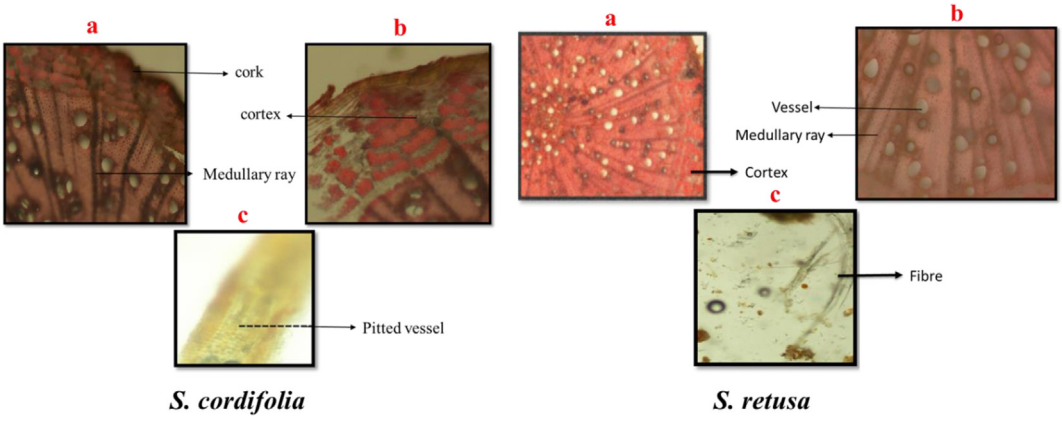

Fig. 2. T.S (a, b) and powder microscopy (c) of *bala*.

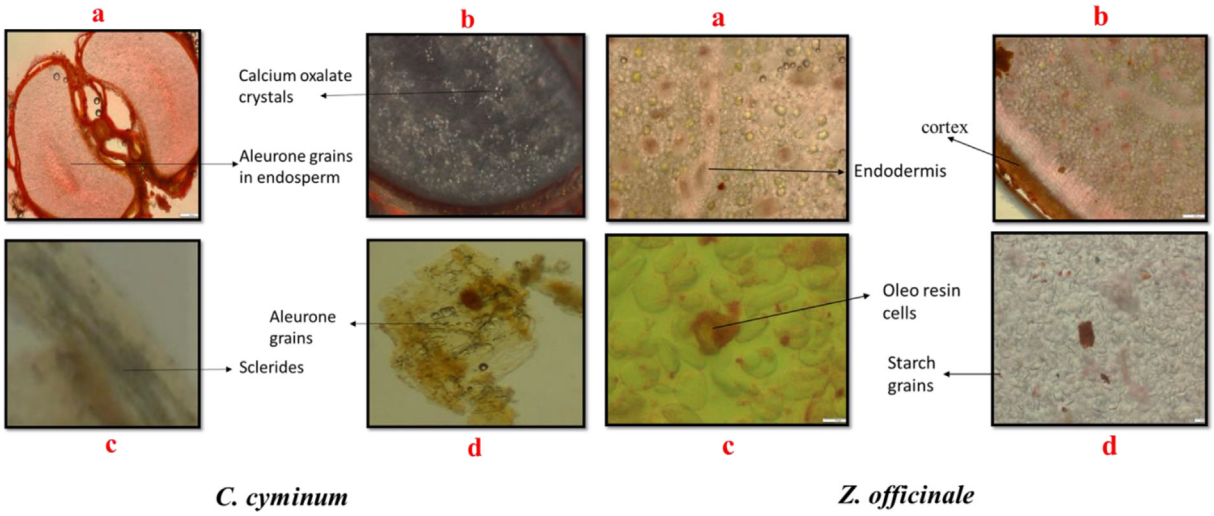

Fig. 3. T.S (a, b) and powder microscopy (c, d) of *jeeraka* and *nagara*.

3.4. Preliminary phytochemical analysis

Preliminary phytochemical analysis of alcoholic extracts of root of B1 and B2, seeds of *jeeraka*, and dried rhizomes of *nagara* revealed the presence of different phytochemicals. The results are given in Table 5.

3.5. Isolation of major phytochemicals

3.5.1. S. cordifolia (B1)

Ethanol extract of the dried and milled roots of B1 was subjected to separation and purification using column chromatography to obtain *β*-sitosterol-*β*-D-*glucopyranoside* and ecdysterone. Structures of the isolated compounds are shown in Fig. 4 and the spectral details of the compounds are depicted in supporting information (S2–S5).

3.5.2. S. retusa (B2)

Ethanol extract of the dried and powdered roots of B2 after repeated column chromatographic separation yielded two common phytosterols such as *β*-sitosterol and stigmasterol. Structures of the isolated compounds are shown in Fig. 5 and the detailed spectral data of the compounds are given in supporting information (S7-S10).

3.5.3. C. cyminum

Four compounds were isolated from the ethanol extract of the seeds after repeated column chromatographic separation include cuminaldehyde, leuteolin, 1-(4-(3-methylbut-1-en-1-yl) phenyl) ethan-1-one and apigenin-7-O-glucoside. Structures of the isolated compounds are shown in Fig. 6 and the detailed spectral data of the compounds are given in supporting information (S12-S15).

Table 5
Preliminary phytochemical evaluation of the individual plants.

Experiment	*S. cordifolia (B1)*	*S. retusa (B2)*	*C. cyminum*	*Z. officinale*
Alkaloids	+	+	+	+
Flavonoids	-	-	+	+
Saponins	-	-	+	+
Tannins	+	+	-	+
Phenols	-	-	+	-
Steroids	-	-	+	+

3.5.4. Z. officinale

Acetone extract of the dried and milled rhizomes of *Z. officinale* yielded 8-shogaol and 6-gingerol as the major compounds after column chromatographic purification. Structures of the isolated compounds are shown in Fig. 7 and the detailed spectral data of the compounds are given in supporting information (S17-S20).

3.6. Physicochemical analysis of NK

The specific gravity of the *kwatha* was found to be 1.00 for all the six samples. The pH was found to be around 6–7 and total solids were 0.04, 0.06, 0.09, 0.1, 0.03 and 0.05 for NKB1-3:2:1, NKB2-3:2:1, NKB1-10:1:1, NKB2-10:1:1, NKB1-1:1:1and NKB2-1:1:1 respectively. The results are given in Table 6.

Table 4
Preliminary physicochemical evaluation of the individual plants.

Parameters	*S. cordifolia* (B1)	*S. retusa (B2)*	*C. cyminum*	*Z. officinale*
Foreign matter	0.5%	0.3%	2%	1%
Moisture	10.01%	9.03%	8.4%	9%
Total ash	1.45%	1.34%	8%	6%
Acid insoluble ash	0.645%	0.541%	1%	1.5%
Alcohol soluble extractive	2.012%	1.076%	7%	3%
Water soluble extractive	4.731%	4%	15%	10%
Fiber content	44%	46.02%	5.06%	8%
Total sugar	1.24%	1.78%	0.08%	1.17%
Reducing sugar	0.543%	0.572%	_	0.09%

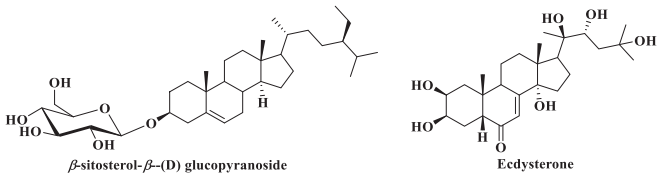

Fig. 4. Structures of the isolated compounds from *S. cordifolia* (B1).

