ISOLATION AND CHARACTERIZATION OF BIOLOGICALLY ACTIVE MOLECULES FROM SPICES AND MEDICINAL PLANTS OF KERALA

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By

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UNDER THE SUPERVISION OF

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January 2016

Dedicated to my beloved parents.....

DECLARATION

I hereby declare that the Ph. D. thesis entitled: "**ISOLATION AND CHARACTERIZATION OF BIOLOGICALLY ACTIVE MOLECULES FROM SPICES AND MEDICINAL PLANTS OF KERALA**" is an independent work carried out by me and it has not been submitted anywhere else for any other degree, diploma or title.

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CERTIFICATE

This is to certify that the work embodied in the thesis entitled:**"ISOLATION AND CHARACTERIZATION OF BIOLOGICALLY ACTIVE MOLECULES FROM SPICES AND MEDICINAL PLANTS OF KERALA**" has been carried out by **Mr. Sajin Francis K**. under my supervision and guidance at the Organic Chemistry Section, Chemical Sciences and Technology Division of the CSIR-National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Thiruvananthapuram and the same has not been submitted elsewhere for a degree.

Mangalam S Nair
(Thesis Supervisor)

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CONTENTS

PREFACE

Kerala with its abundance of luxuriant flora is well known for Ayurveda and is possibly the only state in the country where Ayurveda continues to be practiced in its purest form. The use of plant principles for the treatment of a wide variety of diseases is practiced in most cultures. However, the active ingredients in these plants and their mechanisms of action are poorly understood. Delineation of this knowledge could provide safer, more efficacious treatment of various ailments. Therefore, we have embarked on a study involving the isolation and characterization of biologically active compounds which are being used as drug, herbs or spices in Kerala.

First chapter gives a brief introductory discussion on secondary metabolites and their classification. A brief description of anticancer drugs from natural products as well as the biological significance of reactive oxygen species also given. The use of spices as antioxidants is also outlined in this chapter.

Second chapter describes the detailed phytochemical investigation of fruits and stem bark of the *Myristica fragrans* (Nutmeg). In first part of the chapter, the isolation of chemical constituents from various parts of *M. fragrans* fruit is described. The pericarp, seed and mace from the fruit were separated and used for detailed studies. Part B of the second chapter outlines the isolation of chemical constituents from the *M. fragrans* stem bark.

Third chapter describes the isolation of azadirone, epoxyazadiradione, azadiradione and 17β-hydroxyazadiradione, four major compounds found in *A. indica* seeds. All the compounds were tested for their anticancer activity. Out of four compounds isolated, azadirone showed most potent anticancer activity. The molecular mechanism by which azadirone exerts anticancer effects has been studied for the first time.

Fourth chapter gives phytochemical investigation *P. herbacea* roots. Three known compounds and two novel compounds were isolated and identified from the roots of *P herbacea*. Bharangin, bharanginin and sugiol are the reported compounds isolated from this plant. Two novel molecules named as, pygmaeocinin and bharanginione were also isolated. Anticancer activity of bharangin and its mode action has also been evaluated.

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List of Abbreviations

1	AA	-	Antioxidant activity
2	α	-	Alpha
3	β	-	Beta
4	BC	-	Before Christ
5	BHA	-	Butylated hydroxy anisole

6	BHT	-	Butylated hydroxy toluene
7	br.	-	Broad
8	^{13}C NMR	-	Carbon-13 Nuclear Magnetic Resonance Spectroscopy
9	Cys	-	Cysteine
10	$CDCl_3$	-	Deuterated chloroform
11	^{O}C	-	Degree Celsius
12	DCM	-	Dichloromethane
13	DNA	-	Deoxyribonucleic acid
14	δ	-	Delta
15	DEPT	-	Distortionless Enhancement by Polarization Transfer
16	EI-MS	-	Electron Impact mass spectroscopy
17	EtOAc	-	Ethyl acetate
18	EC_{50}	-	Effective concentration for 50 % inhibition
19	*dd*	-	doublet of doublet
20	*d*	-	Doublet
21	*dq*	-	doublet of quartet
22	GAE	-	Gallic acid equivalent
23	GC-MS	-	Gas chromatography-mass spectroscopy
24	g	-	Gram
25	γ	-	Gamma
26	^{1}H NMR	-	Proton Nuclear Magnetic Resonance Spectroscopy
27	HPLC	-	High performance liquid chromatography
28	Hz	-	Hertz
29	IR	-	Infrared
30	IC_{50}	-	Inhibitory concentration for 50%
31	kg	-	Kilogram
32	L	-	Litre
33	Lys	-	Lysine
34	mg	-	Milligram
35	mL	-	Millilitre
36	*m*	-	Multiplet
37	M^{+}	-	Molecular ion
38	MTT	-	3-(4,5-Dimethylthiazal-2-yl)-2,5-diphenyl terazolium

			bromide
39	NF-κB	-	Nuclear Factor kappa-light-chain-enhancer of activated B cells
40	PE	-	Petroleum ether
41	*p*	-	Para
42	RNA	-	Ribonucleic acid
43	ROS	-	Reactive Oxygen Species
44	Ser	-	Serine
45	*s*	-	Singlet
46	s	-	Sharp
47	TRAIL	-	TNF Related Apoptosis Inducing Ligand
48	TNF-*α*	-	Tumour Necrosis Factor alpha
49	*t*	-	Triplet
50	TLC	-	Thin layer chromatography
51	UV	-	Ultraviolet

CHAPTER 1

Natural Products: An Overview with Special Emphasis on Chemical Diversity and Plant Derived Anticancer Drugs

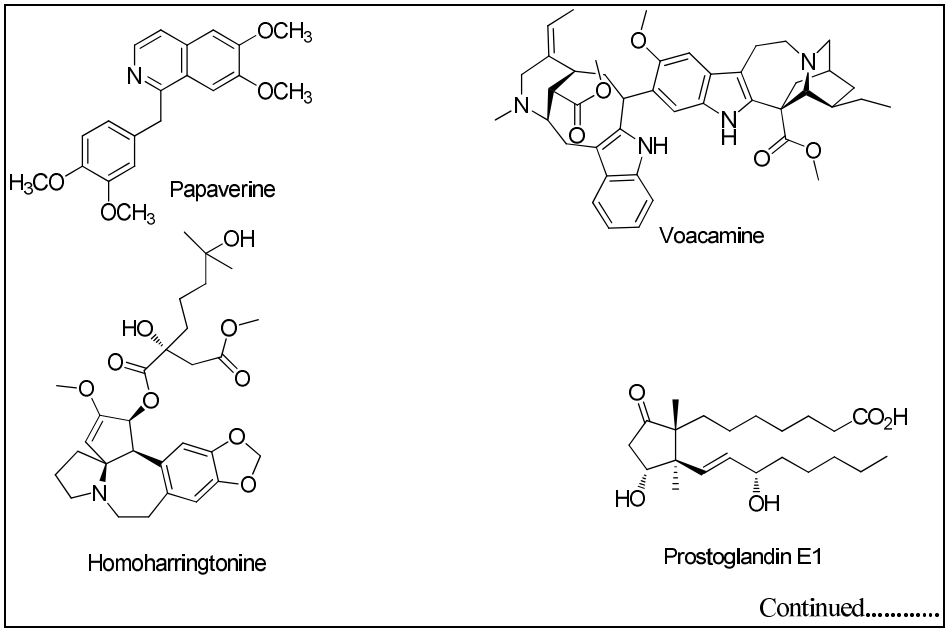
1.1. Introduction

Plants have formed the basis of sophisticated traditional medicine systems we have today, with the earliest records dating from around 2600 BC. The vast chemical-biodiversity of the plant world has been exploited for thousands of years by human cultures not only for food but also to prevent pain, to produce pleasure, for use in religious ceremony, and to cure various human diseases [Luca *et al.*, **2012**]. World Health Organization (WHO) has estimated that nearly 65% of the world's population primarily rely on plant-derived traditional medicines for the treatment of a wide spectrum of diseases [Farnsworth *et al*., **1985**]. The first written record on the medicinal uses of plants appeared in about 2600 BC from the Sumerians and Akkaidians. The "*Ebers Papyrus*", dating from 1500 BC is the earliest known Egyptian pharmaceutical record, which documented over 700 remedies, representing the history of Egyptian medicine. The Chinese "*Materia Medica"*, describes the use of more than 600 medicinal plants, and is well documented with the first record dating from about 1100 BC. Documentation of the Ayurvedic system of medicine as recorded in Susrutha and Charakasamhita dates from about 1000 BC [Kaur *et al.*, **2011**].

Among 2,50,000 higher plant species on earth, more than 80,000 are known to have medicinal properties and are used in traditional medicines. India is one of the world's 12 biodiversity centres with the presence of over 45,000 different plant species [Joy *et al*, **1988**]. India has varying agro-climatic and soil conditions ranging from arid to alpine zones with different types of plants found growing in different climatic conditions leading to a lush and diverse flora. India is therefore rightly called the emporium of medicinal plants [Kapoor *et al.*, **1990**]. The mountain ranges of Himalayas have been

known to be a source of rare, important medicinal plants since time immemorial [Andrew *et al.*, **1996**]. The forests in India are the principal repository of a large number of medicinal and aromatic plants, which have been largely collected as raw materials for the manufacture of drugs and perfumery products.

Natural Products, obtained from plants, animals and minerals have been the basis of treatment of human diseases since time memorial. Many natural products have pharmacological or biological activity and find use in pharmaceutical drug discovery and drug design processes. The term 'natural products' really refers to any naturally occurring substance, but is generally taken to mean a secondary metabolite from the plant/organism- viz., a small molecule that is not essential to the growth and development of that plant/organism. It has been estimated that well over 300,000 secondary metabolites have been identified, and it is thought that their primary function is to increase the likelihood of an organism's survival by repelling or attracting other organisms. Typical examples include, alkaloids such as papaverine, voacamine, homoharringtonine etc., eicosanoids, such as prostaglandin E1; and antibiotics, such as erythromycin, penicillins, geldanamycin, tobramycin etc., terpenoids such as azadirachtin, entangosin etc., marine natural products such as cyclomarine and halichondrin B etc., to name a few (**Figure 1.1**).



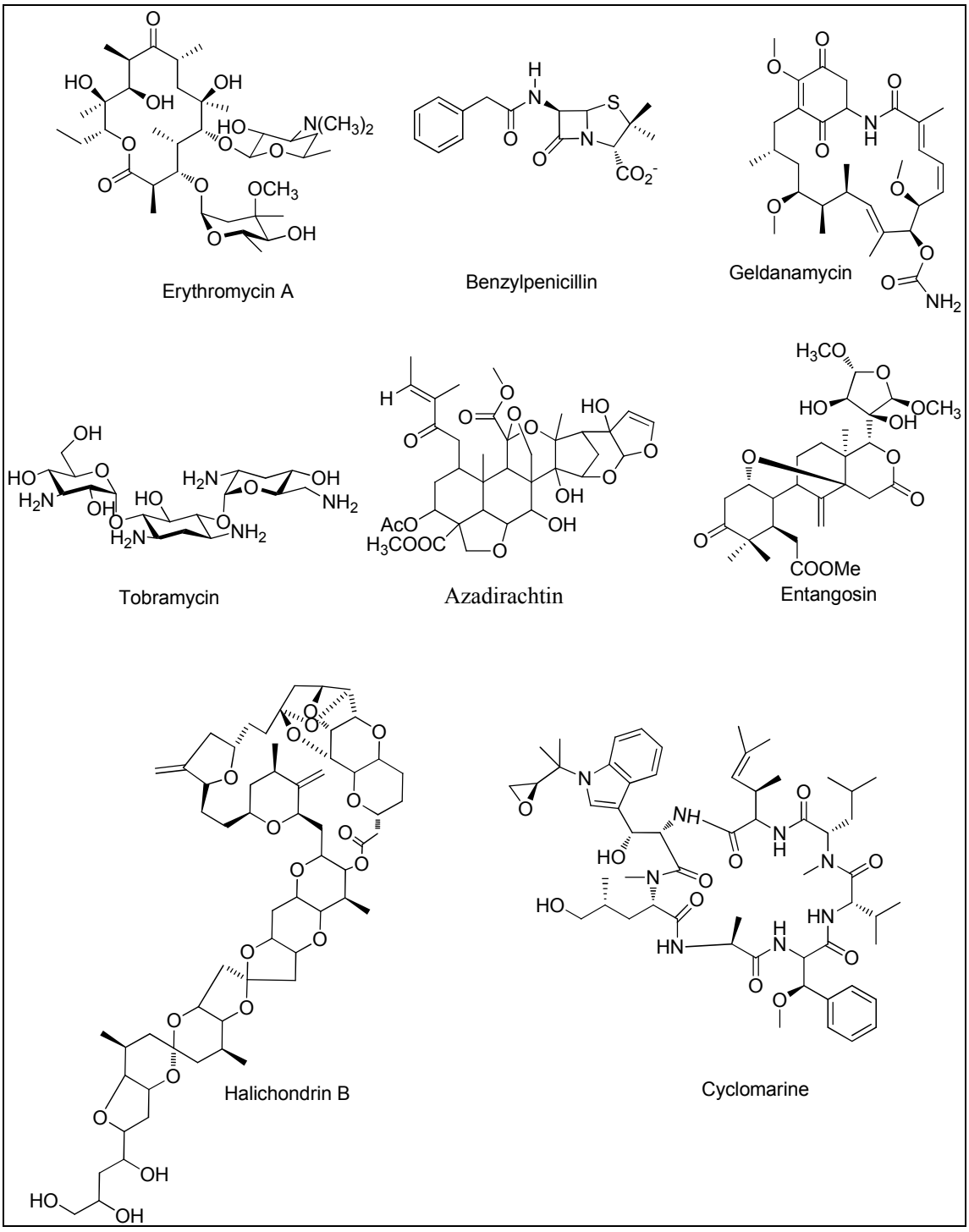


Figure 1.1 : Structures of some different types of natural products

1.1.1. Classification of Natural Products

There is no rigid scheme for classifying natural products as their immense diversity in structure, function and biosynthesis is too great to allow them to fit neatly into a few simple categories. The first categorization in natural products is function based. viz., as primary metabolites and secondary metabolites. Further classification is carried out by a systematic structure oriented organising principle. *Though classification of natural products is fairly common knowledge, it has been incorporated in this introduction as to use it as a platform for showcasing the diversity, excitement and beauty of natural products in a pictorial way.*

Primary metabolites

A primary metabolite is directly involved in normal growth, development and reproduction of all living organisms. It usually performs physiological functions in the organisms. Examples of primary metabolites include energy rich fuel molecules such as sucrose and starch, structural components such as cellulose, informational molecules such as DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), and pigments such as chlorophyll. In addition to having fundamental roles in plant growth and development, some primary metabolites are precursors for the synthesis of secondary metabolites.

Secondary metabolites

Secondary metabolites are organic compounds that are not directly involved in the normal growth, development or reproduction of an organism or plant. They often play an important role in plant defense against herbivory and other interspecies defences. These chemicals are structurally diverse and have been identified in several major classes. Each plant family, genus, and species produces a characteristic mix of these chemicals and they can sometimes be used as taxonomic characters in classifying plants. Some of these compounds have found extensive use as medicines, flavourings and perfumery materials.

Secondary metabolites can be classified on the basis of chemical structure or the pathway by which they are synthesized.

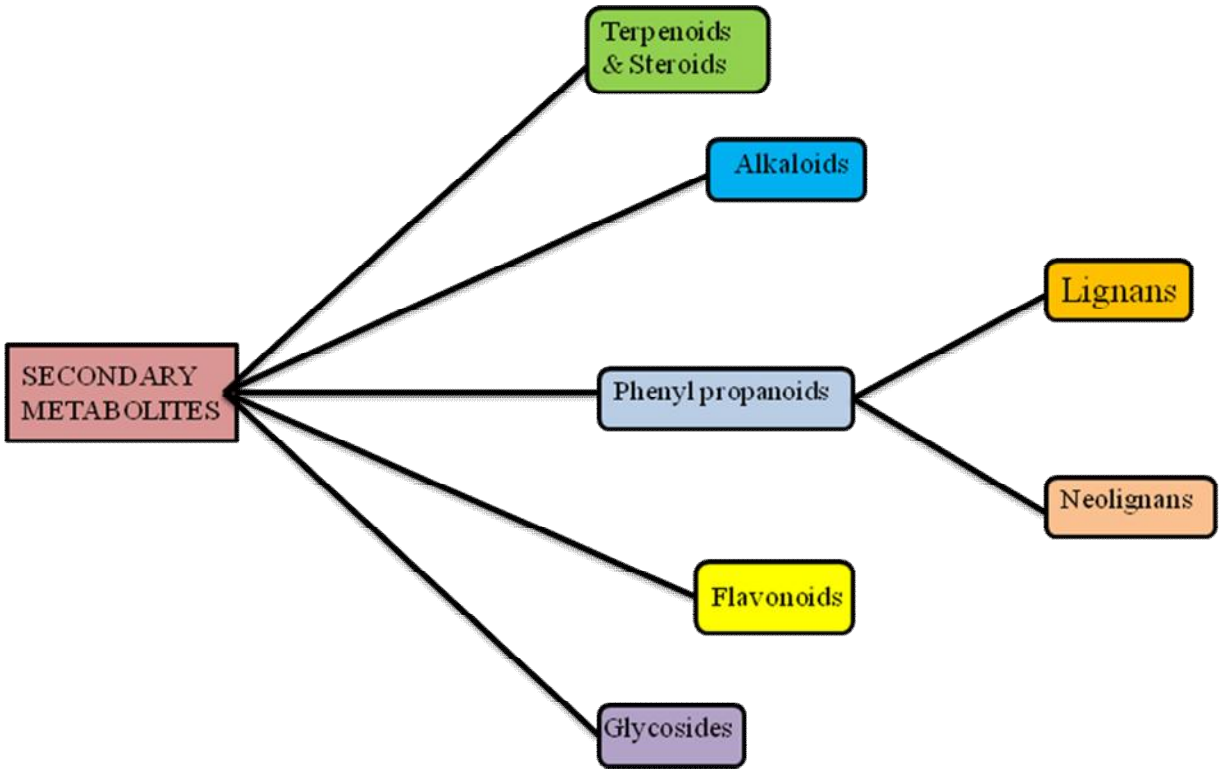


Figure 1.2 : Classification of secondary metabolites

1.1.1.1. Terpenoids and Steroids

Terpenoids are broadly classified on the basis of the number of isoprene units incorporated into a specific unsaturated hydrocarbon terpenoid molecule as, Monoterpenoids (*two* isoprene units -$C_{10}H_{16}$), sesquiterpenoids (*three* isoprene units- $C_{15}H_{24}$), diterpenoids (*four* isoprene units -$C_{20}H_{32}$), triterpenoids (*six* isoprene units - $C_{30}H_{48}$) and tetraterpenoids (*eight* isoprene units -$C_{40}H_{64}$).

However, it is also interesting to note that some terpenoids also occur with one or two carbons less than that required to be termed as sesqui, di or tri terpenoids. So they are usually named as mono seco, di seco, sesqui seco, tri seco terpenes etc.

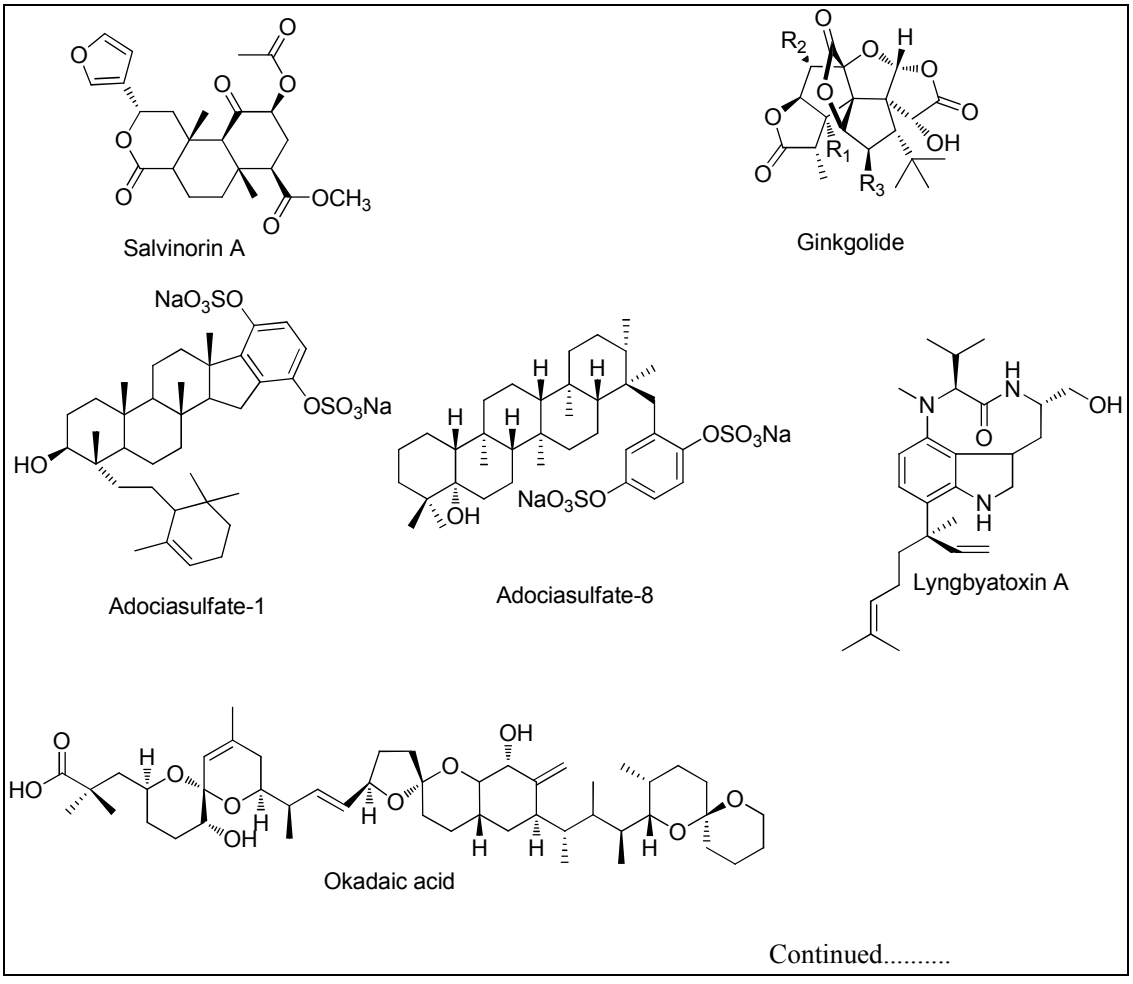
Plant terpenoids are used extensively for their aromatic qualities. Terpenoids contribute to the scent of eucalyptus, the flavours of cinnamon, cloves and ginger, the yellow colour in sunflowers, and the red colour in tomatoes etc. Terpenoids like

citral, menthol, camphor etc., are used extensively in flavourings and perfumery. Terpenoids play a key role in traditional herbal remedies and hundreds of them are used for or are under investigation as antibacterial, anticancer, anti-infammatory or for other pharmaceutical functions. Terpenoids occur with the most fascinating array of structures and biological activities. A few examples of terpenoids with such fascinating structures and biological activities from terrestrial plants include, salvinorin A, found in the plant *Salvia divinorum* [Ortega *et al*., **1982**; Katherine *et. al*, **2013**]; the cannabinoids found in *Cannabis sativa* [Small *et al*., **1973a**; Lambert *et al.*, **2005**]; ginkgolide and bilobalide found in *Ginkgo biloba* [Maruyama *et al*., **1967**; Stromgaard *et al.*, **2004**]. Apart from plants, marine organisms too are rich sources of bioactive terpenoids. Investigation of the sponge *Adocia* sp. (Chalinidae), collected near the Lizard island, Australia, led to the isolation of adociasulfate-1, along with adociasulfates-7 and -8 [Kalaitzis *et al.*, **1999**] which were shown to be active as inhibitors of the proton pump in membrane vesicles. Arthur *et al*, reviewed the possible role of tumor promoting compounds produced by *Lyngbya majuscula* (type of filamentous cyanobacteria) in marine turtle against fibropapillomatosis, a neoplastic disease of marine turtles where turtles develop benign tumors [Arthur *et al.*, **2007**]. It has been suggested that naturally occurring tumor promoters such as okadaic acid or lyngbyatoxin A might play a role in the development of this disease [Landsberg *et al.*, **1999**]. Eleuothrobin is a marine natural product isolated from *Eleutherobia albiflora* (soft-coral) which has shown potential anticancer activity [Lindel *et al.*, **1997**].

Steroids are compounds possessing the skeleton of cyclopenta[*a*]phenanthrene or a skeleton derived from this core structure by one or more bond scissions, ring expansions or contractions. Methyl groups are normally present at C-10 and C-13 positions. most of the steroid skeleton is similar to the structure of cholestane. Additional carbon atoms may be present in the side chain [Moss *et al.*, **1989**]. An alkyl side chain may also be present at C-17. Sterols are steroids carrying a hydroxyl group at C-3 are known as sterols.

There are many steroids and sterols that are important in health and medicine. Some steroids such as estrone are well known hormones and are therefore used as such or in modified forms as medicines. Marine steroids such as certonardosterols were isolated

from the starfish *Certonardoa semiregularis* in 2004. Steroids from marine sources are always highly oxygenated and often occur in very limited amounts. Certonardosterol D2 was found to be cytotoxic on certain human cancer cells with ED_{50} of 0.01-0.15 mg/mL, which was comparable to that of doxorubicin [Wang *et al.*, **2004**]. Campesterol is one of the phytosterols found in various nuts which reduces the cholesterol level [Heggen *et al*., **2010**]. Conessine is one of the steroidal alkaloids found in *Holarrhena floribunda* which acts as histamine antagonist [Duez *et al*., **1987**; Santora *et al*., **2008**]. The structures of a few bioactive terpenoids and steroids are shown in **Figure 1.3**.



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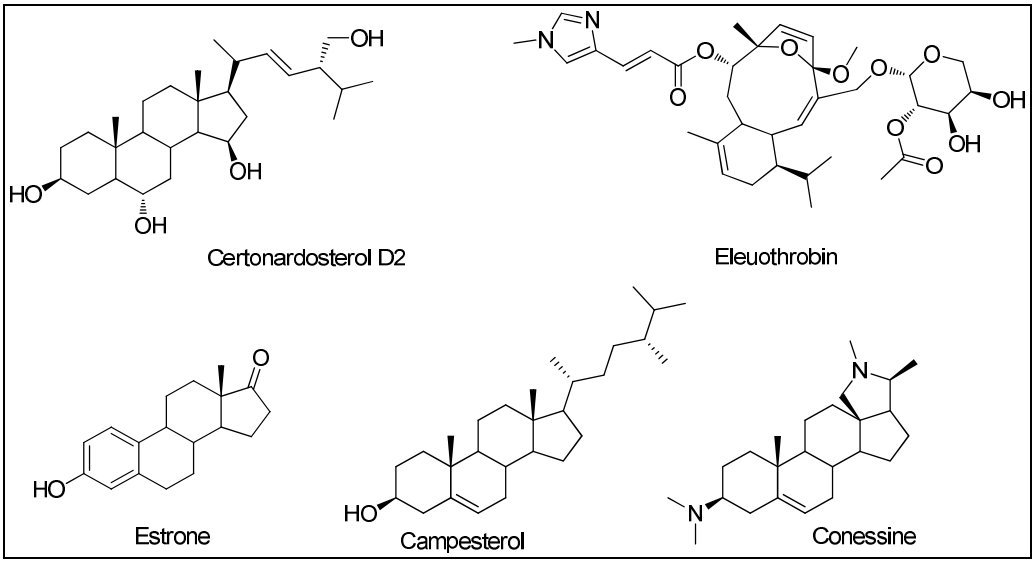


Figure 1.3 : Structures of a few bioactive terpenoids and steroids

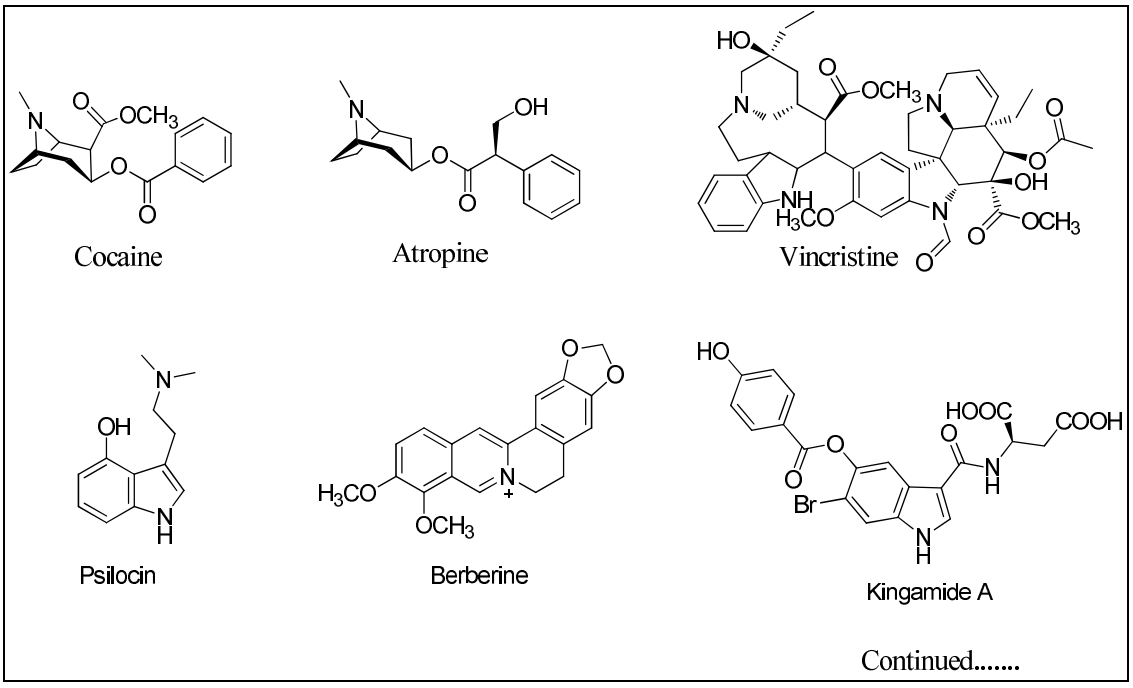
1.1.1.2. Alkaloids

Alkaloids are a group of naturally occurring chemical compounds that contain one or more basic nitrogen atoms. This group also includes some related compounds with neutral and even weakly acidic properties[Wilkinson *et al.*, **1997**]. The nitrogen atom is usually present in a heterocyclic ring, such as in pyrrole, pyridine, pyrrolidine, quinoline, or isoquinoline or found in an aliphatic ring or side chain. In addition to carbon, hydrogen and nitrogen, alkaloids may also contain oxygen, sulphur and more rarely other elements such as chlorine, bromine, and phosphorus. Some synthetic derivatives for example, heroin (diacetyl morphine), lysergic acid diethylamide are also termed as alkaloids. Alkaloids are produced by a large variety of organisms including bacteria, fungi, plants, and animals. They can be purified from crude extracts of these organisms by acid-base extraction. Many alkaloids are toxic to other organisms. They often have pharmacological effects and are used as medications, recreational drugs etc.

Morphine was the first alkaloid isolated in 1804 by Friedrich Serturner, which is generally believed to be the first ever isolation of a natural plant alkaloid in history. Examples of some important biologically active alkaloids are: the local anaesthetic and stimulant-cocaine, the psychedelic-psilocin, the stimulants-

caffeine, nicotine, the analgesic-morphine, the antibacterial-berberine, the anticancer compound-vincristine, the antihypertension agent-reserpine, the cholinomimetic-galantamine, the anticholinergic agent-atropine, the vasodilator-vincamine, the antiarrhythmic compound-quinidine, the antiasthma therapeutic-ephedrine, and the antimalarial drug-quinine.

Some recent additions include, Lirofolines A and B, possessing a novel rearranged ibogane ring system, isolated from *Tabernaemontana corymbosa* and *Tabernaemontana divaricata* from Malaya. Both the compounds showed significant activity in reversing multidrug resistance in vincristine-resistant KB (VJ300) cells [Low *et al.*, **2010**]. Seven cytotoxic indole alkaloids, bruceollines H, I , J, K, L, M, and N were isolated from the ethanol extract of the stems of *Brucea mollis* Wall., distributed in southern China [Chen *et al.*, **2011**]. A new brominated indole alkaloid, kingamide A was isolated from the Australian ascidian *Leptoclinides kingi*. This compound was screened against the breast cancer cell line MDA-MB-231 and a panel of seven prostate cancer cell lines [Liberio *et al.*, **2011**]. Structures of some very interesting and important alkaloids are listed in the **Figure 1.4**.



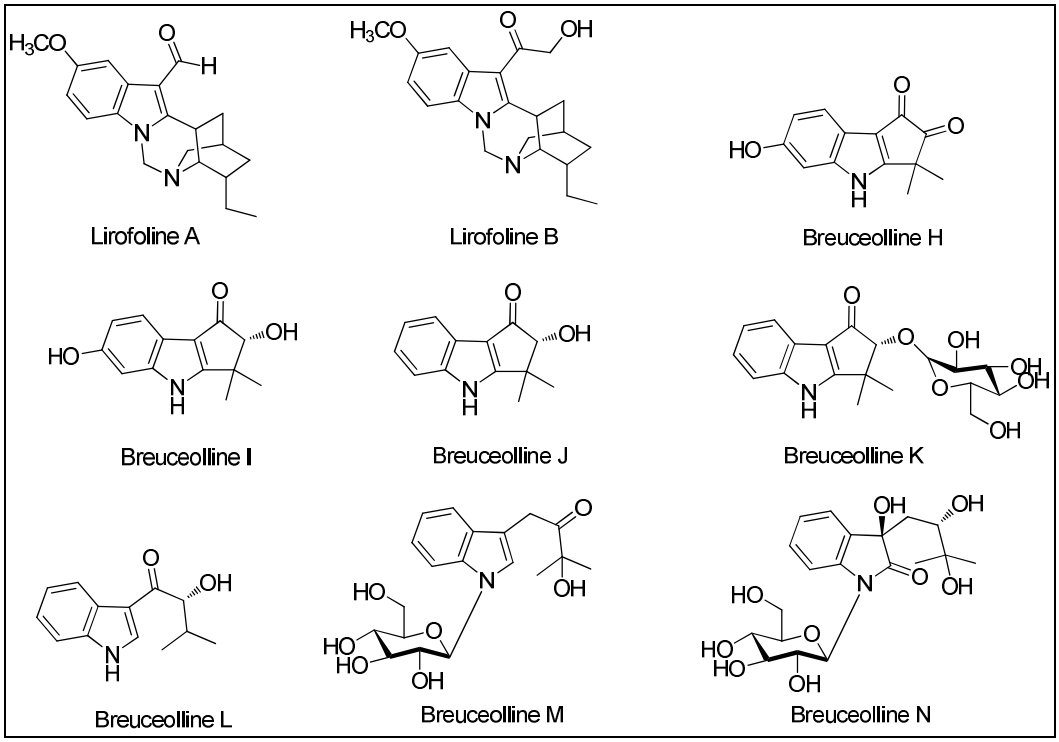


Figure 1.4 : Structures of some bioactive alkaloids

1.1.1.3. Flavonoids

Flavonoids are a large group of polyphenolic secondary metabolites occurring in plants, a group containing more than 8000 known compounds arising from the great structural diversity possible from the various hydroxylation, hydrogenation, methoxylation, malonylation, glycosylation, sulfation and acylation patterns of the core flavonoid structure. Originally, flavonoids were discovered as the pigments responsible for plant colour, ranging from red, orange, yellow to violet in flowering plants. Anthocyanins were the first group of flavonoids documented.

Flavonoids are believed to be endowed with many useful biological activities, such as anti-inflammatory, anti-allergic, anti-ischemic, anti-platelet, immunomodulatory and antitumor activities [Prior *et al.*, **2003**]. They have also been shown to inhibit some enzymes, including lipoxygenases and cyclooxygenases, mono-oxygenases, xanthine oxidase, mitochondrial succinoxidase, reduced nicotinamide-adenine dinucleotide (NADH) oxidase, phospholipase A_2, topoisomerases and protein kinases . The biological

activities of flavonoids are thought to be mainly due to their antioxidant properties, which are displayed by limiting the production of reactive oxygen species (ROS) and/or scavenging them [Pietta *et al.*, **2000**], thus preventing or delaying the onset of many life style diseases.

The flavonoids constitute one of the most characteristic classes of compounds in higher plants and their chemical structures are based on a C15 skeleton with a chromane ring bearing a second aromatic ring B in position 2, 3 or 4. The skeleton can be represented as a C_6-C_3-C_6 system. **Figure 1.5** shows the general structure and the numbering system used to distinguish the carbon positions around the molecule. The left hand ring, which is derived from the acetate malonate pathway, is referred to as the **A** ring. The right hand ring, which is derived from the ring carbons of phenylalanine, is referred to as the **B** ring. The heterocyclic ring between the two rings is referred to as the **C** ring and the free radical scavenging ability of this ring mostly determines the antioxidant potential of the flavonoid.

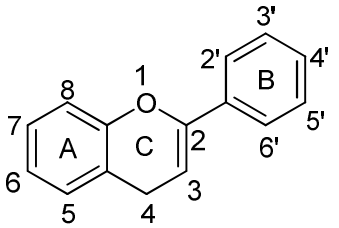


Figure 1.5 : Basic structure of flavonoids

The different ways to classifying flavonoids include, (i) according to their biosynthetic origin, (ii) according to whether the central heterocyclic ring is unsaturated or not, (iii) according to their molecular size etc. The most common way is according to the variation of the heterocyclic C ring. From the flavonoid basic structure, a heterocyclic pyrone C ring can be derivatised in to the flavones, flavonols, flavanones and isoflavones where as a pyran C ring produces the flavonols and anthocyanins [Graf *et al.*, **2005**]. The basic structures of each sub class are shown in **Figure 1.6**.

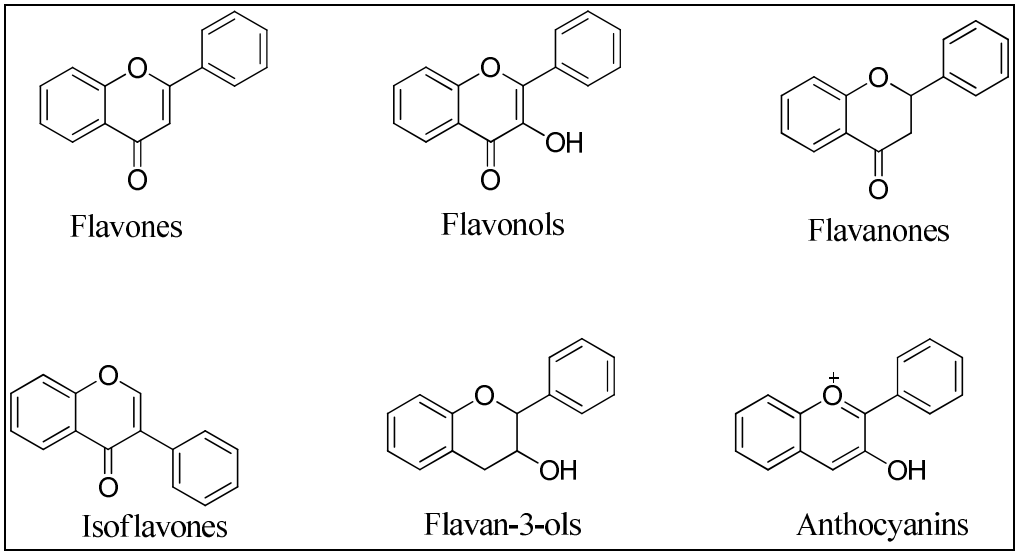
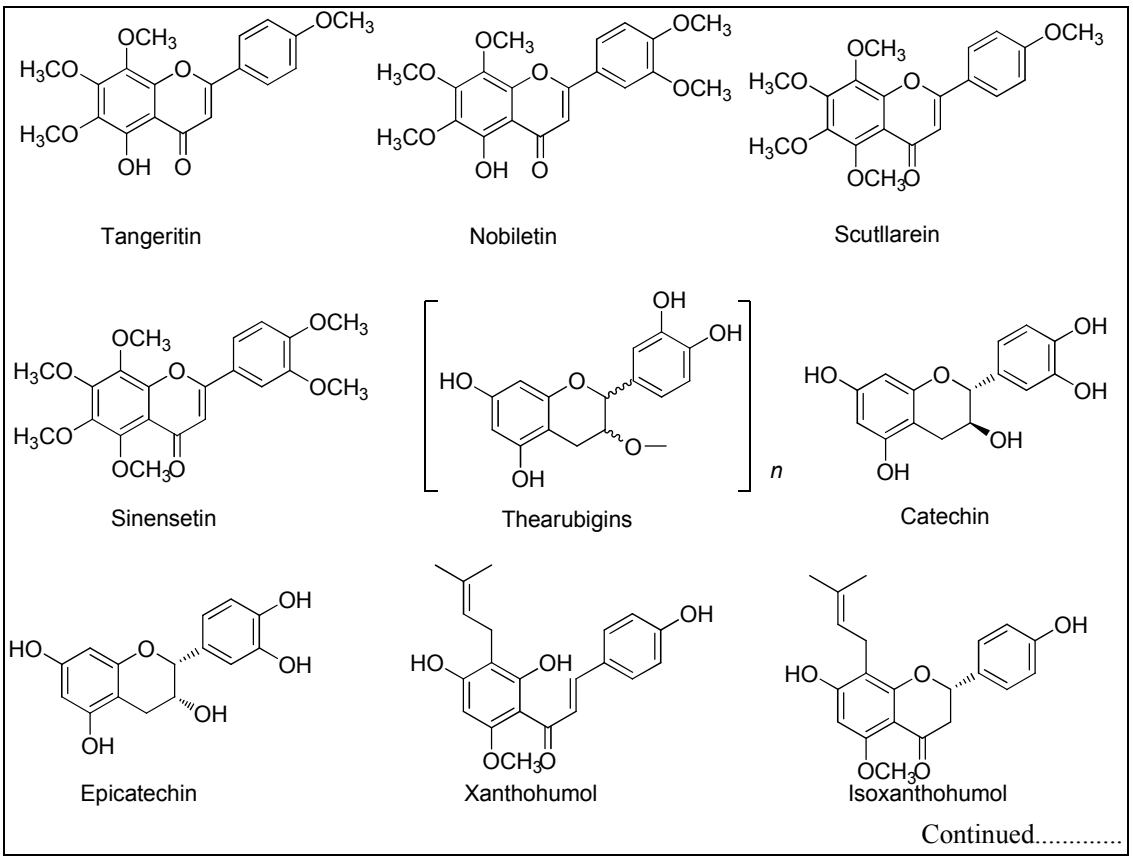


Figure 1.6 : Classification of flavonoids

Fruits, some cereals such as wheat and barley, tea, coffee as well as vegetables are very good sources of flavonoids. Orange juice contains polymethoxylated flavones such as tangeretin, nobiletin, scutellarein and sinensetin, which are found exclusively in citrus species. These compounds are pharmacologically important because of their antioxidant and anticancer activities. Apples (*Malus domestica*) and pears (*Pyrus communis*) are among the main sources of proanthocyanidins in the diet [Santos-buelga *et al.*, **2000**]. Tea, coffee, cocoa, wine and beer are the other significant dietary sources of flavonoids. The brownish water-soluble thearubigins are the major phenolic fraction of black tea. Molecular mass of some flavonoids can extend to 2000 daltons. These flavonoids are called as polymeric proanthocyanidins [Brown *et al.*, **1969**]. Cocoa and chocolate are the sources of untransformed flavan-3-ols and proanthocyanidins. The major polyphenols in fresh coffee beans are (+)-catechin, (-)-epicatechin and oligomeric procyanidins ranging from dimers to decamers. Beer made from fermenting malted grains, usually barley (*Hordeum vulgare*) or wheat (*Triticum vulgare*), hops (*Humulus lupulus*) with yeast (*Saccharomyces spp*.) in water contains a range of phenolic and polyphenolic compounds, derived partly from the barley (70%) and partly from the hops (30%). Hops contain quercetin conjugates and the prenylflavonoid xanthohumol, which during the brewing process undergoes substantial conversion to the flavanone isoxanthohumol,

which predominates in most beers. Other prenylflavonoids found in beers include desmethylxanthohumol, 6- and 8-prenylnaringenin and 6-geranylnaringenin [Steven *et al.*, **1999**]. Soya bean is one of the richest source of isoflavones such as genistein, daidzein and their *β*-glycoside conjugates [Lori *et al*., **1993**]. Genistein is an important anticancer isoflavone that inhibits DNA topoisomerase and tyrosine protein kinase [Akiyama *et al*., **1987**]. The compound also shows cell cycle inhibitor activity and it is currently undergoing phase I/II clinical trials. Among the various fruits, grape is one of the richest source of flavonoids. Most of the flavonoids are found primarily in the outer epidermal cells of fruits (the grape skin), whereas about 60–70% of total polyphenols are stored in grape seeds [Nassiri-Asl *et al*., **2009**]. The most common class of flavonoid found in grapes are anthocyanins. Bomser *et al*, found that grape seed extract contains mainly oligomeric and polymeric proanthocyanidins B1-B4 which show antitumor activity in mouse skin epidermis [Bomser *et al*., **1999**]. From the vast data on flavonoids, structure of few interesting flavonoids are shown as examples in **Figure 1.7**.



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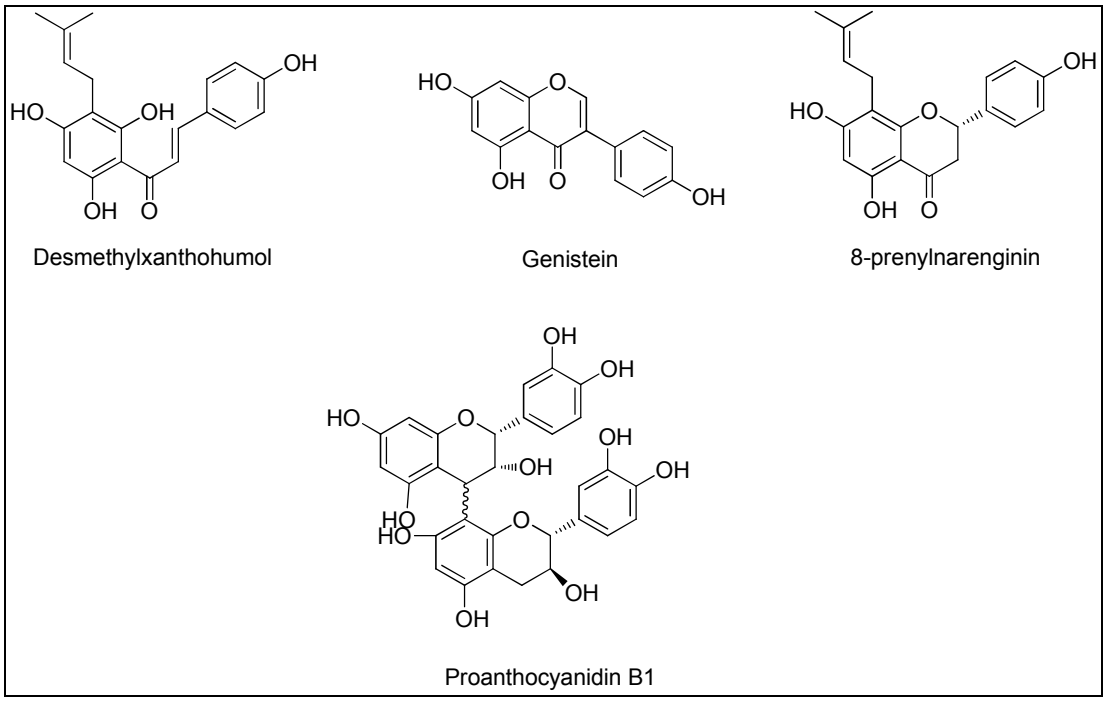


Figure 1.7 : Structures of some important flavonoids

1.1.1.4. Glycosides

The term glycoside is a generic term for natural product that is chemically bound to a sugar. The glycoside composes of two parts: the sugar and the aglycone. The aglycone may be a terpene, a flavonoid, a coumarin, steroid or any other type of natural product. Glycosides therefore show wide chemical diversity. Among the sugars found in natural glycosides, D-glucose is the most abundant one, L-rhamnose and L-fructose also occur quite frequently. Of the pentoses, L-arabinose is more common than D-xylose. The sugar part in glycosides can be disaccharides too.

Because of the cyclic structure of the sugar, two diastereoisomers of the glycoside exist, depending on the configuration of the anomeric carbon. These diastereoisomers are called anomers and are designated as α and β based on the stereochemistry.

Chemically, glycosides are usually mixed acetals in which the hydroxyl group on the anomeric carbon is replaced by a moiety possessing a nucleophilic atom. Thus the sugar moiety of a glycoside can be joined to the aglycone via

- Oxygen atom (*O*-glycosides)

- Carbon atom (*C*-glycosides)
- Nitrogen atom (*N*-glycosides)
- Sulfur atom (*S*-glycosides)

Only very few examples of glycosides are discussed here. Based on the utility and historical importance, the first mention must go to salicin, a *β*-glycoside, which is an anti-inflammatory agent isolated from willow bark (*Salix alba*). Frangulin B is a 6-*O*-(D-apiofuranosyl)-1,6,8-trihydroxy-3-methylanthraquinone isolated from *Rhamnus frangula*, which inhibits selectively collagen-induced aggregation and ATP release in rabbit platelets [Wagner *et al*., **1972**; Teng *et al*., **1973**]. Recently, phytochemical investigation on the plant *Eucomis bicolor* Baker has resulted in the isolation of three triterpenoid oligosaccharides viz., scillasaponin A-C. Their structures are shown in **Figure 1.8** in order to show case that within a single organism too, varied sugar linkage are present. Interestingly, scillasaponin A has a modified spirocyclic side chain and has been found to inhibit cyclic AMP phosphodiesterase at extremely low concentration [Ori *et al.*, **2013**].

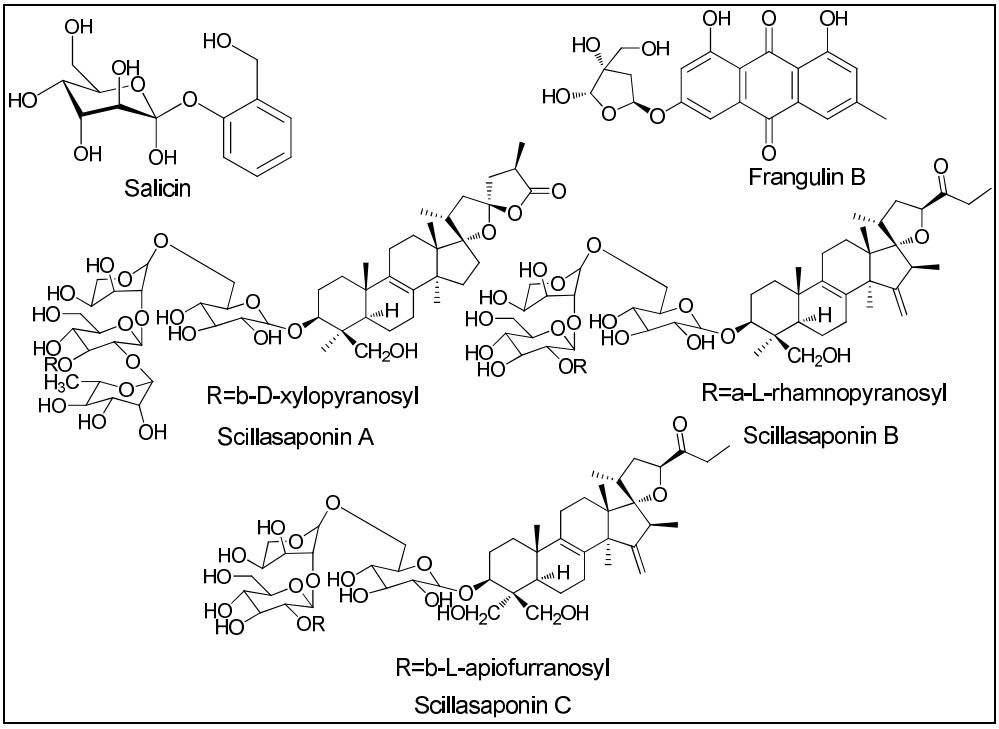


Figure 1.8 : Structures of some glycosides

1.1.1.5. Phenyl propanoids : lignans and neolignans

Lignans and neolignans are a large group of natural products characterised by the coupling of two phenyl propanoids i.e. C_6-C_3 units. When the two C_6-C_3 units are linked by a bond between positions 8 and 8', the compound is referred to and named as a lignan. Plant lignans come from sources such as whole grain cereals, berries, vegetables, flax seeds etc., and have attracted much attention due to their positive health effects. In the absence of the C-8 to C-8' bond, and where the two C_6-C_3 units are linked by a carbon-carbon bond, it is referred to and named as a neolignan. Where there are no direct carbon-carbon bonds between the C_6-C_3 units and they are linked by an ether oxygen atom, the compound is named as an oxyneolignan. The sesquineolignans have three C_6-C_3 units and dineolignans have four C_6-C_3 units. *Since the second chapter of this thesis will deal with many lignans, the biosynthesis of lignans and neolignans is outlined here for a clear perspective.*

1.1.1.5.1. Biosynthesis

As mentioned before, lignans and neolignans are both originated from C_6-C_3 units, thus indicating that these metabolites are biosynthesized through the same pathway in the earlier steps. As seen in **Figure 1.9**, aromatic aminoacids *L*-phenylalanine and *L*-tyrosine which are produced from shikimic acid pathway are then converted in to series of cinnamic acid derivatives. The reduction of these acids via coenzyme A of related esters and aldehydes forms the alcohols *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, that are the main precursors of all lignans and neolignans.

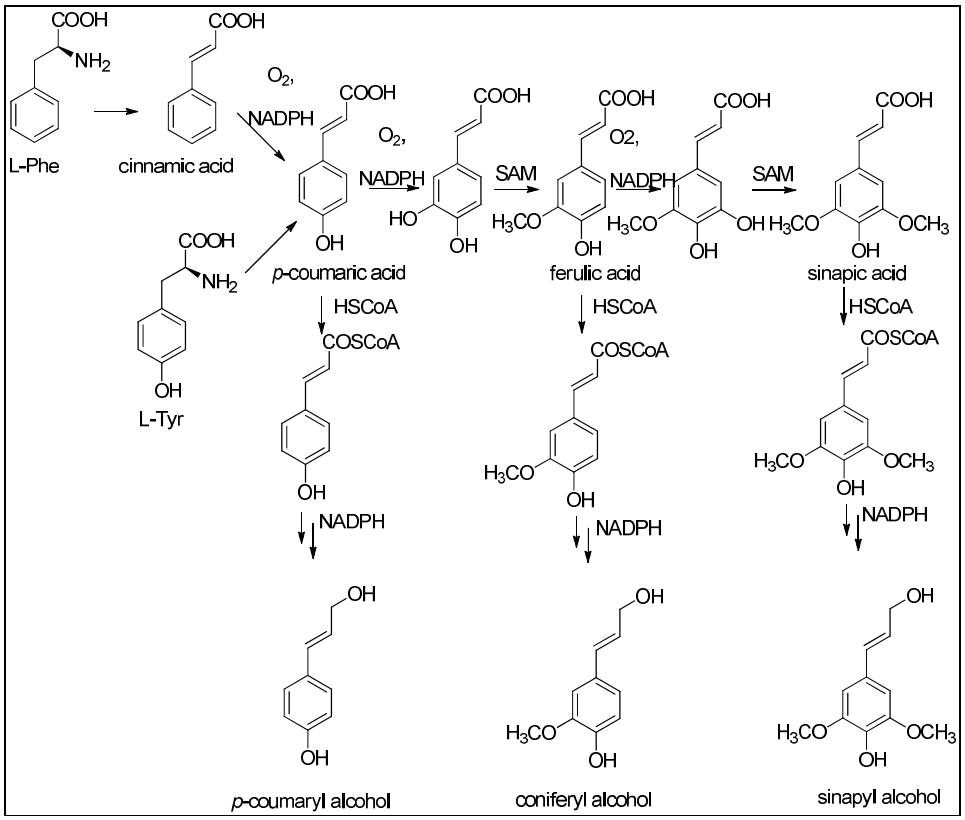


Figure 1.9: Synthesis of phenyl propanoids

Further, the peroxidase enzyme induces one-electron oxidation of the phenol group allowing the delocalization of the unpaired electron through its resonance forms. In these hydroxycinnamyl alcohols, conjugation allows the unpaired electron to be delocalized also into the side chain. After this point, radical pairing of these resonance structures lead to reactive dimeric systems susceptible to nucleophilic attack from hydroxyl groups, thus leading to a wide range of lignans and neolignans.

The biosynthesis of lignans and neolignans involve the enantioseletive dimerization of two coniferyl alcohol monomeric units shown below viz., B and D resonance forms of coniferyl alcohol radical as in **Figure 1.10** and **Figure 1.11** [Dewick, **2002**].

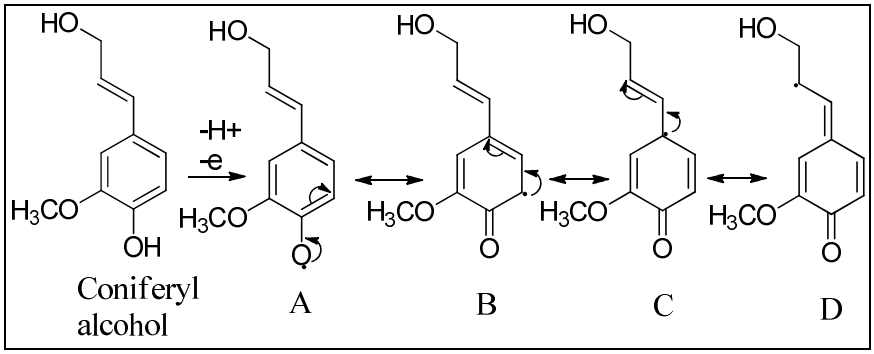


Figure 1.10 : Resonance structures of coniferyl alcohol

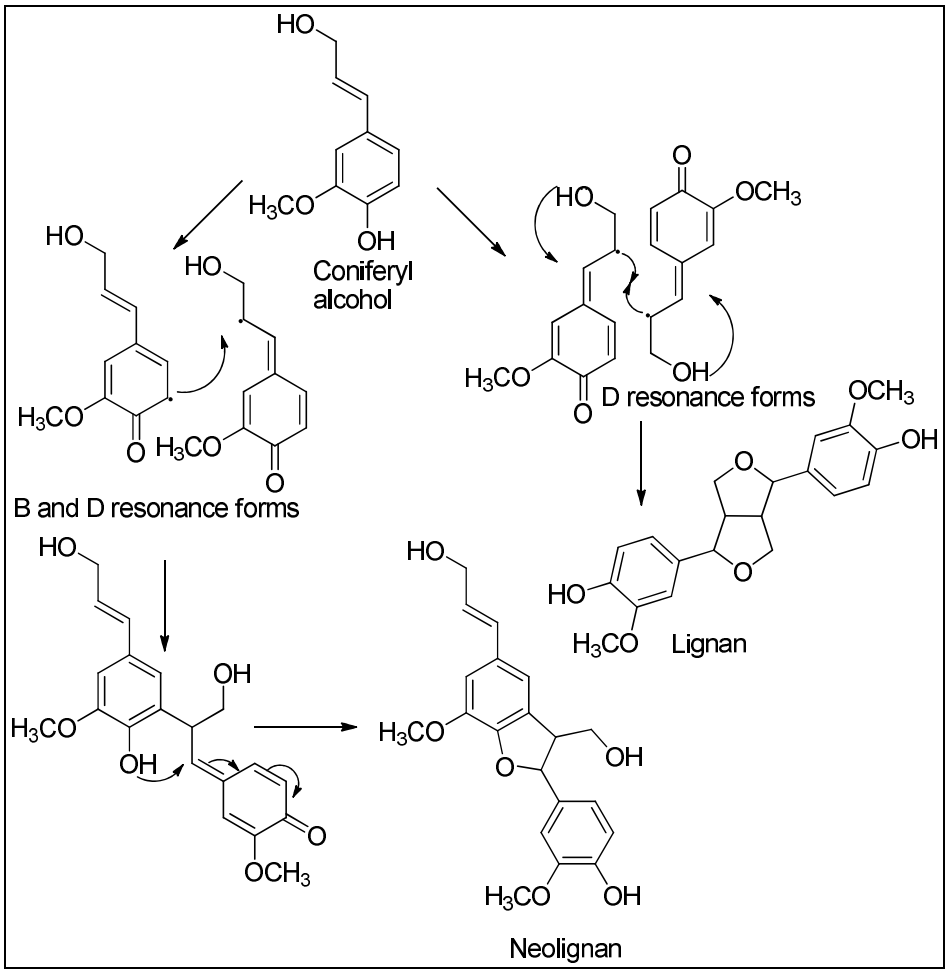
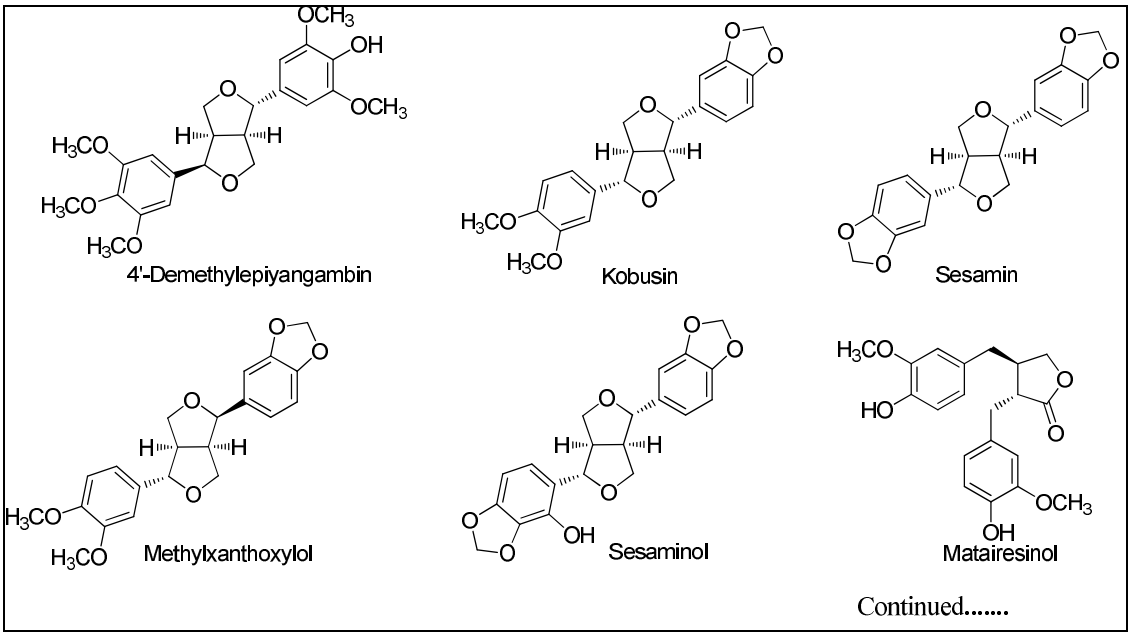


Figure 1.11 : Synthesis of lignans and neolignans

As mentioned earlier, the lignans and neolignans occur with diverse structures due to their C_8-$C_{8'}$ bonding characteristics. Structures of important dietary lignans and biologically active lignans are shown in **Figure 1.12**. The lignans with 2,6-diaryl-3,7-dioxabicyclo[3.3.0]octane structure include sesamin, sesaminol (antioxidants from *Sesamum indicum* seeds, whose intake reduces cholesterol level in humans) [Liang *et al*., **2015**], pinoresinol from *Forsythia intermedia* which is a blood platelet activating factor [Iwakami *et al*., **1992**], 4'-demethylepiyangambin from the leaves of *Ocotea duckei* [Hojo *et al.*, **1996**], Kobusin (methylpiperitol) and methylxanthoxylol isolated from *Samadera bidwillii* [Gibbons *et al*, **1997**].

Two very important lignans with biological activity are secoisolariciresinol and matairesinol. Arylnaphthalene type of lignans have gained much attention after the discovery of podophyllotoxin as an anticancer agent(discussed later). Examples of other arylnaphthalene type lignans include the phyllamyricins C–F isolated from *Phyllanthus myrtifolius*, where they occur along with phyllamyricosides A–C. [Lee *et al.*, **1996**].



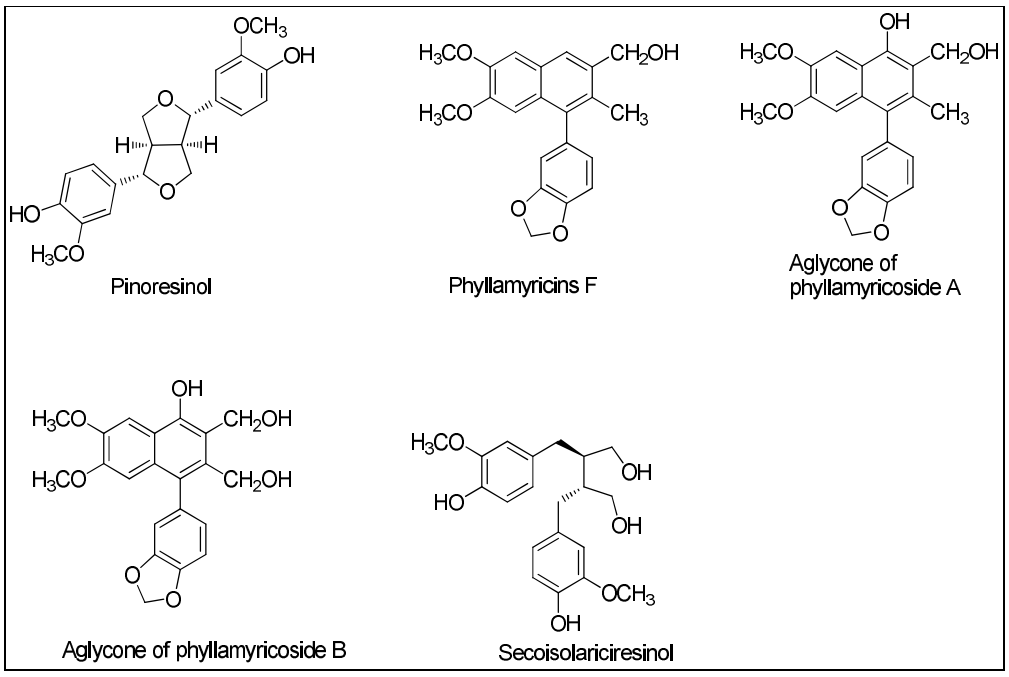


Figure 1.12 : Structures of important lignans and neolignans

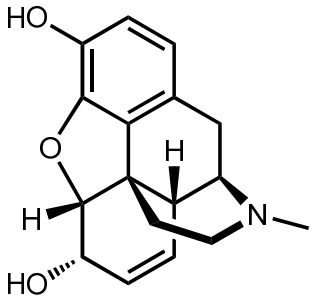
1.1.2. Some important plant derived drugs

Apart from curiosity, the main reason for exploring the plant world in search of novel natural products/ phytochemicals has been for obtaining better drugs. A few of these natural products which are very well known for their utility to man as drugs and few that are important historically in the development of pharmaceutical agents are mentioned below.

Morphine is a potent opiate analgesic drug, which is used to relieve severe pain. It is the most abundant opiate found in opium, the dried latex extracted by shallowly slicing the unripe seedpods of the *Papaver somniferum* known as 'poppy'. Morphine was the first active principle purified from a plant source (Frederich Serturner, 1803-1805) and is primarily used to treat both acute and chronic severe pain [Friedrich *et al.*, **1984** ; David, **2009**]. Annually 45,000 kg of it is used directly for pain relief. Thebaine, also known as codeine methyl enol ether is the main alkaloid extracted from Iranian poppy, *Papaver*

bracteatum. Oxycodone is a semisynthetic opioid synthesized from thebaine which is extensively used clinically.

Papaver somniferum

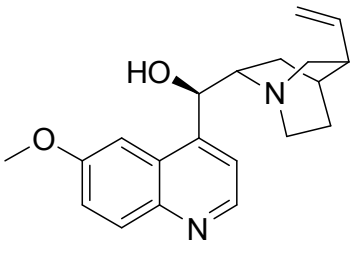


Morphine

Figure 1.13

Quinine is a natural white crystalline alkaloid with bitter taste having antipyretic, antimalarial, analgesic, and anti-inflammatory properties. It is a stereoisomer of quinidine which, unlike quinine, is an antiarrhythmic. Quinine occurs naturally in the bark of the *Cinchona officinalis*, though it is now synthesized in the laboratory in larger quantities. Quinine contains two major fused-ring systems viz., the aromatic quinoline and the bicyclic quinuclidine. Quinine was the first effective treatment for malaria caused by *Plasmodium falciparum*. It remained as the antimalarial drug of choice until the 1940s, when other drugs based on quinine such as chloroquine, mefloquine etc. were developed that had less unpleasant side effects and higher activity on resistant strains of *P. falciparum*

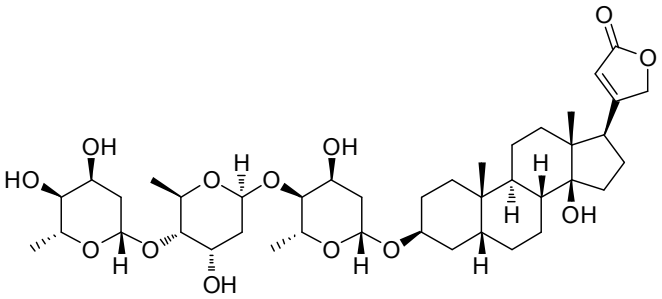
Cinchona officinalis



Quinine

Figure 1.14

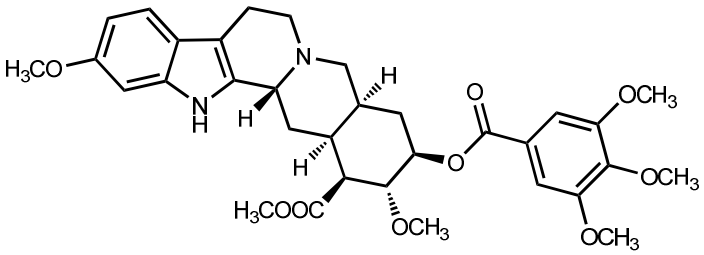
Digitoxin is a cardiac glycoside isolated from *Digitalis purpurea* commonly known as foxglove plant [Deluka *et al.*, **1989** ; Castle, **1975**]. Even though the first description of the use of foxglove dates back to 1775, the active compound was isolated in 1875 by Oswald Schmiedeberg who was able to obtain a pure sample. Digitoxin and related cardenolides also displayed some anticancer activity against a range of human cancer cell lines *in vitro* but the clinical use of digitoxin to treat cancer has been restricted by its narrow therapeutic index as well as side effects.



Digitalis purpurea Digitoxin

Figure 1.15

Reserpine is an indole alkaloid, isolated from the dried roots of the plant *Rauwolfia serpentina* (Indian snakeroot) which is known as Sarpagandha in Sanskrit and has been used for centuries in India for the treatment of insanity, fever and snakebites. Reserpine was used for the control of high blood pressure [Garrett *et al.*, **1968**] for many years but has now been abandoned due to its side-effects. Unfortunately the side effects include nasal congestion, nausea, vomiting, weight gain, gastric intolerance, gastric ulceration (due to increased cholinergic activity in gastric tissue and impaired mucosal quality), stomach cramps and diarrhoea.

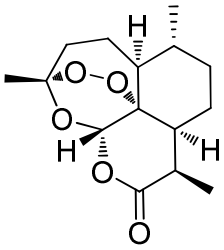


Rauwolfia serpentina Reserpine

Figure 1.16

Artemisinin is a natural antimalarial drug isolated from the herb *Artemisia annua*, a plant that was used by Chinese herbalists for more than a thousand years in the treatment of malaria and fever. Chemically, artemisinin is a sesquiterpene lactone containing an unusual peroxide bridge. This peroxide moiety is believed to be responsible for the drug's mechanism of action. Malaria was traditionally treated by chloroquine or quinine, but with declining success. By the late 1960s, efforts to eradicate Malaria had failed and the disease was on the rise. At that time, Youyou Tu in China turned to traditional herbal medicine to tackle the challenge of developing novel Malaria therapies. From a large-scale screen of herbal remedies in Malaria-infected animals, an extract from the plant *Artemisia annua* emerged as an interesting candidate. However, the results were inconsistent, so Tu revisited the ancient literature and discovered clues that guided her in her quest to successfully extract the active component from *Artemisia annua*. Tu was the first to show that this component, later called Artemisinin, was highly effective against the Malaria parasite *Plasmodium falciparum* , both in infected animals and in humans [Tu *et al.*, **1981**] The discovery of the drug Aremisinin has significantly reduced the mortality rates for patients suffering from Malaria. Artemisinin represents a new class of antimalarial agents that rapidly kill the Malaria parasites at an early stage of their development, which explains its unprecedented potency in the treatment of severe malaria. This discovery has led to her winning the Nobel prize in medicine along with William C. Campbell and Satoshi Ōmura in 2015.

Artemisia annua



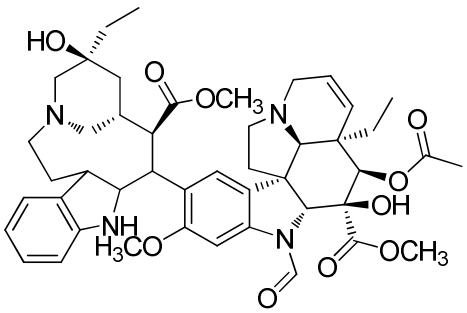
Artemisinin

Figure 1.17

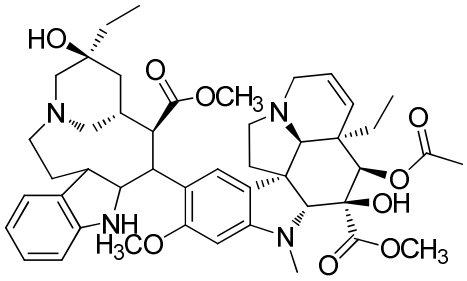
Catharanthus roseus (Madagascar periwinkle) is a traditional herbal medicine used for the treatment of diabetes, malaria and Hodgkin's lymphoma. Alkaloids are the major constituents present in this plant. Vincristine and vinblastine (commonly known as vinca

alkaloids) have been isolated from *Catharanthus roseus*, which have proven to be effective against childhood leukaemia, breast cancer, Hodgkin's disease (cancer of the lymph nodes) and choriocarcinoma [Oleg *et al*., **1963**]. Vicristine and vinblastine exert their anticancer properties by inhibiting mitosis by binding to tubulin, thus preventing the cell from making spindles [Gordon *et al*., **2005**]. Vinorelbine is one of the synthetically derived drugs from vinca alkaloids which has been used against non small-cell lung cancer [Gridelli, **1999**]. Vinorelbine was approved by United States Food and Drug Administration (FDA) in December 1994 and is marketed as a drug since 2003.

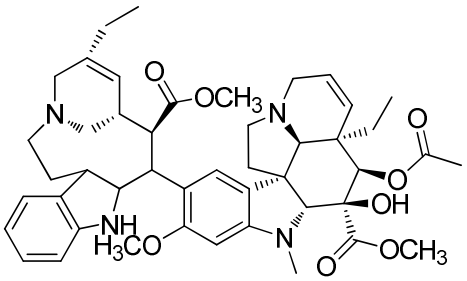
Catharanthus roseus



Vincristine



Vinblastine



Vinorelbine

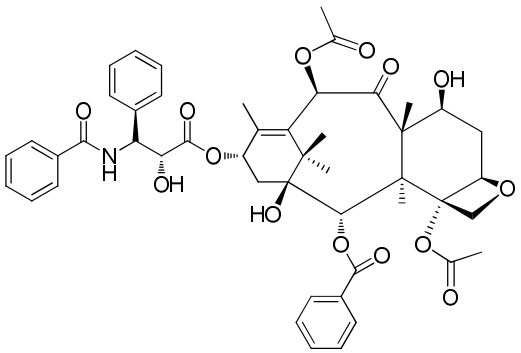
Figure 1.18

Taxol was isolated by Monroe E. Wall and Mansukhlal C. Wani from the bark of the Pacific yew tree, *Taxus brevifolia* [Mansukhlal *et al.*, **1971**]. As a result of long term programme of the National Cancer Institute (NCI), USA, on finding new drugs for the treatment of cancer, taxol too was tested and the world got a new drug called Paclitaxel/Taxol for treating breast cancer. Paclitaxel has basic [$9.3.1.0^{3,8}$] pentadecane, tetracyclic ring system. It has a N-benzoyl-*β*-phenylisoserine side chain attached at the C-

13 hydroxyl as an ester linkage. Taxol is a mitotic inhibitor used in cancer chemotherapy and is used to treat patients with lung, ovarian, breast, head and neck cancer, and advanced forms of Kaposi's sarcoma. Paclitaxel promotes microtubule assembly and stabilizes microtubule polymers, thereby blocking cell replication. This leads to apoptosis. After the discovery of taxol and its unique mechanism of action of stabilizing microtubules, several compounds having the same mode of action were discovered. The discovery that 10-deacetyl baccatin can be readily obtained from the leaves of *Taxus baccata* (European yew, also found in lower Himalayas) has paved way for obtaining large amounts of taxol/paclitaxel through semi synthesis. Paclitaxel is also useful for the prevention of restenosis occuring in heart patients who have stents put in their valves.

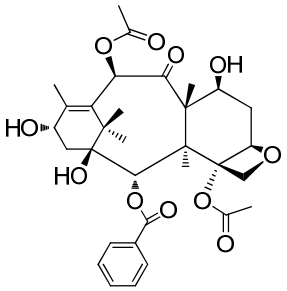
Docetaxel is one of the synthetic derivatives of taxol that is a clinically well-established anti-mitotic chemotherapy medication that also works by interfering with cell division. Docetaxel remains twice as potent as paclitaxel (due to docetaxel's effect on the centrosome of the mitotic spindle), but the two taxanes have been observed to have comparable efficacy *in vivo*. Like paclitaxel, it interferes with microtubule stability, preventing cell division and leading to apoptosis. Clinical data has shown that docetaxel has cytotoxic activity against breast, colorectal, lung, ovarian, prostate, liver, renal, gastric, head and neck cancers, and melanoma.

Taxus brevifolia

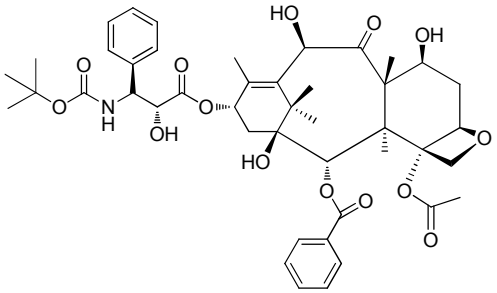


Taxol

Taxus baccata



10-deacetyl baccatin III

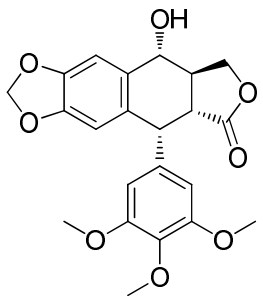


Docetaxel

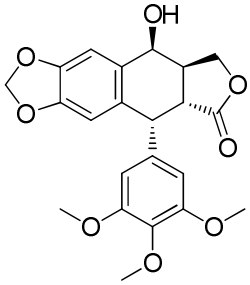
Figure 1.19

Podophyllotoxin is another natural anticancer compound isolated from the rhizomes of *Podophyllum peltatum* (American May apple) [Jackson *et al.*, **1984**]. Podophyllotoxin has an aryl tetralin structure with four consecutive chiral centers. It is used for the treatment of external genital warts, caused by some types of the human papilloma virus (HPV). Two synthetic derivatives of epipodophyllotoxin (an isomer of podophyllotoxin) viz., etoposide and teniposide have reduced adverse effects as compared to podophyllotoxin and improved anticancer activity even though their mechanism of action is different. They are currently used clinically in various chemotherapies for lung cancer, lymphomas, leukamia and testicular cancers. Podophyllotoxin binds reversibly to tubulin and inhibits microtubule assembly whereas, etoposide and teneposide inhibit the enzyme DNA topoisomerase II and subsequently increases DNA cleavage thus preventing DNA synthesis and cell cycle progression.

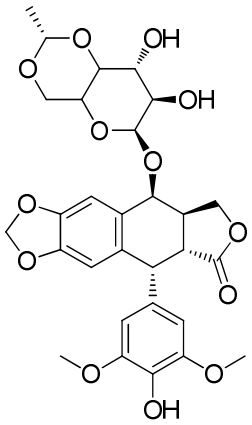
Podophyllum peltatum



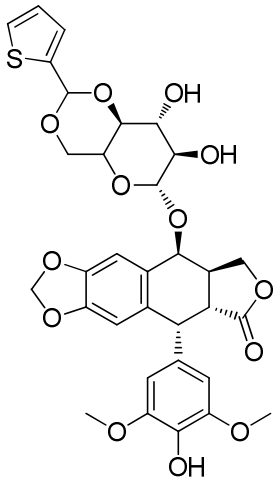
Podophyllotoxin



Epipodophyllotoxin



Etoposide

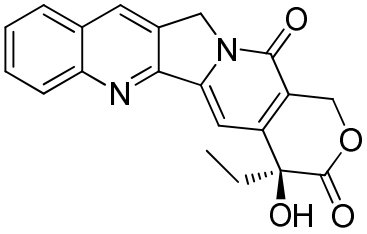


Teniposide

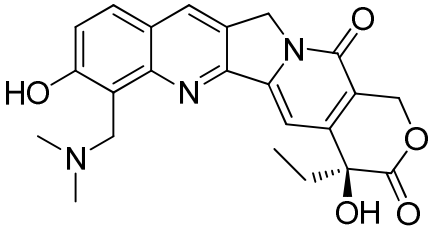
Figure 1.20

Camptothecin is a cytotoxic quinoline alkaloid isolated first from the bark and stem of *Camptotheca acuminata*, a tree native to China [Wall *et al.*, **1966**]. Camptothecin has a planar pentacyclic ring structure, that includes a pyrrole [3,4-*β*]- quinoline moiety, conjugated pyridine moiety and one chiral centre at position 20 within the alpha-hydroxy lactone ring with (*S*) configuration. This compound inhibits the DNA enzyme topoisomerase I (topo I) [Pommier *et al.*, **2009]**. It shows remarkable anticancer activity but has low solubility. Numerous synthetic derivatives of camptothecin have been synthesized and investigated. Two camptothecin analogues have been approved and are used in cancer chemotherapy today. They are topotecan and irinotecan which are used for the treatment of ovarian and lung cancer.

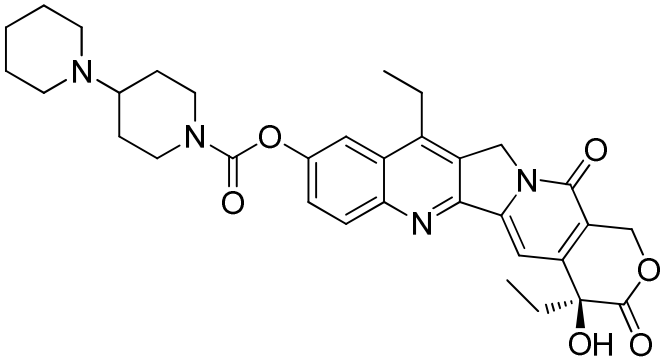
Camptotheca acuminata



Camptothecin



Topotecan



Irrinotecan

Figure 1.21

1.2. Cancer

Cancer is one of the most dreaded diseases of today that causes death of nearly 10 million lives each year worldwide [Reddy *et al*., **2003**]. Cancer is basically a disease of cells characterized by a shift in the control mechanisms that govern cell proliferation and differentiation. Such cells proliferate excessively and form local tumors that can compress or invade adjacent normal structures. A small subpopulation of cells within the tumor can be described as tumor stem cells. They retain the ability to undergo repeated cycles of proliferation as well as to migrate to distant sites in the body to colonize various organs in the process called metastasis. More than 100 different types of cancers are known to occur including tumours of/at/in breast, cervix, prostate, stomach, colon, rectum, lung, mouth, blood/leukaemia, sarcoma of bone, Hodgkin disease etc [Abdulla *et al*., **2000**].

The incidence, geographic distribution, and behaviour of specific types of cancer are related to multiple factors, including sex, age, race, genetic predisposition, and exposure to environmental carcinogens. Of these factors, environmental exposure is probably most

important. Chemical carcinogens (particularly those in tobacco smoke) as well as azo dyes, aflatoxins, asbestos, and benzene have been clearly implicated in cancer induction in humans and animals. Cancers such as colon and breast cancer, often run in families from one generation to another. It is only the predisposition to cancer that is inherited [Doll *et al*., **1981**]. A woman is at increased risk for some gynecological cancers (e.g. breast or uterine cancer) if her system is exposed to too much estrogen, as this stimulates cell proliferation in these tissues. Over exposure to ionizing radiation such as X-rays and nuclear radiation can cause DNA injury that may lead to cancer. Ultraviolet B (UVB) rays damage cell DNA and can cause 90% of all skin cancers. Chemical carcinogens such as asbestos, benzene, formaldehyde and diesel exhaust are dangerous in high concentrations. Many pesticides including organochlorines, chlordane, DDT and indane are tumour promoters [Ditch *et al*., **1997**]. Smoking can cause cancers in the lungs. People who drink alcohol heavily have a higher risk of mouth, throat, oesophagus, stomach and liver cancer. Salted, pickled and smoked foods, such as pickles or smoked fish and meats treated with nitrites are sources of carcinogens. Taking Vitamin C, either through the diet or by supplementation may protect against the cancer-causing effects of carcinogenic foods. A diet with high saturated fat (especially from red meat) is associated with several different types of cancer, including colon cancer, rectum, and prostate cancer. Free radicals can also cause DNA damage and lead to cancer. Antioxidants such as Vitamin A and C are taken through supplementation, or a diet which is high in fruits and vegetables, can reduce the risk produced by free radicals.

1.2.1. Metastasis

Metastasis is a complex process in which cancer cells break away from the primary tumour and circulate through the bloodstream or lymphatic system to other sites in the body [Klein, **2008**; Feller *et al*., **2012**]. At new sites, the cells continue to multiply and eventually form additional tumours comprised of cells that reflect the tissue of origin. Many fundamental questions remain about the clonal structures of metastatic tumours, phylogenetic relationships among metastases, the scale of ongoing parallel evolution in metastatic and primary sites, how the tumour disseminates, and the role that the tumour microenvironment plays in the determination of the metastatic site.

There are a number of treatment options available for cancer. Treatments plans are developed depending on the type of cancer, its location, the extent of the cancer and the stage at which it is diagnosed and the health and well-being of the patient. With present methods of treatment, one third of patients are cured with local modalities (surgery or radiation therapy), which are quite effective when the tumor has not metastasized by the time of treatment. Earlier diagnosis might lead to increased cure rates with such local treatment; however, in the remaining cases, where metastasis is likely to have occurred, a systemic approach such as chemotherapy is required (often along with surgery or radiation) for effective cancer management.

Chemotherapy is the use of anticancer drugs. Anti cancer drugs destroy cancer cells by stopping growth or multiplication at some point in their life cycles. Drugs may be administered intravenously, orally, by injection into a muscle, topically (applied to the skin) or in other ways, depending on the drug and the type of cancer. Chemotherapy is often given in cycles of alternating treatment and rest periods [Brennan *et al*., **2013**].

A major effort to develop anticancer drugs through both empiric screening and rational design of new compounds has been under way for over four to five decades. In the earlier section brief description of a few anticancer agents thus discovered for chemotherapy viz., taxol, podophyllotoxin, vincristine etc. has been given. Recent advances in this field have included the synthesis of peptides and proteins with recombinant DNA techniques and monoclonal antibodies. The drug development program has employed testing in a few well-characterized transplantable animal tumor systems. Simple *in vitro* assays for measuring drug sensitivity of a battery of human tumor cells augment and shorten the testing program and are used currently as the primary screening tests for new agents by the National Cancer Institute and many pharmaceutical firms. After new drugs with potential anticancer activity are identified, they are subjected to preclinical toxicologic and limited pharmacologic studies in animals. Promising agents that do not have excessive toxicity are then advanced to phase I & phase II clinical trials, wherein their pharmacologic and toxic effects are usually tested in patients with advanced cancer.

Ideal anticancer drugs would eradicate cancer cells without harming normal tissues. Unfortunately, no currently available agents meet this criterion, and clinical use of these drugs involves a weighing of benefits against toxicity in a search for a favourable therapeutic index. Classes of drugs that have recently entered clinical development include signal transduction inhibitors, focused on critical signaling pathways essential for cell growth and proliferation; microtubule inhibitors, directed against the mitotic spindle apparatus; differentiation agents, antimetastatic drugs, designed to perturb surface properties of malignant cells and thus alter their invasive and metastatic potential, antiangiogenic agents, designed to inhibit the formation of tumor vasculature etc [Russo *et al*., **2015**]. Brief description of few anticancer agents from marine natural sources, which are under phase I & phase II clinical trials are given below.

Aplidine is a marine derived natural product isolated from Mediterranean tunicate, *Aplidium albicans*. Aplidine is formally known as dehydrodidemnin B and is a cyclic depsipeptide [Rinehart *et al*., **1989**]. Aplidin was placed in to Phase I clinical trials in 1999 for the treatment of both solid tumours and non-Hodgkin's lymphoma. It interferes with the synthesis of DNA and induces G_1-G_2 cell cycle arrest [Ebra *et al*., **2002**]. It underwent Phase II clinical trials against leukaemia and was given orphan drug status against lymphoblastic leukemia [Gomez *et al*., **2003**]. It shows promise in shrinking tumors in pancreatic, stomach, bladder, and prostate cancers. As of 2007, it was undergoing multicenter phase II clinical trials [Adrio *et al*., **2007**; Baudin *et al*., **2014**; Newman *et al*., **2014**].

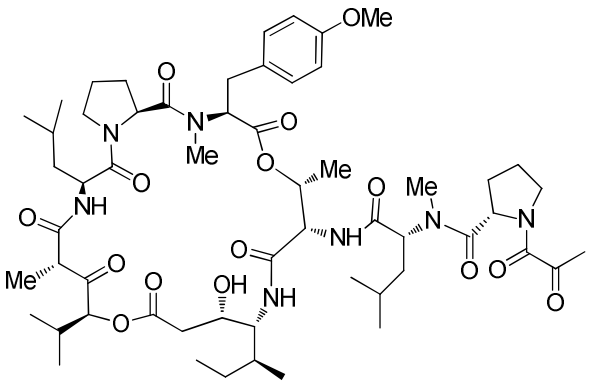


Figure 1.22. Aplidine (Dehydrodidemnin B)

Dolastatin 10 and **dolastatin 15** are marine natural products isolated from the Indian Ocean sea hare *Dollabella auricularia* [Hiroki *et al*., **1997**]. This potent antitumor agent is also isolated from the marine cyanobacterium *Symploca sp. VP642* from Palau [Hendrik *et al*., **2001**; Luesch *et al*., **2001**]. Being small linear peptide molecules, dolastatin 10 and 15 are considered as promising anti-cancer drugs showing potency against breast and liver cancers, solid tumors and some leukemias [Yamada *et al*., **2000**]. Preclinical research indicated potency in experimental antineoplastic and tubulin assembly systems. The dolastatins are mitotic inhibitors. Several dolastatin derivatives have been patented in 2014 [Perez *et al*., **2014**].

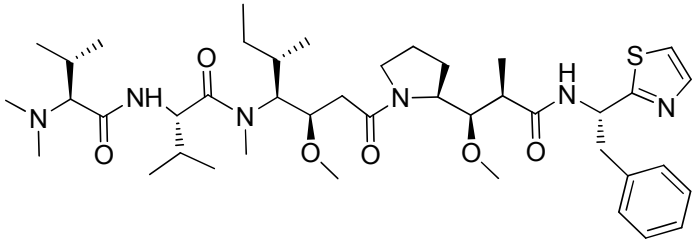


Figure 1.23. Dolastatin 10

Bryostatins are macrocyclic lactones isolated [Pettit *et al*., **1982**; Pettit, **1996**] from marine invertebrate *Bugula neritine*. Among all the bryostatins isolated, bryostatin I is biologically more important one. To date, bryostatin 1 has been in more than 80 human clinical trials, with more than 20 being completed at both the Phase I and Phase II levels [Pettit *et al*., **2002**].

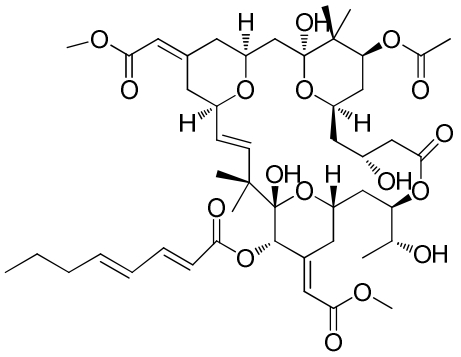


Figure 1.24. Bryostatin 1

KRN-7000 is one of the synthetic derivative of agelasphins, which were isolated from marine sponge *Agelas mauritianus* [Natori *et al*., **1993**]. Agelaphins have been

demonstrated to have antitumor and immonostimulatory activities. These molecules have potent *in vivo* activity against the murine B 16 melanoma, various derivatives were made, leading in the production of KRN-7000 [Motoki *et al*., **1995**]. KRN-7000 entered into Phase I clinical trials in both Asia and Europe for cancer immunotherapy.

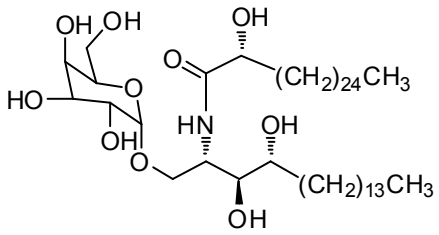


Figure 1.25. KRN-7000

Kahalalide F is marine depsipeptide isolated from *Elysia rufescens*, a marine mollusc found in Hawaii [Hamann *et al*., **1993**]. Kahalalide F showed *in vitro* and *in vivo* selectivity for prostate-derived cell lines and tumours [Suarez *et al*., **2003**]. Phase I clinical trials in patients with androgen-independent prostate cancer have begun.

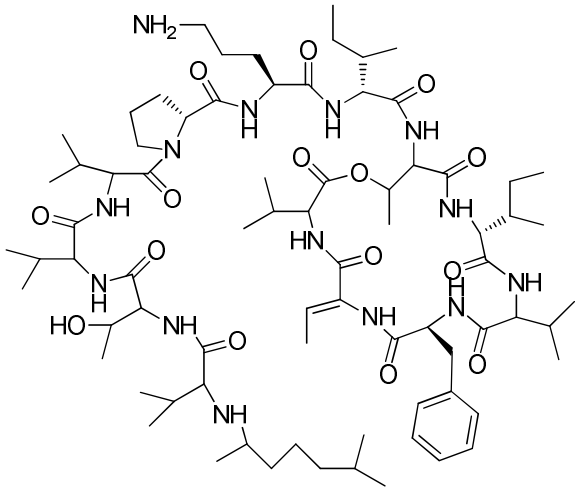


Figure 1.26. Kahalaide F

Natural products thus continue to be an invaluable resource in the search for new anticancer compounds. As cancer as well as many chronic diseases such as cardiovascular diseases, diabetes, arthritis, inflammatory disorders, Alzheimer's disease and Parkinson's disease are mediated through reactive oxygen species, some of the salient features of reactive oxygen species are described in the following sections.

1.3. Reactive oxygen species (ROS)

Oxygen is an element indispensable for life. When cells use oxygen to generate energy, free radicals are produced by the mitochondria. These by-products are generally reactive oxygen species (ROS) that result from the cellular redox process [Tiwari, **2004**]. The free radicals have a special affinity for lipids, proteins, carbohydrates and nucleic acids. A 'free radical' is any chemical species capable of independent existence possessing one or more unpaired electrons.

ROS are also present in the atmosphere as pollutants and can be generated (i) during UV light irradiation, by X-rays and gamma rays (ii) during metal catalyzed reactions (iii) by neutrophils, eosinophils and macrophages during inflammatory cell activation, as by-products of mitochondrial catalyzed electron transport reactions, (v) by cytochrome P450 metabolism and the enzyme xanthine oxidase, which catalyzes the reaction of hypoxanthine to xanthine and xanthine to uric acid [Valko *et al*., **2004**; Sisen, **2014**].

Reactive oxygen species can be classified into oxygen-centered radicals and oxygen-centred non radicals. Various Reactive Oxygen Species are shown in the **Table 1.1**

Superoxide anion radical ($O_2^{-\bullet}$)	One-electron reduction state of O_2, formed in many autoxidation reactions and by the electron transport chain. It is unreactive but can release Fe^{2+} from iron-sulphur proteins and ferritin. This radical undergoes dismutation to form H_2O_2 spontaneously or by enzymatic catalysis.
Hydrogen peroxide (H_2O_2)	Two-electron reduction state, formed by dismutation of $O_2^{-\bullet}$ or by direct reduction of O_2. Hydogen peroxide is lipid soluble and thus able to diffuse across membranes.

Hydroxyl radical ($OH^{\bullet}$)	It is an oxygen centred radical which is produced in presence of oxygen by radical addition to double bonds or by hydrogen abstraction.
Hypochlorous acid (HOCl)	This radical is formed from H_2O_2 by myeloperoxidase. It is highly reactive and lipid soluble. It readily oxidizes protein constituents, including thiol groups, amino groups and methionine.
Peroxynitrite (ONOO-)	Peroxynitrite is formed in a rapid reaction between $O_2^{-\bullet}$ and $NO^{-\bullet}$. It is lipid soluble and has similar in reactivity to hypochlorous acid. Protonation of peroxynitrite forms peroxynitrous acid, which can undergo homolytic cleavage to form hydroxyl radical and nitrogen dioxide.

Table 1.1 : Various Reactive Oxygen Species

1.3.1. Oxidative stress and antioxidants

Oxidative stress is defined as an imbalance between production of free radicals and reactive metabolites, so-called oxidants or reactive oxygen species, and their elimination by protective mechanisms through agents referred to as antioxidants. This imbalance leads to damage of important bio molecules and cells, with potential impact on the whole organism. The harmful effects of ROS are balanced by the action of antioxidants, some of which are enzymes present in the body. Despite the presence of the cell's antioxidant

defence system to counteract oxidative damage from ROS, oxidative damage accumulates during the life cycle and has been implicated in diseases, aging and age dependent diseases such as cardiovascular disease, cancer, neurodegenerative disorders and other chronic conditions [Bhateja, **2012**].

An **antioxidant** is a molecule that inhibits the oxidation of other molecules. When there is deficiency of these antioxidants, damage due to free radicals can become cumulative and debilitating. Antioxidants are capable of stabilizing, or deactivating, free radicals before they attack cells. Well-known antioxidants include enzymes and other substances, such as vitamin C, vitamin E, and beta carotene, which are capable of counteracting the damaging effects of oxidation. Antioxidants are also commonly added to food products such as vegetable oils and prepared foods to prevent or delay their deterioration from the action of air. Antioxidants may possibly reduce the risks of cancer.

Apart from diet, the body also has several antioxidant mechanisms that can protect itself from ROS mediated damage. The antioxidant enzymes – glutathione peroxidase, catalase, and superoxide dismutase (SOD) are such enzymes. They require micronutrient cofactors such as selenium, iron, copper, zinc, and manganese for their activity. It has been suggested that an inadequate dietary intake of these trace elements may also lead to low antioxidant activity [Bhateja, **2012**].

1.3.2. Natural antioxidants

Living tissues can produce antioxidants to control free radicals, lipid oxidation catalysts, oxidation intermediates and secondary breakdown products. These free radicals, reactive oxygen species and pro oxidants generated in living tissues both exogenously (by heat and light) and endogenously (H_2O_2 and transition metals) by the oxidative stress generated within the cells [Nakatani, **2003**; Agati *et al.*, **2007**; Brown *et al.*, **2007**; Chen, **2008**; Iacopini *et al.*, **2008**]. These antioxidant compounds include flavonoids, phenolic acids, carotenoids, and tocopherols that can inhibit Fe^{3+}/AA-induced oxidation, scavenge free radicals and act as reductants [Khanduja, **2003**; Ozsoy *et al.*, **2009**].

Spices and herbs, used in foods for their flavour and in traditional medicinal mixtures for their physiological effects, often contain high concentrations of phenolic compounds that have strong H-donating activity [Lugasi *et al.*, **1995**; Muchuweti *et al.*, **2007**]. Many

of these antioxidants have high ORAC (Oxygen Radical Absorbance Capacity) values. Some plant derived compounds (carnosol, rosmanol, rosmariquinone, and rosmaridiphenol) are better antioxidants than BHA [Richheimer *et al.*, **1996**; Carvalho *et al.*, **2005**].

The major antioxidant plant phenolics can be divided into 4 general groups: phenolic acids (gallic, protochatechuic, caffeic and rosmarinic acids), phenolic diterpenes (carnosol and carnosic acid), flavonoids (quercetin and catechin) and volatile oils (eugenol, carvacrol, thymol, and menthol) [Shan *et al.*, **2005**]. Phenolic acids generally act as antioxidants by trapping free radicals; flavonoids can scavenge free radicals and chelate metals as well [Brewer, **2011**]. Structures of some of the natural antioxidants are shown in **Figure 1.22**.

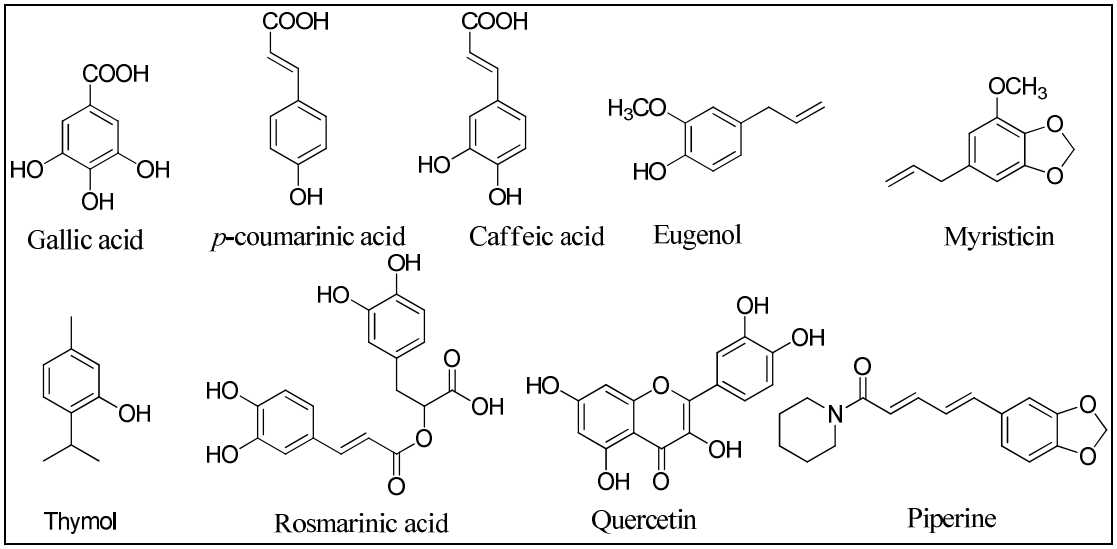


Figure 1.27 : Structures of some natural antioxidants

1.3.3. Spices as antioxidants

Like herbs, spices have significant antioxidant capacity measured using total equivalent antioxidant capacities and total phenolic contents. Major phenolic acids present in spices include caffeic, *p*-coumaric, ferulic, and neochlorogenic acids. Predominant flavonoids found in spices include quercetin, luteolin, apigenin, kaempferol, and isorhamnetin. Spices also possess antibacterial effects. Shan and others found that, of 46 spice extracts evaluated, many exhibited antibacterial activity against foodborne pathogens [Shan *et al.*, **2005**]. The antibacterial activity of the extracts was closely

associated with their phenolic content as well as volatile oils containing many mono and sesquiterpenes. Some of the important spices that are grown in Kerala are discussed below.

1.3.3.1 Cinnamon

Cinnamon (*Cinnamonum zeylanicum*) contains a number of antioxidant components including vanillic, caffeic, gallic, protochatechuic, *p*-hydroxy benzoic, *p*-coumarinic, and ferulic acids and *p*-hydroxybenzaldehyde [Muchuweti *et al*., **2007**]. Of a number of herbs and spices (bay leaves, rosemary, sage, marjoram, oregano, cinnamon, parsley, sweet basil, and mint) evaluated, cinnamon has been found to have the highest polyphenolic compound concentration. Out of 42 commonly used essential oils, cinnamon bark, oregano, and thyme have been found to have the strongest free radical-scavenging abilities [Wen *et al.*, **2009**]. Cinnamon oil shows a very good radical-scavenging activity at 5 mg/ml concentration. The major components responsible for this activity are eugenol, carvacrol, and thymol. Jayaprakasha and co-workers have identified 27 compounds in the volatile oil of cinnamon stalks [Jayaprakasha *et al.*, **2003**]. The volatile oil composed of 44.7% hydrocarbons and 52.6% oxygenated compounds. The antioxidant capability of cinnamon essential oil is stronger than its free radical scavenging capacity [Chen, **2008**]. The essential oil of the cinnamon is a better superoxide radical scavenger than propyl gallate, mint, anise, vanilla, ginger, nutmeg BHA or BHT [Murcia *et al.*, **2004**].

1.3.3.2 Clove

The important components of clove (*Eugenia caryophyllus)* essential oil include, phenylpropanoids such as eugenol, carvacrol, thymol, and cinnamaldehyde [Chaieb *et al.*, **2007**]. Clove consist of a variety of non-volatile compounds (tannins, sterols, flavonoids, and triterpenes) too. Jirovetz *et al*, identified 23 compounds in clove oil, with the main components present being eugenol (76.8%), *β*-caryophyllene (17.4%), *α*-humulene (2.1%), and eugenyl acetate (1.2%) [Jirovetz *et al.*, **2006**]. Clove essential oil can inhibit the hydroxyl radicals and can chelate iron. Khatun and co-workers found that clove has the highest radical scavenging activity than by all spices [Khatun *et al.*, **2006**]. Eugenol present in the clove oil has been reported to possess antioxidant activity equivalent to trolox, carvacrol, and thymol [Dorman *et al.*, **2000**]. The essential oil scavenges free

radicals at concentrations lower than those of eugenol, BHT, and BHA alone. Marinova *et al*, established that in sunflower oil at 100 ^{o}C, myricetin is a more effective and stronger antioxidant than α-tocopherol [Marinova *et al.*, **2008**]. Mixtures of the eugenol and myrcetin exhibited a synergistic effect that was optimized in an equal molar ratio of the eugenol.

1.3.3.3 Nutmeg

The spice 'nutmeg' is obtained from the plant *Myristca fragrans*. Nutmeg oil contains significant amounts of myristicin and safrole, which are responsible for the characteristic aroma of nutmeg. Sabinene, Myristicin, safrole and elemicin constitute 80% of both nutmeg and mace oil. Jukic *et al*, has isolated glycosidically bound volatiles from nutmeg [Jukic *et al.*, **2006**]. Eugenol and terpinen-4-ol are the two major components present in the glycosidically bound and aglycone fractions. The aglycone fraction showed stronger antioxidant properties than that of the free volatiles which obtained from nutmeg oil. Major phytochemical present in *Myristica fragrans* are argenteane which shows higher antioxidant activity [Calliste *et al.*, **2010**]. Bis-*erythro* argenteane (3,3'-dimethoxy-1,1'-biphenyl-4,4'-diol), a dimeric lignan, which has been isolated from nutmeg mace has been found to have free radical scavenging ability [Chatterjee *et al.*, **2007**; Calliste *et al.*, **2010**]. At 180^{o}C, nutmeg oil showed higher free radical-scavenging activity as compared to basil, cinnamon, clove, oregano, and thyme [Tomaino *et al.*, **2005**].

1.3.3.4 Ginger and Turmeric

The rhizome of *Zingiber officinale*, commonly known as ginger is a spice extensively used in food preparations in Asian countries. The dried rhizome called 'Adraka' in Sanskrit and 'chukku' in Malayalam is used in a number of Ayurvedic medicinal preparations. Fresh and dried ginger contain relatively large amounts of the volatile oils such as camphene, *p*-cineole, alpha-terpineol, zingiberene etc [Tiwari *et al.*, **2006**]. Hydrodistillation of ginger produced volatile oil which showed the high phenolc content [Hinneberg *et al.*, **2006**]. Ginger extract has been shown to have higher antioxidant activity as compared to synthetic antioxidants viz., BHA and BHT [Rehman *et al.*, **2003**]. Kikuzaki *et al*, reported that five gingerols and related compounds and eight diarylheptanoids isolated from ginger rhizomes exhibit higher antioxidant activity than α-tocopherol [Terhune *et al*., **1975**; Kikuzaki *et al.*, **2006**].

Turmeric is a spice derived from the rhizomes of *Curcuma longa* plant, which belongs to the ginger family (Zingiberaceae). The bright yellow colour of turmeric is due to fat-soluble, polyphenolic pigments known as curcuminoids, especially curcumin [Priyadarsini *et al.*, **2003**; Anand *et al*., **2008**]. Turmeric consists of curcumin, dimethoxycurcumin, bis-dimethoxycurcumin and 2,5-xylenol [Zhang *et al.*, **2009**]. Curcumin is a phenolic chain-breaking antioxidant, donating free radical from the phenolic groups rather than from the CH_2 group [Ross *et al*, **2000**]. Jayaprakasha *et al*, reported that antioxidant activity of the curcuminoids is of the order curcumin > BHT > dimethoxycurcumin > bisdemethoxycurcumin [Jayaprakasha *et al.*, **2006**]. However, all of these curcuminoids have limited water solubility limiting their absorption from food. Curcumin is highly effective in neutralizing free radicals [Yu *et al.*, **2008**]. Curcumin has higher antioxidant activity than the polyphenol resveratrol [Aftab *et al.*, **2009**] found in grapes. Turmeric oil has been found to have free radical-scavenging ability as comparable with vitamin E and BHT. The major components of turmeric oil responsible for this antioxidant activity are α and β-turmerone, curlone, and α-terpineol [Carolina *et al.*, **2003**].

1.3.3.5 Black pepper

Black pepper (*Piper nigrum*) is a highly valued spice for its distinct hot taste, digestive and other medicinal properties. It is the alkaloid piperine present in the black pepper that stimulates the digestive enzymes of the pancreas, enhancing digestive capacity. The pungency of black pepper is also due to the presence of piperine [Srinivasan, **2007**]. Piperine can quench free radicals and reactive oxygen species and protect against oxidative damage. Piperine acts as a hydroxyl radical scavenger at low concentrations [Mittal *et al.*, **2000**].

Kapoor and others reported that volatile oil of black pepper (*P. nigrum*) contains 54 components [Kapoor *et al*., **2009**]. β-caryophylline is the major component in the volatile oil of black pepper along with limonene, β-pinene, and sabinene. Pepper essential oils also contain α- and β-pinene, cyclohexene, 1-methyl-4-(1-methylethylidene)-2,3-cyclohexen-1-ol, limonen-6-ol, (*E*)-3(10)-caren-4-ol, and β-caryophyllene [Liang *et al.*, **2010**]. The major component of both ethanol and ethyl acetate extracts of black pepper is is piperine (63.9% and 39.0%, respectively) [Liang *et al.*, **2010**]. The oil and oleoresins

obtained from black pepper have been found to have stronger antioxidant activity than BHA and BHT [Kapoor *et al.*, **2009**]. Gurdip *et al*, reported that, piperine, piperolein B, piperamide, and guineensine are the major compounds present in the non volatile parts of black pepper and *β*-caryophyllene, limonene, sabinene, *β*-bisabolene, and *α*-coapene are the predominant compounds in essential oils. [Gurdip *et al.*, **2004**].

1.4. Objectives of the thesis

The above discussion clearly indicates that natural products continue to be a vital source for the pharmaceutical industry as well as spring of drugs and drug leads. Currently, the continuing influence and success of traditional medicines in the health maintenance of a large number of people in India, China and South East Asian countries have re-energised the search for new natural products from medicinal plants used in these countries. Accordingly, a detailed phytochemical study of three plants viz., *Myristica fragrans*, *Azadirachta indica* and *Pygmacopremna herbacea* has been carried out and this forms the subject matter of the thesis.

Among natural products, spices are extensively used in food preparation and traditional system of medicine in India, China, South East Asia, Middle East countries. They are also very good sources of antioxidants and other biologically active compounds. In the category of spice plants, *Myristica fragrans*, belonging to Myristicacea family is one of the plants most widely used in food and medicinal prroperties. Owing to the great biological significance of *Myristica fragrans*, a detailed phytochemical investigation of different parts of this plant has been undertaken. The details of our investigations are presented in the second chapter of the thesis.

Azadirachta indica, belonging to Meliacae is one of the important plants used in Ayurvedic preparations. We have carried out the isolation of azadirone along with other compounds from *Azadirachta indica* seeds. The isolation and anticancer activity studies of azadirone forms the central theme of Chapter 3. Another important source of naturally occurring chemical compounds is *Pygmacopremna herbacae*, a plant belonging to Verbanaceae family. Chapter 4 deals with the phytochemical investigation of *Pygmacopremna herbacea* and anticancer activity studies of bharangin, one of the compounds isolated from this plant.

CHAPTER 2

Phytochemical Investigation on *Myristica fragrans*

As mentioned in the first chapter, plants are a rich source of biologically active molecules with vast structural diversity. Since less than 10% of the world's biodiversity has been studied, many more natural lead compounds await discovery. Among the large number of spices and herbs used by Asians in their food and traditional systems of medicines is *Myristica fragrans* belonging to the Myristicaceae family. *M. fragrans* is cultivated extensively in Kerala and is a source of major revenue of this state. Though there have been many reports on the phytochemical constituents from *M. fragrans*, detailed investigation on *M. fragrans* grown in Kerala has not been reported. It is well known that the same species of plants grown under different climatic conditions can contain different and or different ratios of phytochemicals in them. Therefore we embarked up on the phytochemical investigation of *M. fragrans* currently grown in Kerala. Different plant parts *M. fragrans* such as fruit pericap, bark, aril (mace) and seed kernel were selected for detailed study. Literature survey on Myristicaceae plants in general and *Myristica* specifically was carried out to understand the current status of the genus *Myristica* and the various species in it, which is summarized in the following introduction.

2.1. Myristicaceae

Myristicaceae, the nutmeg family of the magnolia order (Magnoliales), is best known for the fragrant, spicy seeds of nutmeg (*Myristica fragrans*). The most well known genera in this family are *Myristica* and *Virola*. The family contains 17 other genera comprising of 312 species of evergreen trees found throughout moist tropical lowlands. They are found in Brazil, Columbia, Peru, Venezuela, the Caribbean, Guianas, India, Philippines, Malaysia, Indonesia, Papua New Guinea, and Sri Lanka. Most species have fragrant wood and leaves. Some of the important plants belonging to this family which are used extensively either in food preparation and in medicine include *Myristica*

malabarica and *Myristica fragrans* whereas *Gymnacranthera canarica*, *Virola multinervia*, *Virola surinamensis*, *Knema attenuata* etc. Among them, the most important are *M. malabarica* and *M. fragrans* which are used extensively in food preparation and in traditional medicine.

Virola surnamensis extract was found to have strong activity against *Schitstosoma mansoni* infection [Barata *et al.*, **2000**]. In addition, the chloroform extract of aril and hexane extract of seed exhibited highest antimicrobial activity against *Staphylococcus aureus* and moderate antifungal activity against *Candida albicans* [Costa *et al.*, **2008**]. Hydro distillation of *Gymnacranthera canarica* leaves yielded seventy-six constituents (98.1%) with β−caryophyllene (23.4%), linalool (13.4%) and α−humulene (11.3%) as major constituents [Sabulal *et al.*, **2014**]. Bark extract of *Virola multinervia* showed potent antibacterial activity [Filho *et al.*, **1973**].

2.1.1. Important genus of Myristicaceae

Myristicaceae is divided in to 19 genus namely,

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

Table 2.1 : Different Myristicaceae genus

2.1.2. *Myristica*

The genus *Myristica* belonging to the family Myristicaceae contain about 312 species found in different parts of the world. Only very a few of the species are found in India and their habitat varies from the sea level to altitude as high as 2000 m in the Western Ghats and Himalayas. The Myristica species known are listed in the **Table 2.2**.