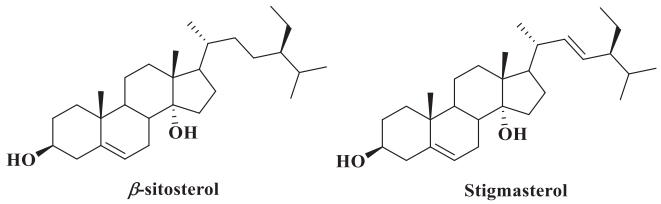

Fig. 5. Structures of the isolated compounds from *S. retusa (B2).*

3.6.1. Quantitative analysis using HPLC

Estimation of the compounds such as β-sitosterol, ecdysterone, luteolin, and 6-gingerol in different ratios of NK were carried out with the aid of HPLC and the results were statistically analyzed and plotted in Fig. 8. Three *kwatha* samples were taken for the quantification of β-sitosterol (NKB2-3:2:1, NKB2-10:1:1 and NKB2-1:1:1) and ecdysterone (NKB1-3:2:1, NKB1-10:1:1 and NKB1-1:1:1). Amount of β-sitosterol was found to be higher in the *kwatha* sample NKB2 in the ratio 3:2:1 and comparatively lower in NKB2-10:1:1 whereas no peaks were detected for NKB2 in the ratio 1:1:1. The *kwatha* sample (NKB1) of the ratio 10:1:1 showed significant amount of ecdysterone when compared to the other two samples. This may be due to the more % w/w of B1 in this proportion. Luteolin was quantified in two sets of three samples in the ratio 3:2:1, 10:1:1 and 1:1:1 with both B1 and B2. Among the *kwathas* made using B2 as the source plant of *bala*, in the ratio 10:1:1 contains significant amount of luteolin while the *kwathas* made using B1 as the source plant of *bala*, the ratio 3:2:1 contains more amount of luteolin. 6-gingerol was also quantified in six samples (three ratios with B1 and B2 are the source plant of *bala*). Amongst the % w/w of 6-gingerol was more in the ratio 1:1:1 both the sample NKB1 and NKB2. Statistical analyses of these four compounds revealed all were found to be significant at a P value < 0.001.

3.6.2. GC–MS analysis

The methanol extract of all the six *kwatha* samples (NKB1-3:2:1, NKB1-10:1:1, NKB1-1:1:1, NKB2-3:2:1, NKB2-10:1:1, NKB2-1:1:1) were subjected to GC-MS analysis. The essential chemical constituents were identified by matching mass spectra with spectra of reference compounds in mass spectral library of NIST and WILEY. The results are given in the following sections.

3.6.2.1. Kwatha NKB1-3:2:1. The chromatogram corresponds to 29 peaks (Fig. 9). The major compounds identified by the chromatogram include gingerol, benzamide, 4-(1-methylethyl)-, stigmasta-5, 22-dien-3-ol (3.beta. 22E) - etc.

3.6.2.2. Kwatha sample of NKB1-10:1:1. The chromatogram corresponds to 31 peaks (Fig. 10).The major compound identified were benzamide, benzoic acid and gingerol.

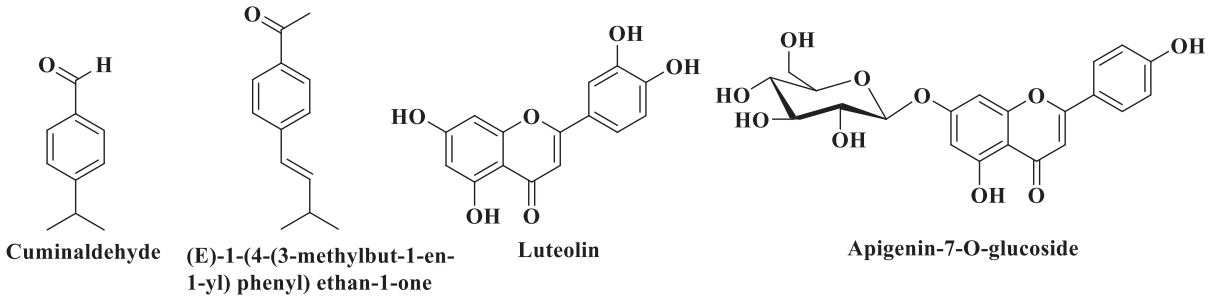

Fig. 6. Structures of the isolated compounds from *C. cyminum*.

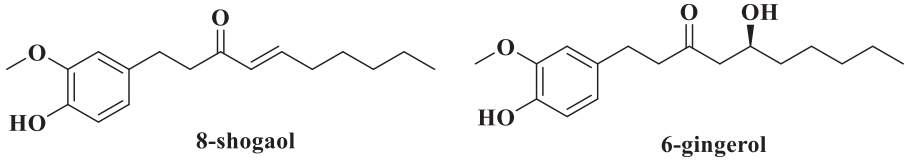

Fig. 7. Structure of the isolated compound from *Z. officinale*.

Table 6
Physicochemical analysis of NK.

Parameter	NKB1-3:2:1	NKB2-3:2:1	NKB1-10:1:1	NKB2-10:1:1	NKB1-1:1:1	NKB2-1:1:1
Specific gravity	1.02	1.05	1.04	1.08	1	1.03
pH	6.56	6.52	6.8	6.65	6.3	6.2
Total solids (g/ml)	0.04	0.06	0.09	0.1	0.03	0.05

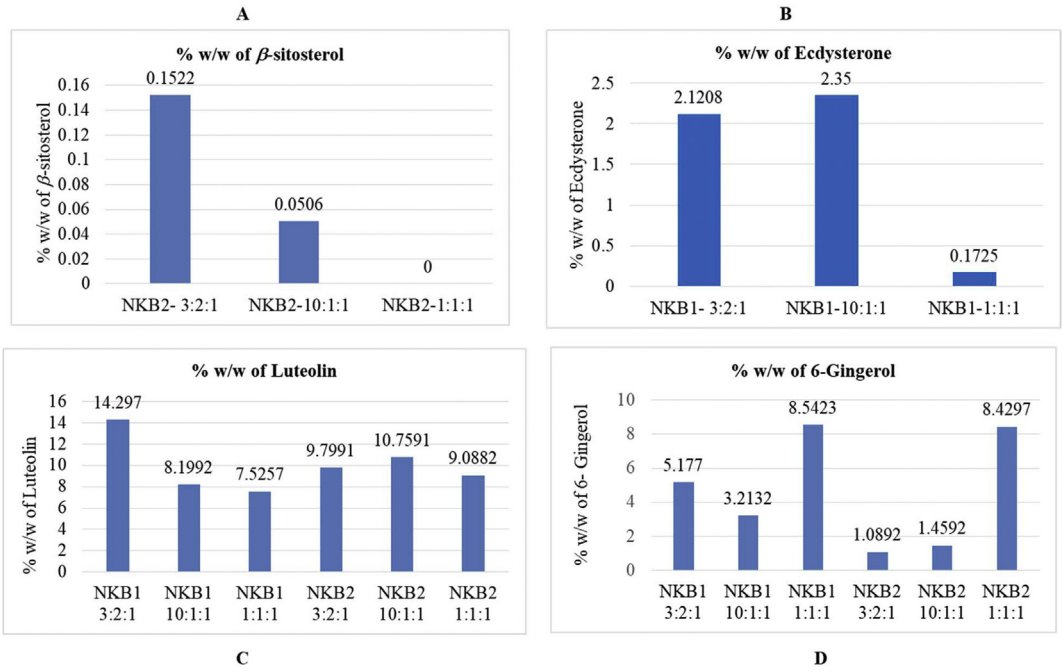

Fig. 8. Mean quantities of the major compounds present in NK.

3.6.2.3. Kwatha NKB1-1:1:1. The chromatogram corresponds to 29 different peaks (Fig. 11). Benzene, gingerol, 2-butanone etc. were identified as the major compounds.