Table 2.2 : *Myristica* species and their distribution

***Myristica* species**	**Distribution**
M. alba	Indonesia
M. ampliata	Queensland and Australia
M. andamanica	India
M. arfakensis	West Papua (Indonesia)
M. argentea	Papua New Guinea
M. atrescens	Papua New Guinea
M. basilanica	Philippines
M. brachypoda	Papua New Guinea
M. brevistipes	Papua New Guinea
M. buchneriana	Indonesia and Papua New Guinea
M. byssacea	Papua New Guinea
M. ceylanica	Sri Lanka
M. cinnamomea	Malaysia, Singapore, and Thailand
M. coacta	Papua New Guinea
M. colinridsdalei	Philippines
M. conspersa	West Papua (Indonesia)
M. corticata	Brunei and Malaysia
M. crassa	Indonesia, Malaysia, and Singapore
M. dactyloides	Sri Lanka
M. depressa	Indonesia and Malaysia
M. devogelii	Indonesia
M. elliptica	Indonesia, Malaysia, and Singapore
M. extensa	Brunei, Indonesia, and Malaysia
M. fasciculata	Papua New Guinea
M. filipes	Papua New Guinea
M. fissurata	Indonesia
M. flavovirens	West Papua (Indonesia)
M. fragrans	India, China, Taiwan, Indonesia, Malaysia, Caribbean, Sri Lanka and South America
M. frugifera	Philippines
M. gigantea	Indonesia and Malaysia
M. gillespieana	Fiji
M. globosa	Papua New Guinea, the Solomon Islands and Australia
M. grandifolia	Fiji
M. guadalcanalensis	Solomon Islands
M. guatteriifolia	Indonesia, Malaysia, Myanmar, Philippines and Vietnam
M. guillauminiana	Fiji and Solomon Islands
M. hollrungii	Papua New Guinea
M. inaequalis	West Papua (Indonesia)
M. incredibilis	Papua New Guinea

M. iners	Indonesia, Malaysia and Singapore
M. inundata	Papua New Guinea
M. irya	Burma, India, Malaysia, Papua New Guinea, Singapore, Solomon Islands, Sri Lanka, Thailand, and Vietnam
M. kalkmanii	Indonesia and Papua New Guinea
M. kjellbergii	Indonesia
M. lasiocarpa	Papua New Guinea
M. leptophylla	Papua New Guinea
M. longipetiolata	Philippines
M. lowiana	Malaysia, and Singapore
M. macrantha	Fiji
M. magnifica	India
M. maingayi	Malaysia and Singapore
M. malabarica	India
M. maxima	Indonesia, Malaysia, and Singapore
M. mediterranea	Indonesia and Papua New Guinea
M. millepunctata	West Papua (Indonesia)
M. nana	Papua New Guinea
M. olivacea	Papua New Guinea
M. ornata	Papua New Guinea
M. ovicarpa	Papua New Guinea
M. pachycarpidia	Papua New Guinea
M. papillatifolia	Indonesia and Papua New Guinea
M. perlaevis	Indonesia
M. petiolata	Solomon Islands
M. philippensis	Philippines
M. pilosella	Papua New Guinea
M. pilosigemma	Philippines
M. polyantha	Papua New Guinea
M. psilocarpa	Manus Island and Papua New Guinea
M. pubicarpa	Indonesia
M. pygmaea	Papua New Guinea
M. robusta	Indonesia
M. sangowoensis	Indonesia
M. sarcantha	West Papua (Indonesia)
M. schlechteri	Papua New Guinea
M. sinclairii	Papua New Guinea
M. sogeriensis	Papua New Guinea
M. succadanea	Indonesia
M. tamrauensis	West Papua (Indonesia)
M. teijsmannii	Indonesia
M. trianthera	West Papua (Indonesia)
M. ultrabasica	Indonesia
M. verruculosa	West Papua (Indonesia)

M. yunnanensis	Southern Yunnan, China, northern Thailand, and Thanh Hóa Province in Vietnam

Of the above, only few species of Myristica has been studied. They include *Myristica malabarica*, *Myristica argentea* and *Myristica fragarns*. The phytochemistry, pharmacological action, medicinal properties and uses of these plants are discussed in the following sections.

2.1.3. *Myristica malabarica*

Myristica malabarica Lam., (commonly known as 'Malabar nutmeg', 'Rampatri' in Hindi and 'Ponnampu' or 'Kattujathi' in Malayalam) is an important medicinal plant which is rare and threatened endemic species [Varghese *et al.*, **2006**] of Western Ghats of South India. The species is red listed [IUCN, **2008**] and is threatened due to extensive damage resulting from unregulated lopping for collecting its fruit, large scale and indiscriminate collection of the wild material [Mathachen *et al.*, **2004**; Daniels *et al.*, **1995**] and habitat shrinkage. It is a medium- sized tree with single-seeded fruits used in traditional medicine for the treatment of indolent ulcers and rheumatism. It is among the highly traded top twenty medicinal plants in India [Ved *et al.*, **1999**] and one of the major ingredients of several Ayurvedic (Indian medicine) preparations. *M. malabarica* is one of the major ingredient in 'Muthu Marunthu' which is a herbal preparation, reported to possess antitumor effect [Palani *et al.*, **1999**]. There are many reports of its anti-oxidant activity [Maity *et al*., **2007**], superoxide scavenging, cytotoxic properties [Khanom *et al.*, **2000**; Pham *et al.*, **2000**], anti-ulcer effect [Maity *et al.*, **2007**] and nematicidal activity [Choi *et al.*, **2008**]. Several classes of therapeutically active compounds including the acylphenols viz., malabaricones [Patro *et al.*, **2005**; Bauri *et al.*, **2006**] and isoflavones like biochanin [Talukder *et al.*, **2000**] have been isolated from *M. malabarica*.

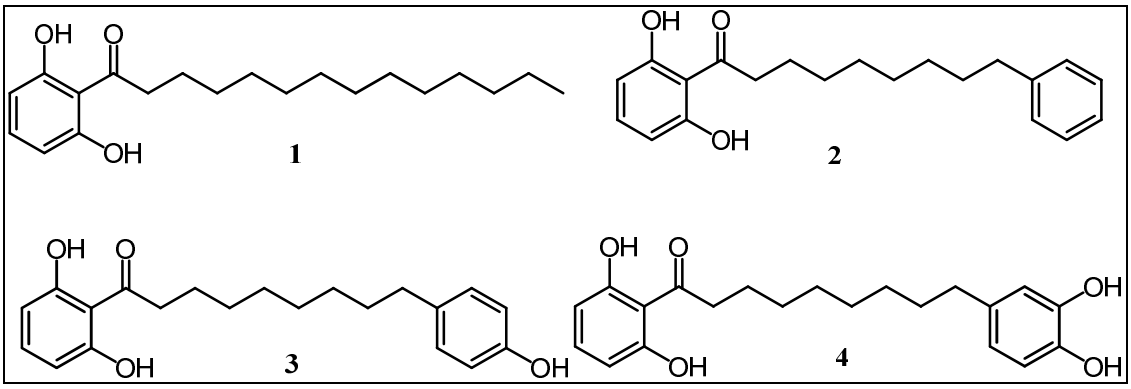
Essential oil from the leaves of *Myristica malabarica* obtained by hydrodistillation was found to contain seventy six constituents (98.5%) with *β*-caryophyllene (27.3%), α-humulene (13.8%) and *α*-copaene (11.5%) identified as major components. [Sabulal *et al.*, **2014**].

The phytochemicals reported from *M. malabarica* are listed in **Table 2.3**.

Table 2.3 : The non volatile phytochemicals isolated from *Myristica malabarica*

Plant	Compounds isolated	No:	Ref:
M. malabarica	1-(2',6'-Dihydroxyphenyl)tetradecan-1-one	**1**	[Patro *et al.*, **2005**]
	Malabaricone A	**2**	
	Malabaricone B	**3**	
	Malabaricone C	**4**	
	Malabaricone D	**5**	
	7,4'-Dimethoxy-5-hydroxyisoflavone	**6**	[Talukdar *et al.*, **2000**]
	Biochanin A	**7**	
	Prunetin	**8**	
	α-2'-Dihydroxy-4,4'-dimethoxydihydrochalcone	**9**	
	1-(2-Hydroxy-4-methoxyphenyl)-3-(3,4-methylenedioxyphenyl) propan-2-ol	**10**	

Figure 2.1 : Structures of phytochemicals isolated from *M. malabarica*



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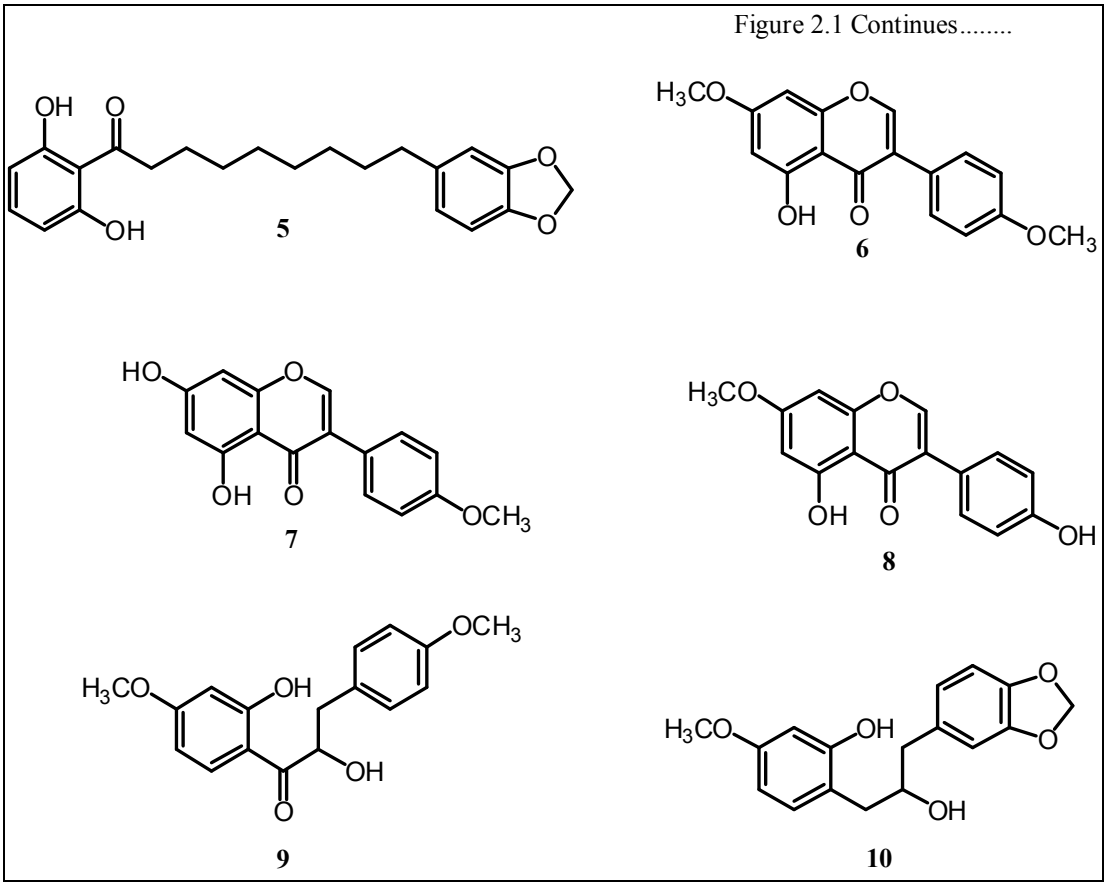


2.1.4. *Myristica argentea*

Myristica argentea Warb. is a tree that grows in the primary rain forests of Papua New Guinea. The plant is known as Macassar nutmeg, female nutmeg, horse nutmeg, long nutmeg, New Guinea nutmeg, Papua nutmeg etc. The fruits are used in Indonasia to treat diarrhoea and to stimulate appetite where it is called 'Pala negri', 'Pala papaoes'. The mace of this plant abounds with a series of diaryldimethylbutane lignans of possible pharmacological value. These lignans include *erythro*-austrobailignan-6, *meso*-dihydroguaiaretic acid and myristargenol A which shows good level of activity against *Streptococcus mutans* [Nakatani *et al.*, **1988**].

Erythro-austrobailignan-6, *meso*-dihydroguaiaretic acid and nectandrin B exert antiproliferative effect on MCF-7 cells as well as antioxidant activity on the DPPH

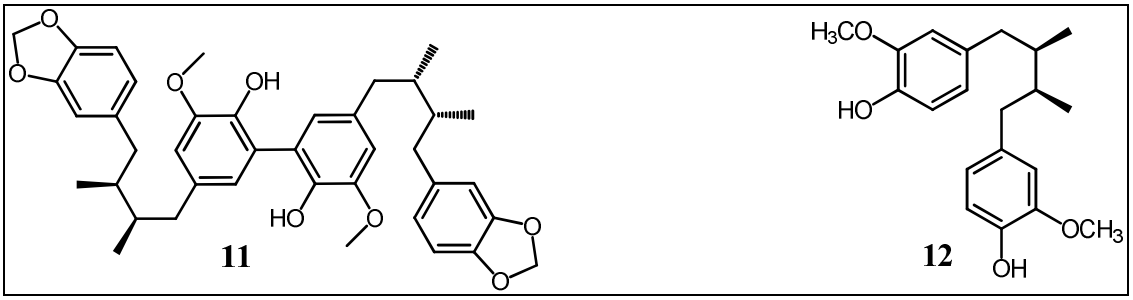
radical. In addition, nectandrin-B inhibits the enzymatic activity of 17β-hydroxysteroid dehydrogenase and shows antiaromatase activities [Filleur *et al.*, **2001**].

The phytochemicals reported from *M. argentea* are listed in **Table 2.4**.

Table 2.4 : The non volatile phytochemicals isolated from *Myristica argentea*

Plant	**Compounds isolated**	**No:**	**Ref:**
M. argentea	Argenteane	**11**	[Calliste *et al.*, **2010**]
	Meso-dihydroguaiaretic acid	**12**	
	Erythro-austrobailignan-6	**13**	
	3,3'-dimethoxy-1,1'-biphenyl-4,4'-diol	**14**	
	Myristargenol A	**15**	[Filleur *et al.*, **2001**]
	Licarin A	**16**	
	Licarin B	**17**	
	Machilin C	**18**	
	Vanillic acid	**19**	
	Safrole	**20**	
	Eugenol methyl ether	**21**	
	Elemicin	**22**	
	Eugenol	**23**	
	Isoeugenol methyl ether	**24**	
	Methoxy eugenol	**25**	
	Nectandrin B	**26**	[Nakatani *et al.*, **1988**]
	Myristargenol B	**27**	

Figure 2.2 : Structures of phytochemicals isolated from *M. argentea*



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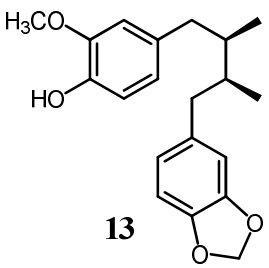

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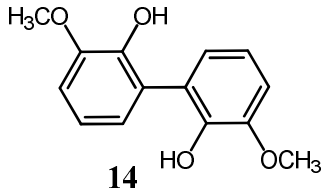

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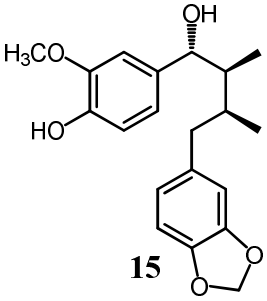

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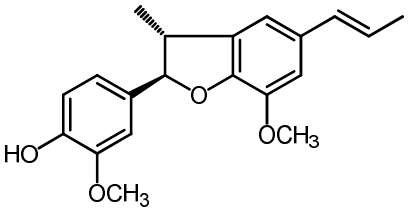

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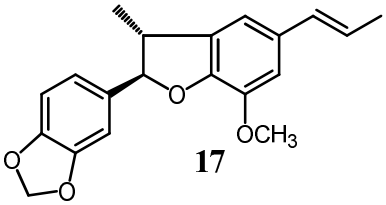

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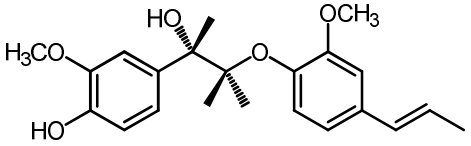

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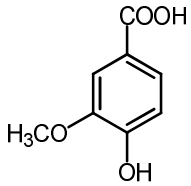

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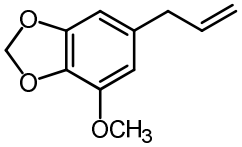

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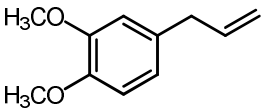

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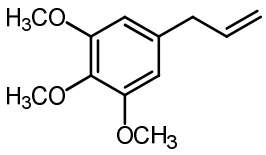

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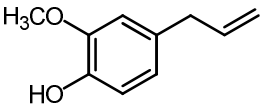

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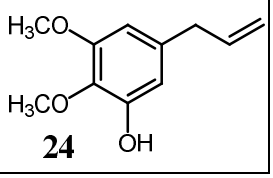

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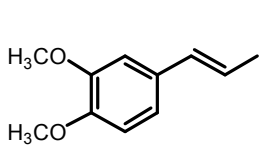

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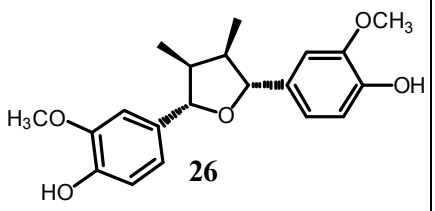

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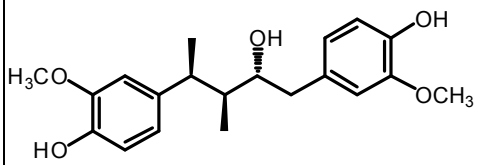

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2.2. Aim and scope of the present investigation

Myristica fragrans Houtt. ("Jathiphala" in Sanskrit and "Jathikka" in Malayalam) is commonly known as “nutmeg”, it produces two spices: mace and nutmeg. Nutmeg is the seed kernel inside the fruit and mace is the red lacy covering (aril) on the kernel. *Myristica* species are natives of Moluccas, India, Indonesia and Sri Lanka and is now cultivated in many tropical countries of both hemispheres as well as in South Africa [Pal *et al.*, **2011**].

M. fragrans is a spreading aromatic evergreen tree usually growing to about 5 to13 m high, occasionally 20 m. The bark contains watery pink or red sap. The pointed dark green leaves (5 to 15 cm × 2 to 7 cm) are arranged alternately along the branches and are borne on leaf stems about 1 cm long. Upper leaf surfaces are shiny. Flowers are usually single sexed; occasionally male and female flowers are found on the same tree.

Female flowers arise in groups of 1 to 3; males in groups of 1 to 10. Flowers are pale yellow, waxy, fleshy and bell-shaped. Male flowers are 5 to 7 mm long; female flowers are up to 1 cm long. The fruits are fleshy, drooping, yellow, smooth, 6 to 9 cm long with

a longitudinal ridge. When ripe, the succulent yellow fruit coat splits into two halves revealing a purplish-brown, shiny seed (nutmeg) surrounded by a red aril (mace).

Seeds (nutmeg) are broadly ovoid (2 to 3 cm long), firm, fleshy, whitish and transversed by red-brown veins. When fresh, the aril (mace) is bright scarlet becoming more horny, brittle and with a yellowish-brown colour when dried.

M. fragrans tree

Fruit (pericarp, mace and seed)

Figure 2.3 : *M. fragrans* tree and fruit

Nutmeg mace is popular as a spice and also possesses various therapeutic properties. Nutmeg has a characteristic pleasant fragrance and a slightly warm taste. It is used to flavour many kinds of baked foods, confections, puddings, meats, sausages, vegetables and beverages. It is also used as components of curry powder, teas and soft drinks or mixed in milk and alcohol [Olaleye *et al.*, **2006**]. For a long time, *M. fragrans* has been used as a folklore medicine for treating diarrhoea, mouth sores and insomnia [Somani *et al.*, **2008**]. Since the Middle Ages, nutmeg has been used as a stomachic, stimulant, carminative as well as for intestinal catarrh and colic. It is used to stimulate appetite, to control flatulence and has a reputation as an emmenagogue and abortifacient [Min *et al.*, **2011**]. The essential oil of nutmeg is used externally for rheumatism and possesses analgesic and anti-inflammatory properties [Santos *et al.*, **1997**; Olajide *et al.*, **1999**]. It also finds extensive use in many Ayurvedic preparations.

2.2.1. *Myristica fragrans* - Literature survey

Nutmeg and mace oils have been analysed since the last century and their composition and organoleptic characteristics have been extensively studied. Essentially these oils contain >80% monoterpenes, >5% monoterpene alcohols, >5% aromatics and

other minor components. Sabinene, myristicin, safrole and elemicin constitute 80% of both nutmeg and mace oil. The oils from nutmeg cultivated in Grenada have considerable amount of α-pinene, β-pinene and sabinene (40%-50%) and are low in safrole and myristicin whereas oils from nutmeg cultivated in Indonasia and other regions of South East Asia have higher amount of myristicin [Purseglove *et al.*, **1981**]. Gas chromatographic analysis of nutmeg and mace oil revealed that α- pinene, β-pinene and sabinene constituted 77.38% and 60.76% in nutmeg and mace respectively. The concentration of myristicin and elimicin are very high in Indian nutmeg oils [Gopalakrishnan, **1992**]. The characteristic flavour of nutmeg is said to be due to the presence of myristicin and elemicin. Mace and nutmeg oils contain ~1.9% and ~0.3% safrole respectively, which has been suspected to be carcinogenic.

The composition of the volatile oil extracted by hydro-distillation from nutmeg pericarp and the component retention indices were determined [Choo *et al.*, **1999**]. The oil contains 16 monoterpenes (60%), nine monoterpene alcohols (29%), eight aromatic ethers (7%), three sesquiterpenes (1%), six esters (1%) and eight other minor components. The components are similar in both nutmeg and mace oils, but differ substantially in their concentrations. The sabinene, myristicin and safrole concentrations are much lower in nutmeg pericarp while the terpinen-4-ol and α-terpineol contents are much higher in nutmeg oil than mace oil.

M. fragrans mace is widely used as a flavouring agent and in traditional medicine. It also possesses antipapillomagenic (cervical cancer), anticarcinogenic [Hussain *et al.*, **1991**] and anti-inflammatory activities [Ozaki *et al.*, **1989**]. Both nutmeg and mace is known to exhibit strong antimicrobial activity against animal and plant pathogens, food poisoning and spoilage bacteria including *Bacillus subtilis*, *Escherichia coli*, *Saccharomyces cerevisiae*, multi-drug resistant *Salmonella typhi* and *Helicobacter pylori* [Orabi *et al*., **1991**]. Methanolic extracts of nutmeg has been found to have anti-bacterial activity against *Micrococcus pyogens* var. aureus [Anonymous, **1995**].

Many biologically active compounds have been isolated from *M. fragrans*. Hattori *et al.* reported the isolation of several tetrahydrofuran lignans, namely fragransins A_2, B_1, B_2, B_3, C_1, C_2, C_{3a} and C_{3b} along with nectandrin B and Verrucosin from the aril of nutmeg [Hattori *et al*, **1987**]. The same group reported the isolation of fragransol C,

fragransol D, myristicanol A and myristicanol B from the neutral fraction of mace methanolic extract [Hattori *et al.*, **1988**]. Lee *et al.* have reported that myristicin (1-allyl-3,4-methylenedioxy-5-methoxybenzene), a naturally occurring alkyl benzene derivative found in nutmeg induces cytotoxicity in human neuroblastoma SK-N-SH cells by an apoptotic mechanism [Lee *et al.*, **2005**]. Macelignan a natural compound isolated from *M. fragrans* enhanced the insulin sensitivity and improved lipid metabolic disorders by activating peroxisome proliferator receptor (PPAR, á/ã) and attenuating endoplasmic reticulum stress, suggesting that it is an antidiabetic agent for the treatment of type 2 diabetes [Han *et al*., **2008**].

In the course of isolation of antibacterial principles against a primary cariogenic bacterium, *Streptococcus mutans*, from the aril of *Myristica fragrans* (collected from Sri Lanka), which is used as a combination of drugs for dental caries prevention in Ayurvedic medical system, some lignans and neolignans have been reported. The major phenolic components isolated, dehydrodiisoeugenol (Licarin A) and 5'-methoxydehydrodiisoeugenol, were demonstrated to have significant antibacterial action against *S. mutans* [Hattori *et al.*, **1986**]. In 2009, Lin *et al.* reported the isolation of cytotoxic and antioxidative phenolic compounds from *M. fragrans* seed (collected from China) (see **Figure 2.4**) viz., (−)-1-(2,6-dihydroxyphenyl)-9-[4-hydroxy-3-(*p*-menth-1-en-8-oxy)-phenyl]-1-nonanone, (7*R*,8*R*)-7,8-dihydro-7-(3,4-dihydroxyphenyl)- 3′-methoxy-8-methyl-1′-(*E*-propenyl)benzofuran, (+)-*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*)-Δ8′-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-dihydroxyphenyl)-9-(3,4-dihydroxyphenyl)-1-nonanone [Lin *et al*., **2009**].

Raphidecursinol B, a lignan isolated from *M. fragrans* mace has been found to have antimalarial activity [Parai *et al*., **2008**; Kumar *et al*., **2008**]. In 2011, Cuong *et al.* reported the isolation of phenolic compounds such as ((*7S*)-8'-(benzo[3',4']dioxol-1'-yl)-

7-hydroxypropyl)benzene-2,4-diol, ((7*S*)-8'-(4'-hydroxy-3'-methoxyphenyl)-7-hydroxypropyl)benzene-2,4-diol and ((*8R,8'S*)-7-(4-hydroxy-3-methoxyphenyl)-8'-methylbutan-8-yl)-3'-methoxybenzene-4',5'-diol from the seeds of *Myristica fragrans*. Anti-inflammatory activity has been evaluated using these isolated compounds [Cuong *et al.*, **2011**]. Recently, Cao *et al.* reported five new 8-*O*-4' type neolignans, myrifralignans A–E, together with five known analogs from the seeds of *Myristica fragrans* Houtt [Cao *et al.*, **2014**]. Among them compounds myrislignan and machilin D were found to have the most potent inhibitory effect on NO production.

Figure 2.4 : Structures of compounds isolated from *M. fragrans*

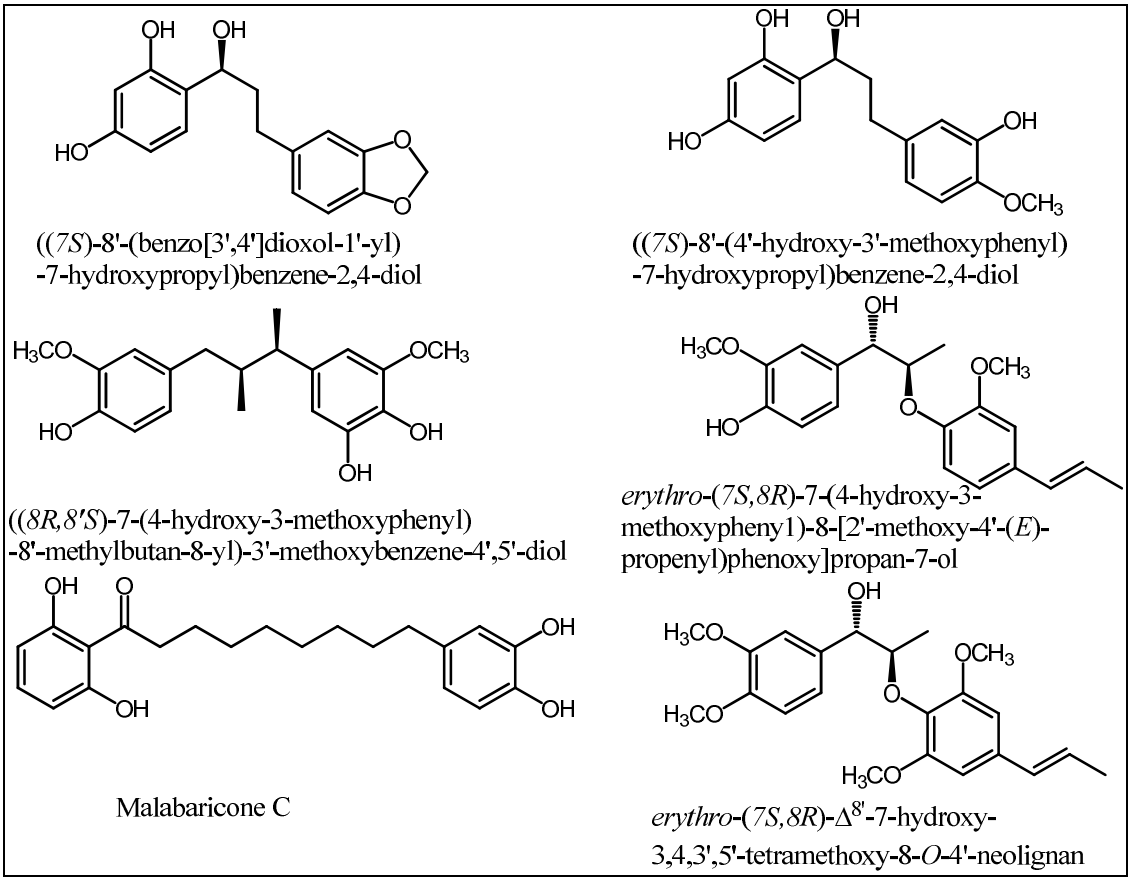
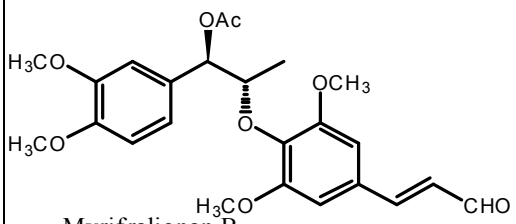
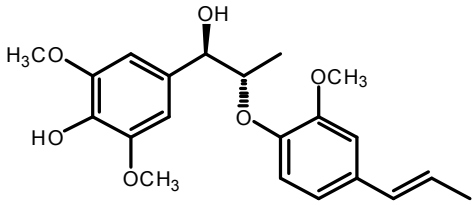


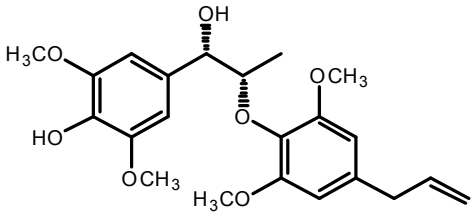
Figure 2.4 continues.....



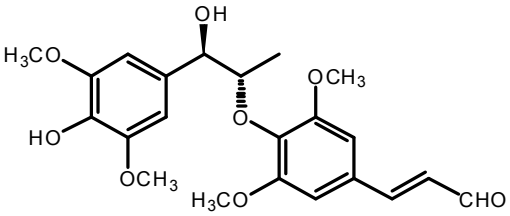
Myrifralignan B



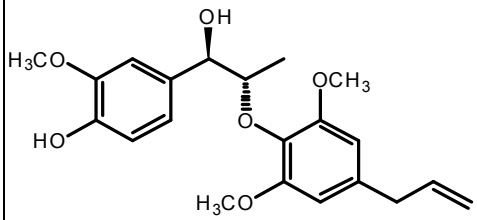
Myrifralignan C



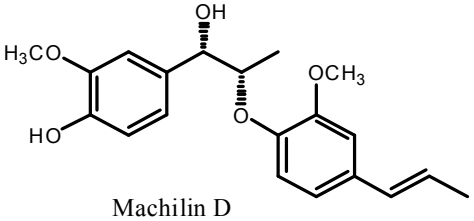
Myrifralignan D



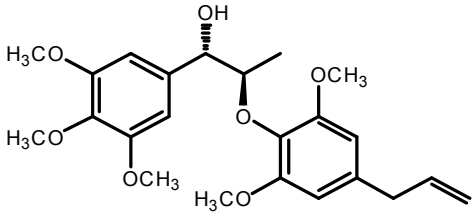
Myrifralignan E



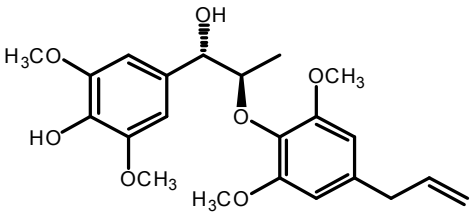
Myrislignan



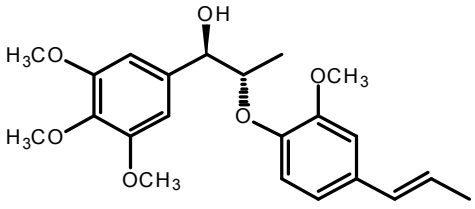
Machilin D



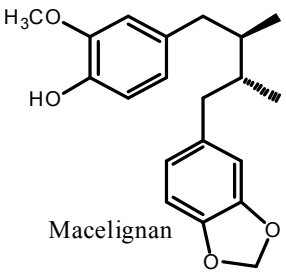
(*7S,8R*)-2-(4-allyl-2,6-dimethoxy -phenoxy)-1-(3,4,5-trimethoxyphenyl)-propan-1-ol



(*7S,8R*)-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3,5-dimethoxyphenyl)-propan-1-ol

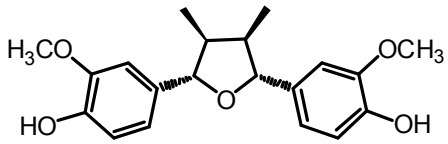


(*7R,8S*)-2-(4-propenyl-2-methoxyphenoxy)-1-(3,4,5-trimethoxyphenyl)-propan-1-ol

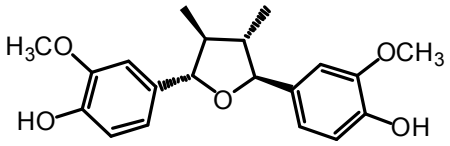


Macelignan

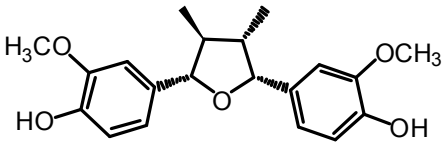
Figure 2.4 continues.....



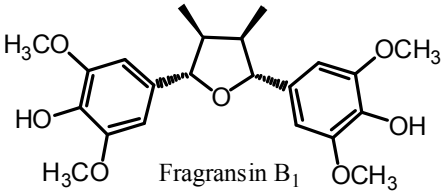
Nectandrin B



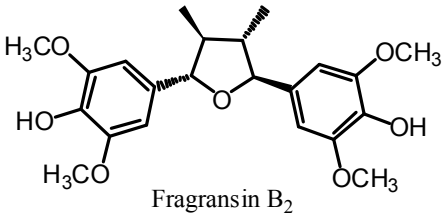
Fragransin A_2



Verrucosin



Fragransin B_1



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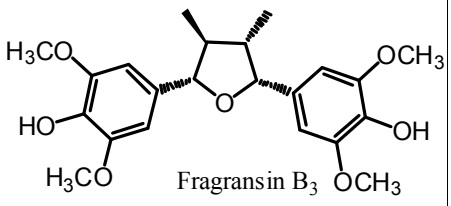
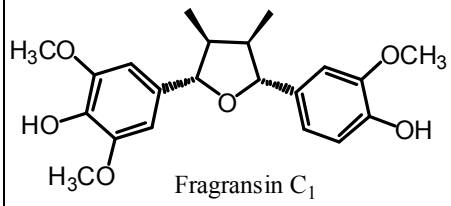
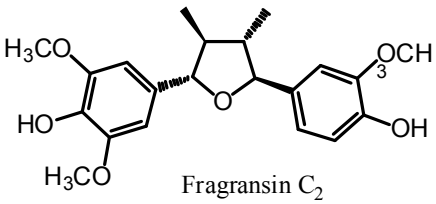


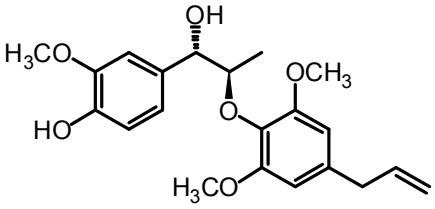
Fragransin B_3



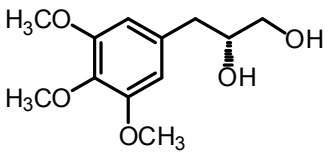
Fragransin C_1



Fragransin C_2

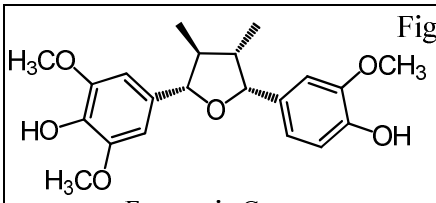


(+)-*erythro*-(*7S,8R*)-$\Delta^{8'}$-4,7-dihydroxy-3,3',5'-trimethoxy-8-*O*-4'-neolignan-8'-ene

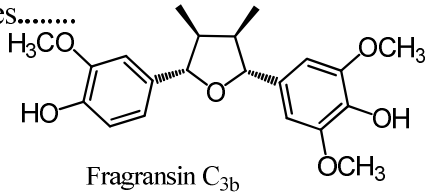


(*2R*)-3-(3,4,5-trimethoxyphenyl)-1,2-propanediol

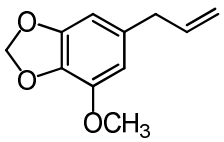
Figure 2.4 continues.....



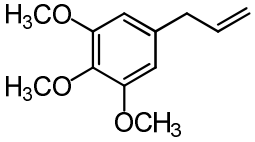
Fragransin C_{3a}



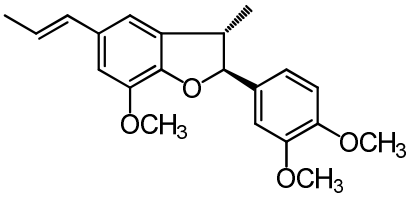
Fragransin C_{3b}



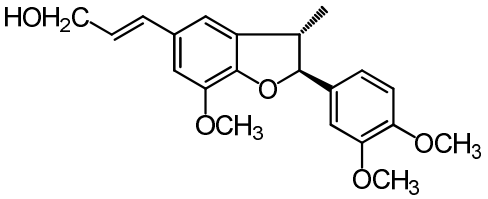
Myristicin



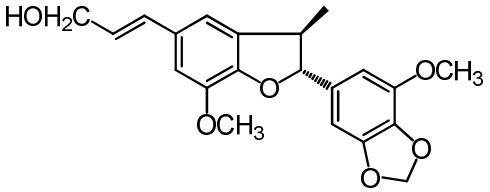
Elemicin



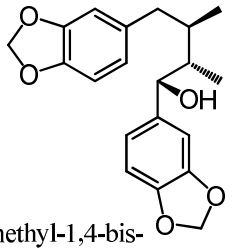
2,3-dihydro-7-methoxy-2-(3,4-dimethoxyphenyl)
-3-methyl-5-(1-(*E*)-propenyl)benzofuran



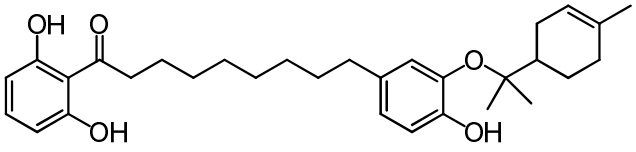
Fragransol C



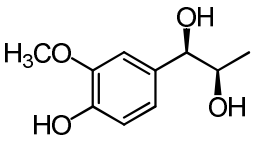
Fragransol D



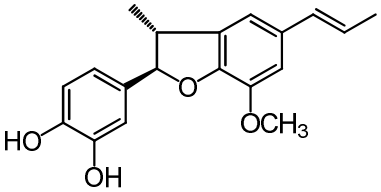
2,3-Dimethyl-1,4-bis-
(3,4-methylenedioxyphenyl)butan-1-ol



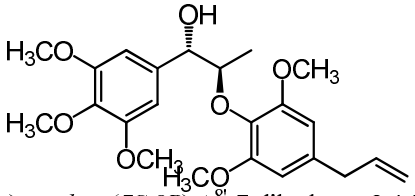
(-)-1-(2,6-dihydroxyphenyl)-9-[4-hydroxy-3-
(*p*-menth-1-en-8-oxy)-
phenyl]-1-nonanone



(*1R,2R*)-1-(4-hydroxy-3-
methoxyphenyl)-1,2-propanediol

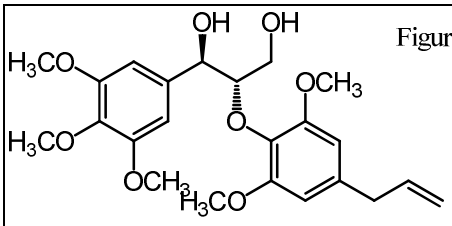


(*7R,8R*)-7,8-dihydro-7-(3,4-dihydroxyphenyl)-
3'-methoxy-8-
methyl-1'-(E-propenyl)benzofuran

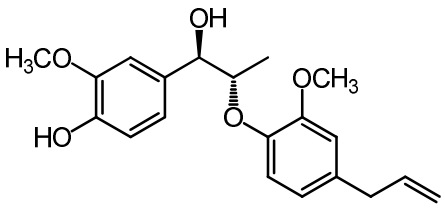


(+)-*erythro*-(*7S,8R*)-$\Delta^{8'}$-7-dihydroxy-3,4,5,3',5'-
pentamethoxy-8-*O*-4'-neolignan-8'-ene

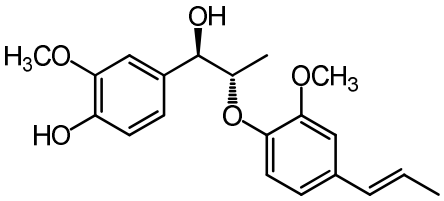
Figure 2.4 continues.....



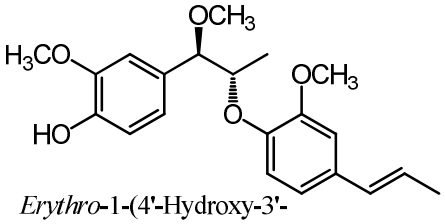
Erythro-2-(4"-allyl-2",6"-dimethoxyphenoxy)
-1-(3',4',5'-trimethoxyphenyl)propan-1,3-diol



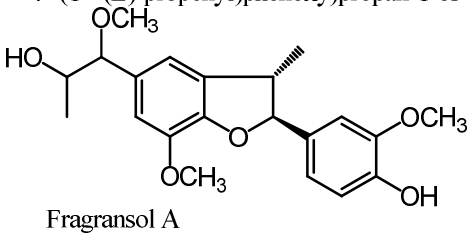
Threo-2-(4"-allyl-2"-methoxyphenoxy)
-1-(3',4',5'-trimethoxyphenyl)propan-1-ol



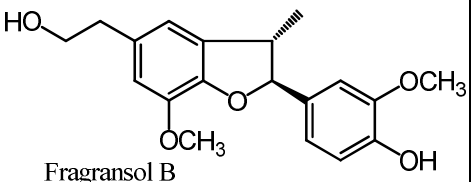
Erythro and *Threo*-1-(4'-Hydroxy-3'-
methoxyphenyl)-2-(2"-methoxy
-4"-(1'"-(*E*)-propenyl)phenoxy)propan-1-ol



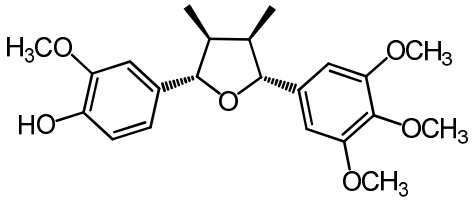
Erythro-1-(4'-Hydroxy-3'-
methoxyphenyl)
-1-methoxy-2-(2"-methoxy
-4"-(1'"-(*E*)-propenyl)phenoxy)propane



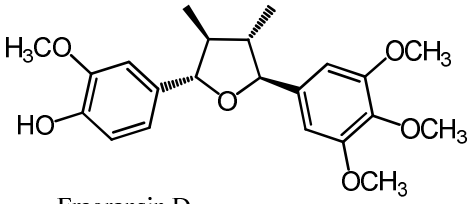
Fragransol A



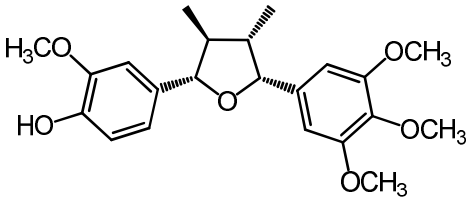
Fragransol B



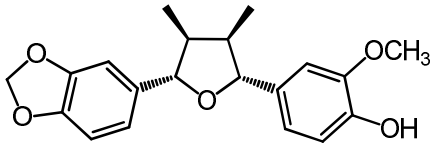
Fragransin D_1



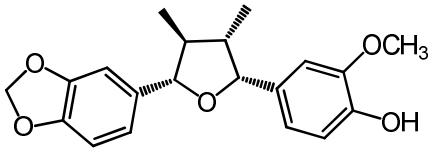
Fragransin D_2



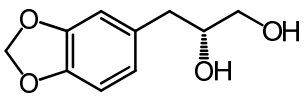
Fragransin D_3



Fragransin E_1

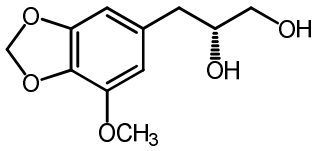


Ausrtobailignan-6

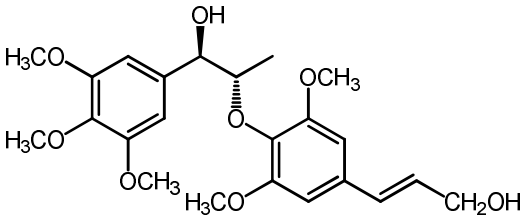


(*2R*)-3-(3,4-methylenedioxyphenyl)
-1,2-propanediol

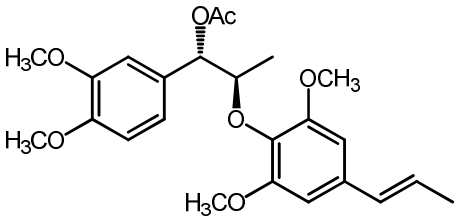
Figure 2.4 continues.....



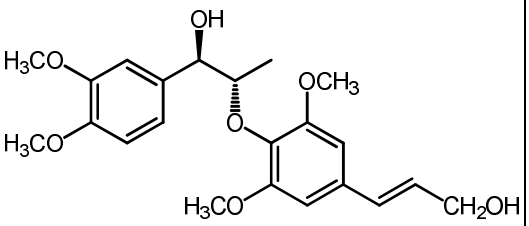
(*2R*)-3-(5-methoxy-3,4-methylenedioxyphenyl)
-1,2-propanediol



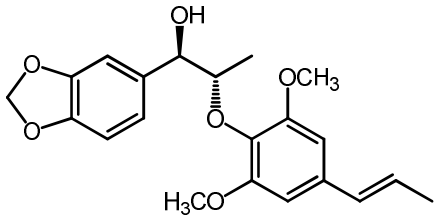
Myristicanol A



erythro-(*7S,8R*)-$\Delta^{8'}$-7-acetoxy-
3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan



Myristicanol B



Myrifralignan A

Mace and seed of *M. fragrans* have been well studied for their bioactive compounds, the fruit pericarp has not been investigated earlier in terms of their phytochemical constituents. Most importantly, even though, *M. fragrans* is grown commercially all over Kerala, the phytochemical constituents of the material and sold from Kerala has not been studied in total. Therefore, such an exercise was undertaken. In this chapter, the isolation of chemical constituents from all parts of *M. fragrans* fruit as well as that from the stem bark are described.

CHAPTER 2A

Phytochemical Investigation on Fruits of *Myristica fragrans*

2a.1. Isolation and characterization of compounds from fruits of *M. fragrans*

As mentioned earlier, the aim of this study was to carry out the isolation and characterization of phytochemicals from various parts of *M. fragrans* viz., fruit pericarp, seed and mace.

2a.1.1. From fruit pericarp

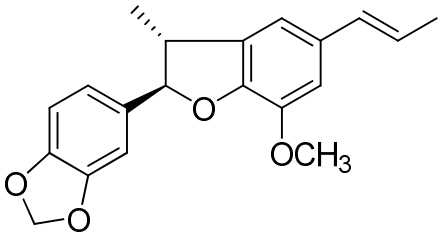
2a.1.1.1. Plant material and extraction

M. fragrans fruits were collected in January 2013, from Ernakulam in Kerala State, India.Voucher specimen (TBGRI-60679) has been deposited in a herbarium of Jawaharlal Nehru Tropical Botanical Garden and Research Institute, Palode, Trivandrum in Kerala State. The pericarp, seed and mace were separated and used for the study. Of the dry and powdered fruit pericarp, 750 g was extracted with acetone (2 L X 3) at room temperature, which yielded 32 g of crude extract. This was then suspended in ethyl acetate and stirred for an hour. The ethyl acetate soluble part (19 g) was subjected to CC on silica gel (100–200 mesh) and eluted with hexane–ethyl acetate mixtures of increasing polarities to give 15 fraction pools.

2a.1.1.2. Isolation and characterization of major compounds

Fraction pools 1–3 (2.05 g) on crystallisation in hexane yielded palmitic acid. Fraction pool 4 (500 mg) was subjected to column chromatography on silica gel (100–200 mesh) using hexane–ethyl acetate (9:1) as eluent and followed by crystallisation in ethyl acetate/hexane afforded compound 1 (280 mg). The ^{1}H NMR (**Figure 2a.1**) spectrum showed the presence of two methyl groups, resonating at δ 1.36 and 1.85. A singlet appearing at δ 3.88 integrating for three protons suggested the presence of a methoxy group. A sharp singlet seen at δ 5.94, integrating for two protons indicated the

presence of a methylenedioxy group. A doublet of doublet at δ 6.34 and a multiplet at δ 6.10 each integrating for one proton with *J* value of 16.5 Hz could be attributed to *trans* olefinic protons. Signal at δ 55.9 in the ^{13}C NMR (**Figure 2a.2**) spectrum confirmed the presence of an -OMe group. The ^{13}C NMR spectrum confirmed the presence of olefinic and aromatic carbons in between δ 101.1-147.9 (14C). The DEPT-135 spectrum confirmed the presence of -O-CH_2-O- and seven quaternary carbons. The mass spectrum of the compound gave molecular ion peak at 325.1435 which is the $(M+H)^+$ peak. Putting these spectral data together and comparing with the data of the compound found in literature, the structure of compound 1 could be identified as licarin B [Coy *et al.*, **2009**] shown below.



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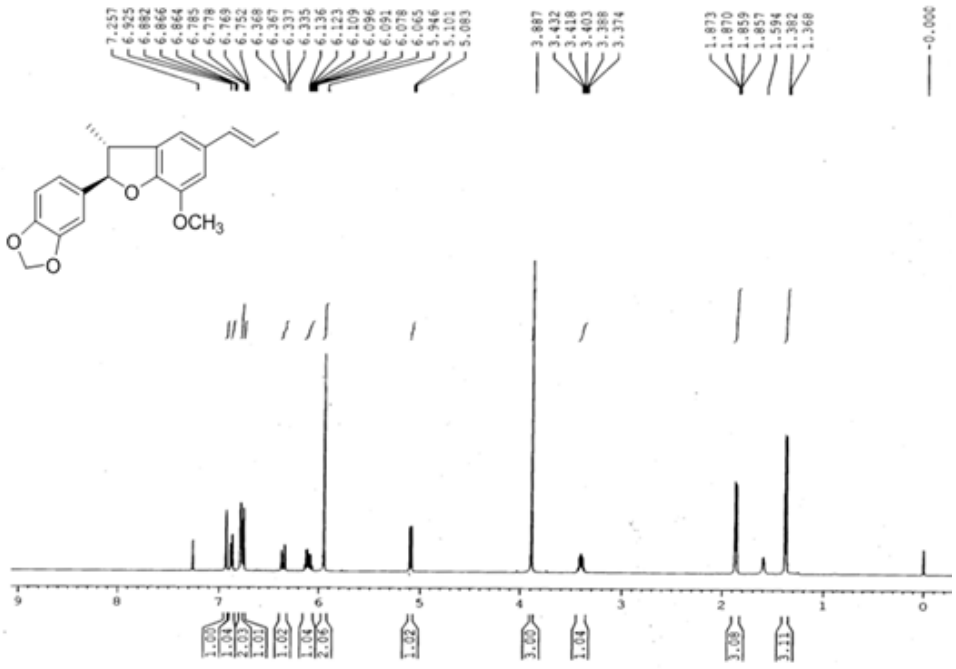


Figure 2a.1: ^{1}H NMR spectrum of Licarin B

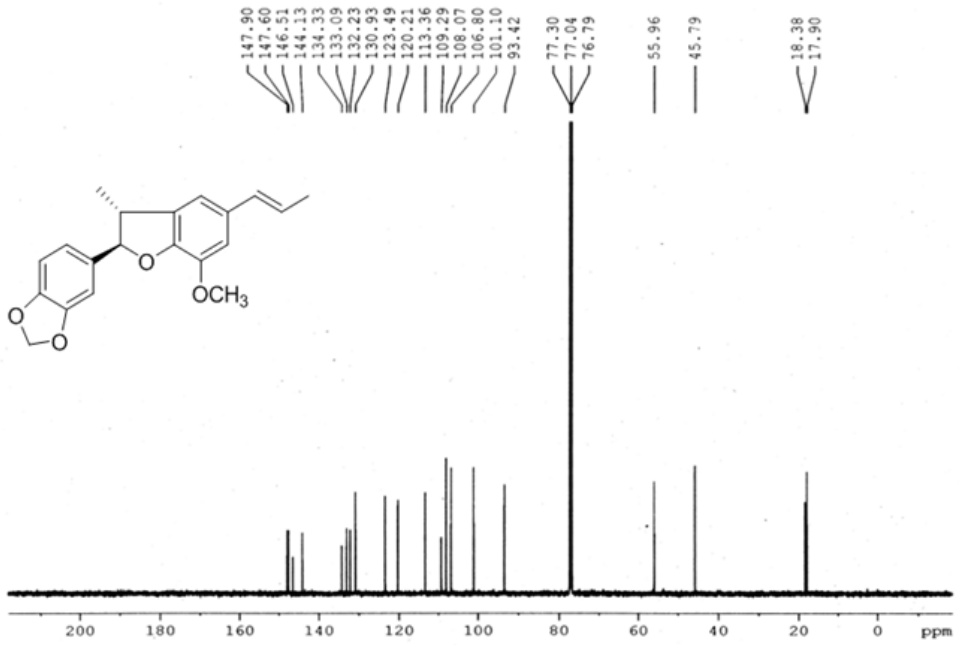
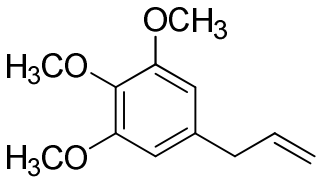


Figure 2a.2: ^{13}C NMR spectrum of Licarin B

Fraction pool 5 (665 mg) was submitted to column chromatography on silica gel by eluting with hexane–ethyl acetate (7:1), which afforded compound 2 (156 mg) as colourless oil. Two singlets at δ 3.82 and 3.85 in the ^{1}H NMR (**Figure 2a.3**) spectrum integrating for three and six protons respectively, suggested the presence of three methoxy groups, among which two of them are identical. Peaks at δ 56.0 and 60.8 in the ^{13}C NMR (**Figure 2a.4**) spectrum confirmed the presence of three -OMe groups. The presence of olefinic and aromatic carbons were confirmed by the peaks at δ 102.8-153.2. The DEPT-135 spectrum confirmed the presence of one $-CH_2-$ group. Mass spectrum of the compound gave molecular ion peak at 209.1180 which is the $(M+H)^+$ peak. Based on these spectral details, compound 2 was confirmed to be elemicin [Base *et al.*, **1993**] and the structure is shown below.



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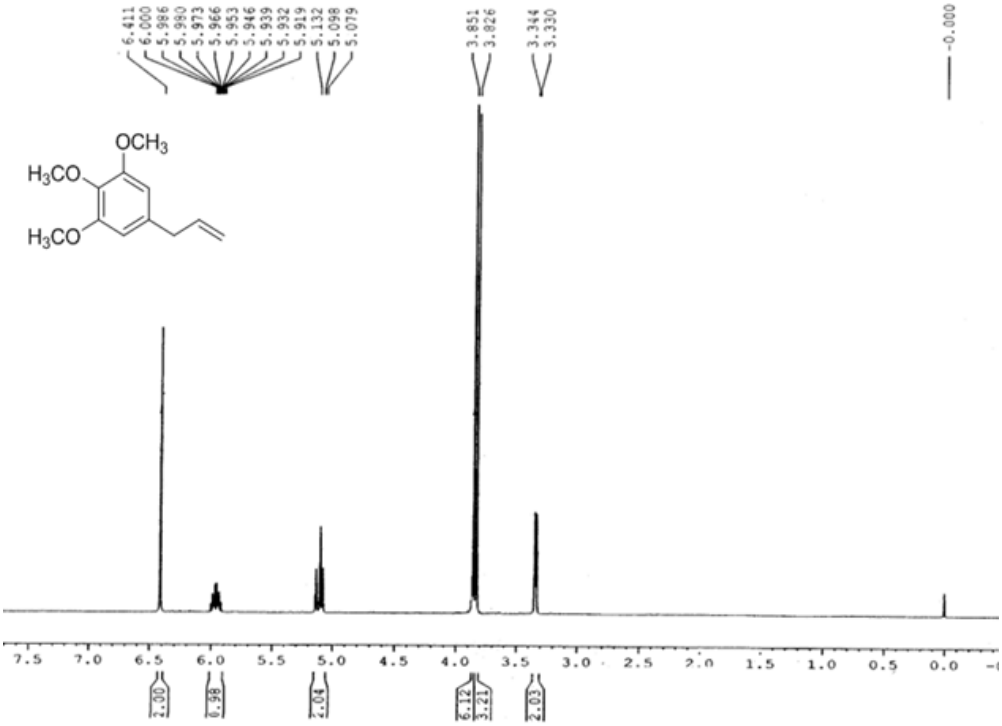


Figure 2a.3: ^{1}H NMR spectrum of Elemicin

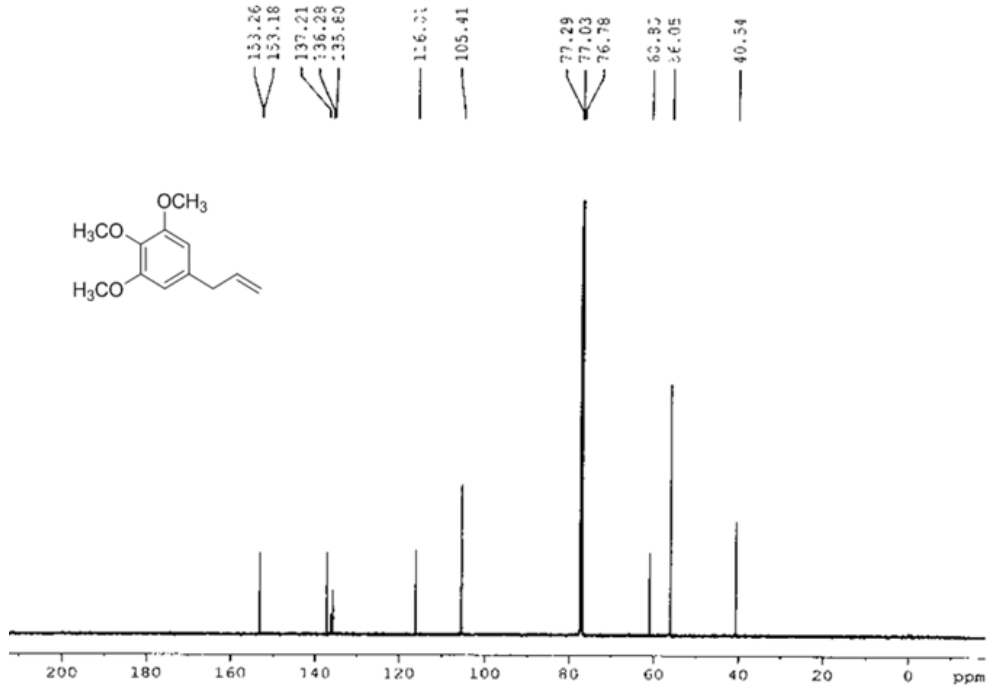
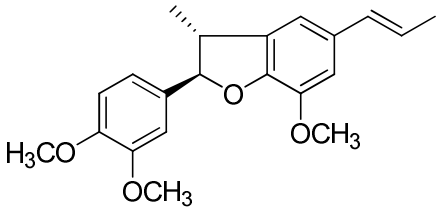


Figure 2a.4: ^{13}C NMR spectrum of Elemicin

Fraction pool 7 (340 mg) when subjected to column chromatography on silica gel by eluting with hexane–ethyl acetate (9:1) gave six sub-fraction pools, named 7a–7f. Fraction 7b on crystallisation in hexane afforded mixture of stigmasterol and *β*-sitosterol. Fraction 7c when subjected to column chromatography on neutral alumina, afforded compound 3 (44 mg) , compound 4 (20 mg) , compound 5 (6 mg) and compound 6 (15 mg).

^{1}H NMR (**Figure 2a.5**) spectrum of compound 3 showed signals at δ 1.37 and 1.86, suggesting the presence of two methyl groups. Three singlets at δ 3.87, 3.88 and 3.89 each integrating for three protons indicated the presence of three methoxy groups. A doublet of doublet at δ 6.35 and a multiplet at δ 6.10 each integrating for one proton with *J* value of 15.5 Hz could be attributed to *trans* olefinic protons. Signals at δ 55.9, 56.0 and 56.1 in the ^{13}C NMR (**Figure 2a.6**) spectrum confirmed the presence of three -OMe groups. Peaks in between δ 110.1-149.5 (14C) in the ^{13}C NMR spectrum confirmed the presence of olefinic and aromatic carbons. Mass spectrum of the compound gave molecular ion peak at 341.1762 which is the $(M+H)^{+}$ peak. Thus the structure of compound 3 was confirmed to be that of acuminatin [Yu *et al.*, **2000**] on comparing its spectral values with those reported in the literature.



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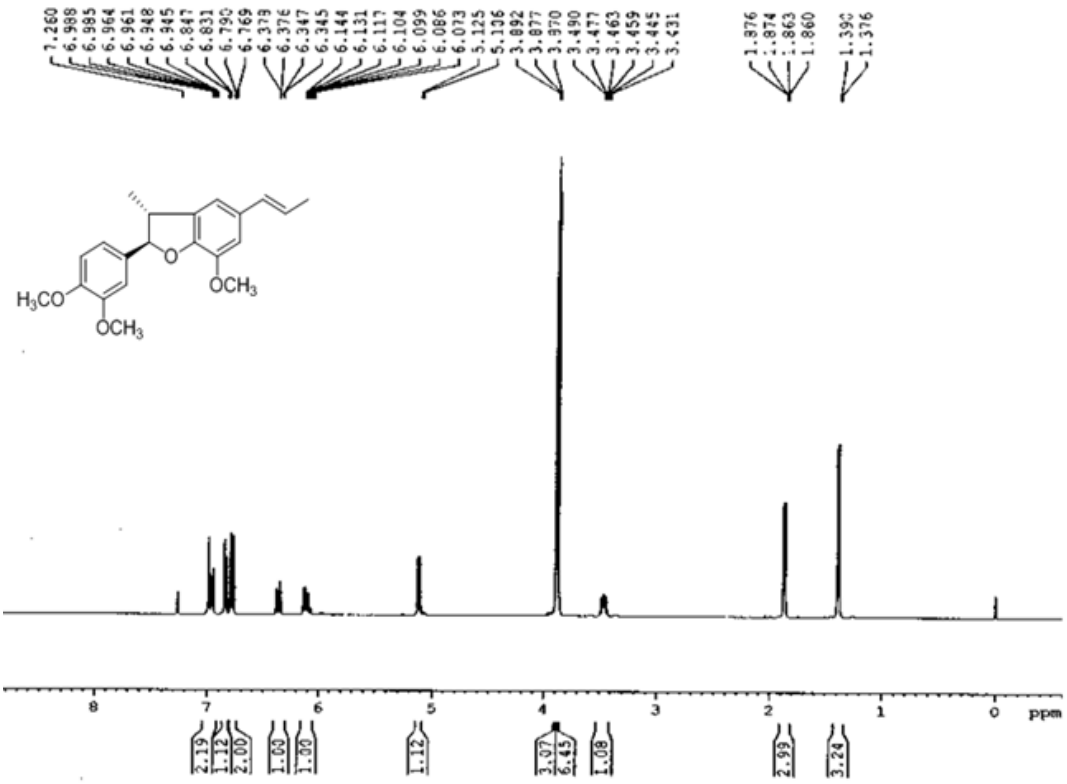


Figure 2a.5: ^{1}H NMR spectrum of Acuminatin

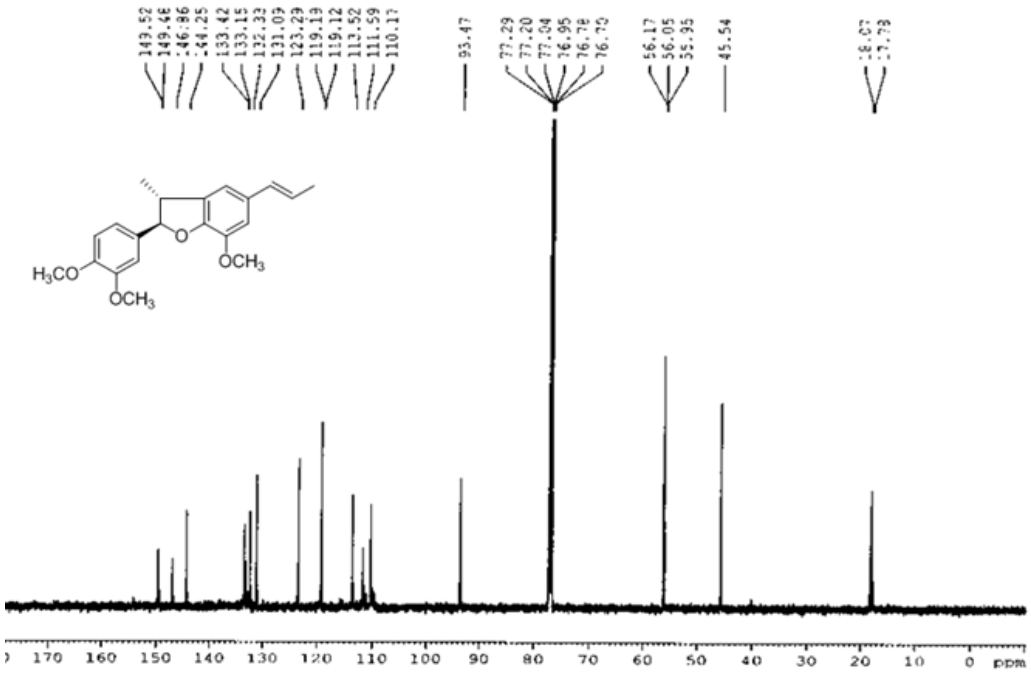
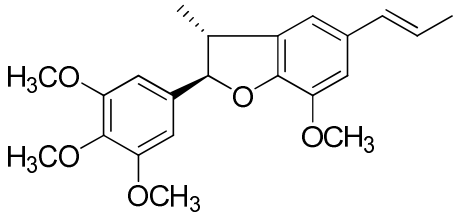


Figure 2a.6: ^{13}C NMR spectrum of Acuminatin

Compound 4 obtained as white crystalline solid was analysed using various spectroscopic data. ^{1}H NMR spectrum of compound 4 showed signals at δ 1.40 and 1.86 suggesting the presence of two methyl groups. Three singlets at δ 3.84, 3.85 and 3.89 in ^{1}H NMR (**Figure 2a.7**) spectrum integrating for three, six and three protons respectively indicated the presence of four methoxy groups, among which two of them are identical. A doublet of doublet at δ 6.35 and a multiplet at δ 6.10 each integrating for one proton with *J* value of 16.0 Hz could be attributed to *trans* olefinic protons. Signals at δ 56.0, 56.2 and 60.7 in the ^{13}C NMR (**Figure 2a.8**) spectrum confirmed the presence of four -OMe groups. Peaks in between δ 103.7-153.3 (14C) in the ^{13}C NMR spectrum confirmed the presence of olefinic and aromatic carbons. The mass spectrum of the compound gave molecular ion peak at 371.1862 which is the $(M+H)^{+}$ peak. From all the above spectral data and comparing with those in the literature, compound 4 was found to be 7-methoxy-3-methyl-5-((*E*)-prop-1-enyl)-2-(3,4,5-trimethoxyphenyl)-2,3-dihydrobenzofuran [Dean *et al.*, **1982**]. The structure of compound 4 was further confirmed from its single crystal X-ray structure (**Figure 2a.9**).



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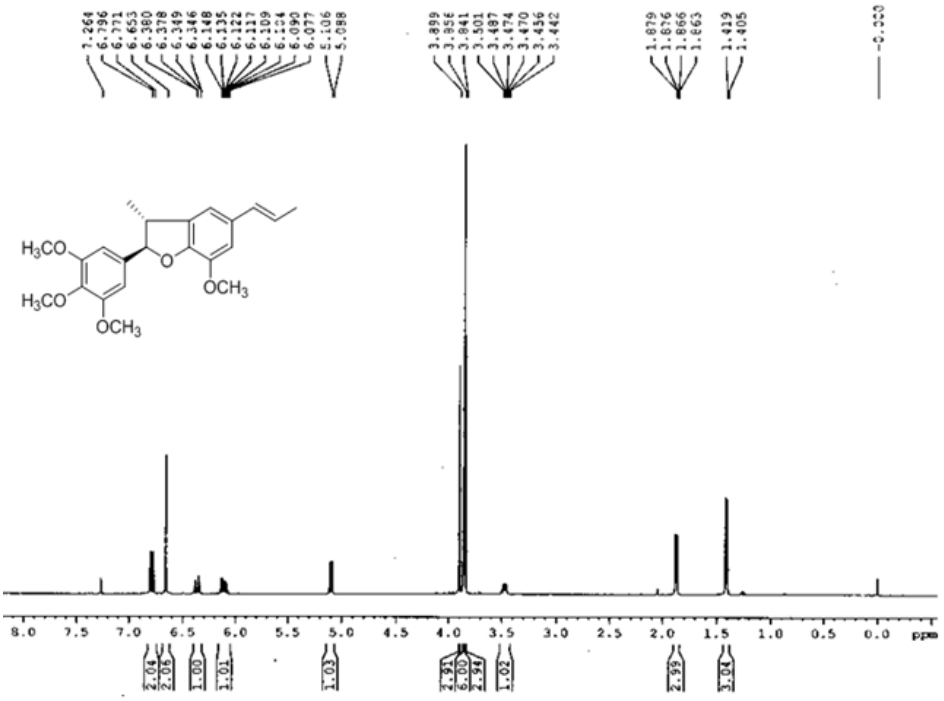


Figure 2a.7: ^{1}H NMR spectrum of 7- methoxy-3-methyl-5-((*E*)-prop-1-enyl)-2-(3,4,5-trimethoxyphenyl)-2,3-dihydrobenzofuran

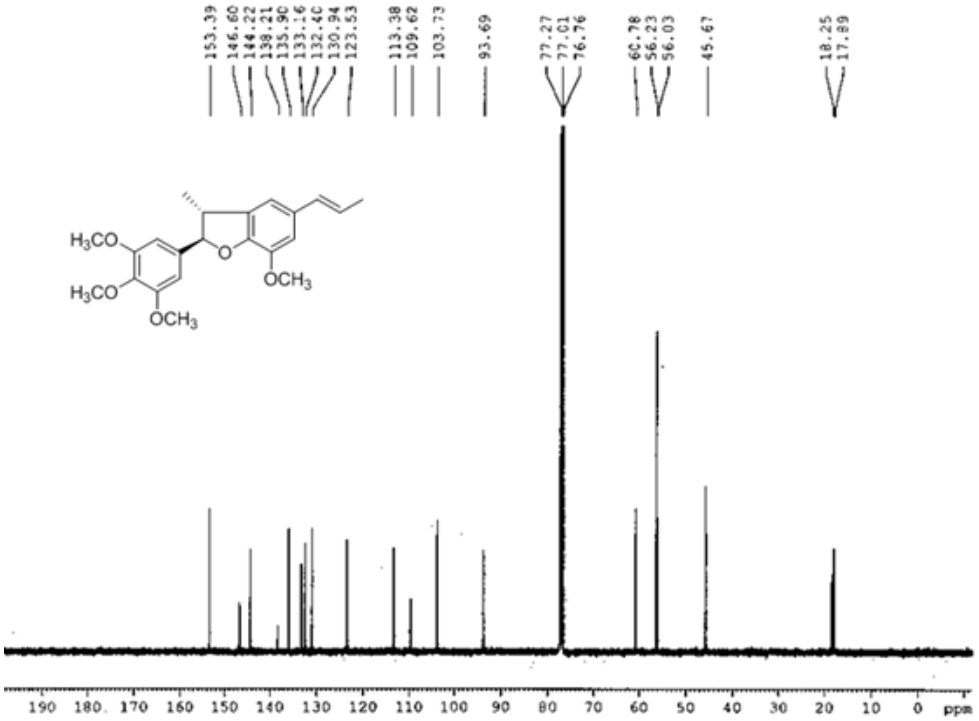


Figure 2a.8: ^{13}C NMR spectrum of 7- methoxy-3-methyl-5-((*E*)-prop-1-enyl)-2-(3,4,5-trimethoxyphenyl)-2,3-dihydrobenzofuran

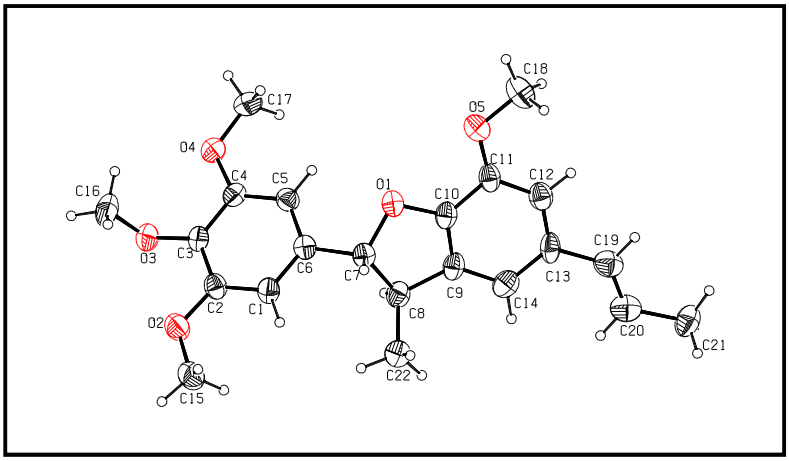
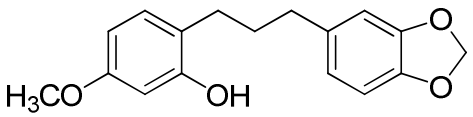


Figure 2a.9 : ORTEP diagram of 7- methoxy-3-methyl-5-((*E*)-prop-1-enyl)-2-(3,4,5-trimethoxyphenyl)-2,3-dihydrobenzofuran

Compound 5 obtained as a white crystalline solid was analyzed using various spectroscopic data. Its IR spectrum showed the presence of a hydroxyl group through the broad absorption at 3416 cm^{-1}. The ^{1}H NMR (**Figure 2a.10**) spectrum of compound indicated the presence of three methylene groups at δ 1.87 (m) and δ 2.54 (t) and 2.58 (t). The peaks at δ 2.54 and δ 2.58 showed a correlation with protons at δ 1.87 ppm in ^{1}H-^{1}H correlation spectroscopy which suggested that there is a propane system attached to two aromatic rings. A sharp singlet at δ 5.91, integrating for two proton indicated the presence of methylenedioxy group. The ^{13}C NMR (**Figure 2a.11**) and DEPT spectra suggested the presence of 17 carbons, which include a methyl, four methylene, six methine, and six quaternary carbons. Among them, the signals at δ 55.3 and 100.7 could be attributed to the methoxy and methylenedioxy carbons respectively. The mass spectrum of the compound gave molecular ion peak at 287.1283 which is the $(M+H)^{+}$ peak Based on the above data, the structure was arrived as virolane and confirmed from comparison with literature value. The compound is being isolated from *M. fragrans* for the first time and has been previously isolated from *Virola surnamensis* [Filho *et al.*, **1973]**.



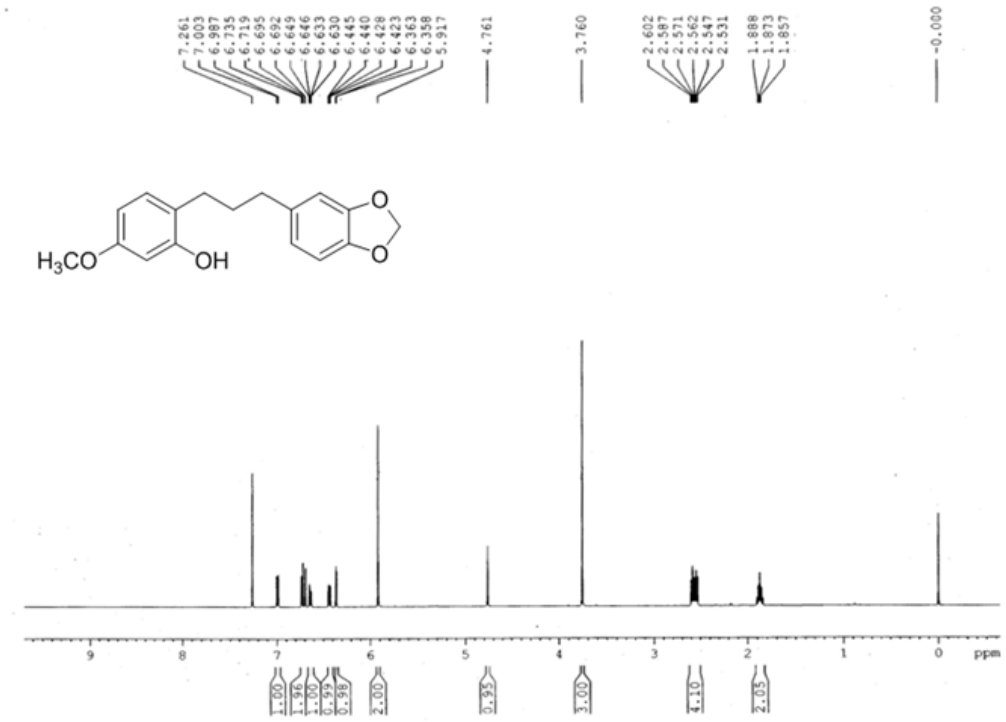


Figure 2a.10 : ^{1}H NMR spectrum of Virolane

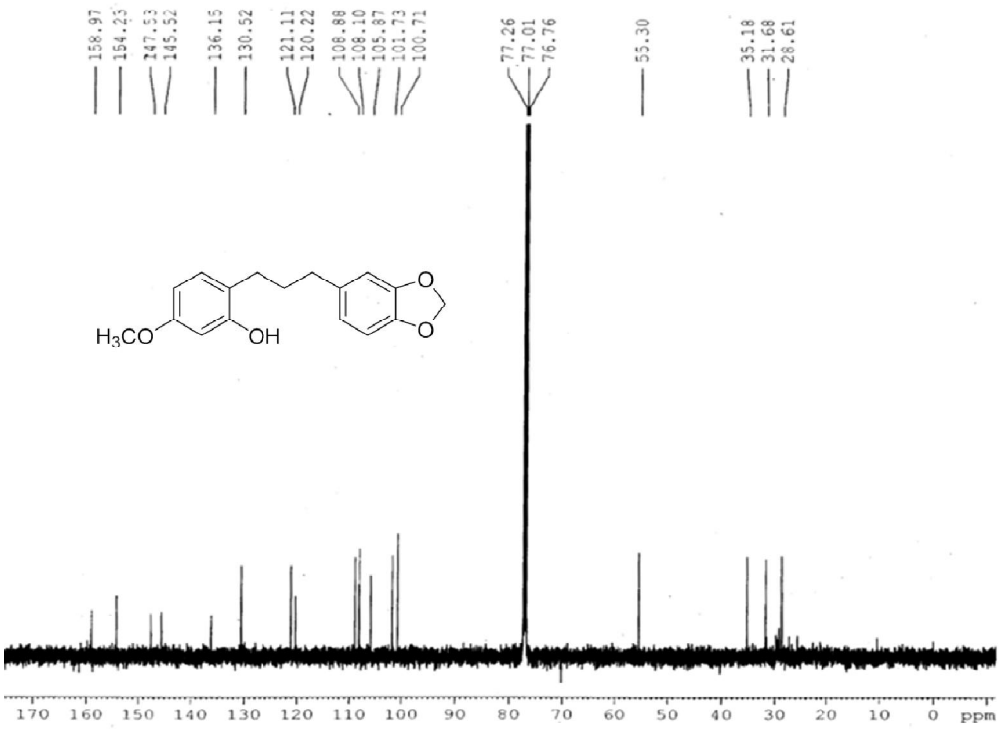
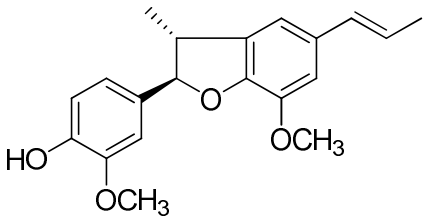


Figure 2a.11: ^{13}C NMR spectrum of Virolane

Compound 6, obtained as a white solid was analysed using various spectroscopic data. IR spectrum showed a broad absorption at 3426 cm^{-1}, which indicated the presence of hydroxyl group. ^{1}H NMR (**Figure 2a. 12**) spectrum of compound 6 showed signals at δ 1.37 and 1.86 indicating the presence of two methyl groups. Two singlets at δ 3.88 and 3.89 in the ^{1}H NMR spectrum each integrating for three protons indicated the presence of two methoxy group. A doublet of doublet at δ 6.35 and a multiplet at δ 6.10 each integrating for one proton with *J* value of 16 Hz could be attributed to *trans* olefinic protons. A sharp singlet at δ 5.63 integrating for one proton confirmed the presence of a phenolic -OH group. Signals at δ 56.0 and 55.9 in the ^{13}C spectrum confirmed the presence of two -OMe groups. Peaks in between δ 108.9-146.7 (14C) in the ^{13}C NMR (**Figure 2a.13**) spectrum confirmed the presence of olefinic and aromatic carbons. The mass spectrum of the compound gave molecular ion peak at 327.1586 which is the $(M+H)^{+}$ peak. From all the above spectral data and comparing with those in the literature, compound 6 was identified as licarin A [Aiba *et al.*, **1973**].



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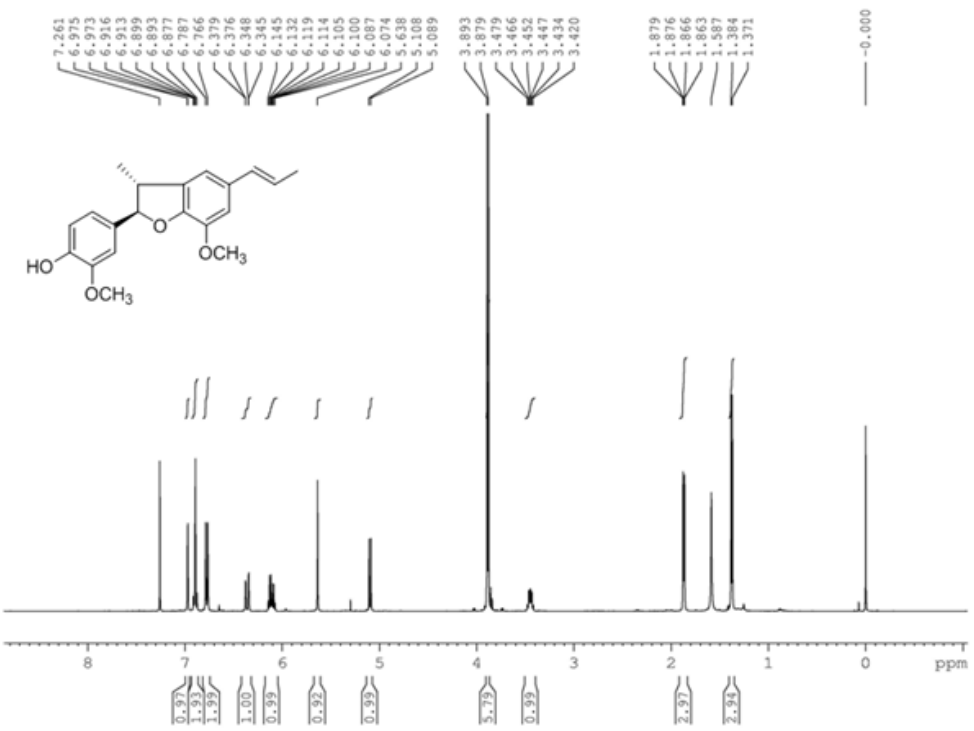


Figure 2a.12: ^{1}H NMR spectrum of Licarin A

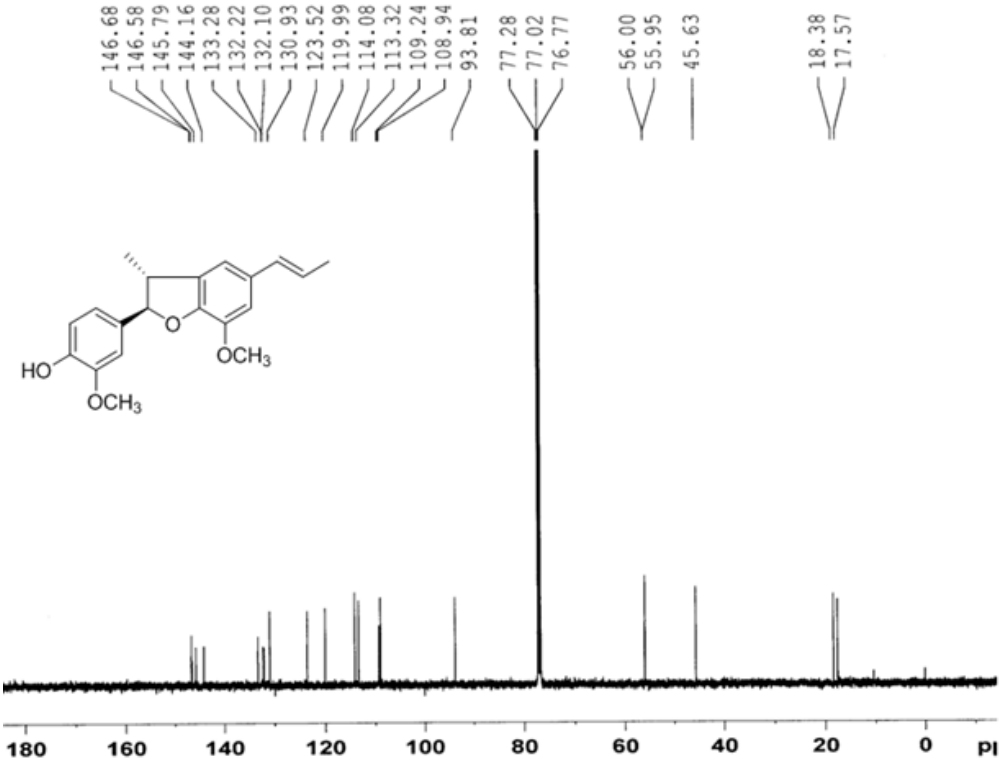
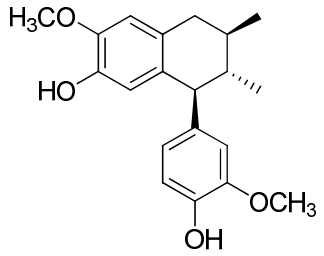


Figure 2a.13: ^{13}C NMR spectrum of Licarin A

Fraction 7d on crystallisation in hexane–ethyl acetate afforded compound 7 (10 mg) as white solid. IR spectrum of compound 7 showed a broad absorption at 3409 cm^{-1}, which indicated the presence of hydroxyl group. Signals at δ 0.84 and 1.06 in the ^{1}H NMR (**Figure 2a.14**) spectrum indicated the presence of two methyl groups. Two singlets at δ 3.81 and 3.84 in the ^{1}H NMR spectrum, each integrating for three protons indicated the presence of two methoxy groups. Two sharp singlets at δ 5.27 and 5.48 integrating for one proton each in ^{1}H NMR spectrum suggested the presence of two phenolic -OH groups. Signals at δ 55.8 and 55.9 in the ^{13}C NMR (**Figure 2a.15**) spectrum confirmed the presence of two -OMe groups. Peaks in between δ 109.9-146.4 (12C) in the ^{13}C NMR spectrum confirmed the presence of aromatic carbons. The mass spectrum of the compound gave the molecular ion peak at 351.1557 which is the $(M+Na)^{+}$ peak. All these spectral data matched with guaiacin [Hattori *et al*., **1986**] and the structure is shown below.



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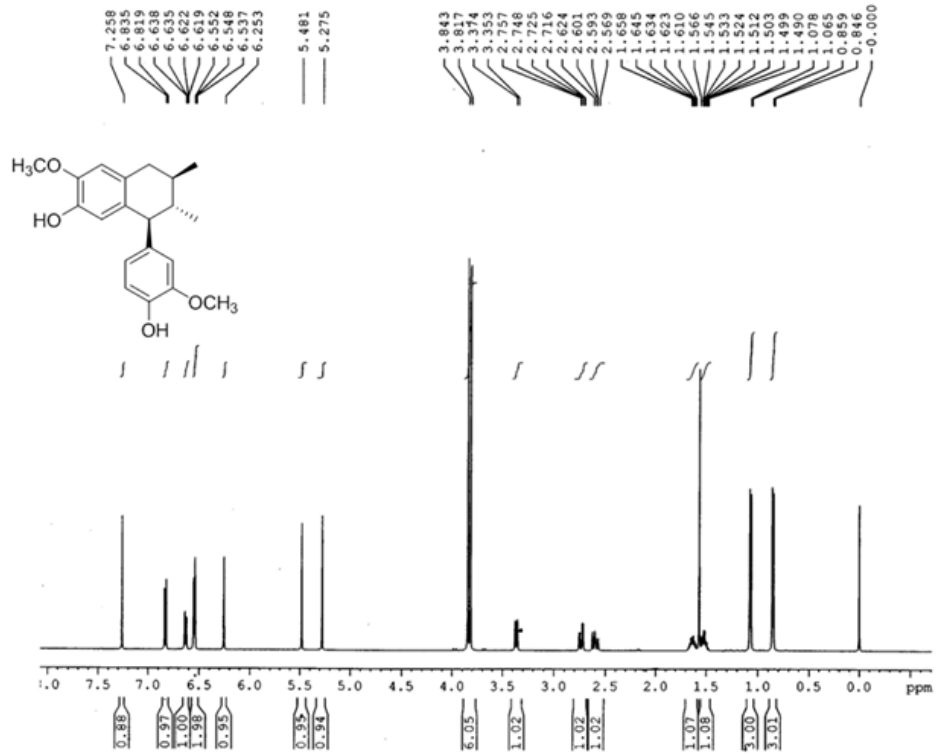


Figure 2a.14: ^{1}H NMR spectrum of Guaiacin

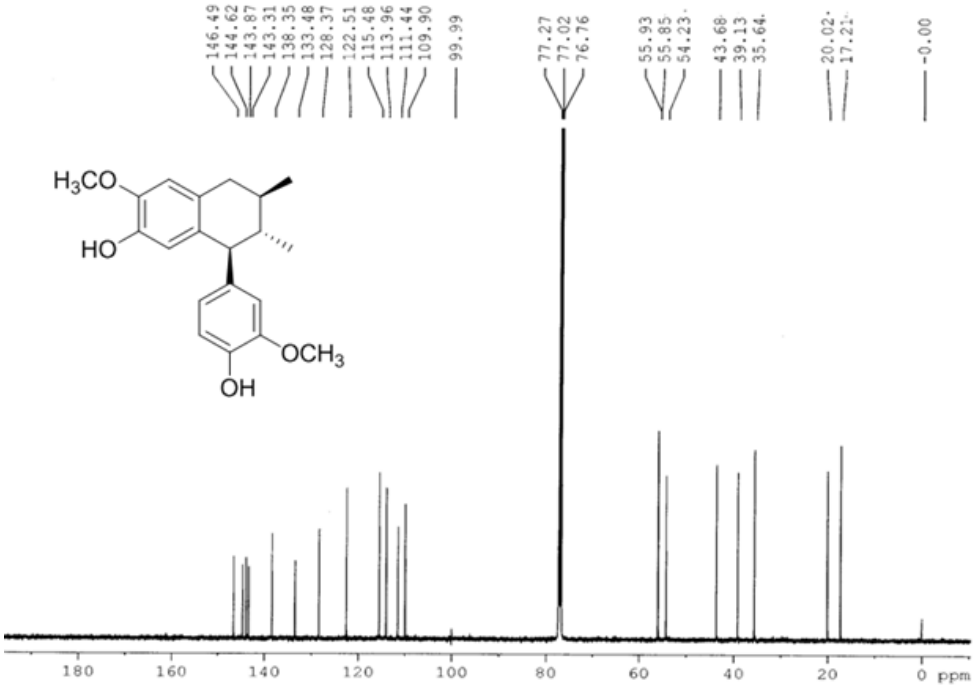
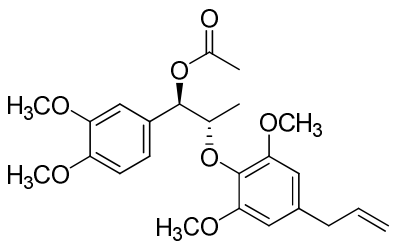


Figure 2a.15 : ^{13}C NMR spectrum of Guaiacin

Fraction 8 (88 mg) when submitted to column chromatography on neutral alumina by eluting with hexane–ethyl acetate (9:1) yielded compound 8 (30 mg) and compound 9 (10 mg).

Compound 8 obtained as colourless viscous liquid was analysed using various spectroscopic data. IR spectrum of the compound showed a strong absorption at 1741cm^{-1}, which indicated the presence of ester carbonyl. Signal at δ 1.28 in the ^{1}H NMR (**Figure 2a.16**) spectrum indicated the presence of a methyl group. A sharp singlet at δ 2.17 which is integrating for three protons indicated the presence of an acetate group. Three singlets at δ 3.77, 3.84 and 3.85 in ^{1}H NMR spectrum, integrating for six, three and three protons respectively, indicated the presence of four methoxy groups in the molecule. Signals at δ 55.8, 55.9 and 56.0 in the ^{13}C NMR (**Figure 2a.17**) spectrum confirmed the presence of four -OMe groups. A doublet at δ 5.86 integrating for one proton and a doublet of quartet at δ 4.44 integrating for one proton having correlation in ^{1}H-^{1}H correlation spectrum with J = 3.5 Hz indicated that the compound is *erythro*. The signal at δ 170.2 could be attributed to the carbon of ester carbonyl. Peaks in between δ 105.5-153.3 (12C) in the ^{13}C spectrum confirmed the presence of olefinic and aromatic carbons. The DEPT-135 spectrum confirmed the presence of two -CH_2- groups. The mass spectrum of the compound gave molecular ion peak at 453.1885 which is the $(M+Na)^+$ peak. From all the above spectral data and comparing with those in the literature, compound 8 was identified as the *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-acetoxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan [Isogai *et al.*, **1973**].



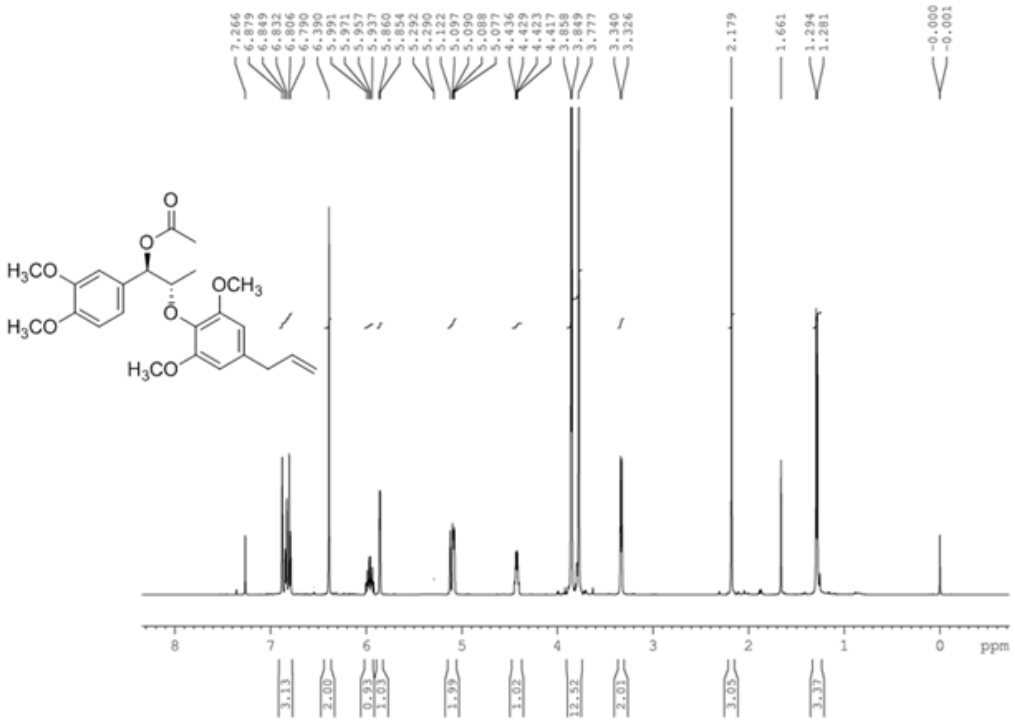


Figure 2a.16 : ^{1}H NMR spectrum of *Erythro*-(*7S,8R*)-$\Delta^{8'}$-7-acetoxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan

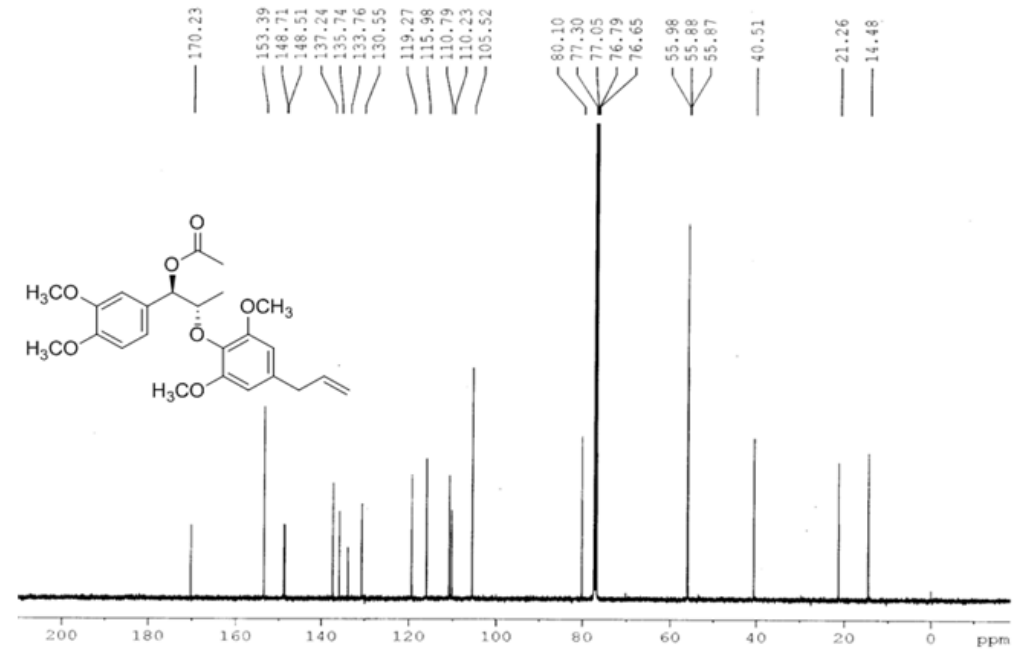
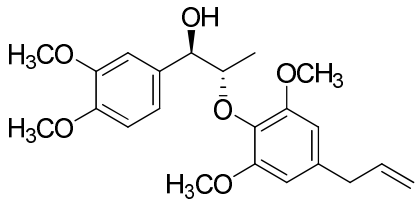


Figure 2a.17 : ^{13}C NMR spectrum of *Erythro*-(*7S,8R*)-$\Delta^{8'}$-7-acetoxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan

IR spectrum compound 9 showed a broad absorption at 3510 cm^{-1} indicating the presence of a hydroxyl group. Signal at δ 1.11in the ^{1}H NMR (**Figure 2a.18**) spectrum indicated the presence of a methyl group. A broad singlet at δ 4.12, integrating for one proton could be assigned to the proton of hydroxyl group. Three singlets at δ 3.85, 3.87 and 3.88 in ^{1}H NMR spectrum which integrating for six, three and three respectively indicated the presence of four methoxy groups. A doublet at δ 4.80 integrating for one proton and a doublet of quartet at δ 4.36 integrating for one proton having correlation in ^{1}H-^{1}H correlation spectrum with J = 1.5 Hz indicated that the compound is *erythro*. Signals at δ 55.8, 55.9 and 56.1 in the ^{13}C spectrum again confirmed the presence of four -OMe groups. Peaks in between δ 105.4-153.4 in the ^{13}C NMR (**Figure 2a.19**) spectrum confirmed the presence of olefinic and aromatic carbons. The DEPT-135 spectrum confirmed the presence of three -CH_2- groups. The mass spectrum of the compound gave molecular ion peak at 411.1775 which is the $(M+Na)^{+}$ peak. From all the above spectral data and comparing with those in the literature, compound 9 was identified as the *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-hydroxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan [Isogai *et al.*, **1973**].



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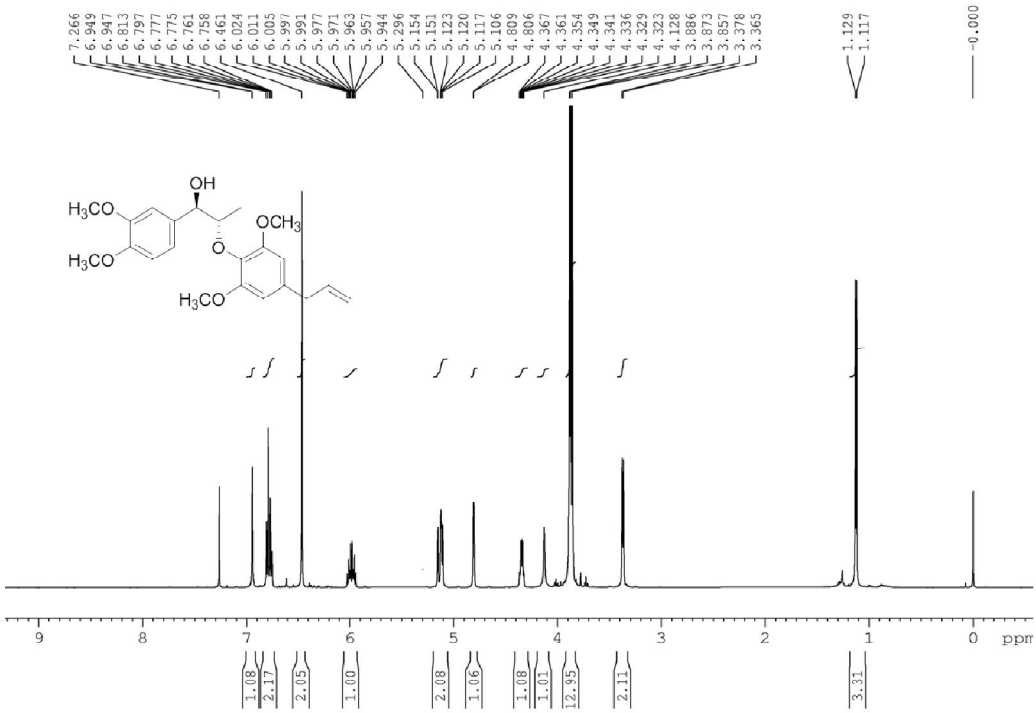


Figure 2a.18 : ^{1}H NMR spectrum of *Erythro-(7S,8R)*-$\Delta^{8'}$-7-hydroxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan

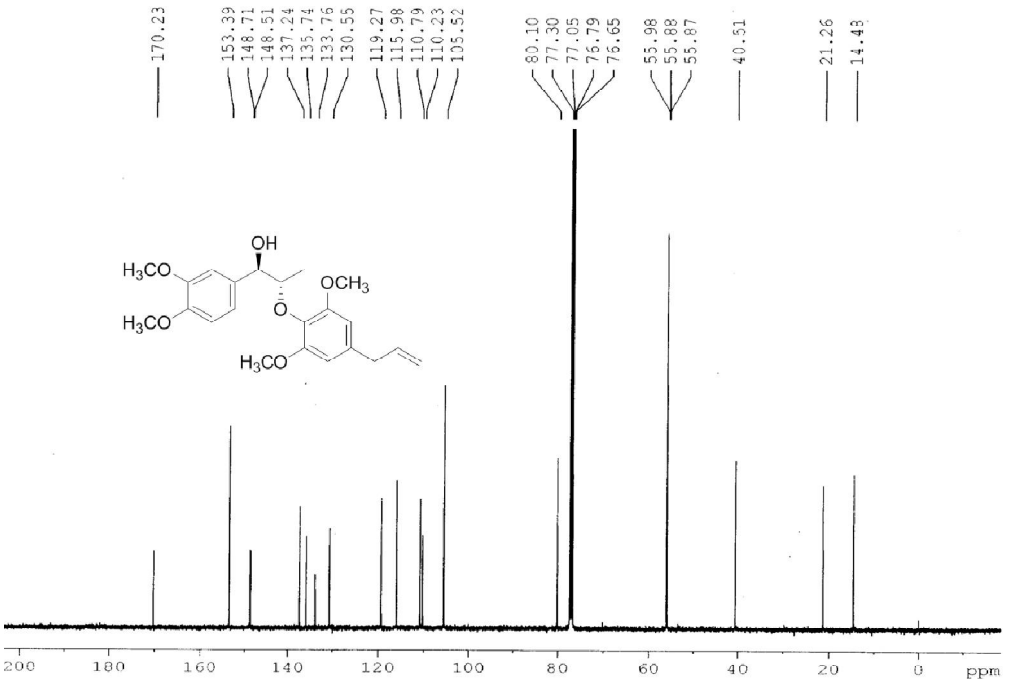
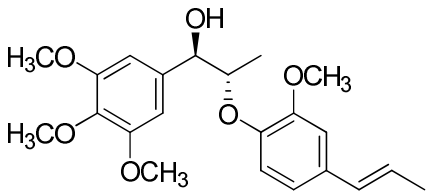


Figure 2a.19 : ^{13}C NMR spectrum of *Erythro-(7S,8R)*-$\Delta^{8'}$-7-hydroxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan

Fraction 9 (531 mg) when submitted to CC on neutral alumina by eluting with hexane–ethyl acetate (8:2) yielded compound 10 (50 mg) as colourless liquid. IR spectrum showed broad absorption at 3501 cm^{-1} suggesting the presence of hydroxyl group. ^{1}H NMR (**Figure 2a.20**) spectrum of compound 10 showed the presence of two methyl groups [δ 1.19 (d), 1.89 (dd)], four methoxy groups [δ 3.82 (s), 3.85 (6H, s), 3.90 (s)], a hydroxyl group [δ 3.59 (s) exchangeable with D_2O], five aromatic protons and two olefinic protons [δ 6.20 (1H, dq), J = 16, 1.5 Hz and δ 6.38 (1H, dd), J = 16, 6.5 Hz]. The protons at δ 1.89 showed a correlation with protons appearing at δ 6.20 ^{1}H-^{1}H correlation spectrum. A doublet at δ 4.82 integrating for one proton and a doublet of quartet at δ 4.36 integrating for one proton having correlation in ^{1}H-^{1}H correlation spectrum with J = 3 Hz indicated that the compound is *erythro*. The ^{13}C NMR (**Figure 2a.21**) and DEPT spectra showed the presence of 22 carbons which include six methyl, eight methylene and six quaternary carbons. Among them signals at δ 55.8, 55.9, 56.0, 56.1 and 60.8 could attributed to methoxy groups. The mass spectrum of the compound gave molecular ion peak at 411.1773 which is the $(M+Na)^{+}$ peak. Thus the structure of the compound was confirmed as *erythro* surinamensin by comparing with the reported data. The compound is being isolated from *M. fragrans* for the first time and has been previously isolated from *V. surnamensis* [Barata *et al.*, **1978**].



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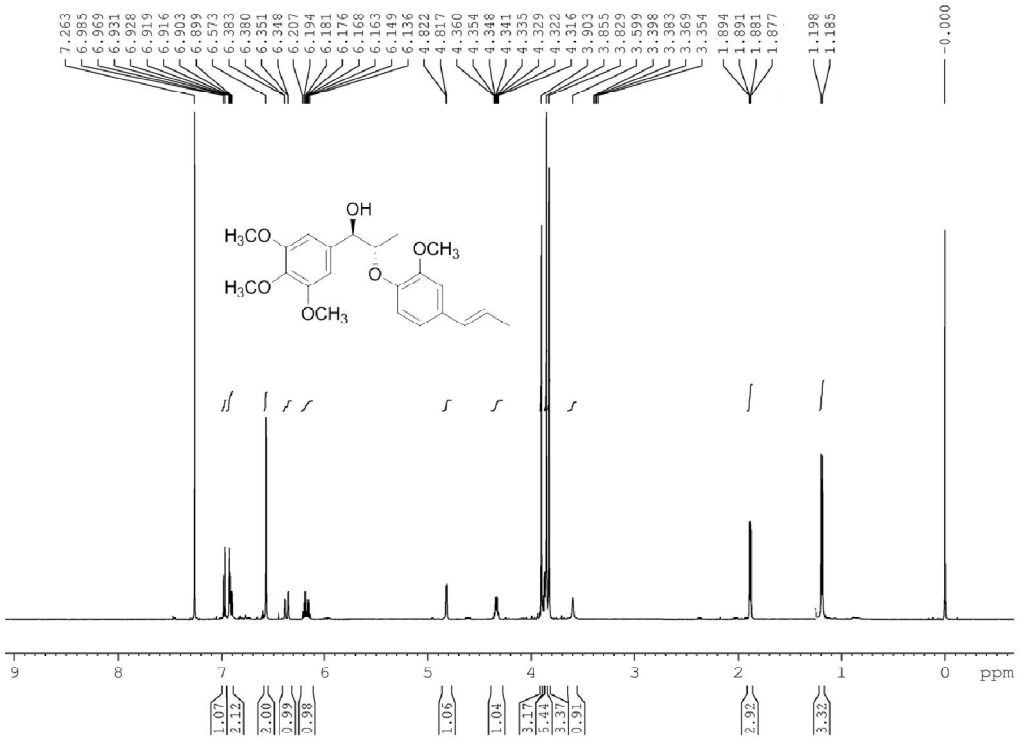


Figure 2a.20 : [1]H NMR spectrum of *Erythro* surinamensin

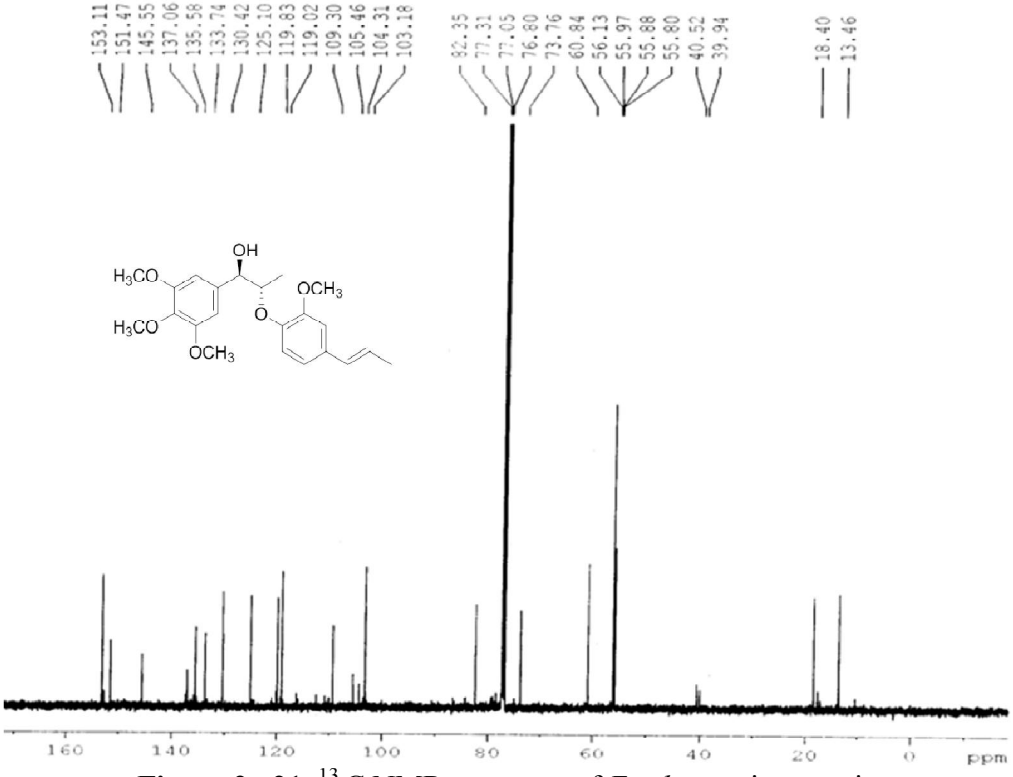


Figure 2a.21: [13] C NMR spectrum of *Erythro* surinamensin

2a.1.1.3. Experimental

2a.1.1.3a. General experimental details

All melting points are uncorrected and were determined on a Fisher-Jones apparatus. The IR spectra were recorded on Bruker FTIR spectrometer. ^{1}H and ^{13}C NMR spectra were recorded at 500 and 125 MHz respectively using deuterated solvents on Bruker AMX 500 MHz spectrometer. Tetramethylsilane was used as internal standard and chemical shifts were expressed in δ-scale. Abbreviations used in ^{1}H NMR are *s* - singlet, *d* - doublet, *dd* - doublet of doublet, *br s* - broad singlet, *q* - quartet and *m* - multiplet. Mass spectra were recorded under ESI/HR-MS at 61,800 resolution using Thermo Scientific exactive mass spectrometer. UV spectral experiments were carried out on a Shimadzu UV-1601 UV-vis spectrophotometer using spectroscopic grade methanol as solvent.

Analytical thin layer chromatography was performed on Merck silica gel 60 F_{254} aluminium sheets. The spots were first checked under UV light and later in an iodine chamber also. Column chromatography was carried out with 100-200 mesh Silica gel and with 60-325 mesh neutral alumina. All the solvents used for chromatography were of commercial grade and were distilled prior to use. The solvents were removed under reduced pressure using Büchi rotary evaporator. Drying of the plant material was carried out in RRLT-NC drier.

2a.1.1.3b. Extraction

The fruits of *Myristica fragrans* were collected from Ernakulam district, Kerala. The pericarp, mace and seeds were separated. The separated pericarp was cleaned, cut in to small pieces, dried in a drier maintained at 50^{o}C and powdered. The powdered pericarp (750 g) was subjected to extraction using acetone (2 L X 3) at room temperature. After extraction, the solvent was removed under reduced pressure using Büchi rotary evaporator. The crude extract (32 g) was then suspended in ethyl acetate and stirred for an hour. The ethyl acetate soluble portion (19 g) thus obtained was then subjected to column chromatographic separation.

2a.1.1.3c. Chromatographic separation of fruit pericarp extract

The extract of the fruit pericarp of *M. fragrans* was dissolved in minimum quantity of ethyl acetate and loaded on the top of silica gel column filled with a slurry of 100-200 mesh silica gel in petroleum ether. The column was eluted successively with gradient mixtures of petroleum ether and ethyl acetate of increasing polarities and finally with pure ethyl acetate. A total of 171 fractions of 150 ml each were collected and they were pooled in to 15 fraction pools according to the similarities in TLC. A pictorial representation of the procedure for the isolation of the compound is shown in **Figure 2a.22**.