3.6.2.4. Kwatha NKB2-3:2:1. The chromatogram corresponds to 30 different peaks (Fig. 12). 6-Octadecenoic acid and 1, 2-benzenedicarboxylic acid was the major compounds obtained.

3.6.2.5. Kwatha NKB2-10:1:1. The chromatogram corresponded to 30 different peaks (Fig. 13). The major compounds obtained were 9-octadecenoic acid, 12-hydroxy-, methyl ester-2-methoxy-4-vinylphenol, p-cymen-7-ol etc.

3.6.2.6. Kwatha NKB2-1:1:1

The chromatogram corresponds to 30 different peaks (Fig. 14). The major compounds obtained were benzene, benzamide, 2-methoxy-4-vinylphenol, gingerol etc.

4. Discussion

Standardization of herbal formulations is inevitable in order to ensure its purity, quality, safety, and efficacy. Herb-based formulations are widely employed to cure various ailments on account of their higher efficacy, safety, and cost effectiveness. One of the major challenges associated with herb-based formulations is its

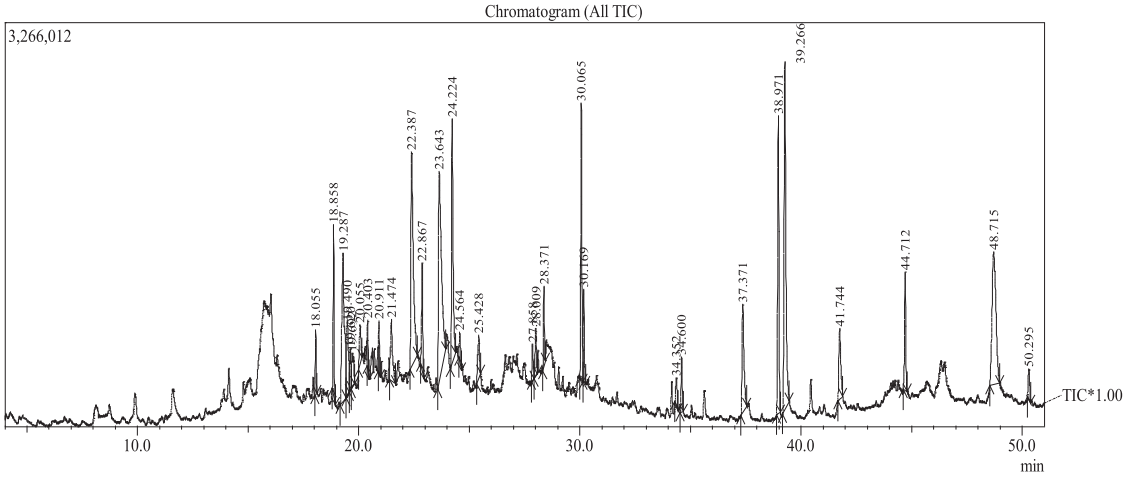

Fig. 9. GC–MS chromatogram of NKB1 3:2:1.

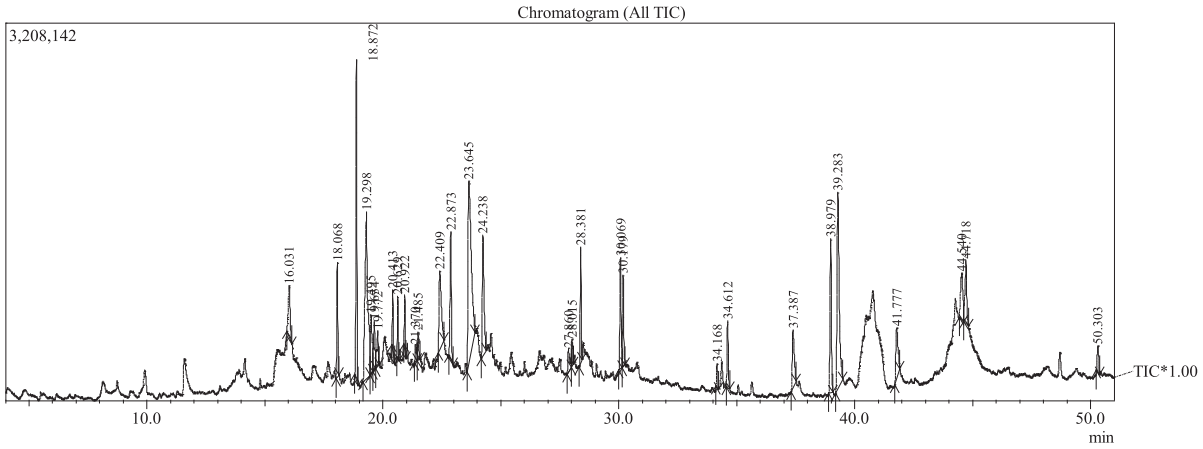

Fig. 10. GC–MS chromatogram of NKB1- 10:1:1.

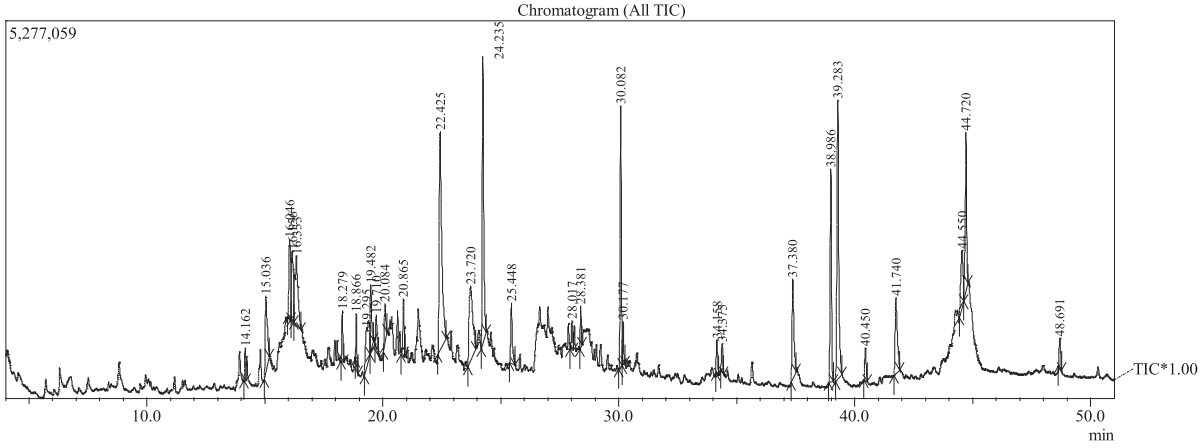

Fig. 11. GC–MS chromatogram of NKB1-1:1:1.

acceptability in modern medicine. Most of the formulations available today in the market have no standardization protocols in terms of marker constituents of the individual herbs used in it. There are some accepted WHO guidelines for the standardization of polyherbal formulations on the basis of several parameters including organoleptic properties, physicochemical analysis, preliminary phytochemical evaluation, and microscopic and macroscopic evaluation of individual plant species used [19]. There is an urgent need to validate basic principles as well as drugs used in the Ayurvedic system of medicine with the help of advanced techniques. In this regard, our efforts are directed towards the development of standardization protocols for the well-known polyherbal formulation NK based on the marker phytoconstituents of the individual herbs.