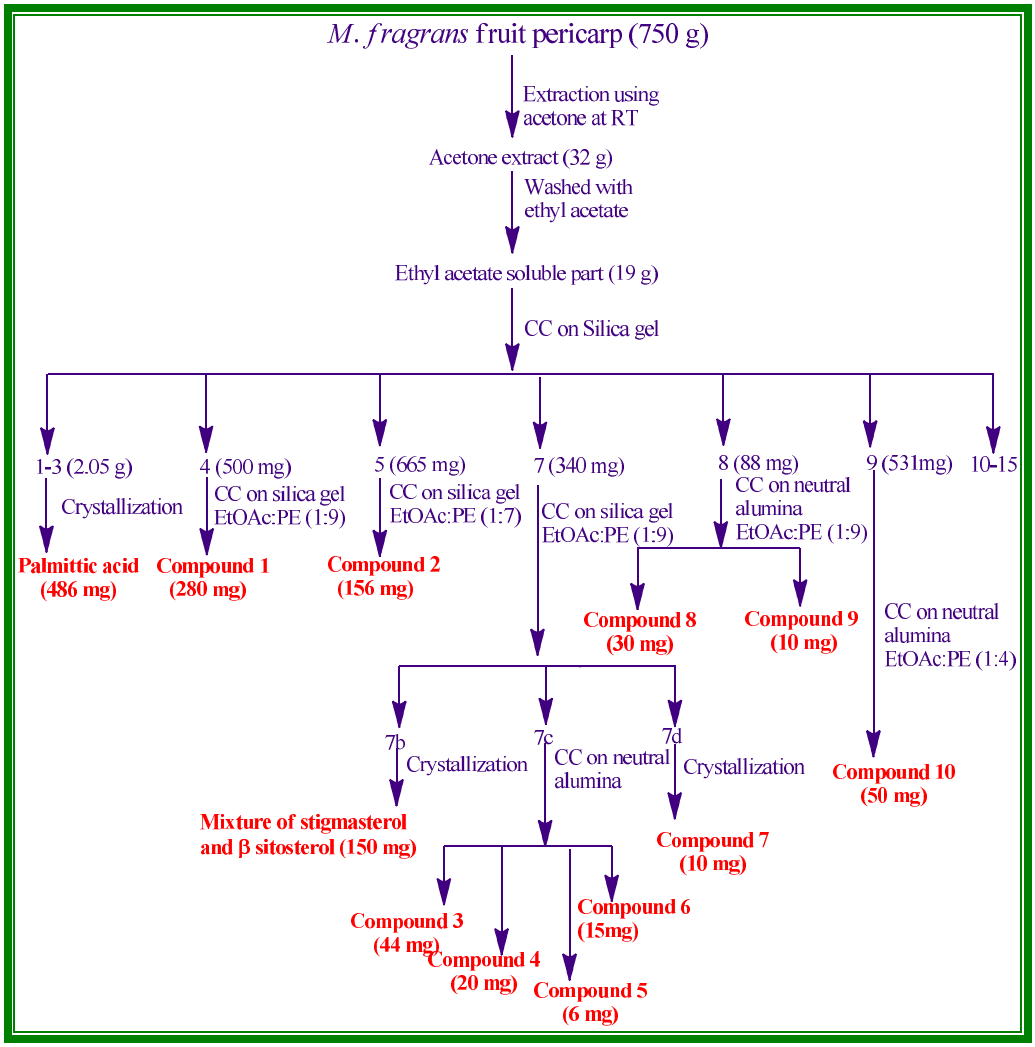
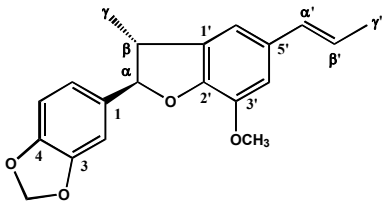


Figure 2a.22 : Pictorial representation of isolation of chemical compounds from *M. fragrans* fruit pericarp

2a.1.1.3d. Isolation of compound 1

The isolation procedure of compound 1 is represented in **Figure 2a.22**. Compound 1 (280 mg) was obtained as a colourless crystals, on eluting the column with 10% ethyl acetate in hexane. IR, ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound, on comparison with literature values, confirmed it to be licarin B.

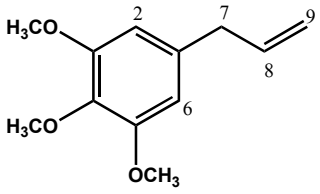


FT-IR(NaCl $\nu_{max,}cm^{-1}$)	:	2921, 1604, 1494, 1247, 1038, 963, 815
^{1}H NMR (500 MHz,$CDCl_3$)	:	δ 6.92 (1H, *s*, H-2), 6.88 (1H, *dd*, J_1 = 1.0 Hz, J_2 = 8.0 Hz, H-6), 6.78 (1H, *d*, *J* = 8.0 Hz, H-5), 6.77 (1H, *br. s*, H-4'), 6.75 (1H, *br. s*, H-6'), 6.36 (1H, *dd*, J_1 = 0.5 Hz, J_2 = 15.7 Hz, H-α'), 6.13 (1H, *dq*, J_1 = 6.5 Hz, J_2 = 15.7 Hz, H-β'), 5.94 (2H, *s*, O-CH_2-O), 5.10 (1H, *d*, *J* = 9.0 Hz, H-α), 3.88 (3H, s, -OCH_3), 3.43 (1H, *dq*, J_1 = 7.0 Hz, J_2 = 9.0 Hz, H-β), 1.87 (3H, *dd*, J_1 = 1.5 Hz, J_2 = 6.7 Hz, H-γ'), 1.38 (3H, *d*, *J* = 7.0 Hz, H-γ)
^{13}C NMR (125 MHz,$CDCl_3$)	:	δ 134.3 (C-1), 106.8 (C-2), 147.6 (C-3), 146.5 (C-4), 108.0 (C-5), 120.2 (C-6), 133.0 (C-1'), 147.9 (C-2'), 144.1 (C-3'), 109.2 (C-4'), 132.2 (C-5'), 113.3 (C-6'), 93.4 (C-α), 45.7 (C-β), 17.9 (C-γ),

		130.9 (C-α'), 123.4 (C-β'), 18.3 (C-γ'), 101.1 (O-CH_2-O), 55.9 (-OCH_3) (C assignments were made as per literature)
	HR-ESIMS *m/z*	: 325.1435 $[M+H]^+$ (calcd for $C_{20}H_{21}O_5$, 325.1440)
	Melting point	: 82-84^oC

2a.1.1.3e. Isolation of compound 2

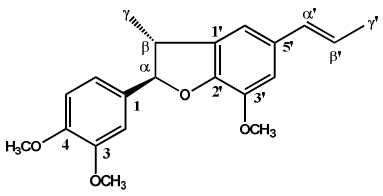
Fifth fraction pool showed the presence of a UV active compound, which was re purified with 22% ethyl acetate in petroleum ether to obtain elemicin in 156 mg yield as colourless oil. The structure of the compound was confirmed as shown below by comparing the IR, ^{1}H NMR, ^{13}C NMR and mass spectral details of the compound with those reported in literature.

	FT-IR (NaCl $\nu_{max,}$ cm^{-1})	: 2923, 1588, 1502, 1458, 1237, 1127, 755
	^{1}H NMR (500 MHz, $CDCl_3$)	: δ 6.55 (2H, *s*, H-2 & H-6), 5.92 (1H, *m*, H-8), 5.07 (2H, *m*, H-9), 3.85 (6H, *s*, 2 x -OCH_3), 3.82 (3H, *s*, -OCH_3), 3.34 (2H, *d*, *J* = 4.5 Hz, H-7)
	^{13}C NMR (125 MHz, $CDCl_3$)	: 135.8 (C-1), 105.4 (C-2 & C-6), 153.2 (C-3 & C-5), 136.2 (C-4), 40.5 (C-7), 137.2 (C-8), 116.0 (C-9), 56.0 (2 x -OCH_3), 60.8 (-OCH_3) (C assignments were made as per literature)
	HR-ESIMS *m/z*	: 209.1180 $[M+H]^+$ (calcd for

$C_{12}H_{17}O_3$, 209.1099).

2a.1.1.3f. Isolation of compound 3

Seventh fraction pool on purification with 10% ethyl acetate in petroleum ether gave four sub fraction pools named 7a-7d. Sub fraction pool 7b on crystallization yielded mixture of β sitosterol and stigmasterol. The sub fraction pool 7c on purification with 10% ethyl acetate in petroleum ether on neutral alumina yielded four compounds. Compound 3 and 6 were obtained as white solids, and compound 4 and 5 were obtained as white crystalline solids. Compound 3 was confirmed as acuminatin based on its spectral details.



FT-IR (NaCl $\nu_{max,}$ cm^{-1})	:	2959, 1601, 1512, 1333, 1265, 1141, 1028, 962
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 6.98 (1H, *s*, H-2), 6.96 (1H, *dd*, J_1 = 1.5 Hz, J_2 = 8 Hz, H-6), 6.84 (1H, *d*, *J* = 8 Hz, H-5), 6.79 (1H, *br. s*, H-4'), 6.76 (1H, *br. s*, H-6'), 6.37 (1H, *dd*, J_1 = 1 Hz, J_2 = 15.5 Hz, H-α'), 6.14 (1H, *dq*, J_1 = 6.5 Hz, J_2 = 15.5 Hz, H-β'), 5.12 (1H, *d*, *J* = 9.5 Hz, H-α), 3.89 (3H, *s*, -OCH_3), 3.87 (6H, *s*, 2 x -OCH_3), 3.45 (1H, *dq*, J_1 = 6.5 Hz, J_2 = 9.5 Hz, H-β), 1.87 (3H, *dd*, J_1 = 1 Hz, J_2 = 6.75 Hz, γ'), 1.30 (3H, *d*, *J* = 7 Hz, H-γ)
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 133.4 (C-1), 111.5 (C-2), 149.5 (C-3), 149.4 (C-4),

119.2 (C-5), 119.1 (C-6), 133.1 (C-1'), 146.8 (C-2'), 144.2 (C-3'), 110.1 (C-4'), 132. 3 (C-5'), 113.5 (C-6') 93.5 (C-α), 45.5 (C-β), 17.7 (C-γ), 131. 1 (C-α'), 123.3 (C-β'), 18.1 (C-γ'), 55.9, 56.0, 56.1 (3 x -OCH_3) (C assignments were made as per literature)

HR-ESIMS *m/z* : 341.1762 $[M+H]^+$ (calcd for $C_{21}H_{25}O_4$, 341.1708

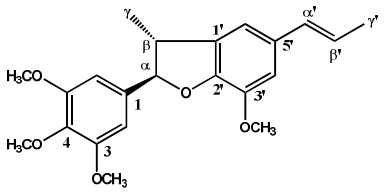
Melting point : 109-110^oC

2a.1.1.3g. Isolation of compound 4

After the isolation of acuminatin, compound 4 obtained in 20 mg as a colourless crystals, on further elution with 10% ethyl acetate in petroleum ether. The spectral studies of the compound matched with those reported for (*2S,3R*)-7-methoxy-3-methyl-5-((*E*)-prop-1-enyl)-2-(3,4,5 trimethoxyphenyl)-2,3-dihydrobenzofuran and the structure is as shown below.

FT-IR (NaCl $\nu_{max,}$ cm^{-1}) : 2935, 1592, 1502, 1457, 1332, 1233, 964

^1H NMR (500 MHz, $CDCl_3$) : δ 6.79 (1H, *s*, H-4'), 6.77 (1H, *s*, H-6'), 6.65 (2H, *s*, H-2 & H-6), 6.38 (1H, *dd*, J_1 = 1.0 Hz, J_2 = 15.7 Hz, H-α'), 6.14 (1H, *dq*, J_1 = 6.5 Hz, J_2 = 15.7 Hz, H-β'), 5.10 (1H, *d*, *J* = 9.0 Hz, H-α), 3.90 (3H, *s*, -OCH_3), 3.85 (6H, *s*, 2 x -OCH_3), 3.84 (3H, *s*, OCH_3), 3.50 (1H, *dq*,

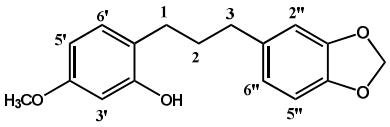


		J_1 = 7.0 Hz, J_2 = 9.0 Hz, H-β), 1.87 (3H, *dd*, J_1 = 1.0 Hz, J_2 = 6.7 Hz, H-γ'), 1.41 (3H, *d*, *J* = 7.0 Hz, H-γ)
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 133.1 (C-1), 103.7 (C-2 & C-6), 153.3 (C-3 & C-5), 138.2 (C-4), 135.9 (C-1'), 146.6 (C-2'), 144.2 (C-3'), 109.6 (C-4'), 132.4 (C-5'), 113.3 (C-6'), 93.6 (C-α), 45.6 (C-β), 17.8 (C-γ), 130.9 (C-α'), 123.5 (C-β'), 18.2 (C-γ'), 56.0 (-OCH_3), 56.2 (2 x -OCH_3), 60.7 (-OCH_3) (C assignments were made as per literature)
HR-ESIMS *m/z*	:	371.1862 $[M+H]^+$ (calcd for $C_{22}H_{27}O_5$, 371.1814
Melting point	:	122-123^{o}C

2a.1.1.3h. Isolation of compound 5

Compound 5 was obtained as a colourless crystals after the isolation of compound 4. The IR, ^{1}H NMR and ^{13}C NMR spectral details were closely related to that of virolane reported earlier from *Virola surinamensis*.

FT-IR (NaCl $\nu_{max,}$ cm^{-1})	:	3416 br (-OH), 2922, 1619, 1503, 1441, 1243, 937
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 7.00 (1H, *d*, *J* = 8.0 Hz, H-6'), 6.73 (1H, *d*, *J* = 8.0 Hz, H-5''), 6.69 (1H, *d*, *J* = 1.5 Hz, H-2''), 6.64 (1H, *dd*, J_1 = 1.5 Hz, J_2 = 8.0 Hz, H-5'), 6.44 (1H, *dd*, J_1 = 2.5Hz, J_2 = 8.0 Hz, H-



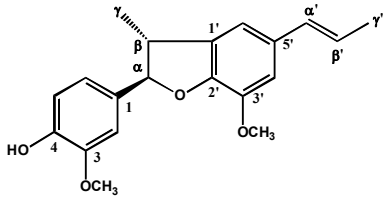
6''), 6.34 (1H, *d*, *J* = 2.5 Hz, H-3'), 5.91 (2H, *s*, O-CH_2-O), 4.76 (1H, *s*, OH), 3.76 (3H, *s*, -OCH_3), 2.58 (2H, *t*, *J* = 7.5 Hz,H-1), 2.54 (2H, *t*, *J* = 7.5 Hz, H-3), 1.87 (2H, *m*, H-2)

^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 31.6 (C-1), 28.6 (C-2), 35.1 (C-3), 120.2 (C-1'), 154.2 (C-2'), 101.7 (C-3'), 158.9 (C-4'), 105.8 (C-5'), 130.5 (C-6'), 136.1 (C-1''), 108.8 (C-2''), 147.5 (C-3''), 145.5 (C-4''), 108.1 (C-5''), 121.1 (C-6''), 55.3 (-OCH_3), 100.7 (O-CH_2-O) (C assignments were made as per literature)
HR-ESIMS *m/z*	:	287.1274 $[M+H]^+$ (calcd for $C_{17}H_{19}O_4$, 287.1283
Melting point	:	99-100^{o}C

2a.1.1.3i. Isolation of compound 6

After the isolation of virolane, compound 6, obtained in 15 mg, as a white solid, on further elution with 10% ethyl acetate in petroleum ether. The spectral studies of the compound matched with those reported for licarin A and the structure is as shown below.

FT-IR (NaCl $\nu_{max,}cm^{-1}$)	:	3426 br (OH), 2924,1614, 1497, 1221, 1123, 956, 860
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 6.97 (1H, *s*, H-2), 6.91 (1H, *d*, *J* = 8.0 Hz, H-6), 6.87 (1H, *d*, *J* = 8.0 Hz, H-5), 6.78 (1H, *br. s*, H-4'), 6.76 (1H, *br. s*, H-6'), 6.37 (1H, *dd*, J_1 = 1.5 Hz,

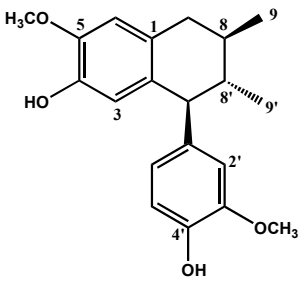


J_2 = 15.5 Hz, H-α'), 6.10 (1H, *dq*, J_1 = 6.5 Hz, J_2 = 15.5 Hz, H-β'), 5.63 (1H, *br. s*, -OH), 5.10 (1H, *d*, *J* = 9.5, H-α), 3.89 (3H, *s*, -OCH_3), 3.88 (3H, *s*, -OCH_3), δ 3.45 (1H, *dq*, J_1 = 7.0 Hz, J_2 = 9.5 Hz, H-β), 1.87 (3H, *dd*, J_1 = 1.5 Hz, J_2 = 6.5 Hz, H-γ'), 1.38 (3H, *d*, *J* = 6.5, H-γ)

^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 132 (C-1), 108.9 (C-2), 146.6 (C-3), 145.7 (C-4), 114.0 (C-5), 119.9 (C-6), 133.2 (C-1'), 146.5 (C-2'), 144.1 (C-3'), 109.1 (C-4'), 132.0 (C-5'), 113.3 (C-6'), 93.8 (C-α), 45.6 (C-β), 17.5 (C-γ), 130.9 (C-α'), 123.5 (C-β'), 18.3 (C-γ'), 55.9, 56.0 (2 x -OCH_3) (C assignments were made as per literature)
HR-ESIMS *m/z*	:	327.1586 $[M+H]^+$ (calcd for $C_{20}H_{23}O_4$, 327.1596)
Melting point	:	119-120^{o}C

2a.1.1.3j. Isolation of compound 7

Fraction 7d on crystallisation in hexane–ethyl acetate afforded compound 7 as white solid. Compound 7 was confirmed as guaiacin based on the spectral details.



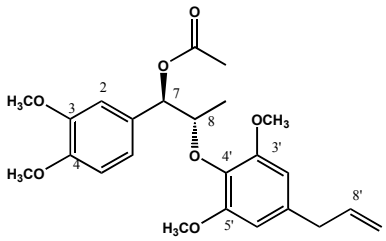
FT-IR (NaCl $\nu_{max,}$ cm^{-1})	:	3409 br (-OH), 2889, 1513, 1276, 1026, 770
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 6.83 (1H, *d*, *J* = 8.0 Hz, H-5'), 6.63 (1H, *dd*, J_1 = 1.5 Hz, J_2 = 8 Hz, H-2'), 6.65 (1H, *d*, *J* = 2 Hz, H-3), 6.53 (1H, *s*, H-2), 6.25 (1H, *s*, H-5), 5.49 (1H, *s*, -OH), 5.28 (1H, *s*, -OH), 3.84 (3H, *s*, $-OCH_3$), 3.82 (3H, *s*, $-OCH_3$), 3.37 (1H, *d*, *J* = 10.5 Hz, H-7'), 2.75 (1H, *dd*, J_1 = 4.5 Hz, J_2 =16.0 Hz, equatorial H-7β), 2.62 (1H, *dd*, J_1 = 12.0 Hz, J_2 = 16.0 Hz, axial H-7α), 1.63 (1H, *m*, H-8'), 1.49 (1H, *m*, H-8), 1.07 (3H, *d*, *J* = 6.5, H-9), 0.85 (3H, *d*, *J* = 6.5, H-9')
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 128.3 (C-1), 143.8 (C-2), 143.8 (C-3), 143.3 (C-4), 115.4 (C-5), 133.4 (C-6), 39.1 (C-7), 43.6 (C-8), 20.0 (C-9), 138.3 (C1'), 111.4 (C-2'), 146.4 (C-3'), 144.6 (C-4'), 113.9 (C-5'), 122.5 (C-6'), 54.2 (C-7'), 35.6 (C-8'), 17.2 (C-9'), 55.8 ($-OCH_3$), 55.9 ($-OCH_3$) (C assignments were made as per literature)
HR-ESIMS *m/z*	:	351.1557 $[M+Na]^+$ (calcd for

$C_{20}H_{24}O_4Na$, 351.1572)

Melting point : 190-192^oC

2a.1.1.3k. Isolation of compound 8

Fraction pool 8 was submitted to column chromatography on neutral alumina by eluting with 10% ethyl acetate in petroleum ether to yield two compounds. Compound 8 and 9 were obtained as colourless liquids. Compound 8 was confirmed as *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-acetoxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan based on the spectral details.



FT-IR (NaCl $\nu_{max,}$ cm^{-1}) : 2937, 1741 s (C=O), 1588, 1461, 1239, 1127, 1026, 817

^1H NMR (500 MHz, $CDCl_3$) : δ 6.87 (1H, *s*, H-2), 6.84 (1H, *d*, *J* = 8.0 Hz, H-6), 6.80 (1H, *d*, *J* = 8.0 Hz, H-5), 6.39 (2H, *s*, H-2' & H-6'), 5.99 (1H, *m*, H-8'), 5.86 (1H, *d*, *J* = 3.5 Hz, H-7), 5.12 (2H, *m*, H-9'), 4.44 (1H, *dq*, J_1 = 3.5 Hz, J_2 = 12.7 Hz, H-8), 3.85 (3H, *s*, -OCH_3), δ 3.84 (3H, *s*, -OCH_3), 3.77 (6H, *s*, 2 x -OCH_3), δ 3.34 (2H, *d*, *J* = 6.5 Hz, H-7'), δ 2.17 (3H, *s*, -OAc), δ 1.29 (3H, *d*, *J* = 6.5 Hz, H-9)

^{13}C NMR (125 MHz, $CDCl_3$) : δ 130.5 (C-1), 110.2 (C-2), 148.7 (C-3), 148.5 (C-4), 110.7 (C-5), 119.2 (C-6), 80.1 (C-7), 76.6 (C-8), 14.4 (C-9),

133.7 (C-1'), 105.5 (C-2' & C-6'), 153.3 (C-3' & C-5'), 135.7 (C-4'), 40.5 (C-7'), 137.2 (C-8'), 115.9 (C-9'), 170.2 (C=O), 21.2 (CO-$\underline{CH_3}$), 55.8 (-OCH_3), 55.9 (-OCH_3), 56.0 (2 x -OCH_3) (C assignments were made as per literature)

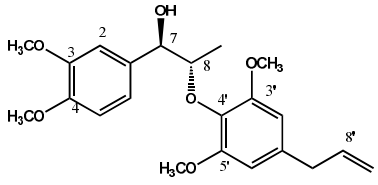
HR-ESIMS *m/z* : 453.1885 $[M+Na]^+$ (calcd for $C_{24}H_{30}O_7Na$, 453.1889)

2a.1.1.3l. Isolation of compound 9

Compound 9 was obtained as colourless liquid after the isolation of compound 8. The spectral studies of the compound matched with those reported for *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-hydroxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan and the structure is as shown below.

FT-IR (NaCl $\nu_{max,}$ cm^{-1}) : 3510 br (-OH), 2937, 1589, 1512, 1231, 1125, 1029, 917

^{1}H NMR (500 MHz, $CDCl_3$) : δ 6.94 (1H, *s*, H-2), 6.79 (1H, *d*, *J* = 8.0 Hz, H-6), 6.76 (1H, *d*, *J* = 8.0 Hz, H-5), 6.46 (2H, *s*, H-2' & H-6'), 5.97 (1H, *m*, H-8'), 5.15 (2H, *m*, H-9'), 4.80 (1H, *d*, 1.5 Hz, H-7), 4.36 (1H, *dq*, J_1 = 1.5 Hz, J_2 = 12.7 Hz, H-8), 4.12 (1H, *br. s*, -OH), 3.87 (3H, *s*, -OCH_3), 3.85 (6H, *s*, 2 x -OCH_3), 3.85 (3H, *s*, -OCH_3), 3.37 (2H, *d*, *J* = 6.5 Hz, H-7'), 1.12 (3H, *d*, *J* = 6.0 Hz, H-9)

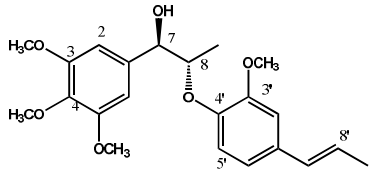


^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 132.6 (C-1), 109.2 (C-2), 148.8 (C-3), 147.8 (C-4), 110.7 (C-5), 118.1 (C-6), 82.3 (C-7), 72.7 (C-8), 12.7 (C-9), 132.9 (C-1'), 105.4 (C-2' & C-6'), 153.4 (C-3' & C-5'), 136.1 (C-4'), 40.5 (C-7'), 137.0 (C-8'), 116.2 (C-9'), 55.8 (-OCH_3), 55.9 (-OCH_3), 56.1 (2 x -OCH_3) (C assignments were made as per literature)
HR-ESIMS *m/z*	:	411.1775 $[M+Na]^+$ (calcd for $C_{22}H_{28}O_6Na$, 411.1784)

2a.1.1.3m. Isolation of compound 10

Fraction 9 when submitted to CC on neutral alumina by eluting with 20% hexane–ethyl acetate, yielded compound 10. The IR, ^{1}H NMR and ^{13}C NMR spectral details were closely related to that of *erythro* surinamensin, which is reported earlier from *Virola surinamensis*. Hence, the structure proposed for this compound is shown below.

FT-IR (NaCl $\nu_{max,}$ cm^{-1})	:	3501 br (-OH), 2937, 1591, 1507, 1229, 1128, 1034, 916
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 6.98 (1H, *d*, *J* = 8.0 Hz, H-5'), 6.91 (1H, *dd*, J_1 = 2.0 Hz, J_2 = 8.0 Hz, H-6'), 6.90 (1H, *d*, *J* = 5 Hz, H-2'), 6.57 (2H, *s*, H-2 & H-6), 6.38 (1H, *dd*, J_1 = 1.5 Hz, J_2 = 16.0 Hz, H-7'), 6.20 (1H, *dq*, J_1 = 6.5 Hz, J_2 = 16.0 Hz, H-8'), 4.82 (1H, *d*, 3 Hz, H-7), 4.36 (1H, *dq*, J_1 =



3.0 Hz, J_2 = 12.7 Hz, H-8), 3.90 (3H, *s*, -OCH_3), 3.85 (6H, *s*, 2 x -OCH_3), 3.82 (3H, *s*, OCH_3), 3.59 (1H, *br. s*, -OH), 1.89 (3H, *dd*, J_1 = 1.5 Hz, J_2 = 6.7 Hz, H-9'), 1.19 (3H, *d*, *J* = 6.5 Hz, H-9)

^{13}C NMR (125 MHz, $CDCl_3$) : δ 133.7 (C-1), 103.1 (C-2 & C-6), 153.1 (C-3 & C-5), 137.0 (C-4), 82.3 (C-7), 73.7 (C-8), 13.4 (C-9), 133.7 (C-1'), 109.3 (C-2'), 151.4 (C-3'), 145.5 (C-4'), 119.0 (C-5'), 119.8 (C-6'), 130.4 (C-7'), 125.1 (C-8'), 18.4 (C-9'), 55.8 (-OCH_3), 55.9 (2 x -OCH_3), 60.8 (-OCH_3) (C assignments were made as per literature)

HR-ESIMS *m/z* : 411.1773 $[M+Na]^+$ (calcd for $C_{22}H_{28}O_6Na$, 411.1784)

2a.1.2. Isolation from nutmeg seed kernel

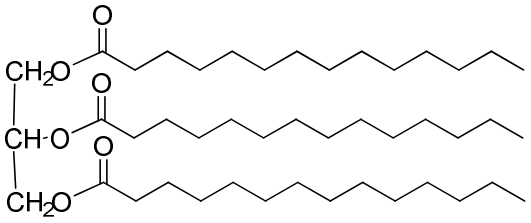
2a.1.2.1. Plant material and extraction

M. fragrans seeds (1 kg) were collected in January 2012, from North Paravur in Kerala state, India. From this, 700 g of seed kernels were obtained. It was dried in an air oven maintained at 50^{o}C for an hour and then powdered mechanically. The powdered material was subjected to extraction with petroleum ether (3 L X 3 days) at room temperature to remove fat and oil. After removing the oil content, it was then subjected to extraction with acetone (2.5 L x 3) at room temperature. 43.14 g of acetone extract was obtained after removing the solvent. The acetone extract (43.14 g) was subjected to CC

on silica gel (100–200 mesh) and eluted with hexane–ethyl acetate mixtures of increasing polarities to give 11 fraction pools.

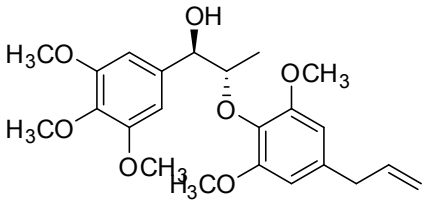
2a.1.2.2.Isolation and characterization of major compounds

Fraction pool 1 (24 g) was subjected to crystallization in acetone yielded 19 g of trimyristin as the major compound.



Fraction pool 6 (350 mg) was subjected to column chromatography on neutral alumina using hexane–ethyl acetate (9:1) to yield compound 11 (200 mg). Compound 11 was confirmed to be licarin A, which was earlier isolated from *M. fragrans* fruit pericarp. Fraction pool 8 (58 mg) was subjected to column chromatography on silica gel using hexane-ethyl acetate (5:1) to yield compound 12 (10 mg) which was characterized as guaiacin. Fraction pool 10 (510 mg) was submitted to column chromatography on neutral alumina using hexane-ethyl acetate (9:1) to yield three compounds namely, compound 13 (200 mg) as *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-acetoxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan, compound 14 (60 mg) as *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-hydroxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan and compound 15 (60 mg) . IR spectrum of compound 15 showed a broad absorption at 3506 cm^{-1}, which indicated the presence of hydroxyl group. Signal at δ 1.12 in the ^{1}H NMR (**Figure 2a.23**) spectrum indicated the presence of one methyl group. A broad singlet at δ 4.15, integrating for one proton indicated the presence of an -OH proton. Three singlets at δ 3.82, 3.84 and 3.88 in ^{1}H NMR spectrum, each integrating for six, six and three protons respectively, indicated the presence of five methoxy groups. A doublet at δ 4.79 integrating for one proton and a doublet of quartet at δ 4.34 integrating for one proton having correlation in ^{1}H-^{1}H correlation spectrum with J = 2.5 Hz indicated that the compound is *erythro*. Signals in between δ 55.8, 55.9, 56.1, 56.2 and 60.8 in the ^{13}C NMR (**Figure 2a.24**) spectrum confirmed the presence of

five -OMe groups. Peaks at δ 102.9-153.5 (14C) in the ^{13}C spectrum confirmed the presence of olefinic and aromatic carbons. The DEPT-135 spectrum confirmed the presence of two -CH_2- groups. The mass spectrum of the compound gave molecular ion peak at 441.1885 which is the $(M+Na)^+$ peak. From all the above spectral data and comparison with those in the literature, compound 15 was identified as the raphidecursinol B [Isogai *et al.*, **1973**].



15

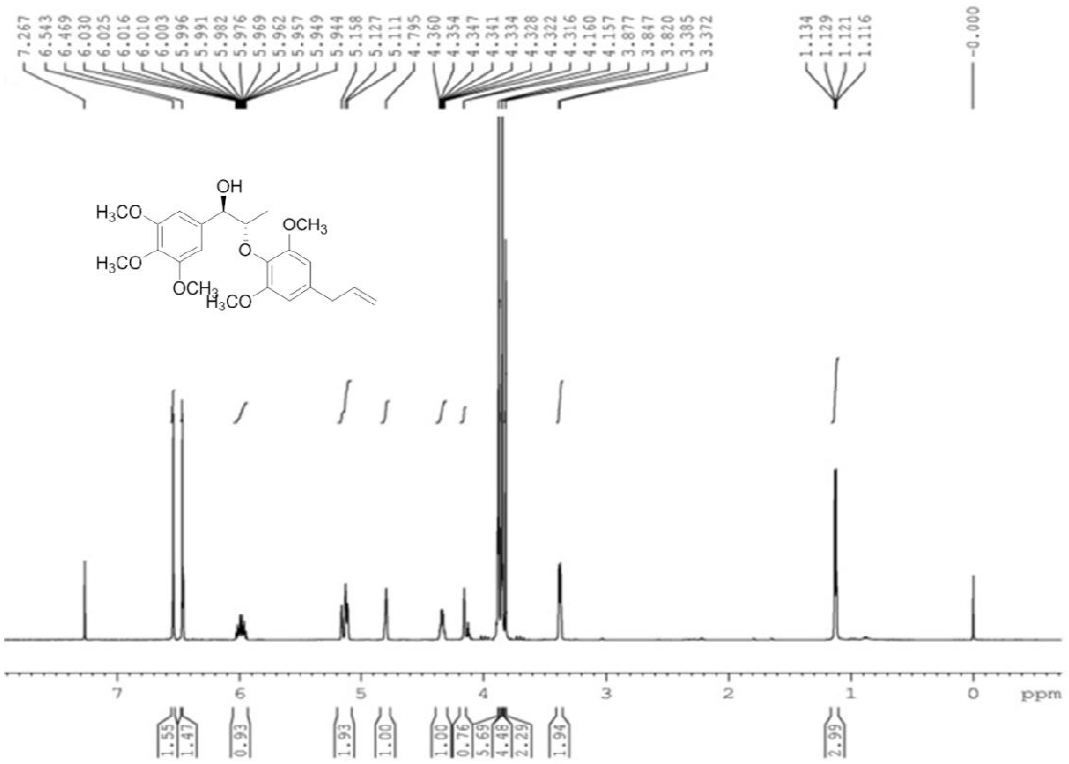


Figure 2a.23 : ^1H NMR spectrum of Raphidecursinol B

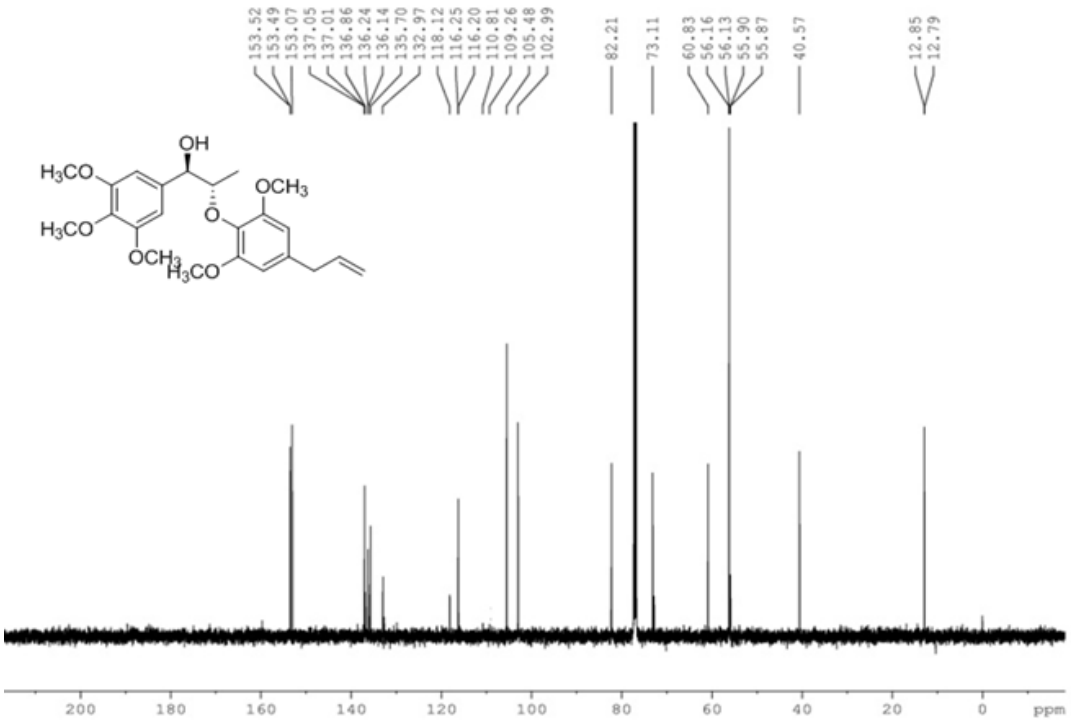
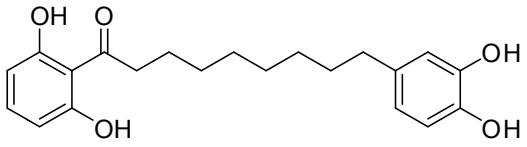


Figure 2a.24 : ^{13}C NMR spectrum of Raphidecurinol B

Fraction pool 11 (120 mg) was subjected to column chromatography in hexane-ethyl acetate (1:1) followed by crystallization yielded compound 16 (76 mg). The IR spectrum of the compound showed absorption at 3350cm^{-1} which suggested the presence of hydroxyl group. The ^{1}H NMR (**Figure 2a.25**) spectrum showed four sets of signals viz; between δ 1.28-1.36, integrating for a total of 8 protons, δ 1.54-1.56 integrating for two protons, δ 1.66-1.72 also for two protons and δ 2.48 integrating for two protons suggesting a total of seven methylene groups. One triplet seen at δ 3.11 integrating for two protons could be attributed to -CO-CH_2 group. The ^{13}C NMR (**Figure 2a.26**) spectrum, showed the presence of peaks at 165.8 ppm, 148.0 and 145.9 ppm indicating the presence of aromatic carbons bearing hydroxyl groups. Peak at δ 212.6 could be attributed to the carbonyl carbon of a ketone. In DEPT-135 spectrum of CH_2 were identified as six downward peaks in between δ 48.6-28.3. The mass spectrum of the compound gave molecular ion peak at 381.4175 which is the $(M+Na)^+$ peak. The structure of the compound 16 was confirmed as malabaricone-C [Patro *et al.*, **2005**]

shown below by comparing the IR, ^{1}H NMR, ^{13}C NMR, UV and DEPT-135 spectral details with those reported in literature. The structure of the compound is shown below.



16

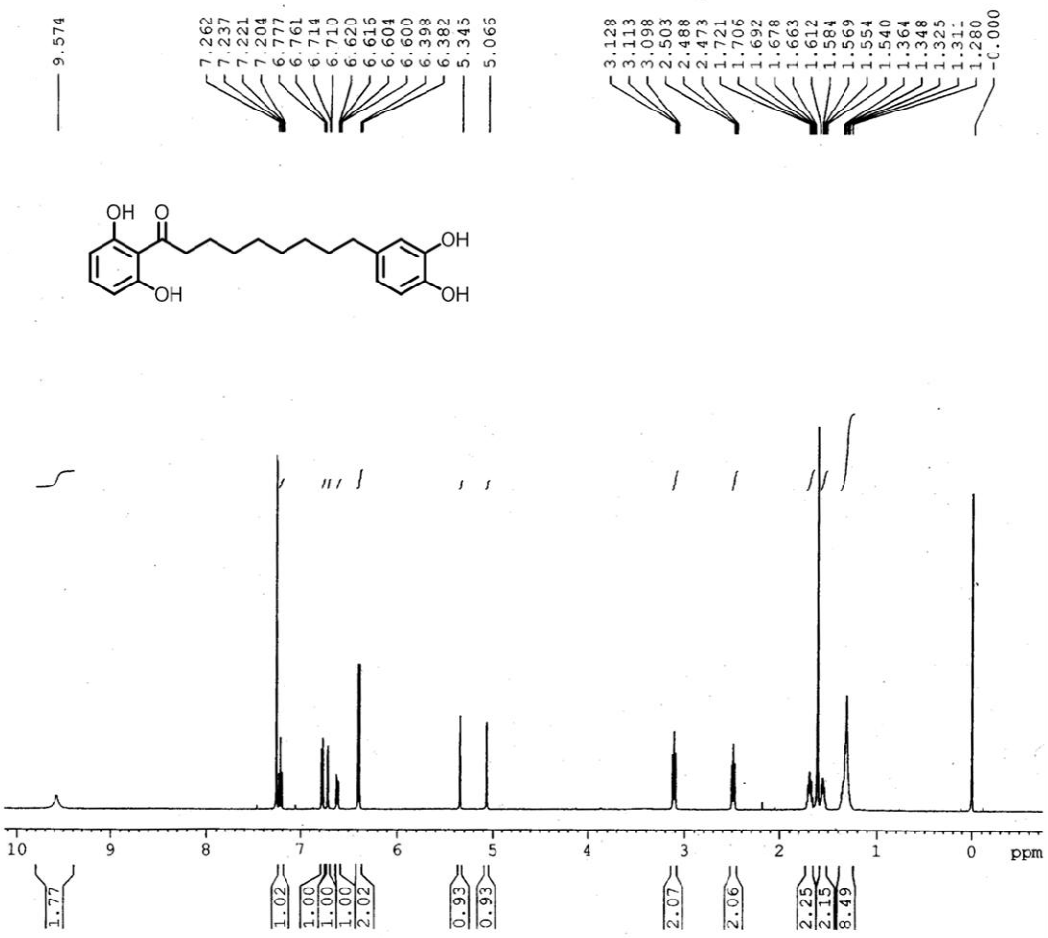


Figure 2a.25: ^{1}H NMR spectrum of Malabaricone C

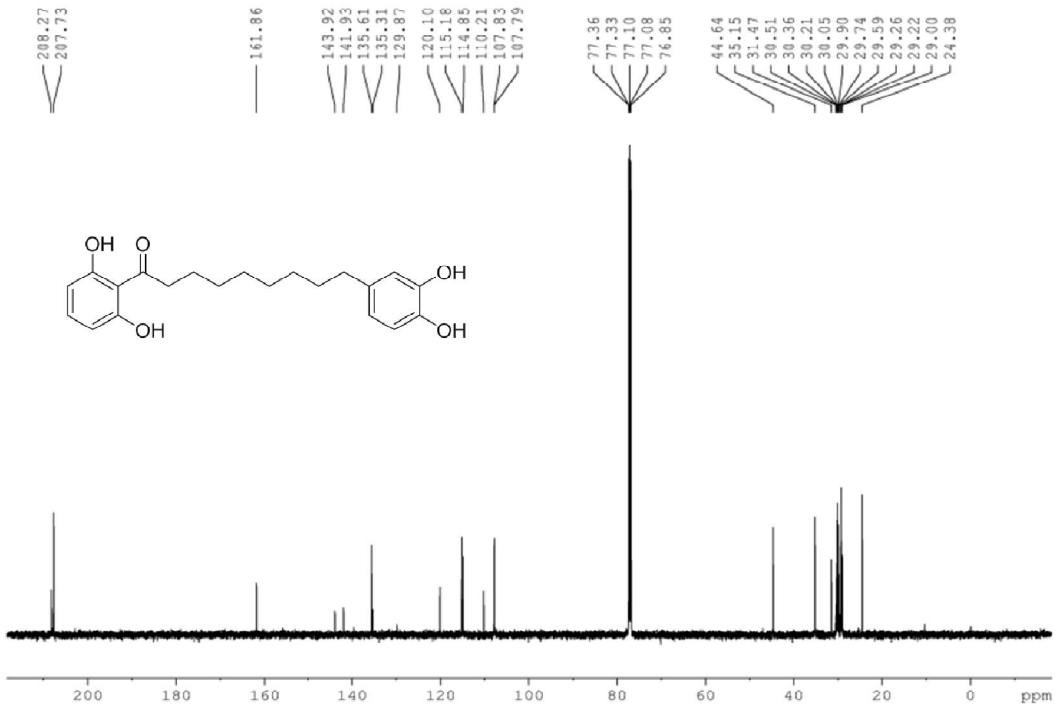


Figure 2a.26 : ^{13}C NMR spectrum of Malabaricone C

2a.1.2.3. Experimental

The seed of *Myristica fragrans* (1 kg) were collected from North Paravur, Kerala. The seed covering was separated from the seed kernel. The seed kernel obtained (750 g) was dried in a drier maintained at 50^{o}C and powdered. The powdered seed kernel was subjected to extraction with 3 L of petroleum ether at room temperature for 3 times in order to remove the fat content. After removing the fat content, it was subjected to extraction with acetone (2.5 L X 3) at room temperature. After extraction, the solvent was removed under reduced pressure using Büchi rotary evaporator. The crude extract (43.14 g) thus obtained was then subjected to column chromatographic separation.

2a.1.2.3a. Chromatographic separation of the acetone extract

The extract of the seed kernel of *M. fragrans* was dissolved in minimum quantity of ethyl acetate and loaded on the top of silica gel column filled with a slurry of 100-200 mesh silica gel in petroleum ether. The column was eluted successively with gradient mixtures of petroleum ether and ethyl acetate of increasing polarities and finally with

pure ethyl acetate. A total of 86 fractions of 150 ml each were collected and they were pooled in to 11 fraction pools according to the similarities in TLC. A pictorial representation of the procedure for the isolation of the compound is shown in the **Figure 2a.27**.

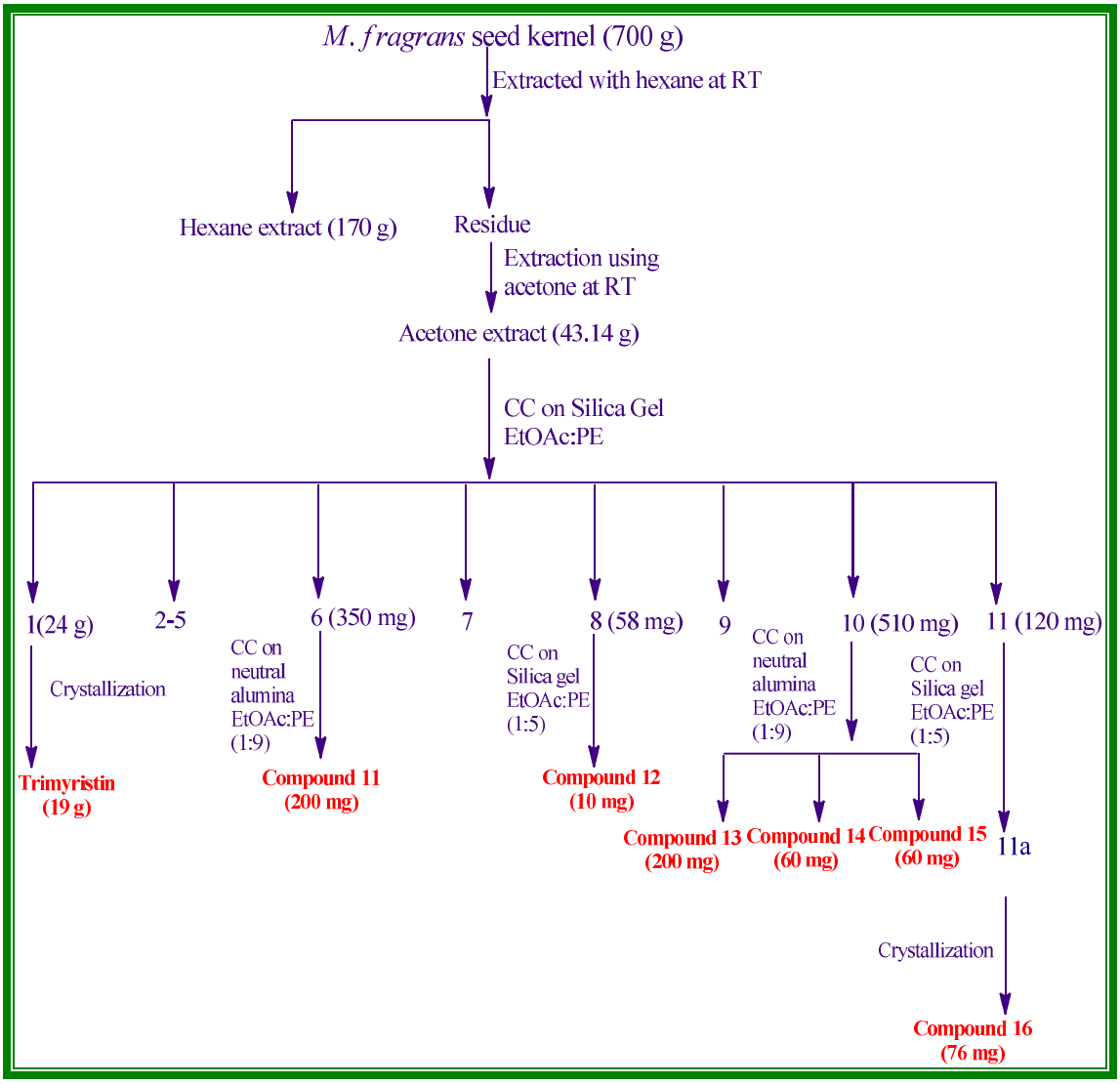


Figure 2a.27 : Pictorial representation of isolation of chemical compounds from *M. fragrans* seed kernel

2a.1.2.3b. Isolation of compound 11

Compound 11 was isolated from the fraction pool 6 as shown in **Figure. 2a.27**. Compound 11 was obtained as a white solid. Spectral data in detail confirmed that the compound 11 is similar to compound 6 (licarin A) obtained earlier.

2a.1.2.3c. Isolation of compound 12

Compound 12 was obtained as white solid by the purification of fraction pool 8 using column chromatography on silica gel, eluting with 20% ethyl acetate in petroleum ether. The compound was confirmed to be guaiacin by the spectral details of the compound 7, which was isolated from *M. fragrans* fruit pericarp.

2a.1.2.3d. Isolation of compound 13

Fraction pool 10 was subjected to column chromatography on neutral alumina eluting with 10% ethyl acetate in petroleum ether to yield three compounds. Compound 13, 14 and 15 were obtained as colourless liquids. Compound 13 is confirmed as *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-acetoxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan, isolated from nutmeg pericarp.

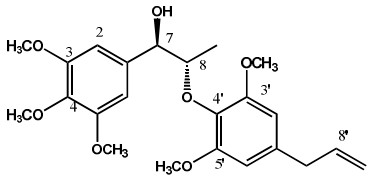
2a.1.2.3e. Isolation of compound 14

After the isolation of compound 13, compound 14 was obtained as colourless liquid on further elution with 20% ethyl acetate in petroleum ether. Compound was confirmed as *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-hydroxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan, isolated earlier.

2a.1.2.3f. Isolation of compound 15

After the isolation of compound 14, compound 15 was obtained as colourless liquid on further elution with 20% ethyl acetate in petroleum ether. Spectral data in detail confirmed that the compound 15 is raphidecursinol B.

FT-IR (NaCl $\nu_{max,}$ cm^{-1})	:	3506 br (-OH), 2938, 2837, 1590,1503, 1228, 1127, 974, 823
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 6.54 (2H, *s*, H-2 & H-6), 6.46 (2H, *s*, H-2' & H-6'), 5.98



(1H, *m*, H-8'), 5.12 (2H, *m*, H-9'), 4.79 (1H, *d*, *J* = 2.5 Hz, H-7), 4.34 (1H, *dq*, J_1 = 2.5 Hz, J_2 = 13 Hz, H-8), 4.15 (1H, *br. s*, -OH), 3.87 (6H, *s*, 2 x -OCH_3), 3.84 (6H, *s*, 2 x -OCH_3), 3.82 (3H, *s*, -OCH_3), 3.38 (2H, *d*, *J* = 6.5 Hz, H-7'), δ 1.12 (3H, *dd*, J_1 = 2.5 Hz, J_2 = 6.5 Hz, H-9)

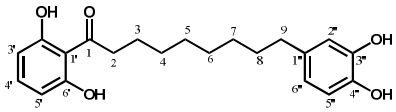
^{13}C NMR (125 MHz,$CDCl_3$) : δ 136.2 (C-1), 102.9 (C-2), 153.1 (C-3), 137.0 (C-4), 153.5 (C-5), 102.9 (C-6), 73.1 (C-7), 82.2 (C-8), 12.8 (C-9), 135.7 (C-1'), 105.5 (C-2'), 153.1 (C-3'), 136.1 (C-4'), 153.5 (C-5'), 105.5 (C-6'), 40.5 (C-7'), 137.0 (C-8'), 116.2 (C-9'), 55.8 (-OCH_3), 55.9 (-OCH_3), 56.1 (2 x -OCH_3), 60.8 (-OCH_3) (C assignments were made as per literature)

HR-ESIMS *m/z* : 441.1885 $[M+Na]^+$ (calcd for $C_{23}H_{30}O_7Na$, 441.1889)

2a.1.2.3g. Isolation of compound 16

Fraction pool 11 was subjected to column chromatography on silica gel, eluting with 50% ethyl acetate in petroleum ether to yield compound 16 as yellow solid. It was characterized as the malabaricone C from the spectral studies. Structure of the compound is shown below.

FT-IR (NaCl $\nu_{max,}$ cm^{-1}) : 3350 (-OH), 2954, 2853, 2358, 1868, 1628, 1594, 1514, 1452, 1346, 1236, 1112



^{1}HNMR(500 MHz, $CDCl_3$) : δ 9.57 (2H, *s*, 2 x -OH), 7.23 (1H, *m*, H-4'), 6.76 (1H, *d*, *J* = 8 Hz, H-5''), 6.71 (1H, *d* , *J* = 2 Hz , H-2''), 6.61 (1H, *dd*, J_1 = 2 Hz, J_2 = 8Hz, H-6''), 6.38 & 6.39 (2H, *d*, *J* = 2 Hz, H-3' & 5'), 5.34 & 5.06 (2H, *s*, 2 x -OH), 3.11 (2H, *t*, *J* = 7.5 Hz, H-2), 2.48 (2H, *t*, *J* = 7.5 Hz, H-9), 1.70 (2H, *m*, H-8), 1.56 (2H, *m*, H-3), 1.32 (8H, *m*, H-4, H-5, H-6 & H-7).

^{13}CNMR (125 MHz, $CDCl_3$) : δ 212.6 (C1), 48.5 (C2), 33.2 (C3 & C7), 32.9 (C4 & C6), 28.3 (C5), 35.4 (C8), 39.0 (C9), 114.1 (C1'), 165.8 (C2' & C6'), 139.6 (C3' & C5'), 33.3 (C4'), 139.1 (C1''), 119.2 (2''), 148.0 (C3''), 145.9 (C4''), 118.8 (C5''), 123.8 (6'') (C assignments were

made as per literature)

HR-ESIMS*m/z* : 381.4175 $[M+Na]^+$ (calcd for $C_{21}H_{26}O_5Na$, 381.4175)

Melting point : 127-128°C

2a.1.3. Isolation from nutmeg mace

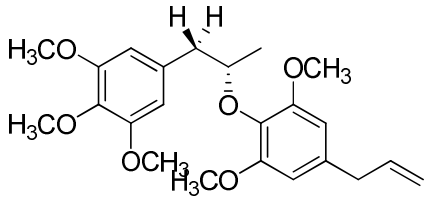
2a.1.3.1. Plant material and extraction

M. fragrans mace (500 g) were collected in December 2010, from North Paravur in Kerala state, India. It was dried in an air oven maintained at 50°C for an hour, and then powdered mechanically. The powdered material was subjected to extraction with acetone (3 L X 3) at room temperature. 120 g of acetone extract was obtained after removing the solvent. The acetone extract (120 g) was fractionated using CC on silica gel (100–200 mesh) by eluting with hexane, followed by 5% ethyl acetate in hexane and finally with acetone. The acetone eluted portion (67.17 g) was subjected to CC on silica gel (100–200 mesh) and eluted with hexane–ethyl acetate mixtures of increasing polarities to give 31 fraction pools.

2a.1.3.2. Isolation and characterization of major compounds

Fraction pool 2 (5.2 g) was subjected to column chromatography on silica gel using hexane–ethyl acetate (20:1) to yield compound 17 (610 mg) as licarin B along with palmitic acid (2 g). Fraction pool 3 (1.7 g) was subjected to column chromatography on silica gel using hexane–ethyl acetate (20:1) to yield compound 18 (554 mg) which was characterized as elemicin. Fraction 10-15 (5.8 g) pooled together according to the similarities in TLC and subjected to column chromatography on neutral alumina using hexane–ethyl acetate (10:1) to yield two sub fraction pools namely 10a and 10b. Sub fraction pool 10a was subjected to column chromatography on neutral alumina using benzene to afford two compounds, namely compound 19 (255 mg) as acuminatin and compound 20 (296 mg) as (*2S,3R*)-7-methoxy-3-methyl-5-((*E*)-prop-1-enyl)-2-(3,4,5 trimethoxyphenyl)-2,3-dihydrobenzofuran. Sub fraction pool 10b was submitted to crystallization in ethyl acetate in hexane mixture to yield compound 21 (1.54 g) as licarin A. Fraction pool 16 (1.4 g) submitted to crystallization in ethyl acetate-hexane mixture yielded the compound 22 (308 mg) as guaiacin. The mother liquor obtained after the

crystallization of guaiacin was submitted to column chromatography on silica gel followed by recycling HPLC yielded compound 23 (57 mg). Signal at δ 1.12 in the ^{1}H NMR (**Figure 2a.28**) spectrum of the compound indicated the presence of a methyl group. Two doublet of doublets at δ 2.74 and 3.13 each having J_2 = 13.5 Hz suggested the presence of two geminal protons. Three singlets at δ 3.79, 3.81 and 3.83 in ^{1}H NMR spectrum, integrating for six, three and six protons respectively indicated the presence of five methoxy groups. Signals at δ 56.0, 56.1, and 60.7 in the ^{13}C NMR (**Figure 2a.29**) spectrum confirmed the presence of five -OMe groups. Peaks in between δ 105.7-153.6 in the ^{13}C spectrum confirmed the presence of olefinic and aromatic carbons. The DEPT-135 spectrum confirmed the presence of three -CH_2- groups. The mass spectrum of the compound gave molecular ion peak at 425.57 which is the $(M+Na)^+$ peak. From all the above spectral data and comparing with those in the literature, compound 23 was identified as the (*8R*)-$\Delta^{8'}$-3,4,5,3', 5'-pentamethoxy-8-*O*-4'-neolignan [Isogai *et al*., **1973**].



23

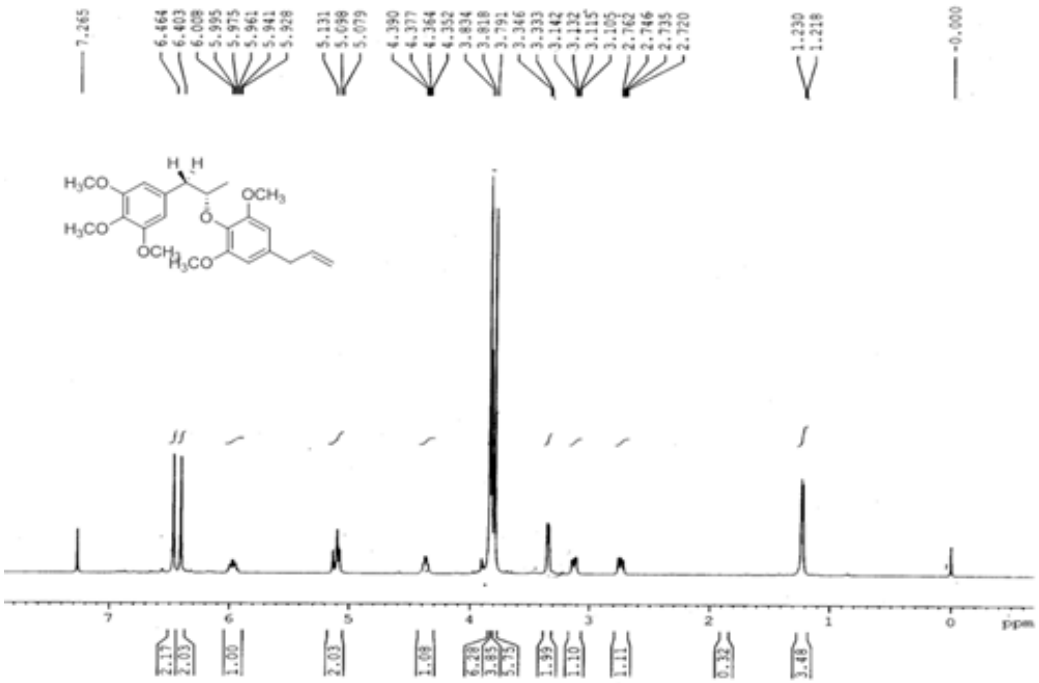


Figure 2a.28 : ^{1}H NMR spectrum of (*8R*)-$\Delta^{8'}$-3,4,5,3', 5'-pentamethoxy-8-*O*-4'-neolignan

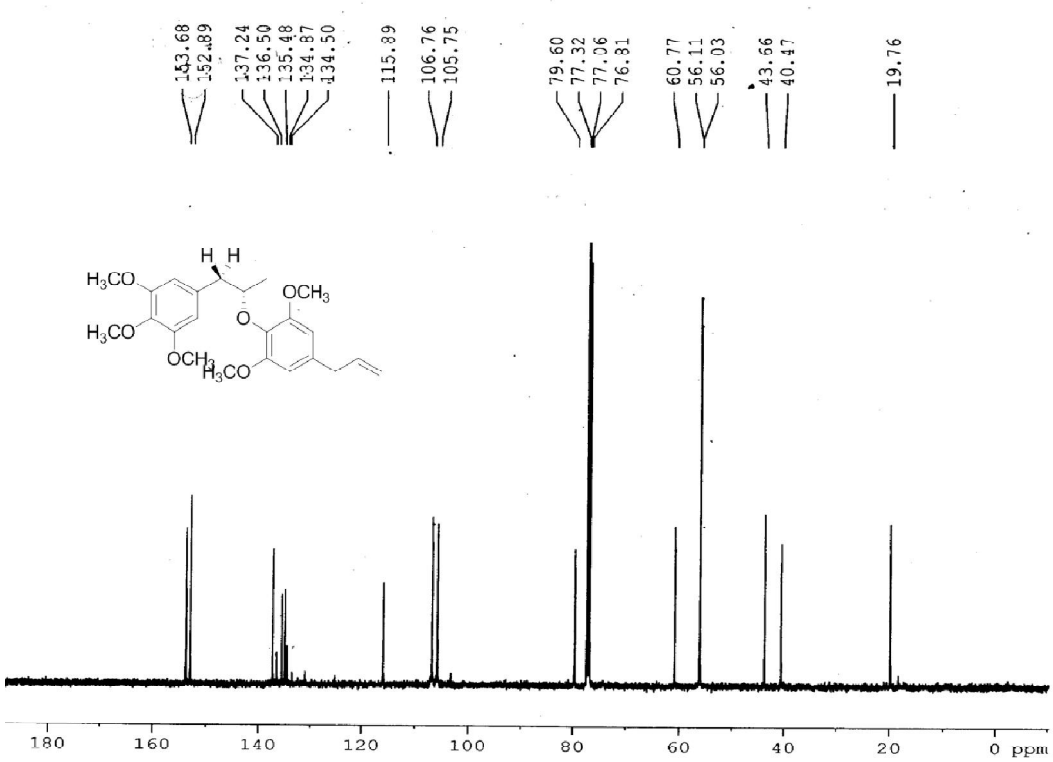
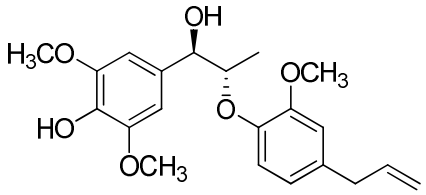


Figure 2a.29 : ^{13}C NMR spectrum of (*8R*)-$\Delta^{8'}$-3,4,5,3', 5'-pentamethoxy-8-*O*-4'-neolignan

Fraction pools 17, 18 and 19 pooled together according to the similarities in TLC which weighed about 8.6 g and subjected to column chromatography on neutral alumina using hexane–ethyl acetate (5:1) to yield two sub fraction pools namely 17a and 17b. Sub fraction pool 17a was subjected to column chromatography on neutral alumina using hexane–ethyl acetate (5:1) to afford three compounds, namely compound 24 (650 mg) as *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-acetoxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan, compound 25 (596 mg) as *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-hydroxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan and compound 26 (200 mg) as raphidecursinol B. Sub fraction pool 17b was subjected to recycling HPLC to yield compound 27 (97 mg). IR spectrum compound 27 showed a broad absorption at 3506 cm^{-1} which indicated the presence of hydroxyl group. Signal at δ 1.11 in the ^{1}H NMR (**Figure 2a.30**) spectrum indicated the presence of a methyl group. A broad singlet at δ 4.12 integrating for one proton indicated the presence of -OH proton. Two singlets at δ 3.86 and 3.88 in ^{1}H NMR spectrum, integrating for six and three protons respectively indicated the presence of three methoxy groups. A doublet at δ 4.79 integrating for one proton and a doublet of quartet at δ 4.34 integrating for one proton having correlation in ^{1}H-^{1}H correlation spectrum with J = 2 Hz indicated that the compound is *erythro*. Signals at δ 55.9 and 56.1 in the ^{13}C NMR (**Figure 2a.31**) spectrum confirmed the presence of three -OMe groups. Peaks in between δ 105.5-153.4 (14C) in the ^{13}C spectrum confirmed the presence of olefinic and aromatic carbons. The DEPT-135 spectrum confirmed the presence of two -CH_2- groups. The mass spectrum of the compound gave molecular ion peak at m/z 397.91 which is the $(M+Na)^{+}$ peak. From all the above spectral data and on comparison with those in the literature, compound 20 was identified as the *erythro*-(*7S,8R*)-$\Delta^{8'}$-4,7-dihydroxy-3,5,3'-trimethoxy-8-*O*-4'-neolignan [Isogai *et al.*, **1973**].



20

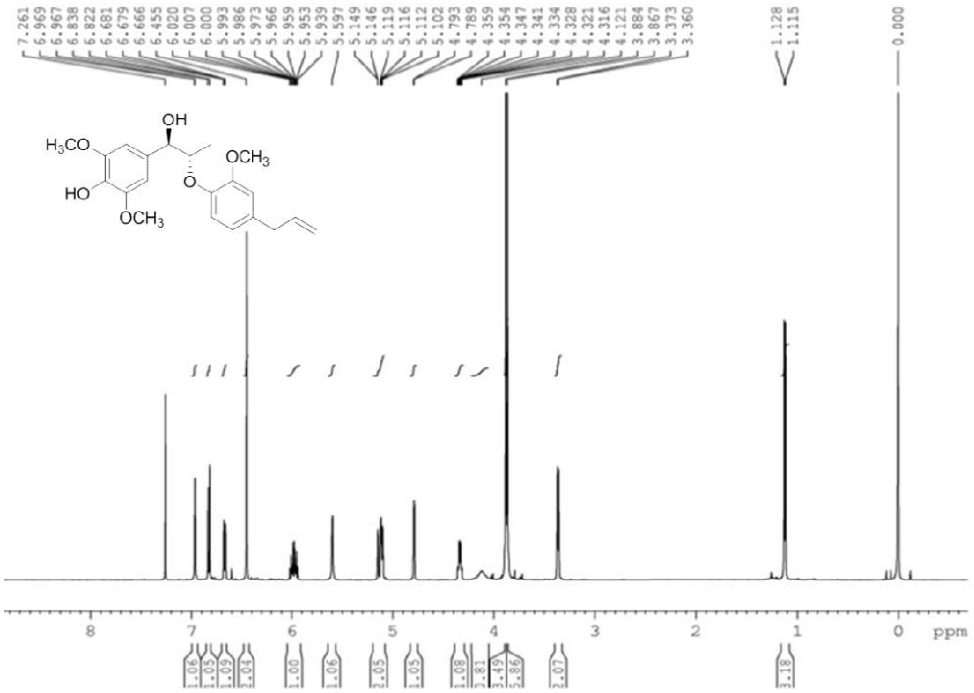


Figure 2a.30 : [1]H NMR spectrum of *Erythro*-(*7S,8R*)-$\Delta^{8'}$-4,7-dihydroxy-3,5,3'-trimethoxy-8-*O*-4'-neolignan

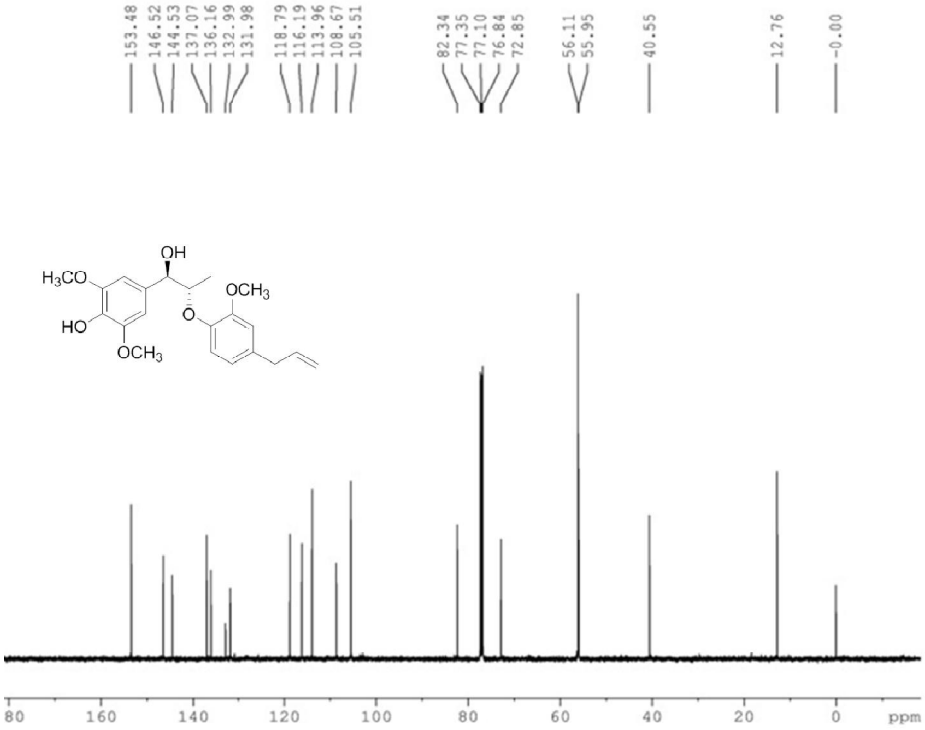


Figure 2a.31: [13] C NMR spectrum of *Erythro*-(*7S,8R*)-$\Delta^{8'}$-4,7-dihydroxy-3,5,3'-trimethoxy-8-*O*-4'-neolignan

Fraction pool 23 (50 mg) was submitted to column chromatography on silica gel eluting with hexane–ethyl acetate (1:1) afforded compound 28 (6 mg) as *erythro* surinamensin.

2a.1.3.3. Experimental

The mace of *Myristica fragrans* (500 g) were collected from North Paravur, Kerala. The material was dried at 50^{o}C and powdered. The powdered mace was subjected to extraction with 3 L of acetone at room temperature for 3 times. After extraction, the solvent was removed under reduced pressure using Büchi rotary evaporator. The crude extract (120 g) thus obtained was then fractionated using column chromatography on silica gel eluting with hexane followed by 5% ethyl acetate in petroleum ether in order to remove the volatile components and fat content. Finally column was eluted with acetone. Acetone eluted part (67.17 g) was subjected to column chromatography on silica gel. The column was eluted successively with gradient mixtures of petroleum ether and ethyl acetate of increasing polarity and finally with pure ethyl acetate. A total of 147 fractions of 150 ml each were collected and they were pooled in to 31 fraction pools according to the similarities of the TLC. The isolation procedure of compounds is depicted in **Figure 2a.32**.

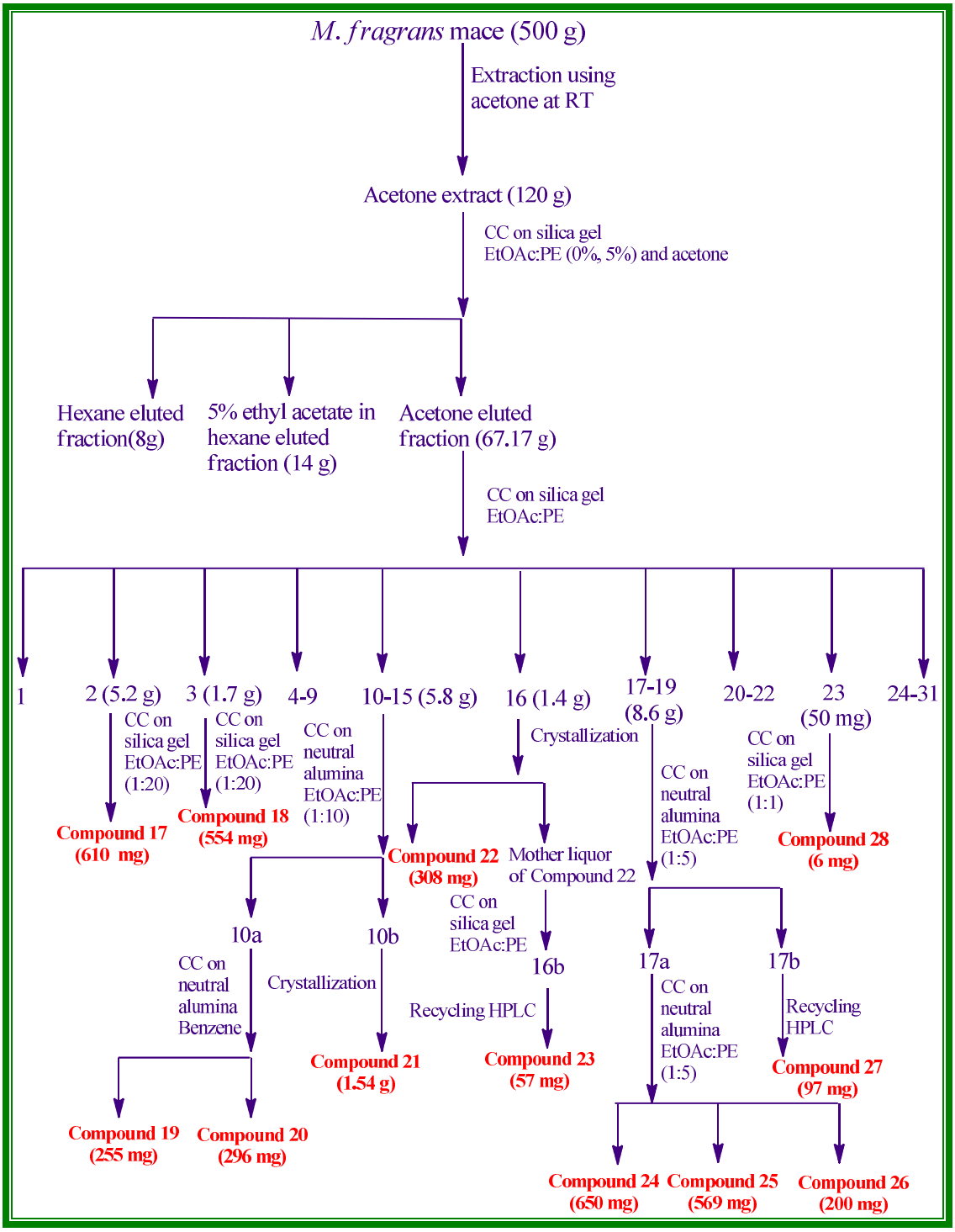


Figure 2a.32 : Pictorial representation of isolation of chemical compounds from *M. fragrans* mace

2a.1.3.3a. Isolation of compound 17

Compound 17 along with palmitic acid was isolated from the mace of *M. fragrans* as represented in figure 2a.32. Compound 17 obtained as colourless crystals by eluting the column with 5% ethyl acetate in petroleum ether. On evaluation of the structure of compound 17 and on comparison with TLC, compound 17 was confirmed as licarin B which was isolated earlier from the fruit pericarp of *M. fragrans* (Compound 1).

2a.1.3.3b.Isolation of compound 18

Figure 2a.32 represents the isolation procedure for compound 18. It is obtained as colourless liquid. Detailed investigation of various spectroscopic data of compound 18 revealed that it was elemicin. Further evidence was made by comparing the TLC with that of compound 2 isolated earlier.

2a.1.3.3c. Isolation of compound 19

Fraction pools 10-15 were combined together and subjected to CC on neutral alumina, eluting with 10% ethyl acetate in petroleum ether yielded two sub fraction pools. The first sub fraction pool (10a) contain two major UV active spots. 10a submitted to CC on neutral alumina eluting with benzene to afford two compounds, namely compound 19 as white solid and compound 20 as colourless crystals. Compound 19 was identified as acuminatin from the spectroscopic details obtained from compound 3.

2a.1.3.3d. Isolation of compound 20

After getting accuminatin, compound 20 was obtained as colourless crystals by further eluting with 10% ethyl acetate in petroleum ether. Compound was confirmed as (*2S,3R*)-7-methoxy-3-methyl-5-((*E*)-prop-1-enyl)-2-(3,4,5trimethoxyphenyl)-2,3-dihydrobenzofuran, isolated earlier.

2a.1.3.3e. Isolation of compound 21

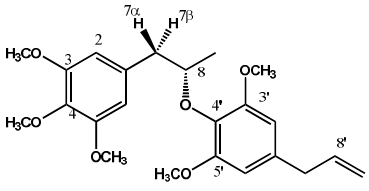
Sub fraction pool 10b was submitted to crystallization in ethyl acetate in hexane mixture to yield the compound 21 as white solid. Spectral data in detail confirmed that compound 21 is similar to the compound 6 (licarin A) obtained earlier.

2a.1.3.3f. Isolation of compound 22

Fraction pool 16 was submitted to crystallization to afford the compound 22 as white solid. The compound was identified as guaiacin which was isolated earlier.

2a.1.3.3g. Isolation of compound 23

Mother liquor obtained after the crystallization of guaiacin was subjected to column chromatography on silica gel by eluting with 50% ethyl acetate in petroleum ether, which on recycling HPLC yielded compound 23 as colourless liquid. It was characterized as the (*8R*)-$\Delta^{8'}$-3,4,5,3', 5'-pentamethoxy-8-*O*-4'-neolignan from the spectral studies. The structure of the compound is shown below.



FT-IR (NaCl $\nu_{max,}$ cm^{-1})	:	2948, 28477, 1586, 1513, 1212, 1129, 975, 830
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 6.46 (2H, *s*, H-2 & H-6), δ 6.40 (2H, *s*, H-2' & H-6'), δ 5.97 (1H, *m*, H-8'), δ 5.09 (2H, *m*, H-9'), δ 4.36 (1H, *dq*, J_1 = 2.5 Hz, J_2 = 13 Hz, H-8), δ 3.83 (6H, s, 2 x -OCH_3), δ 3.81 (3H, *s*, -OCH_3), δ 3.79 (6H, *s*, 2 x -OCH_3), δ 3.33 (2H, *d*, *J* = 6.5 Hz, H-7'), δ 3.13 (1H, *dd*, J_1 = 8 Hz, J_2 = 13.5 Hz, H-7β), δ 2.74 (1H, *dd*, J_1 = 5 Hz, J_2 = 13.5 Hz, H-7α), δ 1.21 (3H, *d*, *J* = 6 Hz, H-9)
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 137.2 (C-1), 105.7 (C-2), 153.6 (C-3), 136.5 (C-4), 152.8 (C-5), 105.7 (C-6), 43.6 (C-7), 79.6 (C-8), 19.7 (C-9), 135.4 (C-1'), 106.7 (C-2'), 153.7 (C-3'), 136.1 (C-4'),

153.7 (C-5'), 106.7 (C-6'), 40.4 (C-7'), 137.2 (C-8'), 115.8 (C-9'), 56.0 (2 x -OCH_3), 56.1 (2 x -OCH_3), 60.8 (-OCH_3) (C assignments were made as per literature)

FAB MS *m/z* : 425.57 $[M+Na]^+$ (calcd for $C_{23}H_{30}O_6Na$, 425.19)

2a.1.3.3h. Isolation of compound 24

Fraction pools 17, 18 and 19 pooled together according to the similarities in TLC and subjected to column chromatography on neutral alumina using 20% ethyl acetate in hexane to yield two sub fraction pools namely 17a and 17b. 17a was subjected to column chromatography on neutral alumina using 20% ethyl acetate in hexane to afford three compounds, namely compound 24 and 25 are colourless oils and compound 28 as colourless viscous liquid. Compound 24 was identified as *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-acetoxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan. It was isolated from nutmeg pericarp.

2a.1.3.3i. Isolation of compound 25

After getting compound 24, compound 25 was obtained as colourless oil further elution with 20% ethyl acetate in petroleum ether. Compound was confirmed as, *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-hydroxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan isolated earlier.

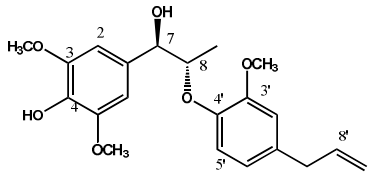
2a.1.3.3j. Isolation of compound 26

After getting compound 25, compound 26 was obtained as colourless viscous liquid further elution with 20% ethyl acetate in petroleum ether. Compound was confirmed as raphidecursinol B, isolated earlier.

2a.1.3.3k. Isolation of compound 27

Sub fraction pool 17b was subjected to recycling HPLC to yield compound 27 as colourless viscous liquid. The spectral studies of the compound 27 matched with *erythro*-(*7S,8R*)-$\Delta^{8'}$-4,7-dihydroxy-3,5,3'-trimethoxy-8-*O*-4'-neolignan.

FT-IR (NaCl : 3506 br (-OH), 2937, 2840,



$\nu_{max,}$ cm^{-1})		1587, 1497, 1273, 1121, 1034
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 6.96 (1H, *d*, *J* = 1 Hz, H-2'), 6.83 (1H, *d*, *J* = 1 Hz, H-5'), 6.68 (1H, *d*, *J* = 8Hz, H-6'), 6.45 (2H, *s*, H-2 & H-6), 5.98 (1H, *m*, H-8'), 5.59 (1H, *s*, -OH at position 4), 5.11 (2H, *m*, H-9'), 4.79 (1H, *d*, *J* = 2 Hz, H-7), 4.34 (1H, *dq*, J_1 = 2 Hz, J_2 = 12.5 Hz, H-8), 4.12 (1H, *br. s*, -OH at position 7), 3.88 (3H, *s*, -OCH_3), δ 3.86 (6H, *s*, 2 x -OCH_3), 3.37 (2H, *d*, *J* = 6.5 Hz, H-7'), 1.12 (3H, *d*, *J* = 5 Hz)
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 136.1 (C-1), 105.5 (C-2), 153.4 (C-3), 132.9 (C-4), 153.4 (C-5), 105.5 (C-6), 72.8 (C-7), 82.3 (C-8), 12.7 (C-9), 131.9 (C-1'), 113.9 (C-2'), 146.5 (C-3'), 144.5 (C-4'), 108.6 (C-5'), 118.7 (C-6'), 40.5 (C-7'), 137.0 (C-8'), 116.1 (C-9'), 55.9 (2 x -OCH_3), 56.1 (-OCH_3) (C assignments were made as per literature)
HR-ESIMS *m/z*	:	397.1625 $[M+Na]^+$ (calcd for $C_{21}H_{26}O_6Na$, 397.1627)

2a.1.3.3l. Isolation of compound 28

Fraction pool 23 was submitted to column chromatography on silica gel eluting with 50% ethyl acetate in hexane to afford compound 28 as colourless liquid. The compound was identified as *erythro* surinamensin, which was isolated earlier.

2a.2. Conclusion

In this chapter, the isolation of chemical constituents from various parts of *M. fragrans* fruit has been carried out. Ten compounds have been isolated from the fruit pericarp of *M. fragrans*, viz., acuminatin, licarin A, elemicin, 7-methoxy-3-methyl-5-((*E*)-prop-1-enyl)-2-(3,4,5-trimethoxyphenyl)-2,3-dihydrobenzofuran, licarin B, guaiacin, *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-acetoxy- 3,4,3',5'- tetramethoxy-8-*O*-4'-neolignan, *erythro*-(*7S,8R*) -$\Delta^{8'}$-7-hydroxy -3,4,3',5'- tetramethoxy- 8-*O*-4'-neolignan, virolane and surinamensin. Of these, the latter two are being isolated for the first time from *M. fragrans*. Similarly, *M. fragrans* mace and seed were subjected to phytochemical investigations. Twelve known compounds viz., licarin B, elemicin, acuminatin, (*2S,3R*)-7-methoxy-3-methyl-5-((*E*)-prop-1-enyl)-2-(3,4,5-trimethoxyphenyl)-2,3-dihydrobenzofuran, licarin A, guaiacin, (*8R*)-$\Delta^{8'}$-3,4,5,3',5'-pentamethoxy-8-*O*-4'-neolignan, *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-acetoxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan, *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-hydroxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan, raphidecursinol B, *erythro*-(*7S,8R*)-$\Delta^{8'}$-4,7-dihydroxy-3,5,3'-trimethoxy-8-*O*-4'-neolignan, *erythro* surinamensin were obtained from mace. Seven compounds namely, trimyristin, licarin A, guaiacin, *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-acetoxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan, *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-hydroxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan, raphidecursinol B and malabaricone C were isolated from seed kernels.

CHAPTER 2B

Phytochemical Investigation on Stem Bark of *Myristica fragrans*

As mentioned in the Part A of chapter 2, phytochemistry of *M. fragrans* stem bark has not been explored anywhere. So in this chapter we are describing the isolation of chemical constituents from stem bark.

2b.1. Isolation and characterization of compounds from stem bark of *M. fragrans*.

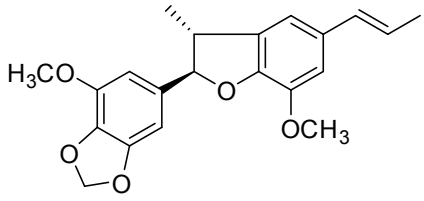
2b.1.1. Plant material and extraction

M. fragrans bark were collected in April 2013, from Ernakulam in Kerala state, India. 2 kg of dry and powdered *M. fragrans* bark was extracted with dichloromethane at room temperature, which yielded 20 g of crude extract. It was subjected to CC on silica gel (100-200 mesh) and eluted with hexane-ethyl acetate mixtures of increasing polarities to give 15 fraction pools.

2b.1.2. Isolation and characterization of major compounds

Fraction pool 2 (485 mg) subjected to CC on neutral alumina using hexane-ethyl acetate (9:1) as eluent followed by crystallization in ethyl acetate-hexane afforded compound 29 (111 mg) which was confirmed to be licarin B, earlier isolated from *M. fragrans* fruit pericarp along with palmitic acid (90 mg). Fraction 3 (1.1 g) submitted to CC on silica gel by eluting with hexane-ethyl acetate (7:1), afforded compound 30 (30 mg) which was characterized as elemicin, isolated earlier, along with compound 31 (650 mg). The ^{1}H NMR (**Figure 2b.1**) spectrum of compound 31 showed the presence of two methyl groups, resonated at δ 1.37 and 1.85. Singlet at δ 3.89 integrating for six protons suggested the presence of two methoxy groups. A sharp singlet at δ 5.95, integrating for two protons indicated the presence of a methylenedioxy group. A doublet of doublet at δ 6.33 and a multiplet at δ 6.11 each integrating for one proton each with a *J* value of 15.5

Hz could be attributed to *trans* olefinic protons. Signal at δ 55.9 and 56.6 in the ^{13}C NMR spectrum confirmed the presence of two -OMe groups. The ^{13}C NMR (**Figure 2b.2**) spectrum confirmed the presence of olefinic and aromatic carbons, resonated between δ 100.6-148.9 (14C). The DEPT-135 spectrum confirmed the presence of methylenedioxy group and seven quaternary carbons. The mass spectrum of the compound gave molecular ion peak at 377.1365 which is the $(M+Na)^+$ peak. Putting these spectral data together and comparing with the data of compounds found in literature, the structure of compound 31 could be identified as (*2S,3R*)-7-methoxy-3-methyl-5-((*E*)-prop-1-enyl)-2-(5-methoxy,3,4-methylenedioxyphenyl)-2,3-dihydrobenzofuran [Dean *et al.*, **1982**] shown below.



31

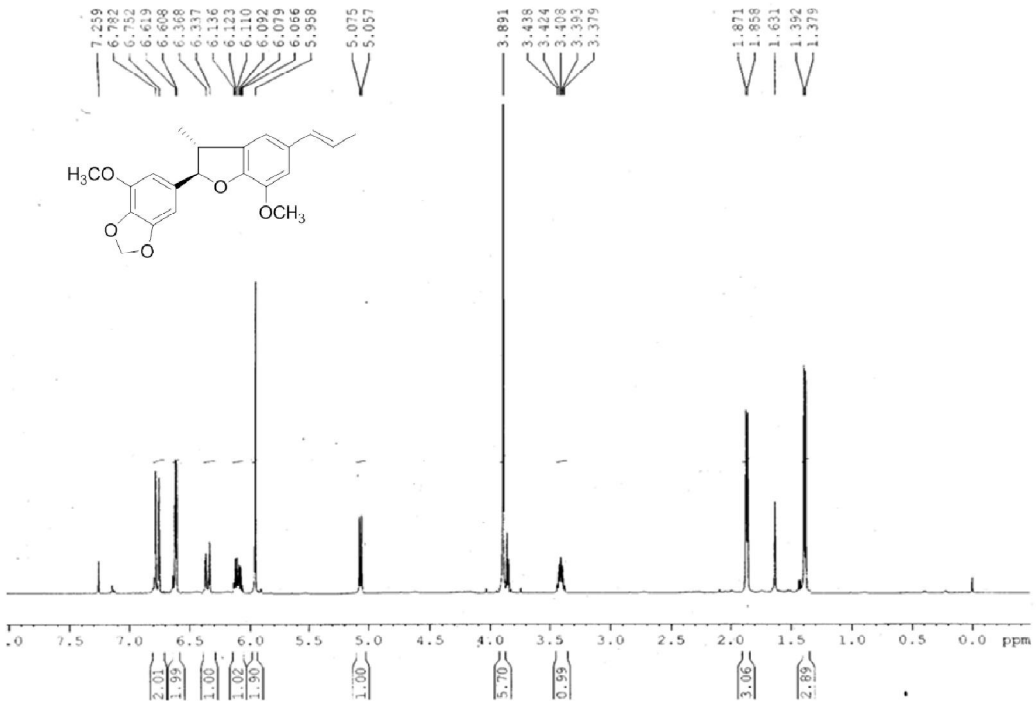


Figure 2b.1: ^1H NMR spectrum of (*2S, 3R*)-7-methoxy-3-methyl-5-((*E*)-prop-1-enyl)-2-(5-methoxy,3,4-methylenedioxyphenyl)-2,3-dihydrobenzofuran

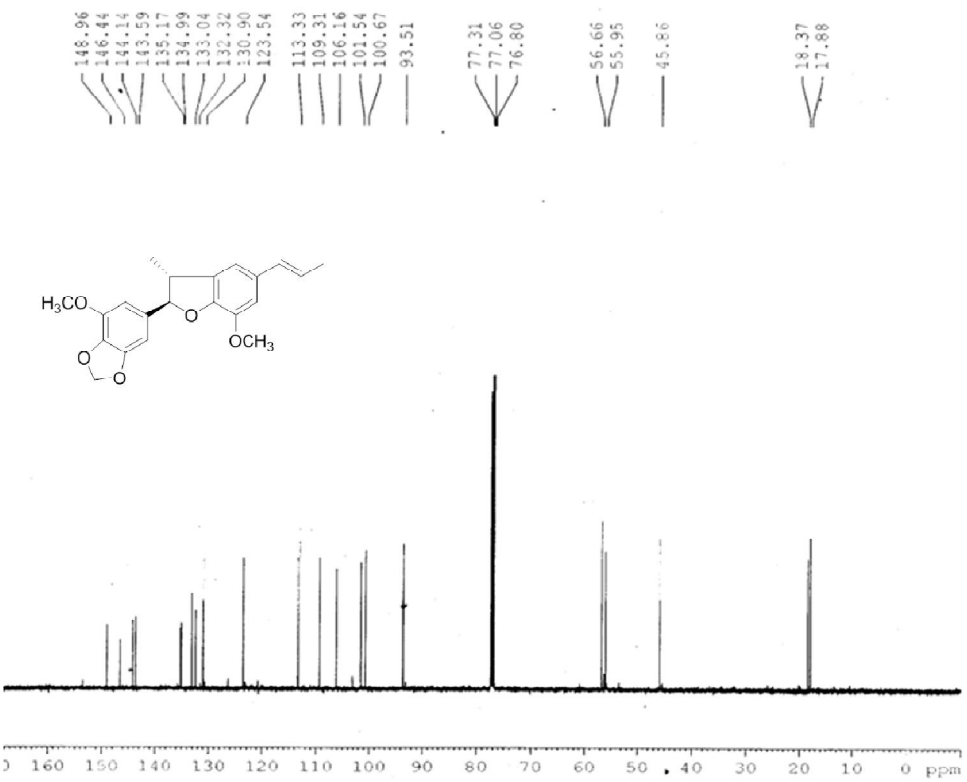
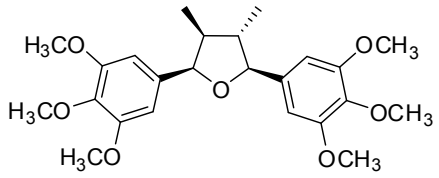


Figure 2b.2: ^{13}C NMR spectrum of (*2S, 3R*)-7-methoxy-3-methyl-5-((*E*)-prop-1-enyl)-2-(5-methoxy,3,4-methylenedioxyphenyl)-2,3-dihydrobenzofuran

Fraction pool 4 (950 mg) submitted to CC on silica gel by eluting with hexane-ethyl acetate (7:1) afforded compound 32 (50 mg) as licarin A and *β*-sitosterol. Fractions 7 and 8 were pooled together (1.2 g) and submitted to CC on silica gel by eluting with hexane-ethyl acetate (5:1), yielded three sub fractions namely 8a, 8b and 8c. Fraction 8a was subjected to CC on silica gel to afford compound 33 (470 mg) as raphidecursinol B. Fraction 8b submitted to CC on neutral alumina yielded compound 34 (78 mg), compound 35 (12 mg) and compound 36 (50 mg) as surinamensin.

Molecular formula of compound 34 was found to be $C_{24}H_{32}O_7$ from HRMS (455.2049 $[M+Na]^+$). The ^{1}H-NMR (**Figure 2b.3**) spectrum showed the presence of two sec-methyls (1.09, d), six methoxy groups (3.84, 6H s, 3.86, 3H, s and 3.89, 9H, s), two methines (1.80 m), two highly deshielded benzylic methines (4.66, d, J = 8.5 Hz) and four magnetically equivalent aromatic protons (6.63, s). The protons at δ 1.80 showed a correlation with protons appearing at δ 1.09 in ^{1}H-^{1}H correlation spectrum and

also with δ 4.65 which indicated that the compound 34 contains a tetrahydrofuran moiety. The chemical shifts of the methyl, methine and benzylic methine protons indicated that 34 has the same stereochemistry as the known compound galbelgin, a type of tetrahydrofuran lignan [Birch *et al*, **1958**]. The ^{13}C NMR (**Figure 2b.4**) and DEPT spectra suggested the presence of 24 carbons, which include eight methyl, eight methine, and eight quaternary carbons. Among them, the signals at δ 56.0, 56.1 and 60.8 could be attributed to the methoxy carbons. The structure was thus arrived at as grandisin first isolated from *Litsea grandis* [David *et al.*, **1974**] on comparison with spectral data reported in literature.



34

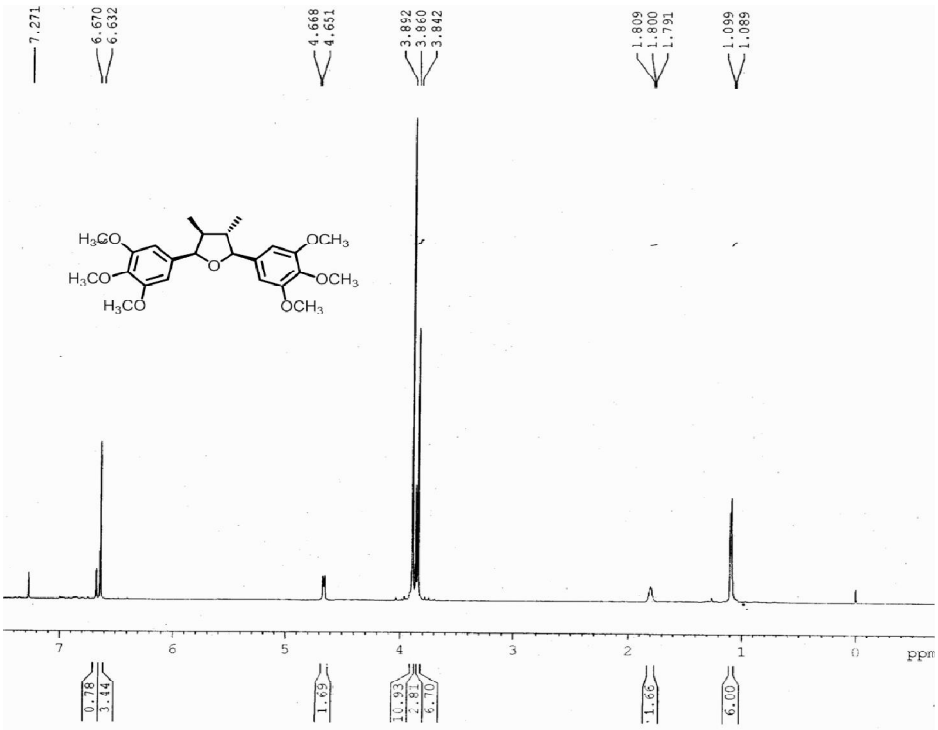


Figure 2b.3 : ^{1}H NMR spectrum of Grandisin

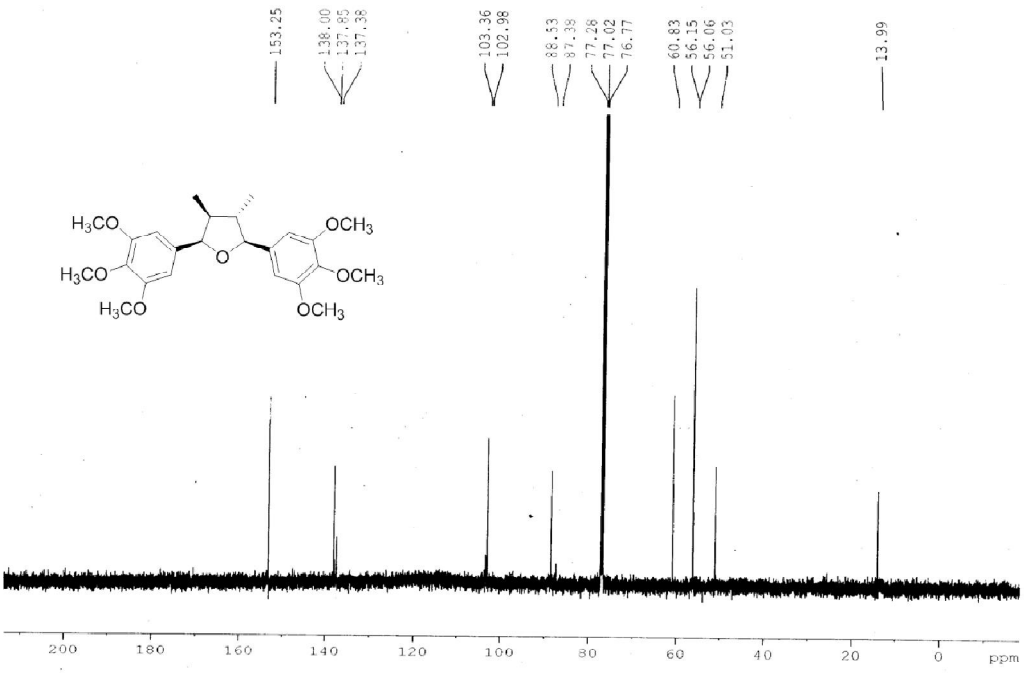
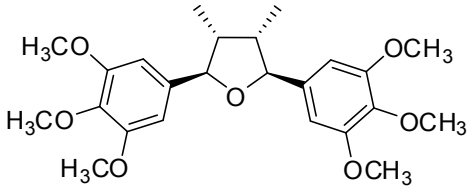


Figure 2b.4: ^{13}C NMR spectrum of Grandisin

Molecular formula of the compound 35 **(**$C_{24}H_{32}O$) (a stereoisomeric form of 34) was established from HRMS (455.2049 $[M+Na]^+$). The ^{1}H NMR (**Figure 2b.5**) spectrum suggested the presence of magnetically equivalent protons in the molecule, as in 34; signals due to two sec-methyls (δ 1.09), two methines (δ 2.36, m), two highly deshielded benzylic methines (δ 4.54, d, J = 6 Hz), six methoxy groups (δ 3.85, 6H, s, δ 3.86, 9H, s and δ 3.90, 3H, s) and four aromatic protons (δ 6.64) were seen. The protons at δ 2.36 showed correlations with protons appearing at δ 1.09 and δ 4.54 in ^{1}H-^{1}H correlation spectrum which indicated that the compound 35 is a tetrahydrofuran system. The signals due to the methyl, methine and benzylic methine protons were essentially similar in chemical shifts and coupling constants to those of a galgravin-type lignan [Blears *et al*, **1968**]. The ^{13}C NMR (**Figure 2b.6**) and DEPT spectra suggested the presence of 24 carbons, which include eight methyl, eight methine, and eight quaternary carbons. Among them, the signals at δ 56.0 and 60.8 could be attributed to the methoxy carbons. Thus, the structure of the compound was confirmed as (*7S,8S,7'R,8'R*)-3,3',4,4',5,5'-

hexamethoxy-7,7', 8,8'-lignan first reported from *Bonamia spectadills* [Kraft *et al.*, **2002**] by comparing with the reported data.



35

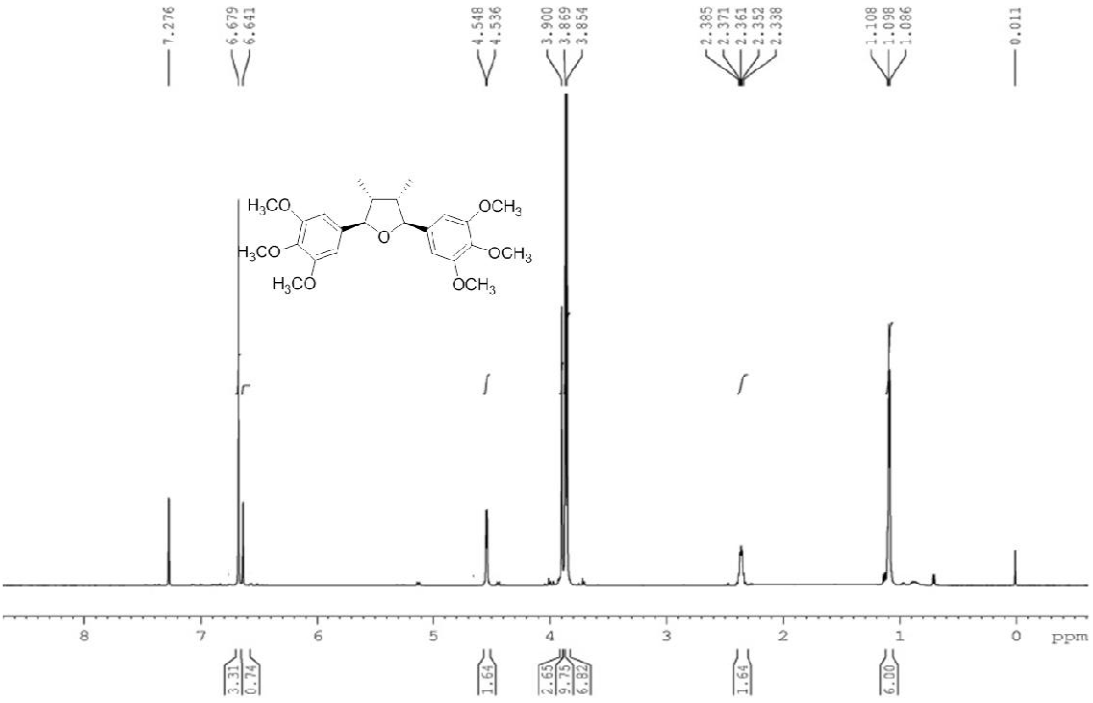


Figure 2b.5: ^{1}H NMR spectrum of (*7S,8S,7'R,8'R*)-3,3',4,4',5,5'-hexamethoxy-7,7', 8,8'-lignan

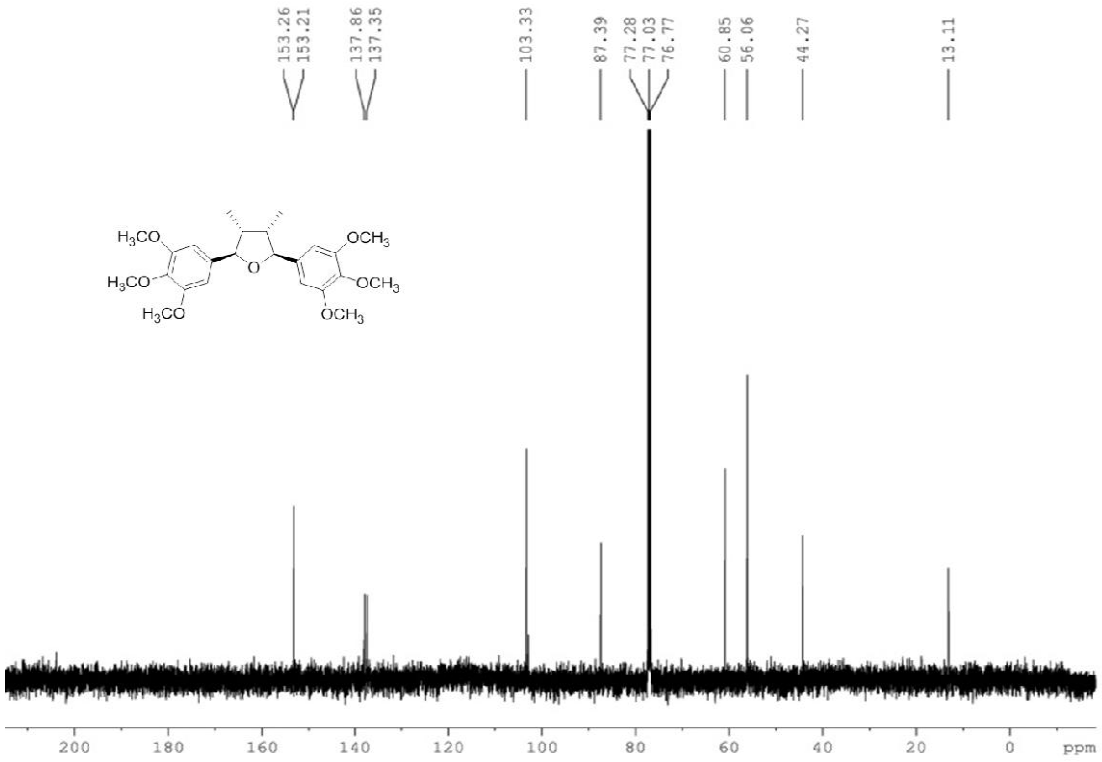
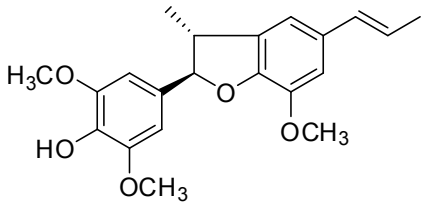


Figure 2b.6: ^{13}C NMR spectrum of (*7S,8S,7'R,8'R*)-3,3',4,4',5,5'-hexamethoxy-7,7', 8,8'-lignan

Fraction 8c was subjected to flash CC by eluting with hexane-ethyl acetate to yield compound 37 (200 mg) and compound 38 (60 mg) *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-hydroxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan

Compound 37, obtained as colourless crystals was analysed using various spectroscopic data. IR spectrum showed a broad absorption at 3432 cm^{-1}, which indicated the presence of hydroxyl group. Signals at δ 1.39 and 1.87 in the ^{1}H NMR (**Figure 2b.7**) spectrum indicated the presence of two methyl groups. Two singlets at δ 3.89 and 3.90 in the ^{1}H NMR spectrum integrating for six and three protons indicated the presence of three methoxy group. A doublet of doublet at δ 6.35 and a multiplet at δ 6.10 each integrating for one proton with *J* value of 16 Hz could be attributed to the *trans* olefinic protons. A sharp singlet at δ 5.58 integrating for one proton confirmed the presence of a phenolic -OH group. Signals at δ 55.9 and 56.3 in the ^{13}C NMR (**Figure 2b. 8**) spectrum

confirmed the presence of three -OMe groups. Peaks in between δ 103.4-147.0 (14C) in the ^{13}C NMR spectrum confirmed the presence of olefinic and aromatic carbons. The mass spectrum of the compound gave molecular ion peak at 357.1708 which is the $(M+H)^+$ peak. From all the above spectral data and comparing with those in the literature, compound 37 was identified as odoratisol A [Giang *et al.*, **2006**]. The structure of compound 37 was further confirmed from its single crystal X-ray structure (**Figure 2b.9**).



37

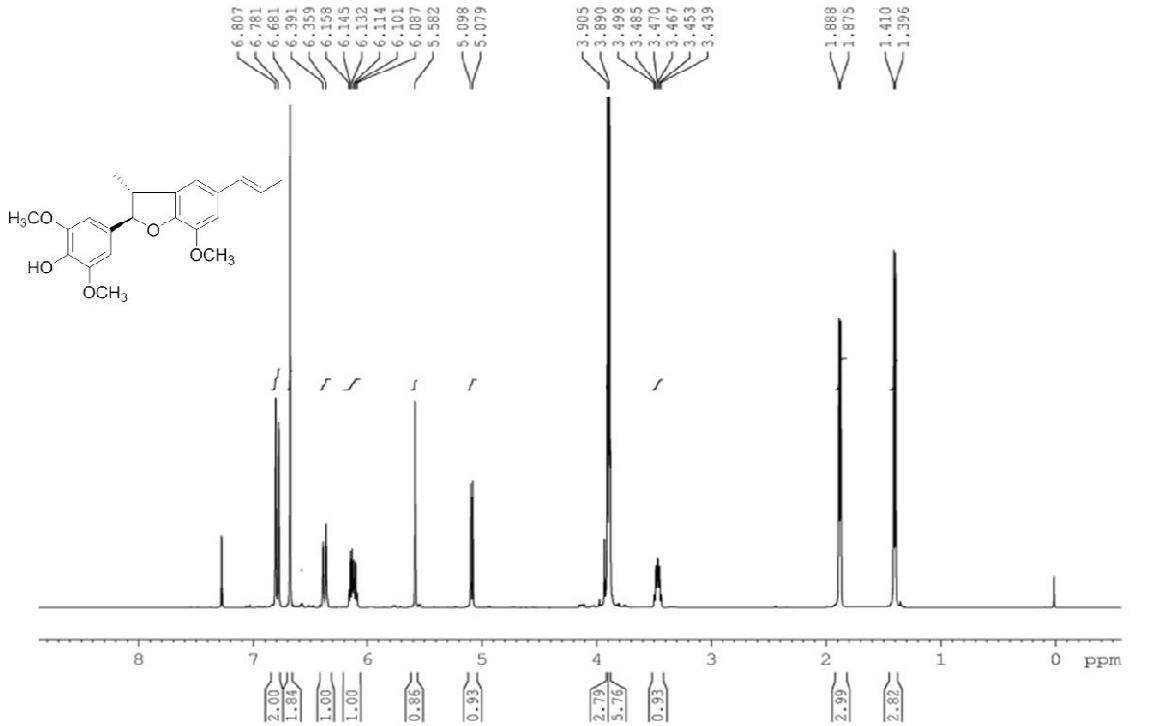


Figure 2b.7: ^1H NMR spectrum of Odoratisol A

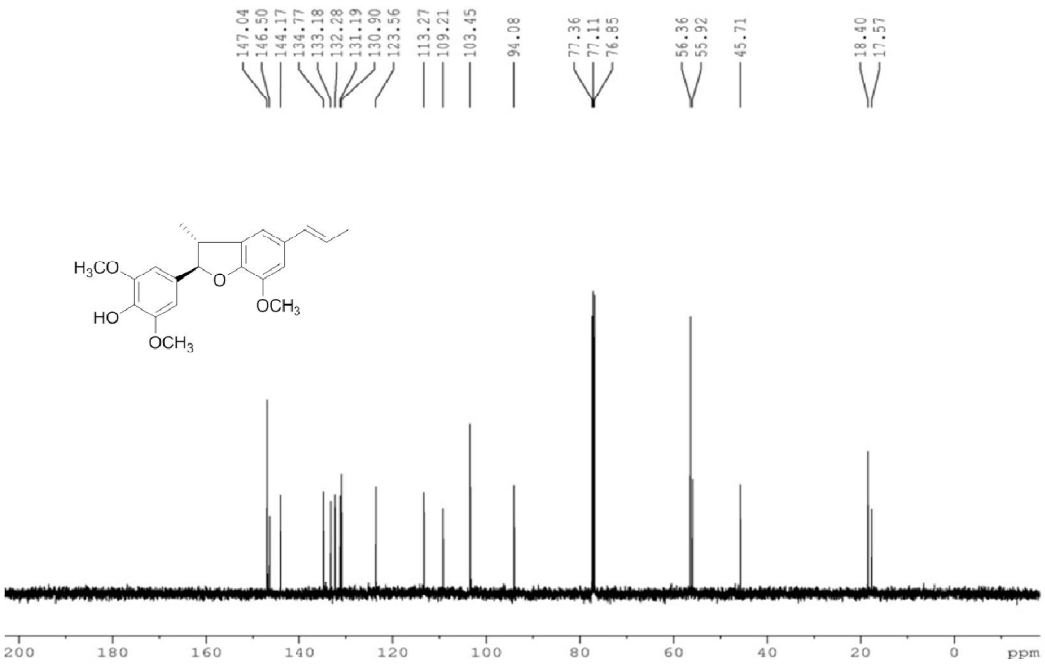


Figure 2b.8: ^{13}C NMR spectrum of Odoratisol A

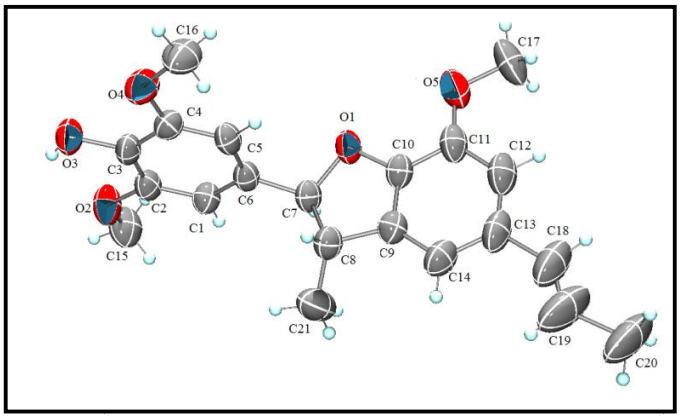
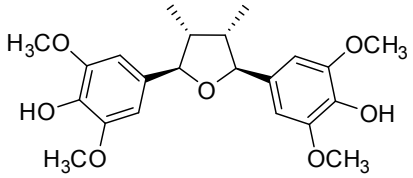


Figure 2b.9: ORTEP diagram of Odoratisol A

Fraction 9 (45 mg) submitted to flash CC by eluting with chloroform-ethyl acetate (20:1), yielded compound 39 (50 mg). IR spectrum of compound 39 showed a broad absorption at 3436 cm^{-1} which indicated the presence of hydroxyl group. The ^{1}H NMR (**Figure 2b.10**) spectrum suggested the presence of a magnetically equivalent element in

the molecule. Signals due to two sec-methyls (δ 1.05), two methines (δ 2.33, m), two highly deshielded methines (δ 4.50, d, J = 6 Hz), four methoxyls (δ 3.87, 12H, s), two hydroxyls (δ 5.55, 2H, br. s.), and four aromatic protons (δ 6.67) were seen. The protons at δ 2.36 showed correlations with protons appearing at δ 1.05 and δ 4.50 in ^{1}H-^{1}H correlation spectrum, which indicated that the compound 39 contains a tetrahydrofuran moiety. The ^{13}C NMR (**Figure 2b.11**) and DEPT-135 spectra suggested the presence of 22 carbons, which include six methyl, eight methine, and eight quaternary carbons. Among them, the signal at δ 56.2 could be attributed to the methoxy carbons. The mass spectrum of the compound gave molecular ion peak at 427.1736 which is the $(M+Na)^{+}$ peak. Thus the structure of the compound was confirmed as fragransin B_1 [Hattori *et al*., **1987**] by comparing with the reported data.