Ayurveda relies on herbal drugs for treatment when compared to other streams of medicine. These herbal products are manufactured primarily from crude drugs. Hence, the quality and purity of crude drugs is an important factor that determines the efficacy of the final product. As a starting point initially the quality of the *kwatha* was analyzed through organoleptic evaluation of individual ingredients used as well as prepared formulations. It revealed that brownish yellow colour, characteristic odour, liquid consistency, and astringent taste were the characteristic features of NK. *Kwatha* samples prepared with different ratios did not show significant differences in organoleptic evaluation apart from the slight colour changes. To ascertain the genuineness of the samples collected, pharmacognostical evaluation was carried out and was compared with the available literature [20]. Organoleptic evaluation of the individual plant species of *kwatha* (*bala*, *jeeraka* and *nagara*) exhibited characteristic features of the plants in terms of dimensions, external surface, fracture, shape, color, odor, and taste. In addition, histological features of the plant parts (roots, seeds and rhizomes) and their powder characteristics were analyzed through compound microscope. Macroscopic evaluation of *bala* revealed that both the roots were cylindrical, tortuous and B2 contains more rootlets than B1. Scent of cumin seeds is the main feature observed and is identical with the features described in pharmacognosy textbook [11,14]. Thus, the collected raw drugs proved to be authentic. In addition, these results can be used to identify the right variety and the adulterants used in formulations. Preliminary physicochemical evaluation based on the parameters such as foreign matter content, moisture content, fiber content, sugar content, total ash, acid insoluble ash, alcohol/water soluble extractive etc. revealed the features of the plant species and are in compliance with the standard values in API. In our analysis, the total fiber content of B1 and B2, *C. cyminum,* and *Z. officinale* was 44%, 46.02%, 5.06%, and 8% respectively. The total sugar content of

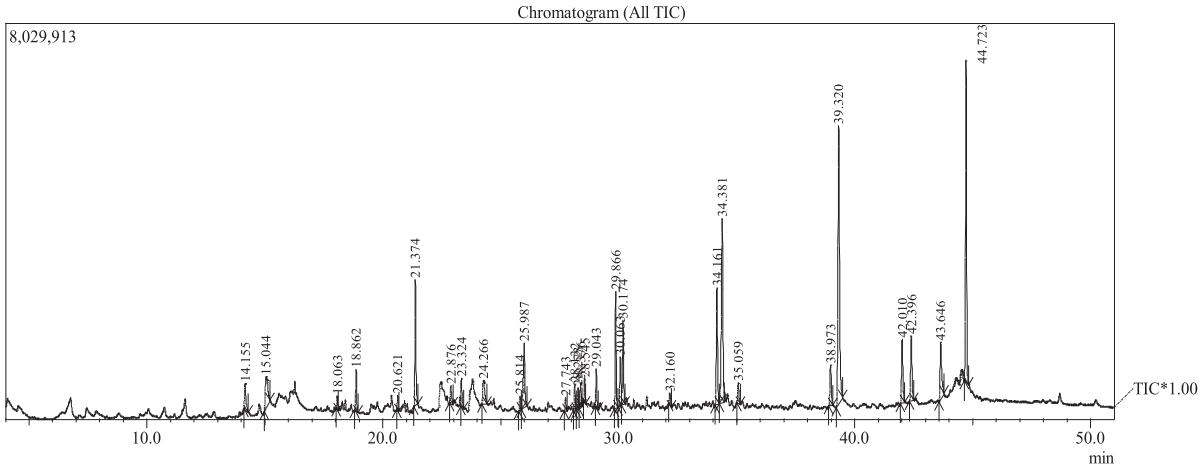

Fig. 12. GC–MS chromatogram of NKB2 - 3:2:1.

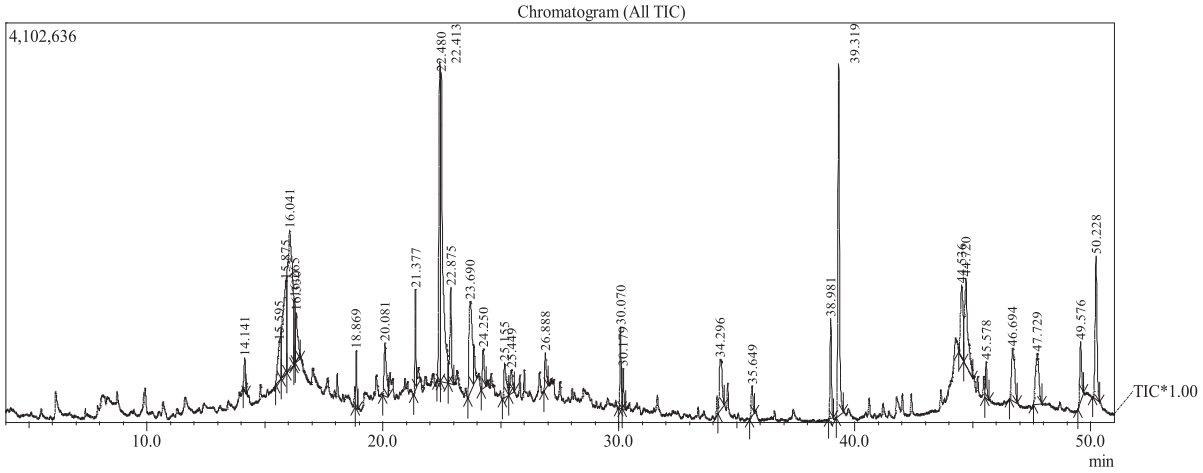

Fig. 13. GC–MS chromatogram of NKB2- 10:1:1.

these drugs was 1.24%, 1.78%, 0.08% and 1.17% respectively. B2 was found to contain maximum fiber and sugar content. The quality and authenticity of the plant species in an Ayurvedic formulation contributes much towards the safety, effectiveness, and acceptability. These basic data obtained can be used for future reference to ensure the authenticity of the plants and to trace out the presence of adulterants. Qualitative analysis of the methanol extract of the ingredients of NK revealed the presence of alkaloid, tannin in *bala;* alkaloid, flavonoid, phenol, steroid, and saponin in *jeeraka*; and steroid, flavonoid, alkaloid, tannin, and saponin in *nagara* and these findings are in accordance with the API standards. Standardization of formulation and its ingredients based on specific marker compounds and its validation is very important. In order to find out the marker compounds of the individual herbs of NK, a detailed phytochemical analysis was carried out. Isolation was carried out with the aid of column chromatography. Alkaloid separation technique was done for getting reported pharmacologically active alkaloids like ephedrine, vasicine, vasicinol etc from B1 and B2 which suggested the presence of these alkaloids in a minimal amount. During the running of column chromatography of cumin, almost all fractions had a smell of cumin. Many of the preliminary fractions contained oil. Gingerol obtained fractions while doing column chromatography created some burning sensation to eyes when kept nearby. Pungency of *Z. officinale* may be due to this compound. The compounds isolated in maximum quantities (major compounds) from each drugs include ecdysterone from B1, β-sitosterol from B2, luteolin from *C. cyminum*, 6-gingerol from *Z. officinale*. Presence of these reported compounds adds to the authenticity of the drugs.

These major compounds were used for comparative quantitative analysis of *N. kwatha*. Six different *kwatha* samples was prepared for the study, based on the standard procedures as per *Sushruta samhita* [15] and these *kwatha* (decoction) were subjected to physicochemical analysis based on its physcicochemical parametres such as specific gravity, pH, and total solids. Specific gravity of the *kwatha* prepared in three ratios was found to be around 1. The formulation NKB2 in the ratio 10:1:1 showed highest specific gravity and NKB1-1:1:1 having lowest specific gravity. pH of the *kwatha* was in an acceptable range for all formulations and was found to be around 6.2–6.8. Altering the drug ratio has no effect on its pH and specific gravity. The sample NKB2 in the ratio 10:1:1 was found to contain more amounts of total solids whereas NKB1 in the ratio 1:1:1 having least amount of total

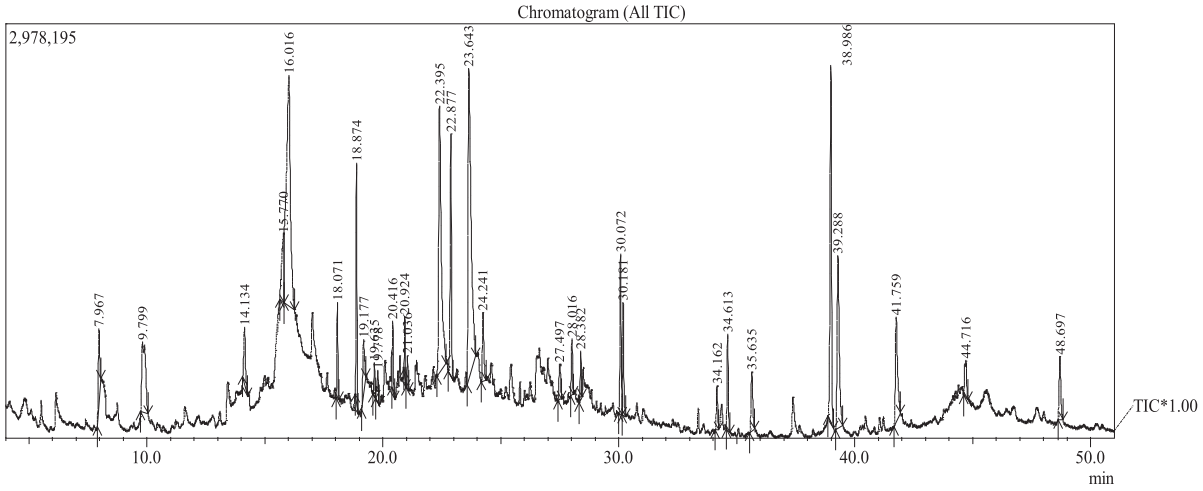

Fig. 14. GC–MS chromatogram of NKB2-1:1:1.

solids. It was found that, altering the ratio of drugs has a slight effect on the total solid contents of NK. These physciochemical parameters will serve as reference for future analysis and furnish information to identify the quality of the formulation.

Quantitative analysis of NK, based on the markers was done using HPLC and identification of volatile components was achieved through GC–MS analysis. Methanol extract of the *kwatha* was chosen for the analysis because it has a polarity similar to that of water. Three *kwatha* samples were taken for the quantification of ecdysterone (NKB1-3:2:1, NKB1-10:1:1, NKB1-1:1:1) and β-sitosterol (NKB2-3:2:1, NKB2-10:1:1, NKB2-1:1:1). The amount of ecdysterone was found to be more in NKB1-10:1:1. This may be due to the more %w/w of *bala* in this proportion. Amount of β-sitosterol was found to be more in NKB2- 3:2:1 which was unexpected. No peaks were detected for NKB2-1:1:1. Among the *kwathas* made using B2, NKB2-10:1:1, contained more amount of luteolin while the *kwathas* made using B1, NKB1-3:2:1, contained more amount of luteolin. Among the six *kwathas*, the % w/w of 6- gingerol was more in NKB1-1:1:1. From the area percentage of standard 6-gingerol was 823525.47 and area percentages of NKB1-1:1:1, NKB2-3:2:1 and NKB2-1:1:1 were more than the standard. This suggests that the amount of 6-gingerol increased in NK than the extract. NKB2-1:1:1 and NKB2- 1:1:1 contained more amount of gingerol than in other samples. Statistical analyes of these four compounds, showed significant at a P value < 0.0001. By analyzing the chromatogram, precise peaks were obtained for luteolin and 6-gingerol.

Pharmacological/therapeutic action of NK can be attributed to the bioactive phytoconstituents present in the individual herbs of the formulation. The constituent *Z. officinale* and its active component 6-gingerol are known to possess potential activity against respiratory disorders and cardiovascular diseases [21]. In our study, it is evident that NK in all drug ratios contains 6-gingerol in appreciable amount and substantially higher in the drug ratio 1:1:1 and there is a notable variation in B1 and B2. Literature suggests that flavonoids including luteolin are beneficial in controlling various respiratory diseases and having anti-allergy potential [22]. Luteolin is also found to present in all drug ratio of NK in significant amount. Thus, these two compounds contribute much towards the therapeutic potential of NK against respiratory problems. In addition, ecdysterone was present in higher amount in the drug ratio of 10:1:1 of NK when B1 was used, in accordance with its weight percent in the ratio. However, in the case of B2, significant amount of β-sitosterol was observed in the drug ratio 3:2:1 and not detected in the ratio 1:1:1 which is unusual. Both ecdysterone and β-sitosterol are pharmacologically important and from this study, we can conclude that B1 can be a better choice when compared to B2 for the NK formulation. Classical drug ratios are very important rather than the ingredients in equal proportion [23,24].

GC–MS analysis of methanol extract of the six *kwathas* was done in qualitative manner in order to find out the volatile components of the formulation. As the technique of GC–MS has limitations in identifying the compound based on the chemical nature, all the compounds were not detected. A total of 29 compounds from NKB1-3:2:1, 31 from NKB1- 10:1:1, 29 from NKB1-1:1:1, 30 from NKB2-3:2:1, 30 from NKB2- 10:1:1, 30 from NKB2-1:1:1. All *kwathas* contain gingerol as a common compound in different percentages. The results will serves as a reference for future studies.

5. Conclusion

NK was analyzed in detail in order to explain the rationale of the particular proportion of ingredients with respect to its phytochemistry. The *kwatha* was characterized in terms of organoleptic evaluation and physcicochemical analysis. Brownish yellow colour, characteristic odour, liquid consistency, and astringent taste were observed as the characteristic features of NK. These preliminary quality parameters can be used for the quality assurance of the formulation as a reference. Physcicochemical parameters of the *kwatha* revealed that altering the drug ratio has no effect on its pH and specific gravity while it will affect the presence of total solids. Authenticity of the individual herbs in the *kwatha* was examined through pharmacognostic, physicochemical, and phytochemical analysis. Ecdysterone from *S. cordifolia*, β-sitosterol from *S. retusa*, luteolin from *C. cyminum,* and 6-gingerol from *Z. officinale* were identified as the marker compounds and were used for HPLC quantification. According to the HPLC analysis, presence of these compounds were identified in the *kwatha* sample in appreciable amount (except the non-detection of β-sitosterol in NKB2-1:1:1), thus contributing to the therapeutic efficacy of the *kwatha*. While analyzing HPLC, luteolin and gingerol were found to be present in more quantities (%w/w) which contribute to its pharmacological action. NK with different drug ratios has different profiles. Also, a notable change was observed in the phytochemical profile of *kwatha* by using two source plants for *bala i.e. S. cordifolia* and *S. retusa*. The present study is the first report of standardization of

NK based on the marker constituents in the individual herbs. This may prove to be a remarkable contribution to the existing knowledge, especially in the field of quality control and standardization. The method developed for HPLC and GC–MS analysis in this study can be used as a reliable technique for standardisation of NK to ensure the purity and quality of raw drugs used.

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Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaim.2021.05.002.

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Novel Flavonoids from the aerial parts of Unexplored and Endangered Wild nutmeg Species *Myristica beddomei* subsp. *spherocarpa* W.J. de Wilde

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ABSTRACT

Chemoprofiling of the aerial parts of the unexplored wild nutmeg species *M. beddomei* subsp. *Spherocarpa* W.J. de Wilde led to the isolation of two new flavonoids, including an isoflavone 5,7-dihydroxy-3-(5'-hydroxybenzo[d](7',9')-dioxol-1'-yl)-4H-chromen-4-one (**1**) and a *β*-hydroxydihydrochalcone; (3(S)-hydroxy-3-phenyl-1-(2',4',6'-trihydroxyphenyl)propan-1-one (**2**) along with eleven known compounds (**3-13**) for the first time. The chemical structures of the isolates were elucidated by various spectroscopic analyses (1D and 2D NMR, HR-ESI-MS and IR). Chemotaxonomic significance of the isolates was discussed in detail. In addition the selected compounds were assessed for their *in vitro* cytotoxicity against human adenocarcinoma cell lines (MCF-7 and MDA-MB-231). Amongst malabaricones (**3, 5-7**) showed promising activity against both the cell lines whereas trimyristin (**10**) efficiently inhibited MCF-7 also partensein (**8**) and promalabaricone B (**9**) were found to be moderately toxic to MDA-MB-231 and MCF-7.