39

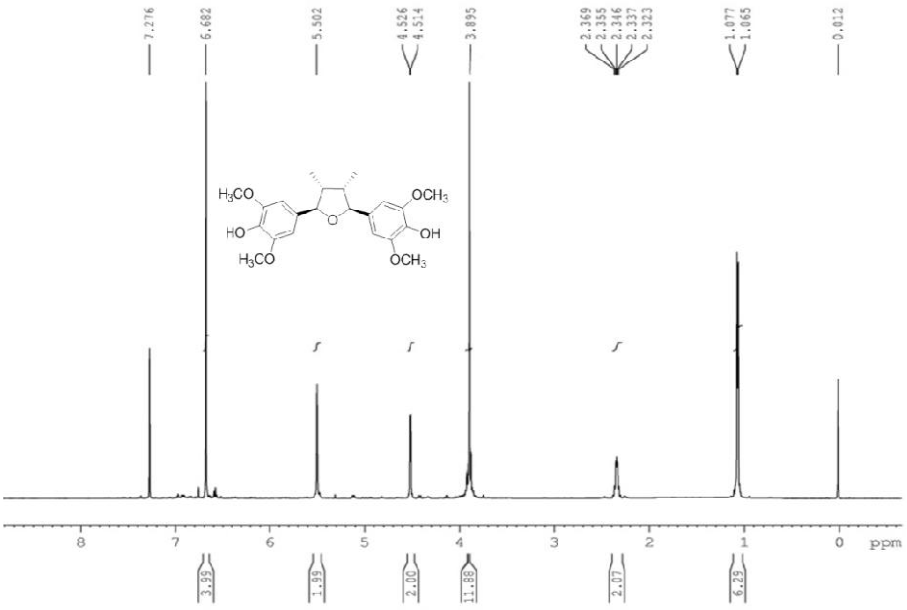


Figure 2b.10: ^{1}H NMR spectrum of Fragransin B_1

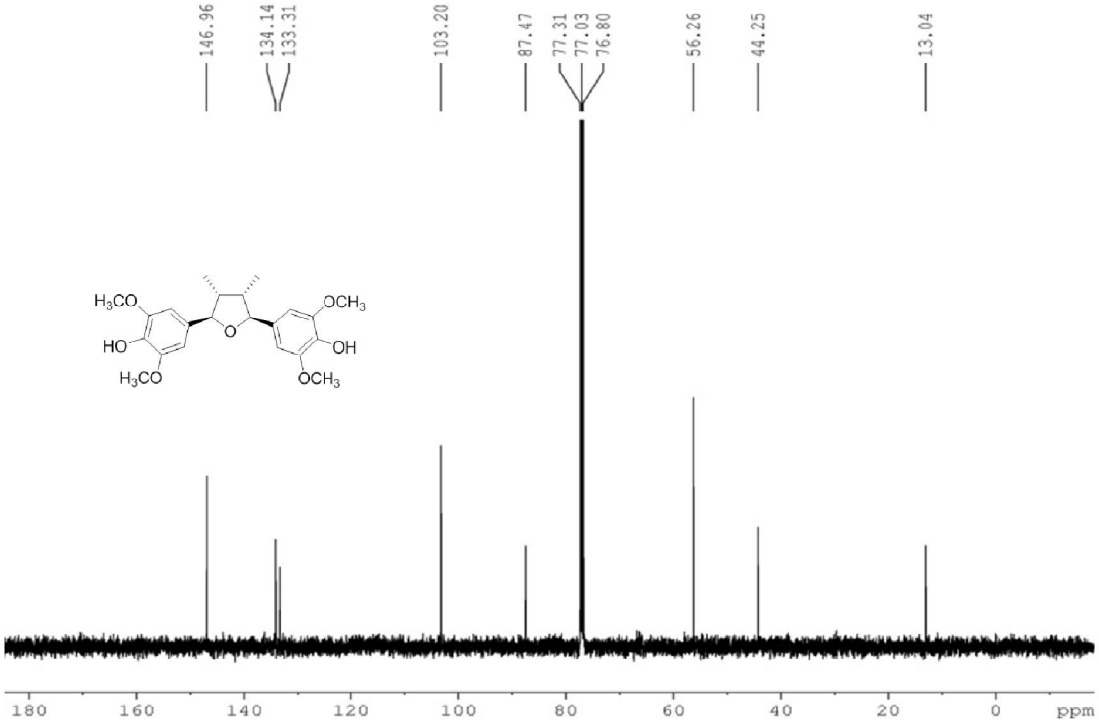


Figure 2b.11: ^{13}C NMR spectrum of Fragransin B_1

Fractions 10 and 11 combined together (79 mg) and subjected to CC on silica gel followed by CC on neutral alumina afforded compound 40 (49 mg) as *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-hydroxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan.

2b.1.3. Experimental

The bark of *Myristica fragrans* were collected from Ernakulam district, Kerala. The bark was cleaned, cut in to small pieces, dried in a drier maintained at 50^{o}C and powdered. The powdered bark (2 kg) was subjected to extraction using dichloromethane (2 L X 3) at room temperature. After extraction, the solvent was removed under reduced pressure using Büchi rotary evaporator. The crude extract (20 g) thus obtained was then subjected to column chromatographic separation.

2b.1.4. Chromatographic separation of the dichloromethane extract

The extract was dissolved in minimum quantity of ethyl acetate and loaded on the top of silica gel column filled with a slurry of 100-200 mesh silica gel in petroleum ether. The column was eluted successively with gradient mixtures of petroleum ether and ethyl

acetate of increasing polarity and finally with pure ethyl acetate. A total of 100 fractions of 150 ml each were collected and they were pooled in to 15 fraction pools according to the similarities of the TLC. The isolation procedure of compounds is depicted in **Figure 2b.12**.

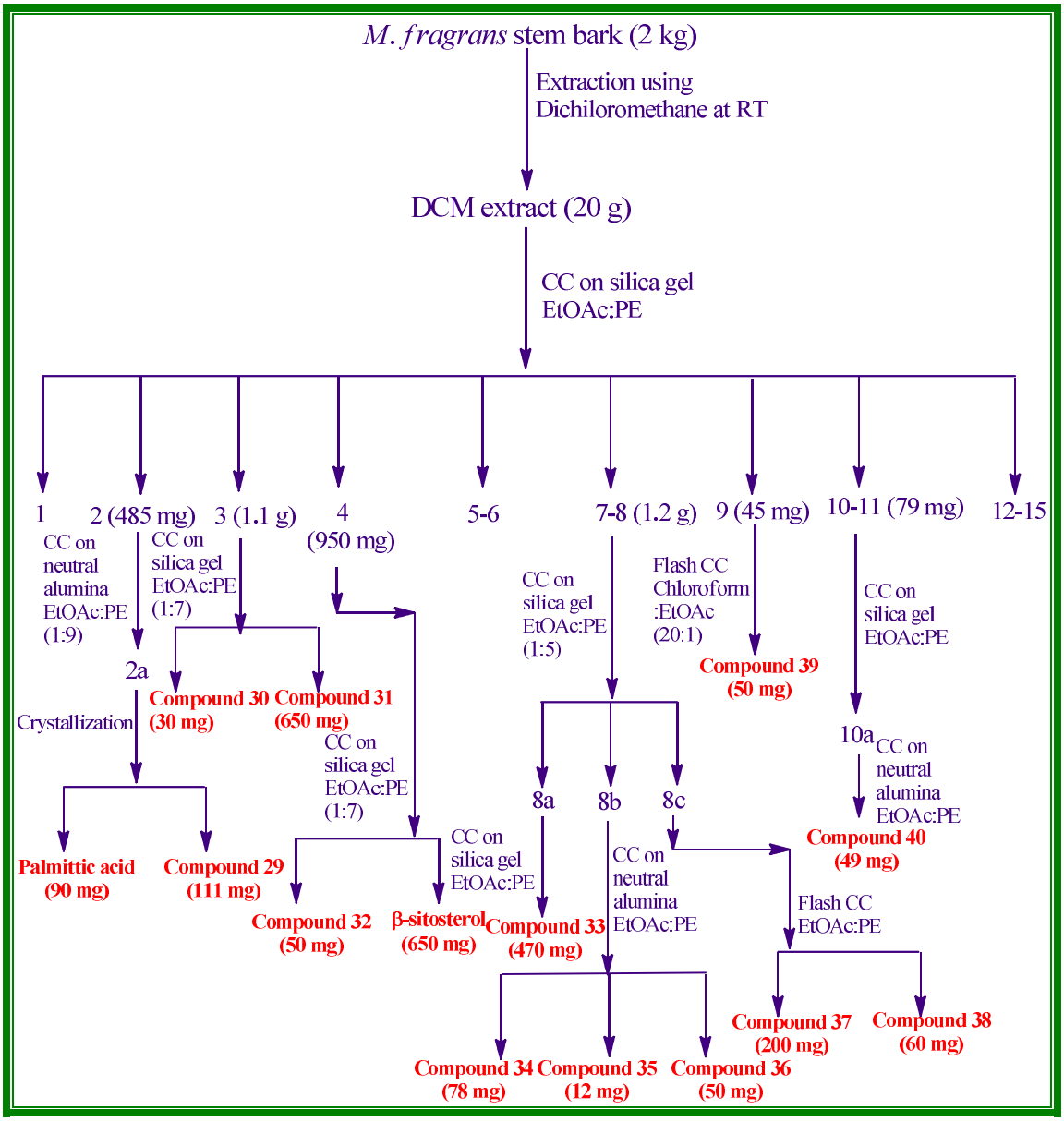


Figure 2b.12: Pictorial representation of isolation of chemical compounds from *M. fragrans* stem bark

2b.1.4a. Isolation of compound 29

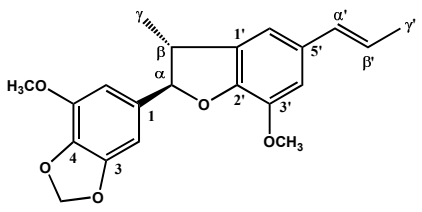
Compound 29 was isolated from the stem bark of *M. fragrans* as represented in **Figure 2b.12**. Compound 29 was obtained as colourless crystals by eluting the column with 10% ethyl acetate in petroleum ether. On evaluation of the structure of compound 29 and on comparison with TLC, it was 29 confirmed as licarin B which was isolated earlier from the fruit pericarp of *M. fragrans* (Compound 1).

2b.1.4b. Isolation of compound 30

Figure 2b.12 represents the isolation procedure for compound 30. It is obtained as colourless liquid. Detailed investigation of various spectroscopic data of compound 30 revealed that it was elemicin. Further evidence was made by comparing the TLC with that of compound 2 isolated earlier.

2b.1.4c. Isolation of compound 31

Compound 31 was obtained as colourless viscous liquid. ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and on comparison to the literature values, confirmed it to be (*2S,3R*)-7-methoxy-3-methyl-5-((*E*)-prop-1-enyl)-2-(5-methoxy,3,4-methylenedioxyphenyl)-2,3-dihydrobenzofuran whose structure is shown below.



FT-IR (NaCl $\nu_{max,}$ cm^{-1})	:	2960, 1677, 1495, 1325, 1204, 1138, 1044, 996, 931
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 6.78 (1H, *s*, H-6), 6.75 (1H, *s*, H-2), 6.61 (1H, *s*, H-4'), 6.60 (1H, *s*, H-6'), 6.36 (1H, *d*, *J* = 15.5 Hz, H-*α*'), 6.11 (1H, *dq*, J_1 = 6.5 Hz, J_2 = 15.5 Hz, H-*β*'), δ 5.95 (2H, *s*, -O-CH_2-O-), 5.07 (1H, *d*, *J* = 9 Hz, H-*α*), 3.89 (6H, *s*, 2 x -OCH_3), 3.42 (1H, *m*, H-*β*), 1.87 (3H, *d*, *J* = 6.5 Hz, H-*γ*'), 1.39 (3H, *d*, *J* = 6.5 Hz, H-γ)

^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 134.9 (C-1), 100.6 (C-2), 143.5 (C-3), 135.1 (C-4), 148.9 (C-5), 106.1(C-6), 133.0 (C-1'), 146.4 (C-2'), 144.1 (C-3'), 109.3 (C-4'), 132.2 (C-5'), 113.3 (C-6'), 93.5 (C-α), 45.8 (C-β), 17.8 (C-γ), 130.9 (C-α'), 123.5 (C-β'), 18.3 (C-γ'), 55.3 (-OCH_3), 56.6 (-OCH_3), 101.5 (-O-CH_2-O-) (C assignments were made as per literature)
HR-ESIMS *m/z*	:	377.1365 $[M+Na]^+$ (calcd for $C_{21}H_{22}O_5$ Na, 377.1365).

2b.1.4d. Isolation of compound 32

Compound 32 along with β-sitosterol was isolated from the fraction pool as shown in **Figure 2b.12**. Compound 32 was obtained as white solid. Spectral data in detail confirmed the compound 32 is similar to that of compound 6 (licarin A) obtained earlier.

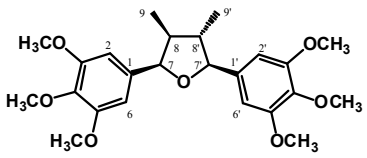
2b.1.4e. Isolation of compound 33

Fraction pools 7 and 8 were pooled together and repurified using column chromatography on silica gel by eluting with 20% ethyl acetate in petroleum ether to yield three sub fraction pools namely, 8a, 8b and 8c. Fraction 8a was subjected to column chromatography on silica gel to afford compound 33. Compound 33 was obtained as colourless oil. Detailed analysis by IR, ^{1}H NMR, ^{13}C NMR and mass spectral studies and by comparing the values from the literature, the compound was confirmed as raphidecursinol B, which was isolated from both *M. fragrans* seed kernel and mace.

2b.1.4f. Isolation of compound 34

Fraction 8b submitted to CC on neutral alumina to yield compound 34 as colourless crystal, compound 35 as colourless liquid and compound 36 as colourless liquid. The IR, ^{1}H NMR and ^{13}C NMR spectral details were closely related to that of grandisin reported

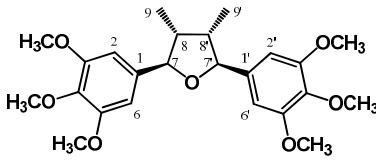
earlier from *Litsea grandis*. Hence, the structure proposed for this compound is as shown below.



FT-IR (NaCl $\nu_{max,}$ cm^{-1})	:	2958, 2837, 1590, 1505, 1353, 1234, 1009, 923,858
^{1}HNMR (500 MHz, $CDCl_3$)	:	δ 6.63 (4H, *s*, H-2, H-6, H-2' & H-6'), 4.66 (2H, *d*, *J* = 8.5 Hz, H-7 & H-7'), 3.89 (9H, *s*, -OCH_3), 3.86 (3H, *s*, -OCH_3), δ 3.84 (6H, *s*, 2 x-OCH_3), 1.80 (2H, *m*, H-8 & H-8'), 1.09 (6H, *d*, *J* = 5 Hz, 2 x -CH_3)
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 88.5 (C7 & C7'), 51.3 (C8 & C8'), 137.3 (C1 & C1'), 103.3 (C2, C6, C2' & C6'), 153.2 (C3, C5, C3' & C5'), 137.8 (C4 & C4'), 56.0 (2 x -OCH_3), 56.1 (2 x -OCH_3), 60.8 (2 x -OCH_3), 13.9 (2 x-CH_3) (C assignments were made as per literature)
HR-ESIMS*m/z*	:	455.2049 $[M+Na]^+$ (calcd for $C_{24}H_{32}O_7Na$, 455.2046)
Melting point	:	128-130^{o}C

2b.1.4g. Isolation of compound 35

After the isolation of grandisin, compound 35 was obtained as colourless liquid on further elution with ethyl acetate in petroleum ether. The spectral studies of the compound matched with those reported for (*7S,8S,7'R,8'R*)-2,2',3,3',4, 4'-hexamethoxy-7,7', 8,8'-lignan and the structure is as shown below.

FT-IR (NaCl $\nu_{max,}$ cm^{-1})	:	2958, 2837, 1590, 1505, 1353, 1234, 1009, 923,858
^{1}HNMR (500 MHz, $CDCl_3$)	:	δ 6.67 (4H, *s*, H-2, H-6, H-2' & H-6'), 4.54 (2H, *d*, *J* = 6 Hz, H-7 & H-7'), 3.90 (3H, *s*, -OCH_3), 3.86 (9H, *s*, 3 x -OCH_3), 3.85 (6H, *s*, 2 x -OCH_3), 2.36 (2H, *m*, H-8 & H-8'), 1.09 (6H, *d*, *J* = 5 Hz, 2 x -CH_3)
	^{13}C NMR (125 MHz, $CDCl_3$)	: δ 87.3 (C7 & C7'), 44.2 (C8 & C8'), 137.3 (C1 & C1'), 103.3 (C2, C6, C2' & C6'), 153.2 (C3, C5, C3' & C5'), 137.8 (C4 & C4'), 56.0 (4 x -OCH_3), 60.8 (2 x -OCH_3), 13.9 (2 x-CH_3) (C assignments were made as per literature)
HR-ESIMS *m/z*	:	455.2049 $[M+Na]^+$ (calcd for $C_{24}H_{32}O_7Na$, 455.2046)

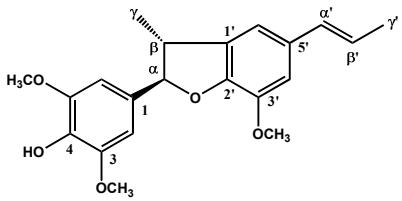
2b.1.4h. Isolation of compound 36

After the isolation of compound 35, compound 36 was obtained as colourless liquid on further elution with ethyl acetate in petroleum ether. Detailed investigation of various spectroscopic data of compound 36 revealed that it was *erythro* surinamensin. Further evidence was made by comparing the TLC with that of compound 10 isolated earlier.

2b.1.4i. Isolation of compound 37

Fraction 8c subjected to flash column chromatography by eluting with hexane-ethyl acetate yielded compound 37 as colourless crystals and compound 38 as colourless liquid.

The spectral studies of the compound 37 matched with those reported for odoratisol A and the structure is as shown below



FT-IR (NaCl $\nu_{max,}$ cm^{-1})	:	3432 br (-OH), 2999, 2934, 1607, 1516, 1516, 1374, 1113, 961, 889
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 6.80 (1H, *s*, H-6'), 6.72 (1H, *s*, H-4'), 6.68 (2H, *s*, H-6 & H-2), 6.35 (1H, *d*, *J* = 16 Hz, H-*α*'), 6.13 (1H, *dq*, J_1 = 6.5 Hz, J_2 = 16 Hz, H-*β*'), 5.58 (1H, *s*, -OH), 5.07 (1H, *d*, *J* = 10 Hz, H-*α*), 3.90 (3H, *s*, -OCH_3), 3.89 (6H, *s*, 2 x -OCH_3), 3.47 (1H, *m*, H-*β*), 1.87 (3H, *d*, *J* = 6.5 Hz, H-*γ*'), 1.39 (3H, *d*, *J* = 10 Hz, H-*γ*)
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 133.1 (C-1), 103.4 (C-2), 147.0 (C-3), 134.7 (C-4), 147.0 (C-5), 103.4 (C-6), 132.2 (C-1'), 146.5 (C-2'), 144.1 (C-3'), 109.2 (C-4'), 131.1 (C-5'), 113.2 (C-6'), 94.0 (C-*α*), 45.7 (C-*β*), 17.5 (C-*γ*), 130.9 (C-*α*'), 123.5 (C-*β*'), 18.4 (C-*γ*'), 56.3 (2 x -OCH_3), 55.9 (-OCH_3) (C assignments were made as per literature)
HR-ESIMS *m/z*	:	357.1708 $[M+H]^+$ (calcd for $C_{21}H_{25}O_5$, 357.1702)

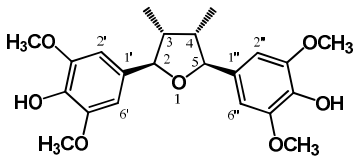
Melting point : 116-118^{o}C

2b.1.4j. Isolation of compound 38

After the isolation of odoratisol A, compound 38 was obtained as colourless liquid on further elution with ethyl acetate in petroleum ether. The spectral studies of the compound 38 matched with those reported for *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-hydroxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan and the compound 20.

2b.1.4k. Isolation of compound 39

Fraction pool 9 submitted to flash column chromatography by eluting with 4% chloroform-ethyl acetate yielded compound 39 as colourless crystals. It was characterized as the fragransin B_1 from the spectral studies. The structure of the compound is shown below.



FT-IR (NaCl $\nu_{max,}$ cm^{-1}) : 3436 br (-OH), 2959, 2875, 1707, 1611, 1426, 1148, 1033

^{1}H NMR (500 MHz, $CDCl_3$) : δ 6.67 (4H, *s*, H-2, H-6, H-2' & H-6'), 5.55 (2H, *br. s*, 2 x -OH), 4.51 (2H, *d*, *J* = 6 Hz, H-7 & H-7'), 3.87 (12 H, *s*, 4 x -OCH_3), 2.34 (2H, *m*, H-8 & H-8'), 1.06 (6H, *d*, *J* = 6.5 Hz, 2 x -CH_3)

^{13}C NMR (125 MHz, $CDCl_3$) : δ 87.4 (C7 & C7'), 44.2 (C8 & C8'), 133.3 (C1 & C1'), 103.2 (C2, C6, C2' & C6'), 146.9 (C3, C5, C3' & C5'), 134.1 (C4 & C4'), 56.2 (4 x -OCH_3), 13.9 (2 x -CH_3)
(C assignments were made as per literature)

HR-ESIMS *m/z* : 427.1736 $[M+Na]^+$ (calcd for

$C_{22}H_{28}O_7Na$, 427.1733)

Melting point : 100-102^oC

2b.1.4l. Isolation of compound 40

Fractions pools 10 and 11 combined together and subjected to column chromatography on silica gel followed by column chromatography on neutral alumina to afford compound 40 which was identified as *erythro*-(*7S,8R*)-$\Delta^{8'}$-4,7-dihydroxy-3,5,3'-trimethoxy-8-*O*-4'-neolignan, isolated earlier from nutmeg fruit pericarp (compound 9).

2b.2. Conclusion

This is the first study being reported on the bark of *M. fragrans* which has resulted in the isolation and characterization of the compounds viz., licarin B, elemicin, (*2S,3R*)-7-methoxy-3-methyl-5-((*E*)-prop-1-enyl)-2-(5-methoxy,3,4-methylenedioxyphenyl)-2,3-dihydrobenzofuran, licarin A, fragransin B_1, odoratisol A, 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'-tetramethoxy-8-*O*-4'-neolignan and surinamensin. In addition, grandisin and *(7S,8S, 7'R,8'R)*-3,3',4,4',5,5'-hexamethoxy-7,7', 8,8'-lignan have also been isolated from *M. fragrans* for the first time.

CHAPTER 3

Isolation of Four Major Phytochemical Components from *Azadirachta indica* Seeds and their Biological Activity Studies

3.1. Introduction

Figure 3.1 : *Azadirachta indica* - Leaves with fruits and dried seeds

Medicinal plants have played a central role in human society's fight against a wide range of diseases. For thousands of years, the beneficial properties of the neem tree (*Azadirachta indica* A. Juss.) have been recognised in India, and it is among the country's most used medicinal plant. Neem has been universally accepted as a wonder tree because of its diverse utility. Since the 1970s, scientists in Europe and the United States have been interested in neem because of its insecticidal properties and its low toxicity to mammals in addition to medicinal properties.

Azadirachta indica is a small to medium-sized tree, usually evergreen, up to 15 m tall, with a round, large crown up to 10 m in diameter; branches spreading; bole branchless for up to 7.5 m, up to 90 cm in diameter, sometimes fluted at base; bark moderately thick, with small, scattered tubercles, deeply fissured and flaking in old trees,

dark grey outside and reddish inside, with colourless, sticky foetid sap. Leaves are alternate, crowded near the end of branches, simply pinnate, 20-40 cm long, light green, with 2 pairs of glands at the base, otherwise glabrous; petiole 2-7 cm long, leaflets 8-19, very short petioluled, alternate proximally and more or less opposite distally, ovate to lanceolate, glossy, serrate; apex acuminate and base unequal. Inflorescence includes an axillary, many-flowered thyrsus, up to 30 cm long; bracts minute and caducous; flowers bisexual or male on same tree, small, white or pale yellow, slightly sweet scented. Fruit-1 (max. 2)-seeded drupe, ellipsoidal, 1-2 cm long, greenish, greenish yellow to yellow or purple when ripe; exocarp thin, mesocarp pulpy, endocarp cartilaginous; seed ovoid or spherical; apex pointed; composed of a shell and a kernel (sometimes 2 or 3 kernels), each about half of the seed's weight.

Various parts of the neem tree have been used in traditional Ayurvedic medicines in India [Verma, **1976**]. Neem seed oil, bark and leaf extracts have been therapeutically used as folk medicine in the treatment and control of leprosy, intestinal helminthiasis, respiratory disorders, constipation, blood morbidity, rheumatism, biliary infections, itching, skin ulcers and many more along with the use as a general health promoter [Kirtikar *et al.*, **1935**: Mitra, **1963**]. A few medicinal applications of various parts of neem are summarised in **Table 3.1**.

However, apart from the Ayurveda records, there are several reports on the biological activity and pharmacological actions of neem based on modern scientific investigation [Brahmachari, **2004**].

The neem tree is native to the Indian subcontinent, Southeast Asia and Africa. There are two closely related species, *A. indica* and *A. azedarch*. The former is popularly known as Indian neem (margosa tree) or Indian lilac, and the latter as the Persian lilac. The taxonomic position of *Azadirachta indica* is as follows:

Division : Mangoliphyta

Order : Sapindales

Family : Meliaceae

Genus : *Azadirachta*

Species : *A. indica*

Part		**Medicinal uses**
Leaf	:	Treatment of leprosy, eye problems, intestinal worms, anorexia, biliousness, skin ulcers
Flower	:	Bile suppression, elimination of intestinal worms and phlegm
Twig	:	Relieves cough, asthma, piles, spermatorrhoea, obstinate urinary disorder, diabetes
Bark	:	Analgesic, antipyretic
Fruit		Relieves piles, intestinal worms, urinary disorder, eye problem, diabetes, wounds and the treatment of leprosy
Gum	:	Effective against skin diseases like ringworms, scabies, wounds and ulcers
Seed pulp	:	Treatment of leprosy and intestinal worms
Oil	:	Treatment of leprosy, intestinal worms, rheumaism
Root, bark, leaf, flower and fruit together	:	Blood morbidity, biliary afflictions, itching, skin ulcer, burning sensation and the treatment of leprosy, rheumatism

Table 3.1 : Different parts of *A. indica* used in traditional medicines and their biological properties

Neem has been found to possess antimalarial activity [Iwalewa *et al.*, **1995**]. Extracts of neem seeds were studied for their effect on *in vitro* and *in vivo* growth and development of the human malarial parasite *Plasmodium falciparum* and found to have highly appreciable results [Dhar *et al.*, **1998**]. Aqueous neem-leaf extract was found to offer protection against paracetamol-induced liver necrosis in rats [Bhanwa *et al.*, **2000**]. An aqueous extract of neem seed kernel (1 mL/100 g body weight daily of a 50 gL^{-1} solution) produces trypsin inhibitory activity as observed in weanling rats [Rao, **1987**].

More than 140 compounds have been isolated so far from different parts of *A. indica*. Indeed, organic chemists, especially natural product chemists are still carrying out research on the active principles of *A. indica*. The compounds found in *A. indica* may be divided into two classes:

1. Isoprenoids like diterpenoids and triterpenoids including protomeliacin, limonoids, azadirone and its derivatives, vilasinin type compounds, nimbin, salanin and azadirachtin.
2. Non isoprenoids which include proteins, aminoacids, carbohydrates, sulphur compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcones, coumarins, tannins, aliphatic compounds etc.

Although a large number of compounds have been isolated from various parts of neem tree, especially from the seeds, only a few of them have been investigated so far for biological activity. Structures of some of the important compounds isolated from *A. indica* are given in **Figure 3.2** [Kraus *et al.*, **1981**; Siddiqui *et al.*, **1986**].

Figure 3.2 : list of some compounds isolated from *A. indica*

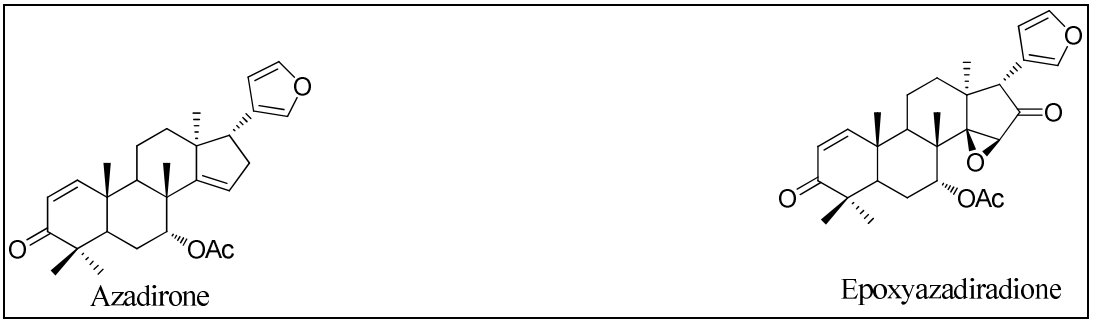
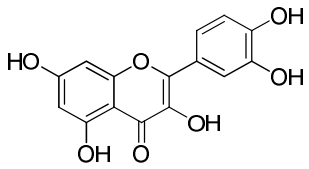
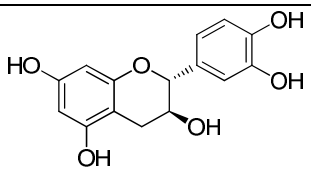


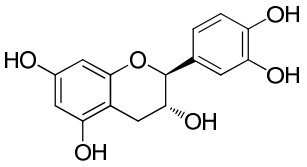
Figure 3.2 continues.....



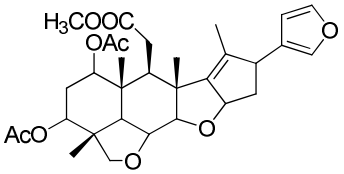
Quercetin



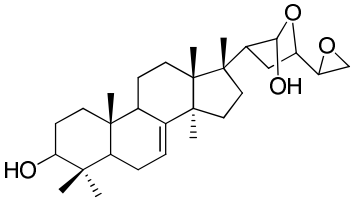
Catechin



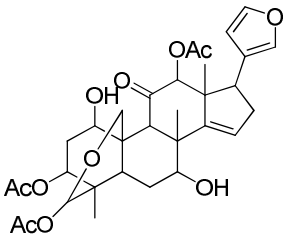
Epicatecin



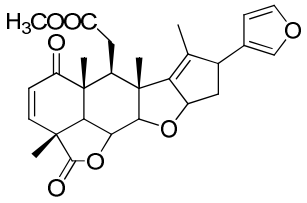
Salanin



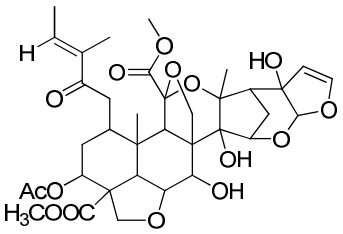
Melianol



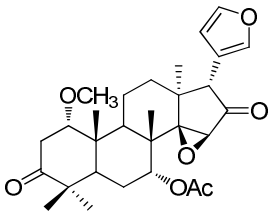
Azadirachtanin



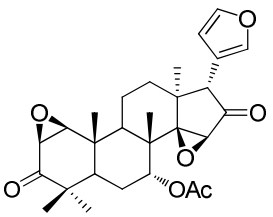
Nimbolide



Azadirachtin

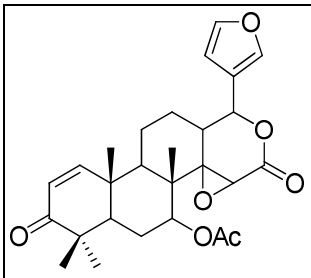


1α-methoxy-12-dihydroepoxyazadirone

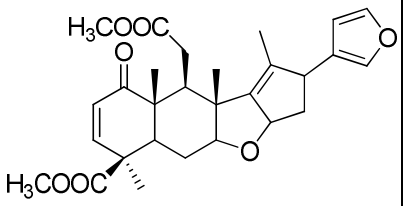


1β, 2β-diepoxyepoxyazadirone

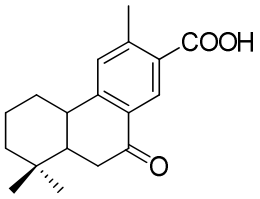
Figure 3.2 continues.....



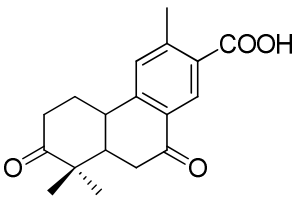
Gedunin



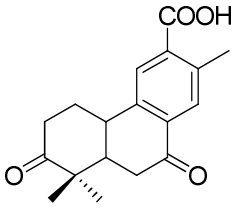
Nimbin



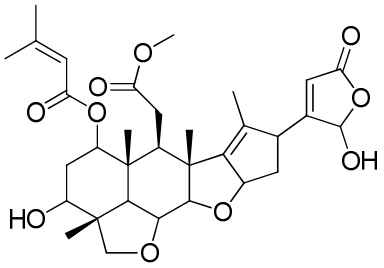
Margalone



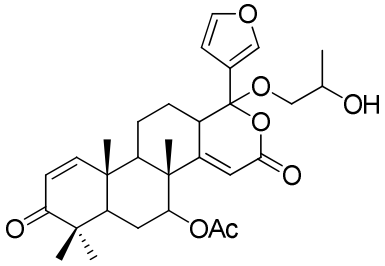
Margalonone



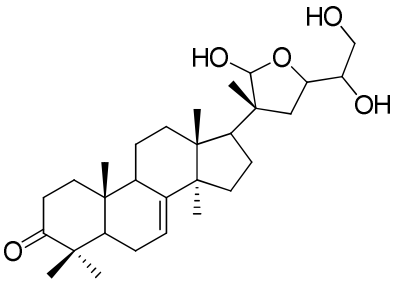
Isomargalonone



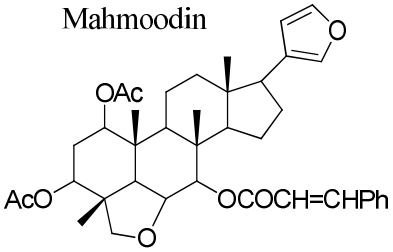
Isoazadirolide



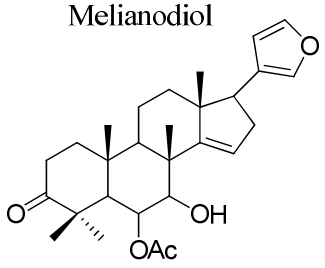
Mahmoodin



Melianodiol

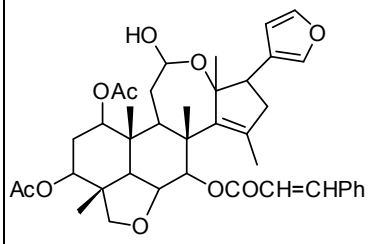


Nimbolin A

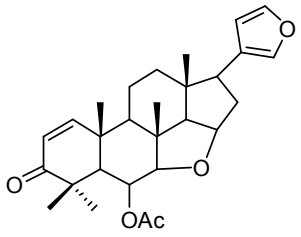


Meldenin

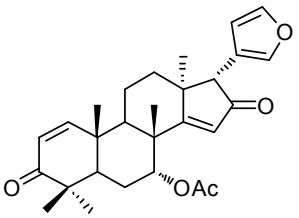
Figure 3.2 continues.....



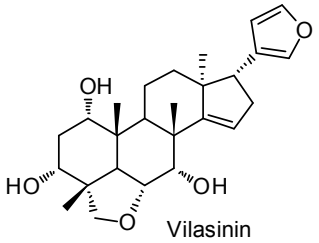
Nimbolin B



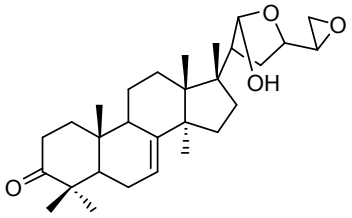
Vepinin



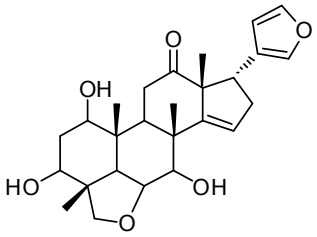
Azadiradione



Vilasinin



Melianone



Nimbidin

As already mentioned, various parts of the *A. indica* tree are used in Indian systems of medicine. Although a large number of compounds have been isolated from various parts of this plant, especially from seeds, only a few of them have been investigated so far for their biological activity. Nimbolide isolated from neem leaves has been found to have anticancer activity against N1E-115 (mouse neuroblastoma), 143B.TK$^-$ (human osteosarcoma) with an IC_{50} ranging from 4 to 10 μM and averaging 6 μM for three cell lines. Other limonoids of decreasing potency with their average IC_{50} values are epoxyazadiradione 27 μM, salanin 112 μM and nimbin, deacetylnimbin and azadiractin each greater than 200 μM (practically nontoxic) [Cohen *et al.*, **1996**]. Gedunin, a tetranortriterpenoid isolated from *A. indica* seeds has been found to have

anticancer activity against breast cancer cell lines (MCF-7 and SKBr3) [Gary *et al*., **2008**]. Several papers related to the anticancer activity of nimbolide, the major compound obtained from *A. indica* leaves have been reported from this laboratory [Gupta *et al*., **2013**; Gupta *et al*., **2010**]. *In continuation of our interest in the biological activity studies on A. indica, the isolation of major compounds from A. indica seeds was envisaged. This chapter therefore gives the details of the isolation of the major compounds of A. indica seeds and the anticancer studies conducted on one of them viz., azadirone.*

3.2. Isolation of major chemical constituents of *A. indica* seeds

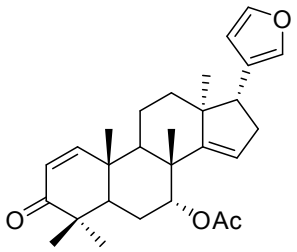
3.2.1. Extraction

1.5 kg of *Azadirachta indica* seeds were purchased from registered Ayurvedic vendor in Trivandrum. It was then dried in an air oven at 50^{o}C and then coarsely powdered. The powdered material was subjected to extraction using (2.5 L x 2) of hexane in order to remove the oil content. Then the material was further subjected to extraction using acetone (2.5 L X 3) at room temperature. Removal of acetone under reduced pressure gave 18.5 g of crude extract. About 300 g of silica gel was packed in a 1000 mL glass column using petroleum ether as solvent and 18.5 g of the crude extract was loaded on the column and eluted initially with petroleum ether. Further, polarity was gradually increased using petroleum ether- ethyl acetate mixtures and total of 214 fractions of 150 ml each were collected. According to the similarities in TLC, they were pooled together to get 13 fractions pools.

3.2.2. Isolation of phytochemicals constituents

TLCs of fraction pool 6 (110 mg) showed a single UV active spot. After concentration, it was subjected to crystallization in dichloromethane in petroleum ether solvent system to yield compound 41 as colourless crystals. The compound was analyzed using various spectroscopic techniques. Compound 41 showed a strong absorption at 1732 cm^{-1} in IR spectrum indicating the presence of an ester carbonyl and a strong absorption at 1662 cm^{-1} suggesting the presence of six membered α, β unsaturated ketone. A doublet observed at δ 7.16 in the ^{1}H NMR (**Figure 3.3**) spectrum, integrating for one proton indicates β hydrogen and another at δ 5.85 indicates an α hydrogen of an α, β unsaturated ketone. The signals at δ 0.79, 1.08, 1.20 and 1.23 each integrating for three, six, three and three protons each respectively indicated the presence of five methyl

groups. The three proton singlet at δ 1.96 could be attributed to acetyl group. The peaks at δ 204.7 and 170.2 in ^{13}C NMR (**Figure 3.4**) spectrum confirmed the presence of α, β unsaturated ketone and an ester carbonyl. Peaks in between δ 111.1-158.8 (8C) in ^{13}C NMR spectrum are due to alkenyl carbons. The mass spectrum showed that the molecular ion peak is at 437.97 which is the M^+ peak. On analyzing these spectral data, structure of the compound was confirmed as azadirone [Lavie *et al.*, **1971**].



41

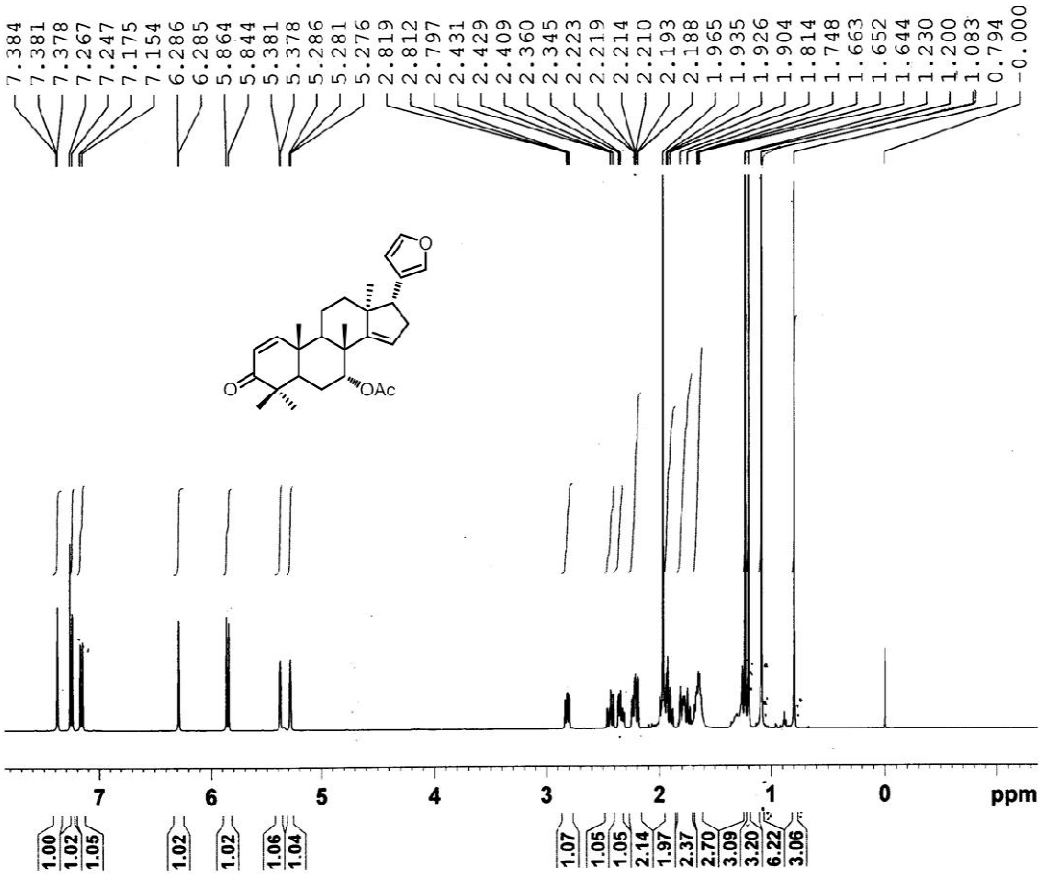


Figure 3.3: ^{1}H NMR spectrum of Azadirone

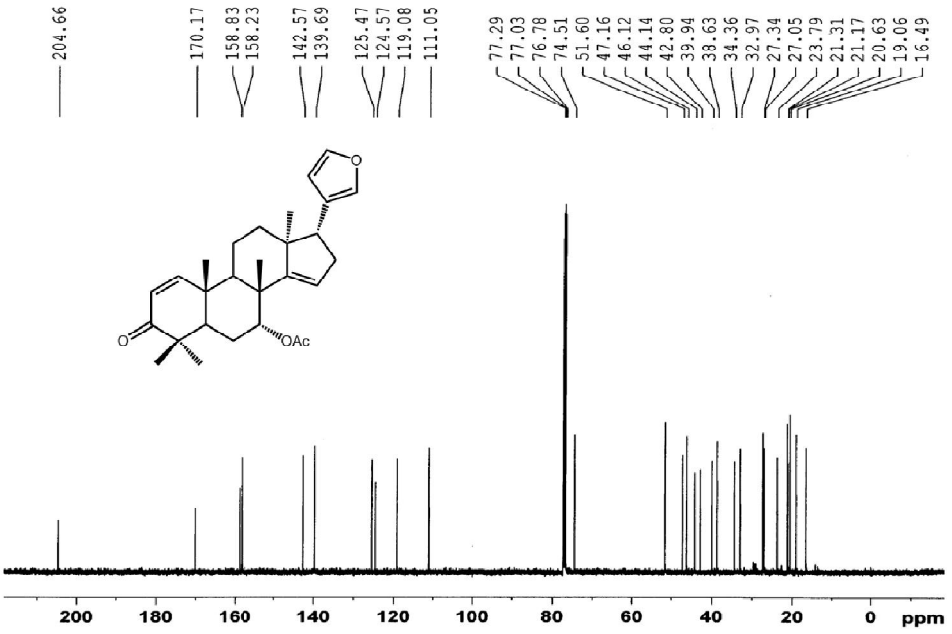
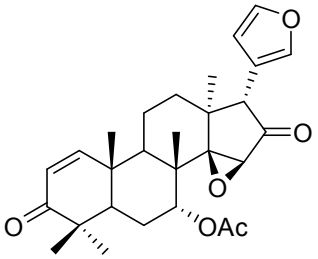


Figure 3.4 : ^{13}C NMR spectrum of Azadirone

Fraction 9 (248 mg) when subjected column chromatography on silica gel by eluting with ethyl acetate in petroleum ether yielded 11 fraction pools based on the TLC analysis, named as sub fractions 9a to 9l. Sub fraction 9b was subjected to crystallization in ethyl acetate-hexane mixture to afford compound 42 as colourless crystals. IR spectrum of the compound 42 showed a strong absorption at 1730 cm^{-1} indicating the presence of carbonyl group and a strong absorption at 1668 cm^{-1} suggesting the presence of six membered α, β unsaturated ketone. The strong absorption at 1747 cm^{-1} in IR spectrum indicated the presence of an ester carbonyl. A doublet present at δ 7.17 in the ^{1}H NMR (**Figure 3.5**) spectrum integrating for one proton could attributed to β hydrogen and another at δ 5.88 to an α hydrogen of the α, β unsaturated ketone. The signals at δ 1.04, 1.07, 1.21 and 1.22 each integrating for three, six, three and three protons respectively indicated the presence of five methyl groups. The three protons corresponds to acetyl group appeared at δ 2.03. The peaks at δ 208.4, 204.3 and 169.8 in ^{13}C NMR (**Figure 3.6**) spectrum confirmed the presence of a ketone, α, β unsaturated ketone and ester carbonyl respectively. Peaks in between δ 110.9-157.5 (6C) in ^{13}C NMR spectrum due to alkenyl carbons too were observed. The mass spectrum showed the molecular ion

peak at 466.92 is the M^+ peak. Further, by comparison of the spectroscopic data and with the values reported earlier [Lavie *et al.*, **1971**] it was confirmed that the compound 42 was epoxyazadiradione whose structure is shown below.



42

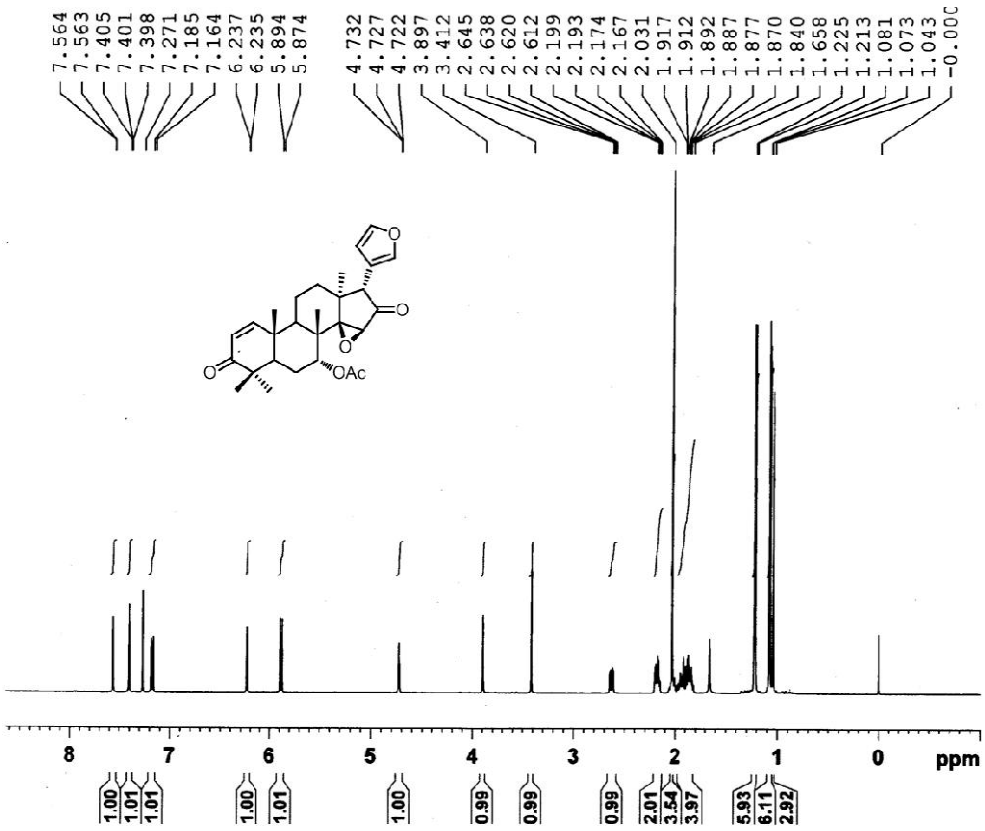


Figure 3.5 : ^{1}H NMR spectrum of Epoxyazadiradione

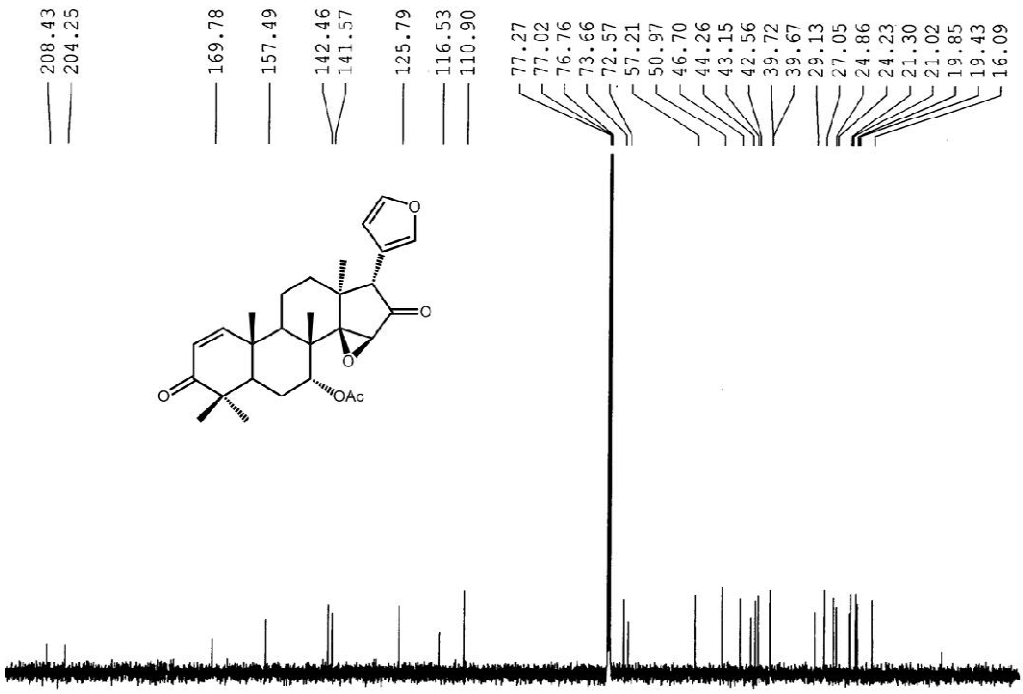
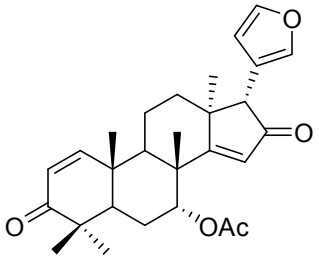


Figure 3.6 : ^{13}C NMR spectrum of Epoxyazadiradione

Fraction 9h showed two UV active spots, which on repurification on neutral alumina by eluting with ethyl acetate-hexane (1:5) yielded two compounds namely compound 43 as colourless crystals and compound 44 as white solid. IR spectrum of the compound 43 showed strong absorption at 1728 cm^{-1}, which indicated the presence of an ester carbonyl. A strong absorption at 1691 cm^{-1} in IR spectrum indicated the presence of five membered α, β unsaturated ketone and a strong absorption at 1666 cm^{-1} was suggestive of six membered α, β unsaturated ketone. The ^{1}H NMR (**Figure 3.7**) spectrum showed singlets at δ 1.03, 1.25, 1.10, 1.09 and 1.34 each integrating for three protons were indicative of five tertiary methyl groups. The doublet at δ 7.12 in the ^{1}H NMR spectrum integrating for one proton indicated the presence β hydrogen and another at δ 5.89 indicated an α hydrogen of an α, β unsaturated ketone. Singlet appearing at δ 1.95 in ^{1}H NMR spectrum which integrating for three proton corresponds to protons of acetyl group. ^{13}C NMR (**Figure 3.8**) spectrum showed 28 peaks. The peaks at δ 205.1 and 204.1 indicated the presence of two keto carbonyls and the peak at 169.6 showed the presence of ester carbonyl. The peaks at δ 20.9, 19.0, 26.9, 23.4, 15.8 and 26.3 could be attributed to methyl carbons. The peaks observed at δ 125.9, 192.5, 118.4, 123.2, 141.61,

111.2 and 142.8 shows the presence of alkenyl carbons. The FAB MS showed a molecular ion peak at 450.68. On analyzing these spectral data the compound was confirmed as azadiradione [Lavie *et al.*, **1971**; Siddiqui *et al*., **1986]** 43 shown below.



43

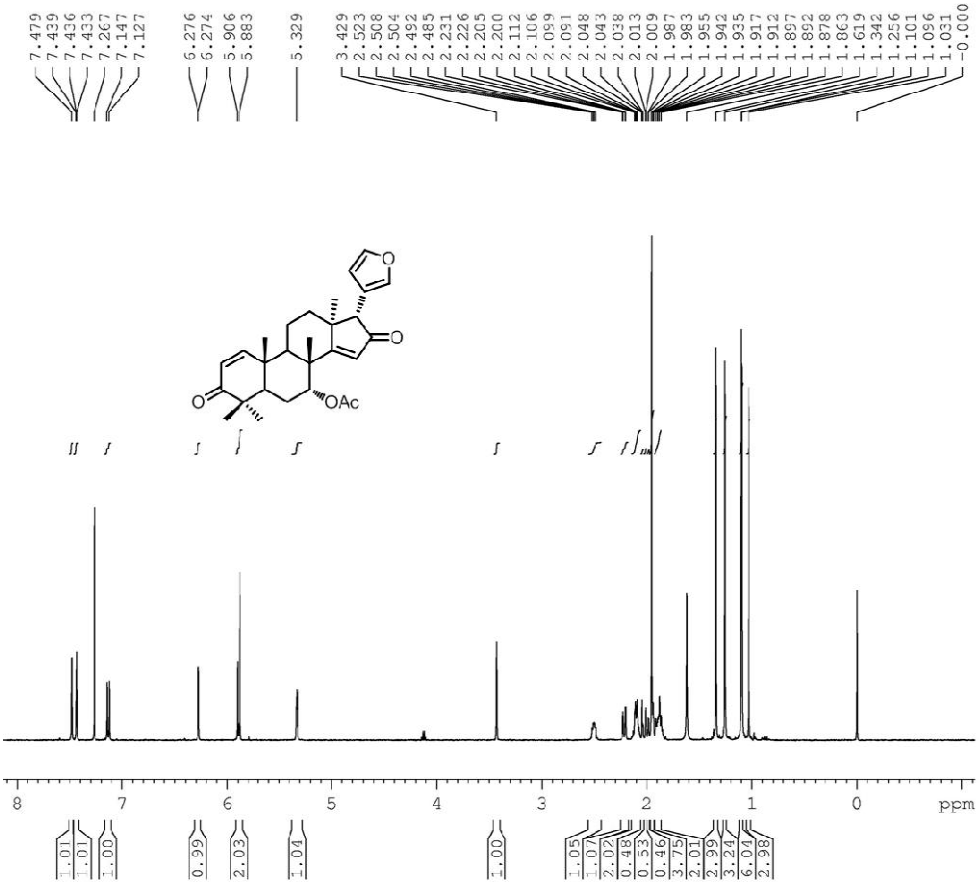


Figure 3.7 : ^{1}H NMR spectrum of Azadiradione

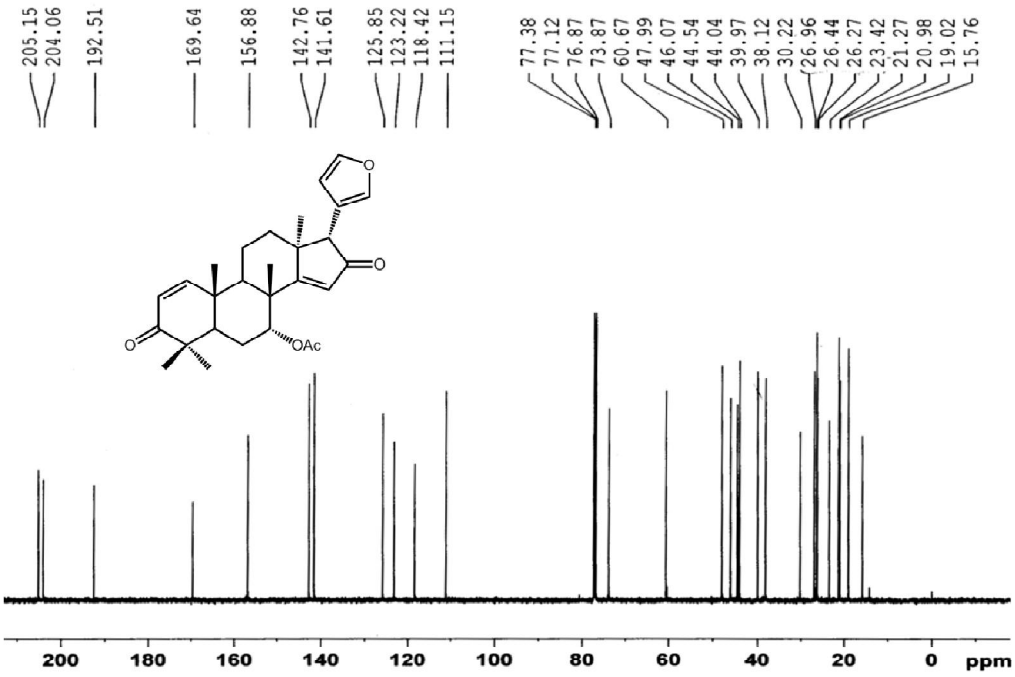
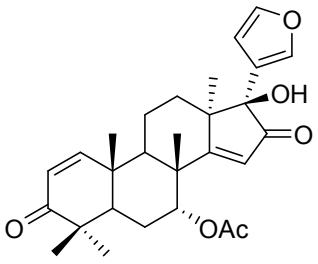


Figure 3.8 : ^{13}C NMR spectrum of Azadiradione

Compound 44 showed a broad absorption at 3437 cm^{-1} in the IR spectrum, which indicated the presence of hydroxyl group. The strong absorption at 1738 cm^{-1} in IR indicated the presence of an ester carbonyl. Another two strong absorptions at 1709 cm^{-1} and 1669 cm^{-1} in the IR spectrum indicated the presence of both five membered α, β unsaturated ketone and six membered α, β unsaturated ketone functionalities. The doublet at δ 7.16 in the ^{1}H NMR (**Figure 3.9**) spectrum integrating for one proton indicates β hydrogen and another at δ 5.89 the α hydrogen of an α, β unsaturated ketone. The singlets at δ 0.98, 1.09, 1.10, 1.25 and 1.36 each integrating for three protons which indicated the presence of five methyl groups. The three protons corresponding to acetyl group appeared at δ 1.95. ^{13}C NMR (**Figure 3.10**) spectrum showed 28 peaks. The peaks at δ 205.9 and 204.2 indicated the presence of two keto carbonyls and the peak at 169.6 showed the presence of ester carbonyl. The peaks seen at δ 109.5, 120.3, 122.5, 125.9, 141.5, 142.8, 157.1 and 193.6 shows the presence of eight alkenyl carbons. The HR MS showed a molecular ion peak at 489.2252 which is $(M+Na)^{+}$ peak. On analyzing these spectral data, the structure of the compound was confirmed as 17-β hydroxy azadiradione [Lee *et al.*, **1988**] 44, shown below.