1. Introduction

Myristicaceae, the primitive angiosperm and pantropical flowering plant family entails approximately 21 genera and 520 species are widespread in the subtropical and tropical regions of the world, especially in Asia (Herve and Annick, 2003; Christenhusz and Byng, 2016). It is characterized by species that are highly endemic to the Western Ghats of India having significant ecological and ethnobotanical importance. Diversity among the species and its abundance make them a unique family. In India, the family represents five genera, *Horsfieldia* Willd., *Endocomia* Wilde., *Gymnacranthera* Warb., *Knema* Lour. and *Myristica* Gronov. of which the latter three are distributed in evergreen forests of the Western Ghats (Nayar et al., 2014). *Myristica* is more specious and constitutes 175 species worldwide (Mabberley, 2018). Amongst India signifies only five species *viz*. *M. andamanica* Hook. F, *M. beddomei* King., *M. fragrans* Houtt., *M. magnifica* Bedd. and *M. malabarica* Lam. (Banik et al., 2017). Of which, *M. beddomei, M. magnifica* and *M. malabarica* are strictly endemic to Western Ghats and *M. fragrans* is native to Mollucas Islands and under cultivation in India (Nayar et al., 2014 & Banik et al., 2017). Most of the wild nutmeg species of this genus are still unexplored.

Myristica beddomei King commonly known as "bitter nutmeg'' is endemic to the evergreen and semi evergreen forests of south Western Ghats of Kerala, possess three subspecies *M. beddomei* subsp. *beddomei, M. beddomei* subsp. *ustulata* and *M. beddomei* subsp. *spherocarpa*. Apart from the other two species, *M. beddomei* subsp. *spherocarpa* not gained adequate attention. No studies on taxonomy, ecology & population structure of this sub species is done so far. But its restricted distribution was reported by Wilde (Wilde, 1997). According to IUCN (2020), this species is under endangered category. It is an evergreen, dioecious tree thrives only at high altitude (>900 m) and is reported only from Agasthyamala biosphere reserve which is considered as the habitat for 2,000 varieties of medicinal plants, of which at least 50 are rare and endangered species. Due to limited distribution on high altitude, dense forest and strict phenology made research about this species difficult. Morphologically, this subspecies shows noticeable variation from other subspecies of *M. beddomei*. Thus a detailed chemical investigation will

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aid to solve taxonomical problems of these particular taxa. To date, there is no report on the isolation of phytochemicals from this sub species. Herein we establish a complete chemoprofiling of the species and identification of two new flavonoids along with the *in vitro* anti-proliferative potential of the isolates against human adenocarcinoma cell lines, MCF-7 and MDA-MB-231.

2. Results and discussion

Detailed phytochemical analysis of the aerial parts of *M. beddomei* subsp. *spherocarpa* led to the isolation of thirteen compounds (Compounds **1-13**, Fig. 1) including two novel chemical structures for the first time. The structures of the isolates were identified and confirmed by the interpretation of various spectroscopic data (IR, HRESIMS, ^{1}H NMR, ^{13}C NMR, 2D NMR, UV and CD) and in comparison with the relevant data from the literature. They were identified as a new isoflavone; 5,7-dihydroxy-3-(5'-hydroxybenzo[d](7',9')-dioxol-1'-yl)-4H-chromen-4-one (**1**), a new *β*-hydroxydihydrochalcone; (3(S)-hydroxy-3-phenyl-1-(2',4',6'-trihydroxyphenyl)propan-1-one (**2**) along with six known acylphenols; [malabaricone A (**3**), malabaricone B (**5**), malabaricone C (**6**) and malabaricone D (**7**) (Purushathaman et al., 1977), promalabaricone B (**9**) (Pham Van et al., 2000) and 1-(2, 6-dihydroxyphenyl) tetradecan-1-one (**4**) (Cooray et al., 1987)] and a triglyceride trimyristin (**10**) (Imanuel and Marsela, 2014) and two isoflavones; partensein (**8**) (Braz et al., 1976) and biochanin A (**13**) (Talukdar et al., 2000) along with two phenylpropanoids (2R)-3(3, 4-methylenedioxyphenyl) 1, 2-propanediol (**11**) (Duan et al., 2009 & Rukachaisirikur et al., 2000) and 2R'-methyl-3-[3, 4-(methylenedioxy) phenyl] 1'-propanol (**12**) (Jaensch et al., 1989).

New compounds were identified from different parts (fruits & stem bark) of the species which indicates the organ specific appearance of secondary metabolites in plants. Synthesis and accumulation of various secondary metabolites resulted from the numerous biosynthetic pathways which in turn depend on the environmental and physiological factors responsible for growth and development. So each part of the plant generally differs in their phytoconstituents.

2.1. Structural elucidation of novel compounds

Compound **1** was obtained as a white powder. The IR absorption bands at 3390, 3371 and 1649 cm^{-1} specifies the presence of hydroxyl and carbonyl carbon functionalities. The ^{1}H NMR spectrum displayed three broad singlets at (δ_H 12.86, 9.70, 8.57 ppm) confirmed the presence of three hydroxyl groups. The one proton singlet at (δ_H 12.86 ppm) without carbon correlation in HMQC clearly indicates the presence of an *enolisable* hydroxyl group and the characteristic proton resonance of an isoflavone skeleton (Mabry et al., 1970) was observed at (δ_H 8.02 (1H, s, H-2) ppm. Besides the ^{1}H NMR also exhibited two sets of doublets at [δ_H 6.29 (1H, d, $J = 2$ Hz, H-8), δ_H 6.16 (1H, d, $J = 2$ Hz, H-6) ppm] and [δ_H 6.64 (1H, d, $J = 1$ Hz, H-2'), δ_H 6.57 (1H, d, $J = 1$ Hz, H-6') ppm] indicate the presence of *meta* coupled aromatic protons of the isoflavone and another two proton singlet at δ_H 5.86 ppm for the methylenedioxy group. The ^{13}C NMR spectrum of the compound showed sixteen carbon resonances comprising of a carbonyl carbon (δ_C 180.5 ppm), a methylenedioxy carbon (δ_C 101.2 ppm), *olefinic* carbons (δ_C 153.9 and 122.9 ppm) and aromatic carbons (δ_C 164.2, 163.0, 158.1, 148.9, 140.6, 134.4, 125.1, 112.3, 105.2, 101.7, 99.0, 93.6 ppm). ^{1}H NMR, ^{13}C NMR and HMBC correlation data of the compound **1** is summarized in Table 1. The HMBC spectra displayed a long range ^{1}H-^{13}C correlation of H-2 to carbonyl carbon (C-4), C-9 and C-3. The hydroxyl group at position 5 showed correlation with C-5, C-10 and C-6. The protons H-6 is correlated with C-5 and also has long range correlation with C-8 & C-10. Similarly H-8 has correlations with C-7/C-9 and C-6/C-10. The proton H-6' also has notable long range correlation with C-3 &C-2'. In addition

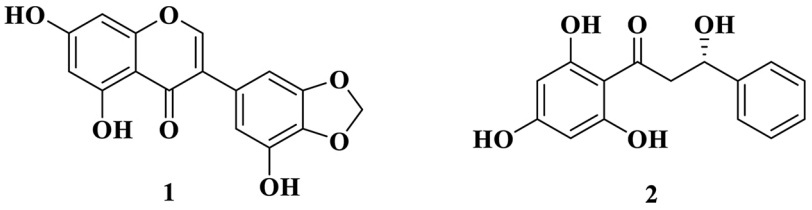

Fig. 1. Chemical structures of the isolated compounds **1** and **2**.

Table 1
^{1}H and ^{13}C NMR Spectral data of the Compound **1** & **2** (δ in ppm, J in Hz).

Compound 1 (in CD_3COCD_3)				Compound 2 (in $CDCl_3$)			
Position	δ_H (mult. J, Hz)	δ_C	HMBC Correlations	Position	δ_H (mult. J, Hz)	δ_C	HMBC Correlations
2	8.02, s	153.9	158.1,122.9, 180.5, 125.1	1		195.8	
3	–	122.9		2	3.09, dd, (13, 3) 2.83, dd, (17, 3)	43.3	195.8, 79.2, 138.3
4	–	180.5		3	5.43, dd, (13, 3)	79.2	138.3, 128.9
5	12.86, s	163.0	163.02, 105.2, 99.0	1'		103.2	
6	6.16, d, (2)	99.0	163.02, 105.2, 93.6	2'	12.04 (s, 1 H)	164.3	103.2, 164.3
7	9.70, s	164.2		3'	6.01 (s,1 H)	96.8	164.2, 103.2
8	6.29, d,(2)	93.6	164.2,158.1, 105.2, 99.0	4'		164.8	
9	–	158.1		5'	6.01 (s,1 H)	95.5	163.2, 103.2
10	–	105.2		6'		163.2	
1'	–	125.1		1''		138.3	
2'	6.64, d, (1)	112.3	140.6,134.4, 122.9, 101.7	2''		128.9	138.3
3'	–	140.6		3''		128.9	
4'	–	134.4		4''	7.45–7.42 (m, 4 H)7.41	126.2	
5'	8.57, s	148.9		5''	–7.39 (m, 1 H)	128.9	
6'	6.57, d, (1)	101.7	148.9,134.4, 122.9, 112.3	6''		128.9	138.3
8'	5.86, s	101.2	134.4				

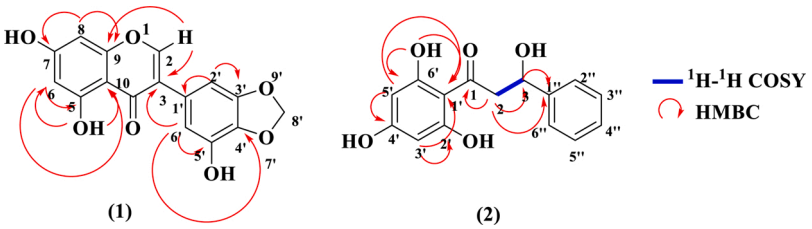

Fig. 2. Selected HMBC and COSY interactions of **1** and **2**.

^{1}H-^{1}H COSY correlations of aromatic protons was also observed (Fig. 2). The molecular formula was assigned as $C_{16}H_{10}O_7$ on the basis of HRESIMS (positive-ion mode) ion peak at *m/z* 337.03302 $[M + Na]^+$ (calcd for $C_{16}H_{10}O_7Na$, 337.0319) together with NMR data. The structure was elucidated by the detailed 2D NMR analysis (COSY, HMQC and HMBC). To the best of our knowledge, there were no reports on the structure shown below (Fig. 2), and this molecule can be considered as a novel one.

Compound **2** afforded as a pale yellow solid with specific rotation $[\alpha]^{25.5}_D$ +106.6 (c 0.1277, $CHCl_3$). The molecular formula $C_{15}H_{14}O_5$ was established on the basis of HRESIMS (positive ion mode) molecular ion obtained at *m/z* 297.07603 $[M + Na]^+$ (calcd for $C_{15}H_{14}O_5$, 297.0733). The IR spectrum showed characteristic absorption for hydroxyl group (3178 cm^{-1}), hydrogen bonded hydroxyl group (2885 cm^{-1}), carbonyl groups (1634 cm^{-1}) and aromatic moiety (1459 cm^{-1}). UV spectrum absorption maxima of the compound at 240 and 289 nm revealed a *β*-hydroxydihydrochalcone skeleton (Lois et al., 2009 & Ozbek et al., 2016). A sharp one proton singlet at (δ_H 12.04 ppm) in the ^{1}H NMR spectrum confirms the presence of an intramolecularly hydrogen bonded hydroxyl group. The ^{1}H NMR also displayed two doublet of doublets at [δ_H 3.09 (1H, dd, $J_1 = 13$ Hz, $J_2 = 3$ Hz H-2a) and δ_H 2.83 (1H, dd, $J_1 = 17$ Hz, $J_2 = 3$ Hz H-2b) ppm] indicates the two *diasterotopic* protons of the methylene group. The –CH proton of position **3** obtained as a doublet of doublets at δ_H 5.43 (1H, dd, $J_1 = 13$ Hz, $J_2 = 3$ Hz H-3) ppm. Besides the presence of an aromatic ring with 2, 4, 6-tri substitution confirmed from the signals at δ_H 6.01 (2H, s) ppm. The protons of the second aromatic ring obtained at [δ_H 7.45−7.42 (4H, m) and 7.41−7.39 (1H, m) ppm]. The ^{13}C NMR spectrum of the compound exhibited fifteen carbon resonances including a carbonyl carbon (δ_C 195.8 ppm), a methylene carbon (δ_C 43.3 ppm), carbon bearing a hydroxyl group (δ_C 79.2 ppm) and aromatic carbons (δ_C 164.8, 164.3, 163.2, 138.3, 128.9, 128.9, 126.2, 103.2, 96.8, 95.5 ppm). In addition the DEPT 135 spectrum confirms the presence of a methylene carbon at δ_C 43.3 ppm. ^{1}H NMR, ^{13}C NMR and HMBC correlation data of the compound **2** is summarized in Table 1. The connectivity of the protonated carbons C_2-C_3 was established from ^{1}H-^{1}H COSY (Fig. 2) interactions. In addition ^{1}H-^{1}H COSY correlations of aromatic protons was also observed. The key HMBC correlations (Fig. 2) between H-2 and C-1, H-2 and C-3, H-2 and C-1"as well as correlations between H-3 and C-1'' suggest that a *β*-hydroxyl carbonyl moiety is flanked between two aromatic rings. The compound appears to be the first report on the occurrence of a *β*-hydroxydihydrochalcone in *M. beddomei*. *β*-hydroxydihydrochalcones are a rare subclass of flavonoids. Only few examples of these types from natural resources are reported, while *α* and *β*-dihydroxyhydrochalcones are still less common (Dang et al., 2018 & Wilmer et al., 2019).

The UV and ECD spectra of compound **2** were measured (Fig. S21) and the ECD spectra exhibited negative Cotton effects at 255−305 nm and positive Cotton effects at 305–366 nm. The absolute configuration of the *β*-stereocenter was deduced through circular dichroism (CD) spectrum. Based on the comparison of experimental and theoretical CD spectrum (Supplementary Information) the compound was found to have (S) configuration at the *β* carbon.

2.2. *In vitro* cytotoxicity

Among the isolates, ten compounds were screened for their *in vitro* cytotoxicity against human adenocarcinoma cell lines MCF-7 and MDAMB-231 along with the normal cell line WI 38 by thiazolyl blue tetrazolium bromide (MTT) assay and the results are summarized in Table 2. Among the tested compounds malabaricones (**3, 5–7)** showed promising activity in both cell lines but showed moderate toxicity in normal cells. The IC_{50} was comparable or better than that of standard Doxorubicin. Trimyristin (**9**) efficiently inhibited MCF-7 while promalabaricone B (both cell) and partensein (MDAMB 23) showed moderate toxicity. The result indicated that in the case of malabaricones, the resorcinol moiety did not contribute towards its cytotoxicity while the substitutions of the second aromatic ring have an effect on its cytotoxic potential (Birija et al., 2010 & Pham Van et al., 2000). Detailed study of the compounds is needed to understand the mechanism of action.

Table 2
In vitro cytotoxicity assay result showing IC_{50} values of compounds.

Compounds	IC_{50} (μg/mL)		
	MCF-7	MD-AMB -231	WI 38
Malabaricone A (3)	15.46	28.58	25.37
Malabaricone B (5)	22.92	14.67	35.13
Malabaricone C (6)	36.25	31.25	>100
Malabaricone D (7)	20.58	32.87	31.80
Trimyristin (9)	34.90	>100	>100
Partensein (7)	>100	97.29	>100
Promalabaricone B (8)	74.41	86.12	>100
1-(2,6-dihydroxyphenyl tetradecan-1-one (4)	>100	>100	>100
5,7-dihydro 3-(7-hydroxy benzol [d] [1,3] dioxol 5-yl) 4H chromen-4-one (1)		>100	>100
Biochanin A (12)		>100	>100
Doxorubicin	>100	31.90	

3. Materials and methods

3.1. Collection of plant material

For the present study the species is procured from Chemunji hills, Bonaccadu region of Thiruvananthapuram District, Kerala, India in April 2019. The plant material was authenticated by Dr. Mathew Dan, Senior Scientist, Plant Genetic Resource Division KSCSTE-Jawaharlal Nehru Tropical Botanic Garden & Research Institute, Palode, Thiruvananthapuram, Kerala, India and a voucher specimen **[*M. beddomei* subsp. *spherocarpa*: Chemunji hills, Bonaccadu, Thiruvananthapuram, Kerala April 2019, Govind** 91046 **(TBGT)]** was deposited in the herbarium of Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode. A geographical map (SI-Fig. S1) of the species was constructed based on the collection and distribution sites using QGIS software.

3.2. General experimental procedure

All the chemicals and solvents were of the best grade commercially available and were used without further purification. Analytical thin layer chromatography (TLC) was performed on Merck silica gel 60 F_{254} aluminium sheets; visualization was effected with UV and/or by staining with Enholm yellow/Mc-Gill solution. Melting point was determined on a Fisher Johns melting point apparatus and is uncorrected. The nuclear magnetic resonance spectra (NMR) were recorded on Bruker AMX 500 spectrometer ($CDCl_3$ & CD_3COCD_3 as solvents). Chemical shifts for ^1H NMR are reported as δ in units of parts per million (ppm) downfield from $SiMe_4$ (δ 0.0). Multiplicities were given as: s (singlet); brs (broad singlet); d (doublet); t (triplet); m (multiplet); dd (doublet of doublet). Coupling constants are reported as J value in Hz. Mass spectra were recorded under ESI/HRMS at 61800 resolution using Thermo Scientific Exactive Mass Spectrometer. IR spectra were recorded on Bruker Alpha FT-IR spectrometer and specific rotation was recorded using Jasco P-2000 polarimeter. The electronic absorption spectra were recorded on a Shimadzu UV-2600 UV–vis scanning spectrophotometer equipped with peltier thermostatic cell holders. Circular dichroism (CD) measurements were performed on JASCO 810 spectrometer equipped with peltier thermostatic cell holders. CD spectra, recorded as θ in millidegrees, and converted into $\Delta\varepsilon$ using the equation $\Delta\varepsilon = \theta/(33982\ cl)$ where, $\Delta\varepsilon$ is the difference in the molar absorptivity for oppositely polarized light in M^{-1} cm^{-1}, c is the concentration in M and l is the path length in cm. Theoretical ECD spectra were recorded using time dependent density functional theory (TDDFT) calculations by using B3LYP/6311++G**\\.

3.3. Extraction and isolation

Aerial parts of the species (fruit and stem bark) were collected; seeds & rinds were separated from the fruit and air-dried. The ground materials (800 g rind, 800 g seed and 600 g stem bark) were then extracted sequentially with different solvents of varying polarity includes hexane (5 L × 3 d), dichloromethane (3 L × 3 d), acetone (5 L × 3 d), Ethanol (3 L × 3 d) and water at room temperature. The supernatant liquid was decanted, filtered and concentrated in a Heidolph Rotary Evaporator under reduced pressure afforded corresponding crude extract.

Acetone extract of the rinds (50 g) were fractionated over 100–200 mesh sized silica gel by gradient elution with Hexane/EtOAc (100:0 to 0:100, v/v) afforded 63 fractions. Based on the similarity in TLC analysis they were combined into 12 fraction pools. Fraction pool 2 on CC separation using Hexane/EtOAc (9:1, v/v) eluent afforded compound **1** (80 mg, 0.01 %, white powder). Fraction pool 3–12 on repeated CC separation using Hexane/EtOAc gradient solvent system (9.5:0.5 to 6:4, v/v) led to the isolation of compound **3** (1 g, 0.125 %, Pale yellow crystals), compound **4** (800 mg, 0.1 %, pale yellow crystalline solid), compound **5** (1 g, 0.125 %, pale yellow solid), compound **6** (2 g, 0.25 %, yellow crystalline solid), compound **7** (650 mg, 0.8125 %, white powder), compound **8** (10 mg, 0.00125 %, white powder) and compound **9** (200 mg, 0.025 %, pale yellow crystalline solid).

Crude dichloromethane extract of the seeds (55 g) were fractionated over silica gel column chromatography (CC) by gradient elution with Hexane/EtOAc (100:0 to 0:100, v/v) as eluent to acquire 55 fractions. Hexane eluted fractions 1–3 afforded compound **10** (100 mg, 0.0125 %, white amorphous solid) after recrystallization in acetone. CC separation of Fractions 4–43 over silica gel using gradient mixtures of Hexane/EtOAc led to the isolation of compounds **3–7** in excellent yield. Fractions 44–47 were purified with Hexane/EtOAc (5:5 v/v) afforded compound **11** (10 mg, 0.00125 %, white solid). Compound **12** (7 mg, 0.00087 %, white powder) was obtained from the fraction 48–54 after silica gel column chromatography (CC) using Hexane/EtOAc (6:4, v/v).

Acetone extract of the stem bark (20 g) were fractionated over silica gel by gradient elution with Hexane/EtOAc (100:0 to 0:100, v/v) afforded 52 fractions. Fraction pool 46–52 subjected to CC separation on silica gel using Hexane/EtOAc (6.5:3.5, v/v) afforded the compound **2** (5 mg, 0.00083 %, pale yellow solid). Compound **13** (10 mg, 0.0016 %, white powder) was purified from the fraction 40–45 with Hexane/EtOAc (6.5:3.5, v/v) as eluent. Compounds **3–5** were also isolated from the CC separation of the fractions 6–39 using gradient elution of Hexane/EtOAc.

3.4. Cell culture and treatment condition

The human cancer cell line MCF-7 (breast cancer) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Human lung cell line WI-38 was kindly gifted from Indian Institute of Chemical Biology (CSIR-IICB), Kolkata, India. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10 % fetal bovine serum and 5 % CO_2 at 37 °C. Cells were cultured in glass-bottom, 96-well plates, T25 flasks and T75 flasks, for the purpose of cytotoxicity experiments.

3.5. In vitro cytotoxicity assays

The growth inhibition capacity of all compounds were evaluated in cancer cell and normal cell lines initially using the MTT assay as previously reported (Joseph et al., 2013). This assay measures cell viability by assessing the cleavage of tetrazolium salt by mitochondrial dehydrogenase. The absorbance was measured at 570 nm using a microplate spectrophotometer (BioTek Power Wave XS) after incubation for 24 and 48 h with test compound.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phytol.2021.07.007.

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