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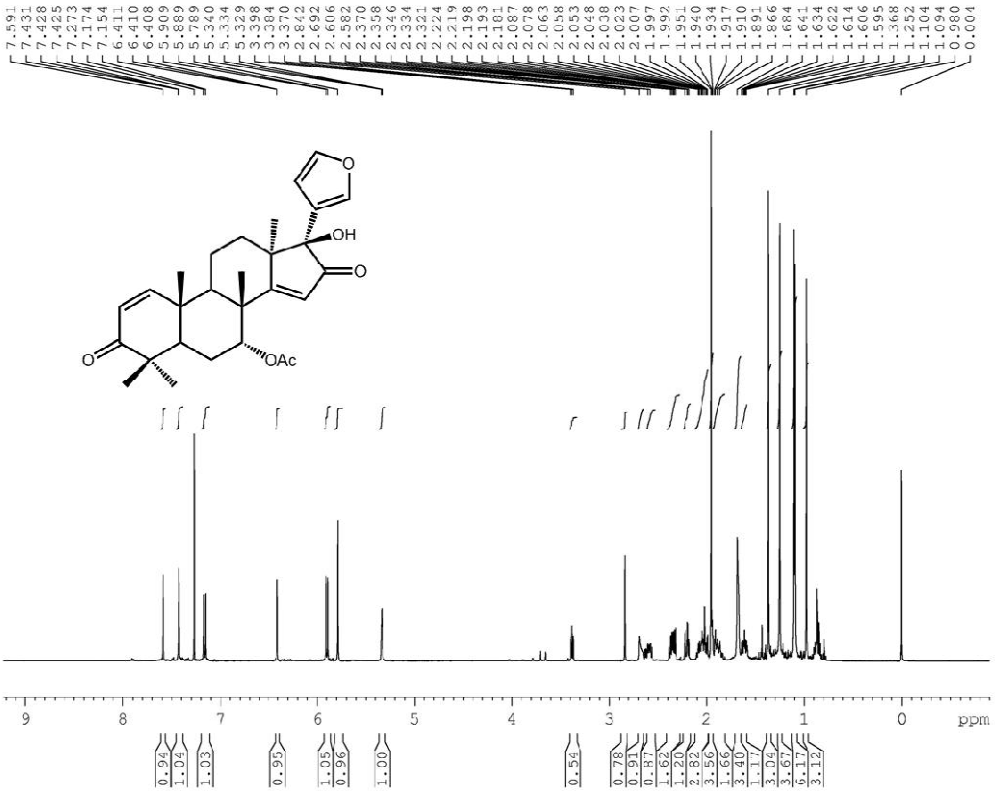


Figure 3.9 : ^{1}H NMR spectrum of 17-β hydroxy azadiradione

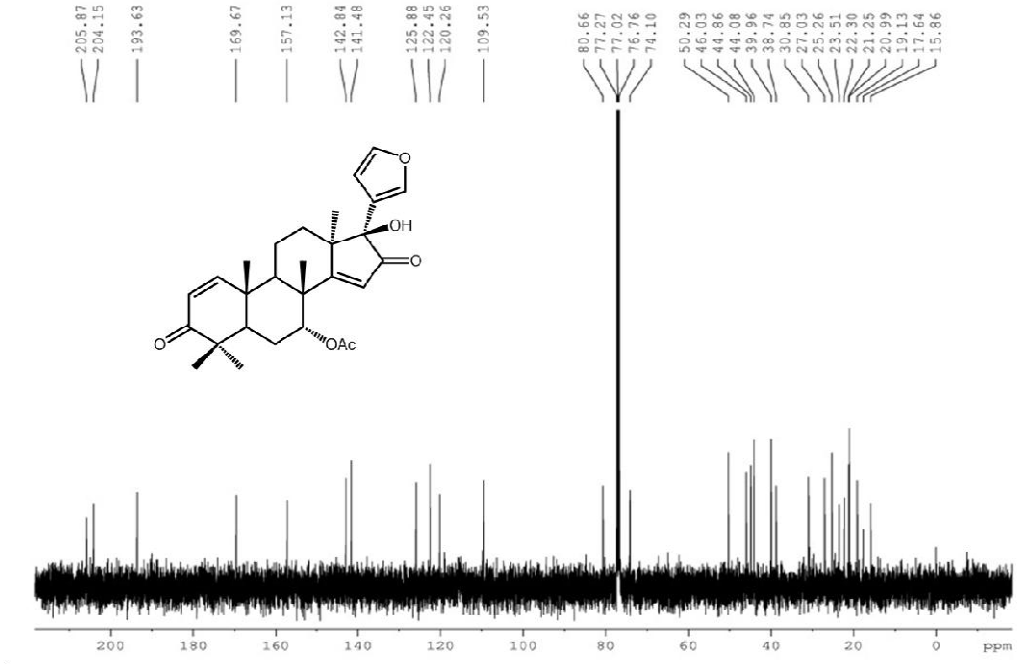


Figure 3.10 : ^{13}C NMR spectrum of 17-β hydroxy azadiradione

Although many compounds are reported from neem seed extract, we observed that only these four major compounds could be readily isolated. It was also observed by us that neem seed obtained in Kerala is not very fresh and some of the compounds therefore may be decomposed/destroyed. As our interest was in obtaining these compounds in larger quantity for anticancer activity studies, the procedure [Nicoletti *et al.,* **2012**] reported for obtaining the compounds viz., azadirone, epoxyazadiradione, azadiradione and 17-β hydroxy azadiradione from neem seed cake was followed.

3.3. Isolation of chemical constituents from *A. indica* seed cake

3.3.1. Extraction

1 kg of *Azadirachta indica* seed cake were purchased from registered Ayurvedic vendor in Trivandrum. It was then dried in an air oven maintained at 50^{o}C and then coarsely powdered. The powdered material was subjected to extraction (24 hours) using (2.5 L X 2) of hexane in order to remove the oil content. Then the material was further subjected to extraction using acetone (2.5 L X 3) at room temperature. Removal of acetone under reduced pressure gave 36 g of crude extract. About 300 g of silica gel was

packed in a 1000 ml glass column using petroleum ether as solvent and 18.5 g of the crude extract was loaded on the column and eluted initially with petroleum ether. Further, polarity was gradually increased using petroleum ether-ethyl acetate mixtures and total of 171 fractions of 150 ml each were collected. According to the similarities in TLC, they were pooled together to get 22 fraction pools.

3.3.2. Isolation of phytochemicals constituents from *A. indica* seed cake

The fraction pool 7 (200 mg) showed single UV active spot with minor impurities. It was subjected to crystallization in dichloromethane in petroleum ether solvent system to yield azadirone (123 mg) as colourless crystals. The fraction pool 8 (2 g) was subjected to crystallization in ethyl acetate in petroleum ether yielded epoxyazadiradione (1.3 g). The fraction pool 14 (530 mg) showed two major UV active spots. The fraction was subjected to purification on neutral alumina using hexane and ethyl acetate as eluant to afford azadiradione (314 mg) and 17-β hydroxyazadiradione (150 mg). Thus through extraction and isolation of neem seed cake, sufficient quantity of the above compounds were obtained.

3.4. Anticancer activity studies of azadirone isolated from *A. indica* seeds

Azadiradione and epoxyazadiradione have been shown to have promising antitumor activities on triple negative breast cancer cell lines. The detailed work in progress. Anticancer activity of azadirone has been studied against various cancer cell lines viz., HCT-116 and HT-29 (colon adenocarcinoma), U-266 (multiple myeloma), A293 (embryonic kidney carcinoma), AsPC-1 (pancreatic adenocarcinoma), MDA-MB-231 and MCF-7 (breast adenocarcinoma), and H1299 (lung adenocarcinoma). The mechanism of anticancer activity has been studied in collaboration with Dr. Bharath B. Aggarwal, University of Texas, USA.

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in cancer cells while it exhibits little or no toxicity in normal cells [Ashkenazi *et al*, 1999]. However, development of resistance to TRAIL by certain cancer types is a major concern. Numerous mechanisms have been identified by which tumour cells develop resistance to TRAIL. One potential mechanism for the development of TRAIL resistance involves down-regulation of death receptor DR4 and DR5 [Kurbanov *et al.*, **2007**; van Geelen *et al.*, **2011**]. A second mechanism of TRAIL resistance involves up-

regulation of antagonistic decoy receptors that bind TRAIL but do not contain the functional domains necessary to transduce apoptotic signals [Bouralexis *et al.*, **2003**; Sheridan *et al*., **1997**]. A third mechanism of TRAIL resistance is up-regulation in cell survival proteins such as Bcl-xL , Bcl-2 , XIAP, survivin, cellular FLICE-like inhibitory protein (c-FLIP, a caspase-8 inhibitor also known as I-FLICE), and mMcl-1, and down-regulation in proapoptotic proteins. It is also possible that cancer cells may simultaneously exhibit multiple mechanisms of TRAIL resistance [Ndozangue-Touriguine *et al.*, **2008**]. Thus eliminating TRAIL resistance by therapeutic agents is an attractive strategy for cancer therapy.

Azadirone can sensitize cells to TRAIL by modulating signaling molecules that regulate apoptosis [Song *et al.*, **2007**; Lavie *et al.*, **1971**; Nanduri *et al.*, **2003**]. Azadirone does sensitize cells to TRAIL through ROS-ERK-CHOP-mediated up-regulation of DR5 and DR4, down-regulation of cell survival proteins and up-regulation of proapoptotic proteins. The binding of TRAIL to DR5 and DR4 triggers cell death through two apoptotic pathways: the extrinsic pathway and the intrinsic pathway. The signaling initiated by the extrinsic pathway involves recruitment of Fas-associated death domain (FADD) and procaspase-8 in a death-inducing signaling complex (DISC) [Chaudhary *et al.*, **1997**]. Pro-caspase-8 is then processed into caspase-8 in the death-inducing signaling complex, which in turn induces apoptosis either by direct activation of caspase-3 or through cleavage of Bid to truncated Bid (tBid). The truncated Bid is then translocated to the mitochondria where the intrinsic pathway of apoptosis is initiated. Numerous signaling molecules are known to trigger DR5 and DR4 induction, including activation of mitogen-activated protein kinases (MAPKs) and the binding of CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) transcription factor to *DR5* promoter [Yamaguchi *et al.*, **2004**]. Reactive oxygen species (ROS), which are a by product of normal metabolic processes and generated by exogenous sources are integral components of cell signaling pathways. Important downstream mediators of ROS-induced signaling are the MAPKs, such as JNK, p38MAPK and ERK. ROS have also been shown to induce CHOP expression [Guyton *et al.*, **1996**]. Thus the agents that can modulate the expression of these signaling molecules can induce DR5 and DR4 expression and might offer potential as anticancer agents.

The main objective of this study were to determine whether azadirone can sensitize human cancer cells to TRAIL-induced apoptosis and if so, how azadirone mediates such an effect.

The effects of azadirone on the cytotoxic potential of TRAIL were assessed by cytotoxic assay, live/dead assay, clonogenic assay which was done to determine the ability of cells in a given population to undergo unlimited division and form colonies, Propidium Iodide (PI) staining assay for Luciferase activity, RNA isolation and RT-PCR, transcription with siRNA, Western Blot Analysis, assay for cell surface expression of DR5 and DR4, measurement of intracellular ROS by membrane-permeable DCFH-DA dye to measure intracellular ROS generation by flow cytometry.

The results showed that azadirone potentiates TRAIL-induced apoptosis in human colon cancer cells, induces expression of TRAIL receptors DR5 and DR4 which is required for sensitization of TRAIL-induced apoptosis, suppresses expression of cell survival proteins, induces expression of proapoptotic proteins, generates ROS for up-regulation of DR5 and DR4 upregulates DR5 and DR4 by activation of MAPKIs, azadirone-induced up-regulation of DR5 is mediated through induction of CHOP which is essential for potentiation of TRAIL-induced apoptosis by azadirone.

In summary, this study provides evidence for the sensitization of cells to TRAIL-induced apoptosis through ROS-, ERK-, and CHOP-mediated up-regulation of DR5 and DR4 by azadirone. This study also provides evidence for the down-regulation of cell survival proteins and for the up-regulation of proapoptotic proteins by azadirone. This study suggests that the combinations of azadirone and TRAIL are an effective approach for anticancer therapy.

3.5. Experimental

General experimental details are given in Chapter 2.

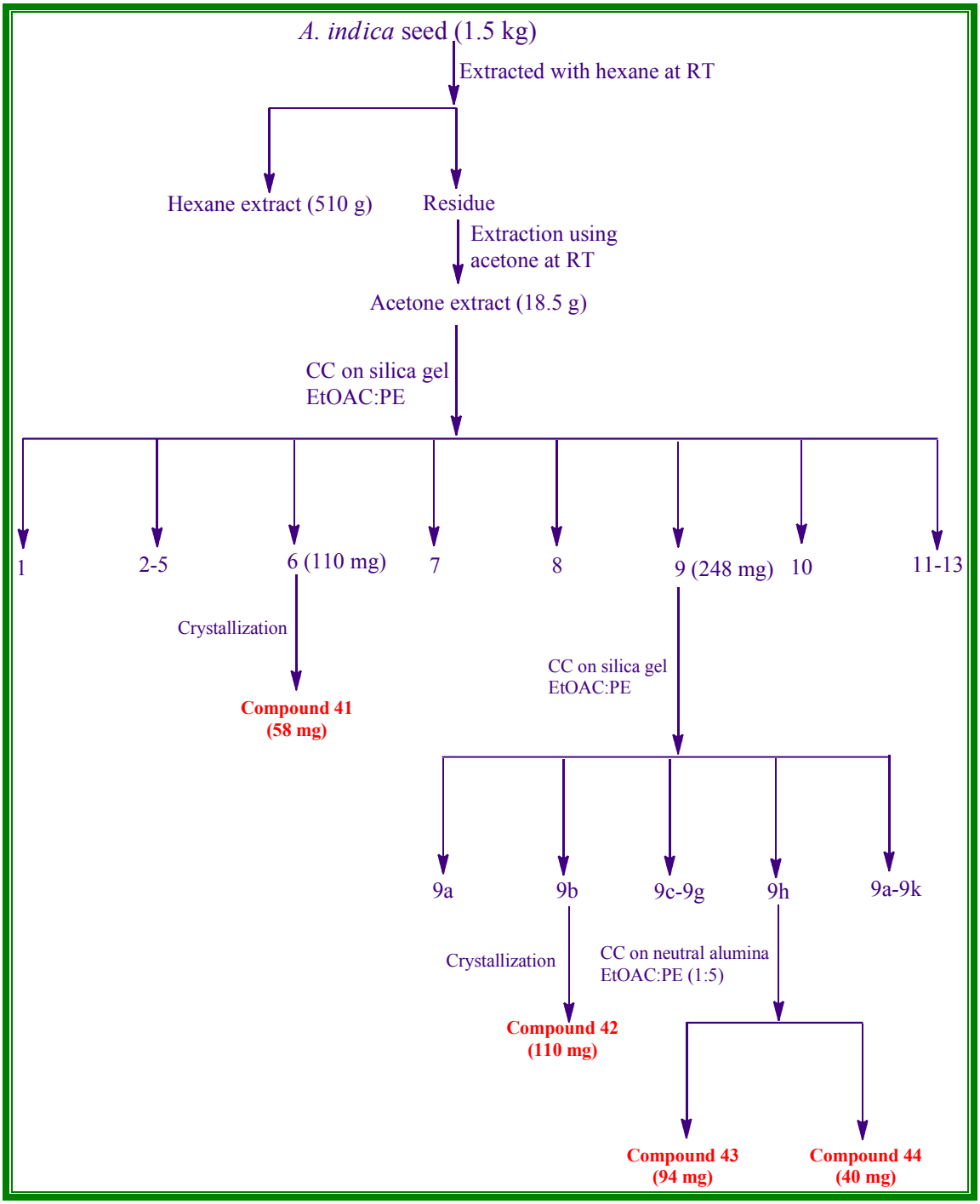


Figure 3.11 : Pictorial representation of chemical compounds from *A. indica* seeds

3.5.1. Extraction

Azadirachta indica seeds (1.5 kg) were purchased from local market in Trivandrum. It was then thoroughly cleaned, dried in an air oven for 24 hours at 50°C and coarsely powdered. The powdered seeds were subjected to extraction at room temperature with hexane followed by acetone. Each extract was then concentrated under reduced pressure. Acetone extract was subjected to column chromatography on silica gel (100-200 mesh) by eluting with ethyl acetate in hexane. The pictorial representation of the isolation procedure of compounds are depicted in **Figure 3.11** Extraction procedure of *A. indica* seed cake are shown **Figure 3.12**.

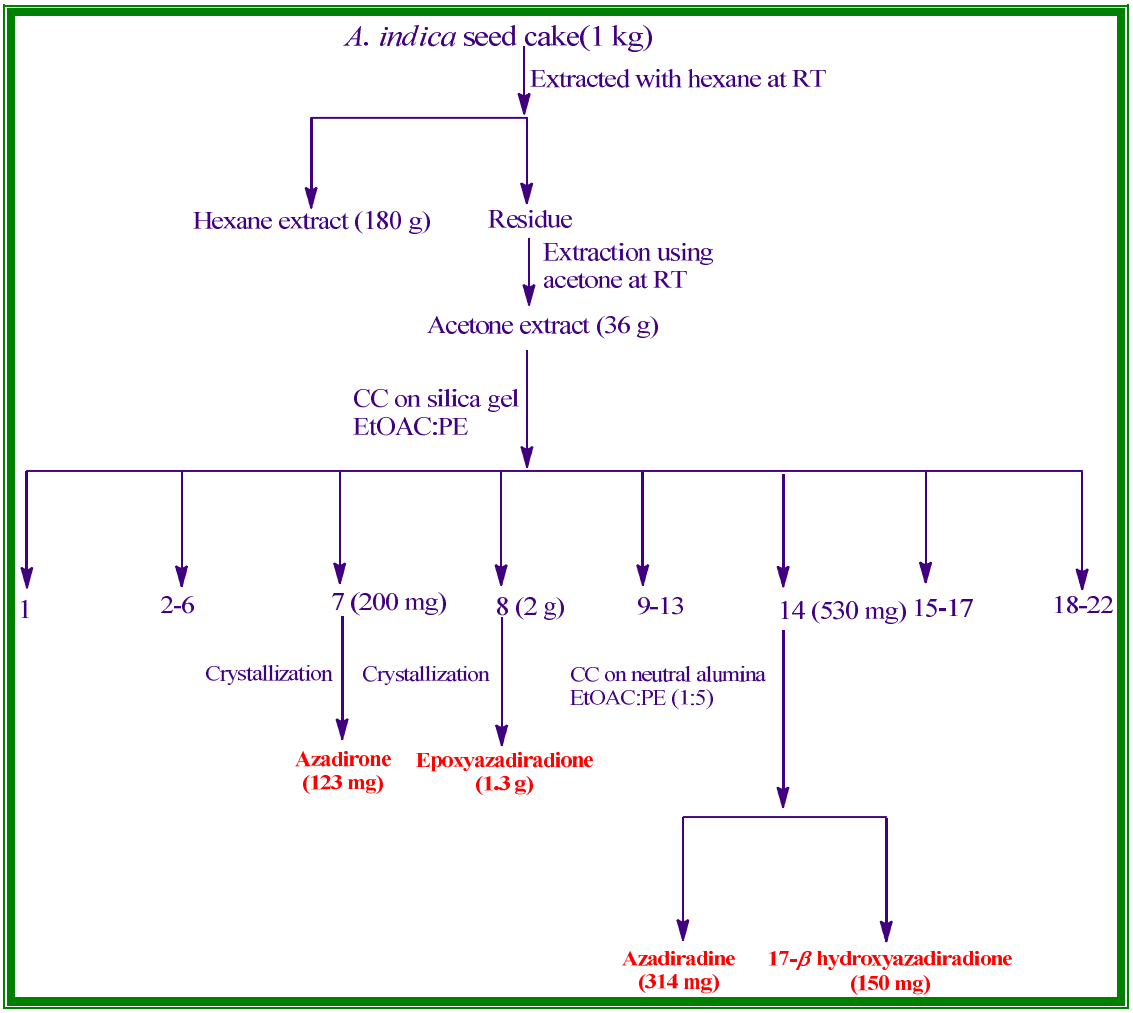


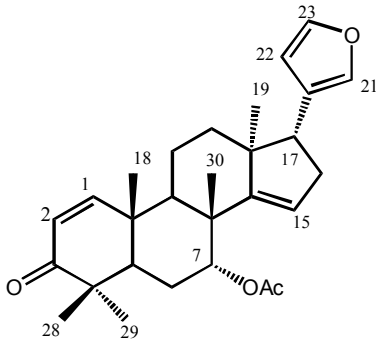
Figure 3.12 : Pictorial representation of chemical compounds from *A. indica* seed cake

3.5.2. Isolation of compound 41

Sixth fraction pool showing one major compound which was subjected to crystallization in dichloromethane-hexane mixtures to obtain compound 41 in 58 mg yield as colourless crystals. The compound 41 was identified as azadirone by comparing the IR, ^{1}H NMR and ^{13}C NMR spectral details shown below with those reported in literature.

FT-IR (KBr $\nu_{max,}$ cm^{-1}) : 2962, 2927, 1732 s (C=O), 1662 s (C=O), 869

^{1}H NMR (500 MHz, $CDCl_3$) : δ 7.38 (1H, *s*, H-21), 7.26 (1H, *d*, *J* = 10 Hz, H-23), 7.15 (1H, *d*, *J* = 10.5 Hz, H-1), 6.28 (1H, *d*, *J* = 0.5 Hz, H-22), δ 5.86 (1H, *d*, *J* = 10 Hz, H-2), 5.38 (1H, *d*, *J* = 1.5 Hz, H-15), 5.28 (1H, *t*, *J* = 2.5 Hz, H-7), 2.81 (1H, *m*, H-17), 2.43-1.64 (10H, *m*), 1.96 (3H, *s*, -OAc), 1.23 (3H, *s*, H-30), 1.20 (3H, *s*, H-19), 1.08 (6H, *s*, H-28 & H-29, 0.79 (3H, *s*, H-18)



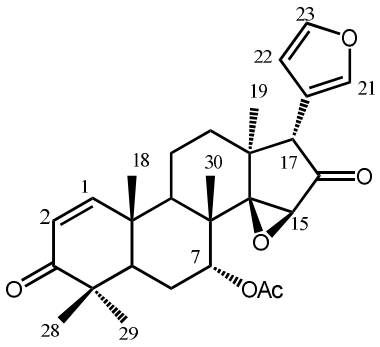
^{13}C NMR (125 MHz, $CDCl_3$) : δ 158.8 (C-1), 125.5 (C-2), 204.7 (C-3), 44.1 (C-4), 46.1 (C-5), 23.8 (C-6), 74.5 (C-7), 42.8 (C-8), 38.6 (C-9), 39.9 (C-10), 16.5 (C-11), 32.9 (C-12), 47.2 (C-13), 158.8 (C-14), 119.0 (C-15), 34.4 (C-16), 51.6 (C-17), 19.1 (C-18), 20.6 (C-19), 119.1 (C-20),

		111.1 (C-21), 139.7 (C-22), 142.6 (C-23), 21.2 (C-28), 21.3 (C-29), 27.3 (C-30) 170.2 (-<u>CO</u>CH_3), 21.2 (-CO<u>CH_3</u>), (C assignments were made as per literature)
FAB MS	:	437.94 $[M+H]^+$ (calcd for $C_{28}H_{37}O_4$, 436.26).
Melting point	:	130-132^oC

3.5.3. Isolation of compound 42

Ninth fraction pool submitted to column chromatography on silica gel yielded 11 fraction pool namely, 9a to 9k. 9b contain a single UV active compound. which on crystallization yielded compound 42 as colourless crystals. The compound 42 was identified as epoxyazadirone by comparing the IR, ^1H NMR and ^{13}C NMR spectral details shown below with those reported in literature.

FT-IR (KBr ν_{max}, cm^{-1})	:	2970, 2937, 1747 s (C=O), 1730 s (C=O), 1668 s (C=O), 1244, 1029, 829
^1H NMR (500 MHz, $CDCl_3$)	:	δ 7.56 (1H, *s*, H-21), 7.40 (1H, *s*, H-23), 7.18 (1H, *d*, *J* = 10 Hz, H-1), 6.23 (1H, *s*, H-22), 5.89 (1H, *d*, *J* = 10.5 Hz, H-2), 4.73 (1H, *t*, *J* = 2.5 Hz, H-7), 3.89 (1H, *s*, H-17), 3.41 (1H, *s*, H-15), 2.64-1.84 (8H, *m*), 2.03 (3H, *s*, OAc), 1.04 (3H, *s*, H-18), 1.22 (3H, *s*, H-30), 1.21 (3H, *s*, H-19), 1.08 (6H, *s*, H-28 & H-29)



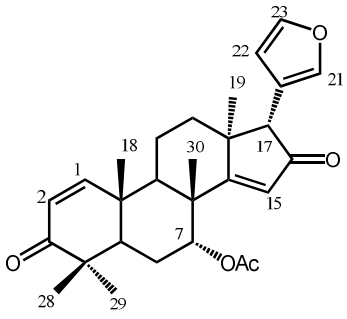
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 157.5 (C-1), 125.8 (C-2), 204.3 (C-3), 43.2 (C-4), 46.7 (C-5), 24.9 (C-6), 73.6 (C-7), 44.3 (C-8), 39.7 (C-9), 39.7 (C-10), 21.0 (C-11), 29.1 (C-12), 27.1 (C-13), 57.2 (C-14), 72.6 (C-15), 208.4 (C-16), 50.9 (C-17), 19.4 (C-18), 19.9 (C-19), 116.5 (C-20), 110.9 (C-21), 141.6 (C-22), 142.5 (C-23), 21.0 (C-28), 21.3 (C-29), 16.1 (C-30), 169.8 (-$\underline{CO}CH_3$), 24.2 (-CO$\underline{CH_3}$), (C assignments were made as per literature)
FAB MS	:	466.92 $[M]^+$ (calcd for $C_{28}H_{34}O_6$, 466.56).
Melting point	:	202-203^{o}C

3.5.4. Isolation of compound 43

Sub fraction pool 9h was repurified using column chromatography on neutral alumina using 20% ethyl acetate in hexane to yield compound 43 as colourless crystals and compound 44 as white solid. The compound 43 was identified as azadiradione by comparing the IR, ^{1}H NMR and ^{13}C NMR spectral details shown below with those reported in literature

FT-IR (KBr $\nu_{max,}$ cm^{-1})	:	2947, 1728 s (C=O), 1691 s (C=O), 1666 s (C=O), 1249, 1153, 873
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 7.47 (1H, *s*, H-21), δ 7.43 (1H, *t*, *J* = 1.5 Hz, H-23), δ

7.14 (1H, *d*, *J* = 10 Hz, H-1), δ 6.27 (1H, *d*, *J* = 1 Hz, H-22), δ 5.90 (1H, *d*, *J* = 10 Hz, H-2), δ 5.88 (1H, *s*, H-15), δ 5.32 (1H, *t*, *J* = 2 Hz, H-7), δ 3.42 (1H, *s*, H-17), δ 2.50-1.61 (8H, *m*), δ 1.95 (3H, *s*, -OAc), δ 1.34 (3H, *s*, H-30), δ 1.25 (3H, *s*, H-19), δ 1.09 (6H, *s*, H-28 & H-29), δ 1.03 (3H, *s*, H-18)



^{13}C NMR (125 MHz, $CDCl_3$) : δ 156.9 (C-1), 125.9 (C-2), 204.1 (C-3), 44.5 (C-4), 46.1 (C-5), 26.4 (C-6), 73.9 (C-7), 47.9 (C-8), 44.0 (C-9), 38.1 (C-10), 15.8 (C-11), 30.2 (C-12), 26.9 (C-13), 192.5 (C-14), 123.2 (C-15), 205.2 (C-16), 60.7 (C-17), 19.0 (C-18), 26.4 (C-19), 118.4 (C-20), 111.2 (C-21), 141.6 (C-22), 142.8 (C-23), 20.9 (C-28), 21.3 (C-29), 26.3 (C-30), 169.6 (-$\underline{CO}CH_3$), 23.4 (-$CO\underline{CH_3}$), (C assignments were made as per literature)

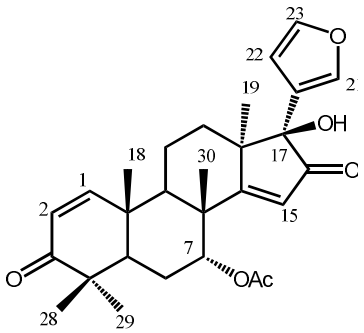
FAB MS : 450.68 $[M]^+$ (calcd for $C_{28}H_{34}O_5$, 450.56).

Melting point : 204-205^{o}C

3.5.5. Isolation of compound 44

After the isolation of azadiradione, compound 44 obtained as white solid on further elution with 20% ethyl acetate in petroleum ether. The compound 44 was identified as 17-β hydroxy azadiradione by comparing the IR, ^{1}H NMR and ^{13}C NMR spectral details shown below with those reported in literature.

FT-IR (KBr $\nu_{max,}$ cm^{-1})	:	3437 br (OH), 2960, 2931, 1738 s (C=O), 1709 s (C=O) 1669 s (C=O), 1242, 1030, 873
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 7.59 (1H, *s*, H-21), 7.42 (1H, *t*, *J* = 1.5 Hz, H-23), 7.17 (1H, *d*, *J* = 10 Hz, H-1), 6.41 (1H, *t*, *J* = 0.5 Hz, H-22), 5.90 (1H, *d*, *J* = 10 Hz, H-2), 5.78 (1H, *s*, H-15), 5.32 (1H, *t*, *J* = 3 Hz, H-7), 3.39 (1H, *t*, , *J* = 7 Hz, H-17), 2.84 (1H, *s*, -OH) 2.69-1.59 (8H, *m*), 1.95 (H, *s*, -OAc), 1.36 (3H, *s*, H-30), 1.25 (3H, *s*, H-19), 1.10, 1.09 (6H, *s*, H-28 & H-29), 0.98 (3H, *s*, H-18)
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 157.2 (C-1), 125.8 (C-2), 204.2 (C-3), 44.8 (C-4), 46.0 (C-5), 25.2 (C-6), 74.1 (C-7), 50.3 (C-8), 44.1 (C-9), 38.7 (C-10), 15.9 (C-11), 30.8 (C-12), 27.0 (C-13), 193.6 (C-14), 122.5 (C-15), 205.9 (C-16), 80.7 (C-17), 19.1 (C-18),



		27.0 (C-19), 120.3 (C-20), 109.6 (C-21), 141.4 (C-22), 142.7 (C-23), 20.9 (C-28), 21.2 (C-29), 23.5 (C-30), 169.7 (-<u>CO</u>CH_3), 22.3 (-CO<u>CH_3</u>), (C assignments were made as per literature)
HR-ESIMS *m/z*	:	489.2252 $[M+Na]^+$ (calcd for $C_{28}H_{34}O_6Na$, 489.2253)
Melting point	:	175-177^oC

3.6. Conclusion

We have carried out the isolation of epoxyazadiradione, azadiradione and 17*β*-hydroxy azadiradione, four major compounds found in *A. indica* seeds as well as seed cake. All the compounds were tested for their anticancer activity. Out of four compounds isolated, azadirone showed most potent anticancer activity. The molecular mechanism by which azadirone exerts anticancer effects has been studied for the first time. Based on the anticancer studies, we hypothesize that azadirone can sensitize tumour cells to TRAIL by modulating signalling molecules that regulate apoptosis. Results indicate that azadirone does sensitize tumour cells to TRAIL through ROSERK-CHOP–mediated up-regulation of DR5 and DR4, down-regulation of cell survival proteins, and up-regulation of proapoptotic proteins.

CHAPTER 4

Phytochemical Investigation on *Pygmacopremna herbacea* Roxb.

4.1. Introduction

Both man and animals depend on the plants for their very existence. Our environment is characterized by richly diversified plant life. In this regard, one of these genus is *Premna* belonging to Verbanaceae family. The *Premna* genus has been used in traditional systems of medicine for treating various ailments like rheumatism, asthma, dropsy, cough, fever and scrofulous disease [Thirumalai *et al.*, **2011**].

The genus *Premn*a consists of trees, shrubs, rarely herbs and climbers. The genus, now contains about 200 species worldwide, which are mainly distributed in tropical and subtropical Asia, Africa, Australia and the Pacific Islands.

4.2. Taxonomical classification

Division	: Tracheophyta
Order	: Lamianae
Family	: Verbenaceae
Genus	: *Premna*

4.3. Some important species of *premna* genus

Scientific Name	Common Name	Traditional uses
Premna tomentosa	Kolukkattai-thekku, Kattutekka, Kampu gummadi	Used as diuretic, for the treatment of diarrhoea
Premna latifolia	Aranika, Nappa, Pachamullai, Gohar	Diuretic, for the treatment of dropsy, fever, liver complaints etc.
Premna mucronata	Jay, Sriparna, Aggibatthu, Ganiyar, Bakar, Bakaru, Minni, Chedi munnai, Kondamanga	Used for curing boils
Premna obtusifolia	Agetha, Ustabunda, Agnimantha, Munna	Used for the treatment of fever, used as stomachic
Premna integrifolia/*Premna serratifolia*	Arni, Ganniari, Ganikarica, Appel, Bhutbhiravi	Used for the treatment of head ache, beriberi, used as eye lotion
Premna herbacea/Pygmacopremna herbacea	Bharangi, Gantubarangi, Sirutekku, Nelaneredu	Used for the treatment of dropsy, cough, asthma, fever, rheumatism and cholera

Chart 4.1. Important species of *Premna* genus and their uses

In order to understand the current status of knowledge on the genus *Premna*, a detailed literature survey of its various species found around the world was carried out. The compounds so far isolated from various *Premna* species are listed in the following table and the structures of important compounds are given in **Figure 4.1**.

Species	**Compounds**	**References**
Premna tomentosa	Icetexane-1	[Hymavathy *et al.*, **2009**]
	Icetexane-2	
	Icetexane-3	
	Icetexane-4	
	Coniferaldehyde	
	Syringaldehyde	
	Lupeol	
	Betulin	
	2-(4-methoxyphenyl)-2-butanone	
	Icetexatriene-1	[Sridhar *et al.*, **2012**]
	Icetexatriene-2	
	Acetoxy syringaldehyde	
	4-(4-methoxy phenyl)-2-butanone	
	Premnone A	[Chin *et al.*, **2006**]
	Premnone B	
	Premnone C	
	Ursolic acid	
Premna latifolia	Latifolionol	[Suresh *et al.*, **2011**]
	Dihydrolatifolionol	
	Latiferanol	
	Premnalatifolin A	[Suresh *et al.*, **2011**]

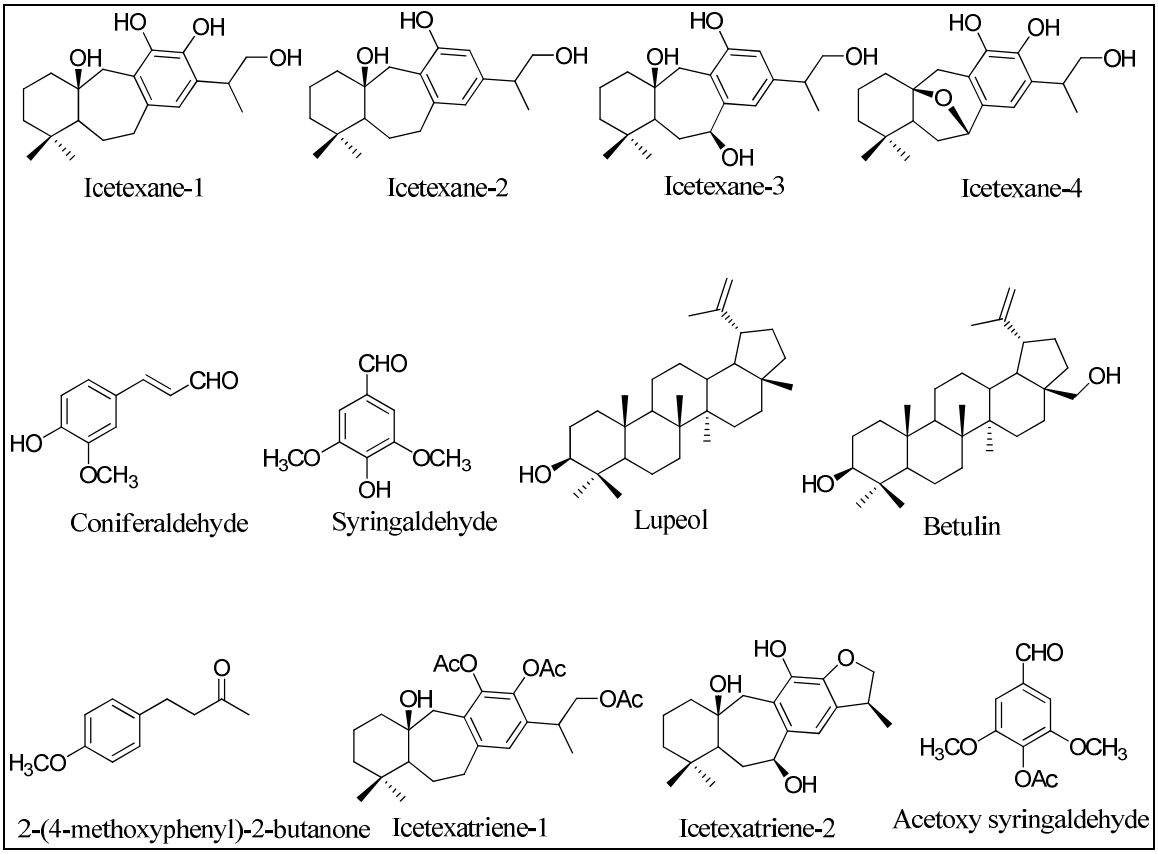
Premna obtusifolia	Obtusinone D	[Salae *et al.*, **2013**]
	Obtusinone E	
	Isopimara-7,15-dien-1β,3β-diol.	[Salae *et al.*, **2012**]
	Isopimara-7,15-dien-1β,19-diol	
	3-*epi*-5,15-rosadien-3α,11β-diol	
	Abietatrien-1β-ol	
	1β-hydroxyferruginol	
	6α,11,12-trihydroxy-7β,20-epoxy-8,11,13-abietatriene	
	5α,11,12-trihydroxy-6-oxaabieta-8,11,13-trien-7-one	
	11,12-dihydroxy-6,8,11,13-icetexatatraen-1-one	
	Obtusinone A	
	Obtusinone B	
	Obtusinone C	
	Lambertic acid	
	Ferruginol	
	17-*O*-methyl ferruginol	
	Sugiol	
	Royleanone	
	Horminone	
	Montbretrol	

	14-deoxycoleon	
	Ttaxodion	
	Arucadiol	
	12-hydroxy-6,7-secoabieta-8,11,13-triene-6,7-dial	
	Salvicanaraldehyde	
	5,6-dihydro-6α-hydroxysalviasperanol	
	Salviasperanol	
	11,12-dihydroxy-8,11,13-icetexatrien-1-one	
	4β,5β-dihydroxy-10-*epi*-eudesmane	
	4β,10β-dihydroxyaromadendrane	
Premna integrifolia/*Premna serratifolia*	1β,3α,8β-trihydroxy-pimara-15-ene	[Yadav *et al.*, **2010**]
	6α,11,12,16-tetrahydroxy-7-oxoabieta-8,11,13-triene	
	2α,19-dihydroxy-pimara-7,15-diene	
	Premnaodoroside A	[Hang *et al.*, **2009**]
	10-*O*-*trans*-*p*-methoxycinnamoylcatalpol	
	Premnadimer	[Yadav *et al.*, **2013**]
	4β-hydroxyasarinin-1-*O*-β-	

	glucopyranoside	
	Acteoside	[Bose *et al.*, **2013**]

Chart 4.2. List of isolated compounds from *Premna* genus

Figure 4.1. Structures of isolated compounds from *Premna* species



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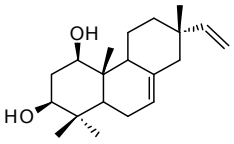
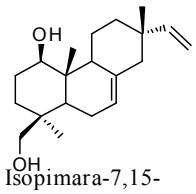
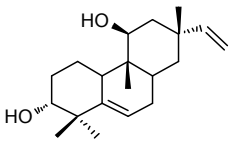


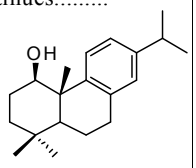
Isopimara-7,15-dien-1 ,3 -diol



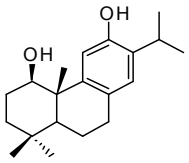
Isopimara-7,15-dien-1 ,19-diol



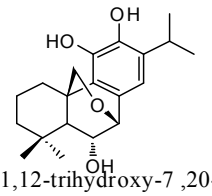
3-epi-5,15-rosadien-3 ,11β-diol



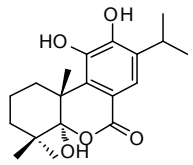
Abietatrien-1 -ol



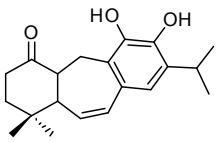
1 -hydroxyferruginol



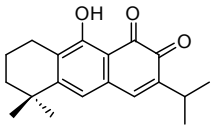
6 ,11,12-trihydroxy-7 ,20-epoxy-8,11,13-abietatriene



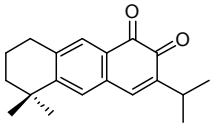
5 ,11,12-trihydroxy-6-oxaabieta-8,11,13-trien-7-one



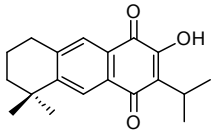
11,12-dihydroxy-6,8,11,13-icetexatatraen-1-one



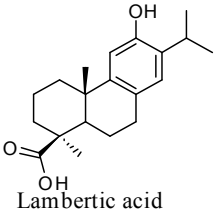
Obtusinone A



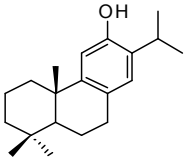
Obtusinone B



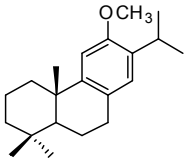
Obtusinone C



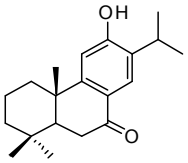
Lambertic acid



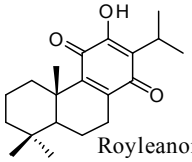
Ferruginol



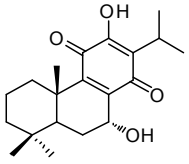
17-*O*-methyl ferruginol



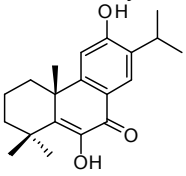
Sugiol



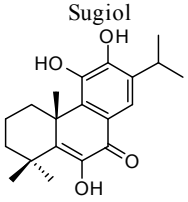
Royleanone



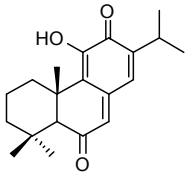
Horminone



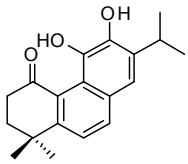
Montbretrol



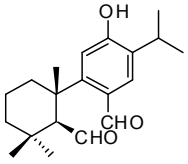
14-deoxycoleon



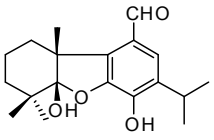
Taxodion



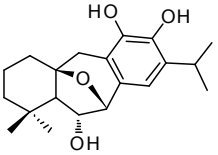
Arucadiol



12-hydroxy-6,7-secoabieta-8,11,13-triene-6,7-dial

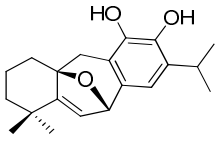


Salvicanaraldehyde

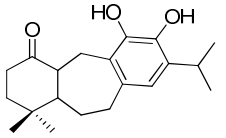


5,6-dihydro-6α-hydroxysalviasperanol

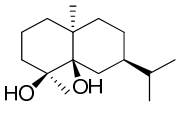
Figure 4.1 Continues.....



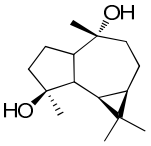
Salviasperanol



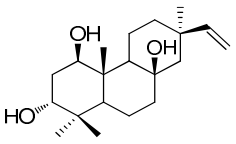
11,12-dihydroxy-8,11,13-icetexatrien-1-one



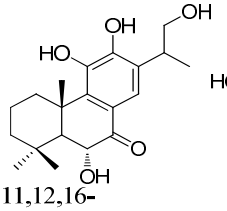
4β,5β -dihydroxy-10-epi-eudesmane



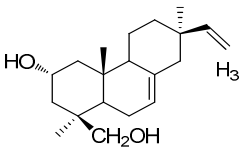
4β,10β -dihydroxyaromadendrane



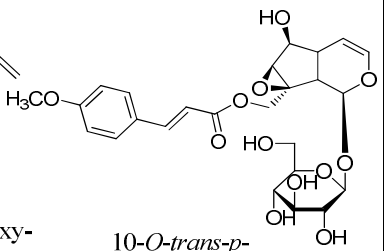
1 β,3 α,8 β -trihydroxy-pimara-15-ene



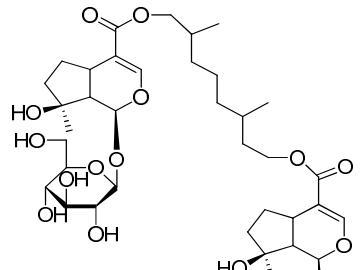
6 α,11,12,16-tetrahydroxy-7-oxoabieta-8,11,13-triene



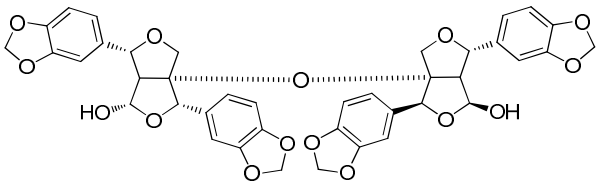
2 α,19-dihydroxy-pimara-7,15-diene



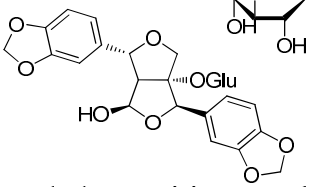
10-*O*-*trans*-*p*-methoxycinnamoylcatalpol



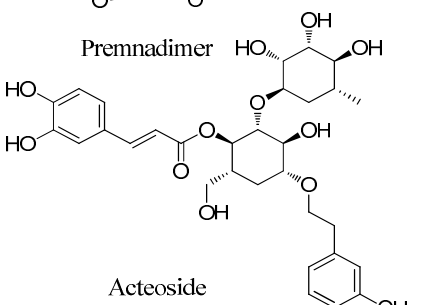
Premnaodoroside A



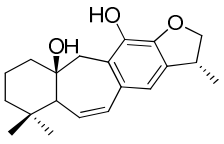
Premnadimer



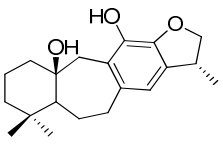
4β -hydroxyasarinin-1-*O*-β-glucopyranoside



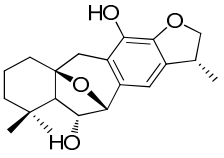
Acteoside



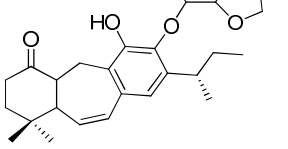
Latifolionol



Dihydrolatifolionol

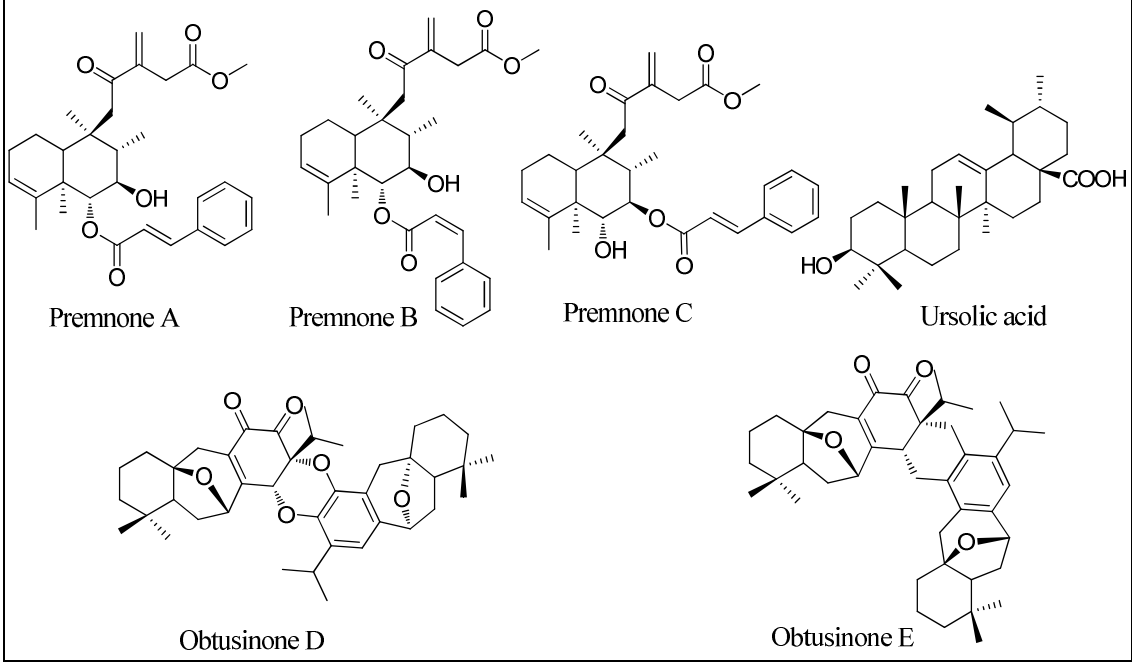


Latiferanol



Premnalatifolin A

Figure 4.1 Continues.....



4.4. *Premna herbacea* (*Pygmacopremna herbacea*)-Literature survey

Figure 4.2 : *P. herbacea* plant and root nodules

Premna herbacea Roxb. or *Pygmacopremna herbacea* (Roxb.) Moldenke. is an important medicinal plant belonging to the family Verbanaceae. It is commonly known as Siru Thekku in Tamil. This plant is usually found in the sub-tropical Himalayas and in

Assam, extending southwards through West Bengal, Bihar, Orissa into Deccan Peninsula and further into western Ghats. Roots and leaves of *P. herbacea* are used for the treatment of asthma and rheumatism and is called Bharangi. Often, roots are confused with those of *Clerodendrum serratum*.

Phytochemical studies on the *P. herbacea* has first carried out by Sankaram *et al.* who identified the compounds bharangin [Sankaram *et al.,* **1988**], bharanginin [Sankaram *et al.*, **1988**] and isobharangin [Sankaram *et al.*, **1989**]. In 2006 Murthy *et al*, reported the anti microbial activity of bharangin isolated from *P. herbacea* roots [Murthy *et al.*, **2006**]. Cytotoxic, diterpenoid quinonemethides were isolated from the roots of *P. herbacea* by Satish *et al.* [Satish *et al.*, **2011**]. Detailed bioactivity studies of phytochemicals from *P. herbacea* has not been reported earlier. Structures of compounds known from *P. herbacea* are shown in **Figure 4.3**. Therefore, the isolation of phytochemicals from *Pygmacopremna herbacea* roots and their anticancer evaluation was carried out and are explained in this chapter.

Figure 4.3. Structures of isolated Compounds from *P. herbacea*

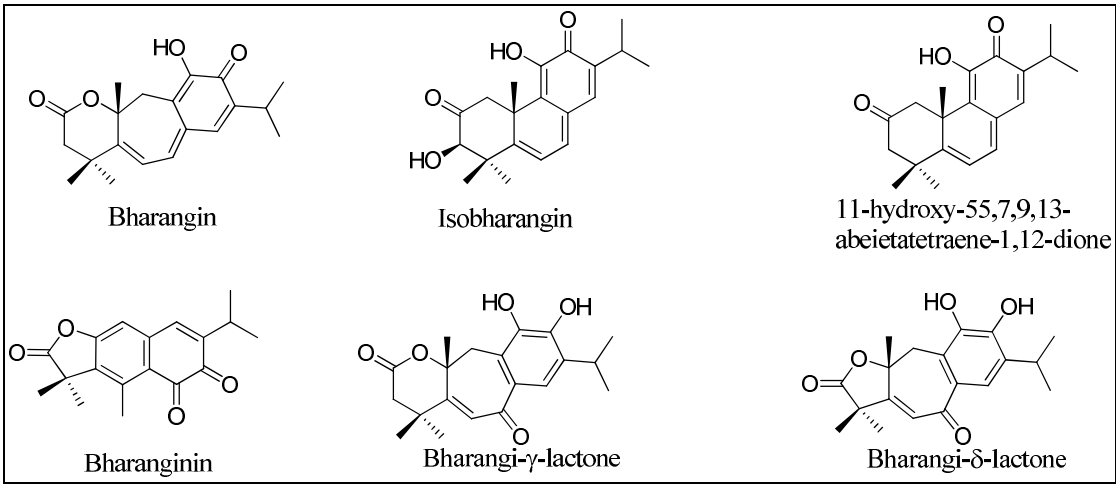
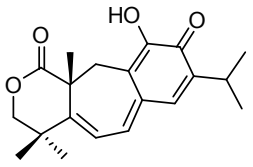
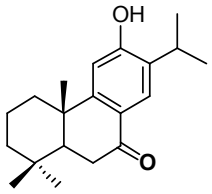


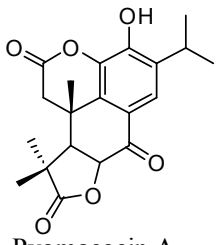
Figure 4.3 continues.....



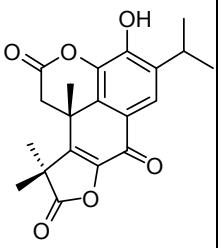
Neo bharangin



Sugiol



Pygmaeocin A



5,6-didehydopygmaeocin A

4.5. Isolation of chemical constituents from *P. herbacea* roots

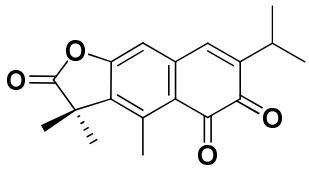
4.5.1. Extraction

2 kg of *Premna herbacea* roots were purchased from an authorized Ayurvedic medical plant vendor in Trivandrum. It was identified by Ayurvedic physicians and compared with herbarium sample by a taxonomist before use. The roots were dried in an air oven at 50^{o}C and then coarsely powdered. The powdered material was subjected to extraction using acetone (2.5 L X 3) at room temperature, which yielded 20 g of the crude extract. The crude extract was subjected to column chromatography on silica gel using petroleum ether-ethyl acetate mixtures. About 200 fractions (150 mL each) were collected. According to the similarities in TLC, they were pooled together to get 21 fraction pools.

4.5.2. Isolation of phytochemical constituents

Fraction pool 12 (100 mg) obtained was subjected to crystallization using ethyl acetate-hexane mixtures to yield the compound 45 (16 mg) as orange solid. IR spectrum of the compound 45 showed a strong absorption at 1679 cm^{-1} and 1660 cm^{-1} indicating the presence of two different keto carbonyl groups and a strong absorption at 1810 cm^{-1} was suggestive of lactone ring. A septet at δ 3.03, integrating for one proton and doublet at 1.18, integrating for six protons in ^{1}H NMR (**Figure 4.4**) spectrum indicated the presence of an isopropyl moiety. Singlets at δ 1.64 (6H) and 2.72 (3H) suggested the presence of three quaternary methyls. The peaks at δ 181.0, 180.7 and 179.6 in ^{13}C NMR (**Figure 4.5**) spectrum confirmed the presence of two ketones and a lactone

carbonyl respectively. Peaks in between δ 110.7-156.7 (8C) in ^{13}C NMR spectrum were due to alkenyl carbons. The mass spectrum showed the molecular ion peak at 299.11, which is the $(M+1)^+$ peak. Further, by comparison of the spectroscopic data with the values reported earlier it was confirmed that the compound 45 was bharanginin [Sankaram *et al.*, **1988**], the structure of which is shown below.



45

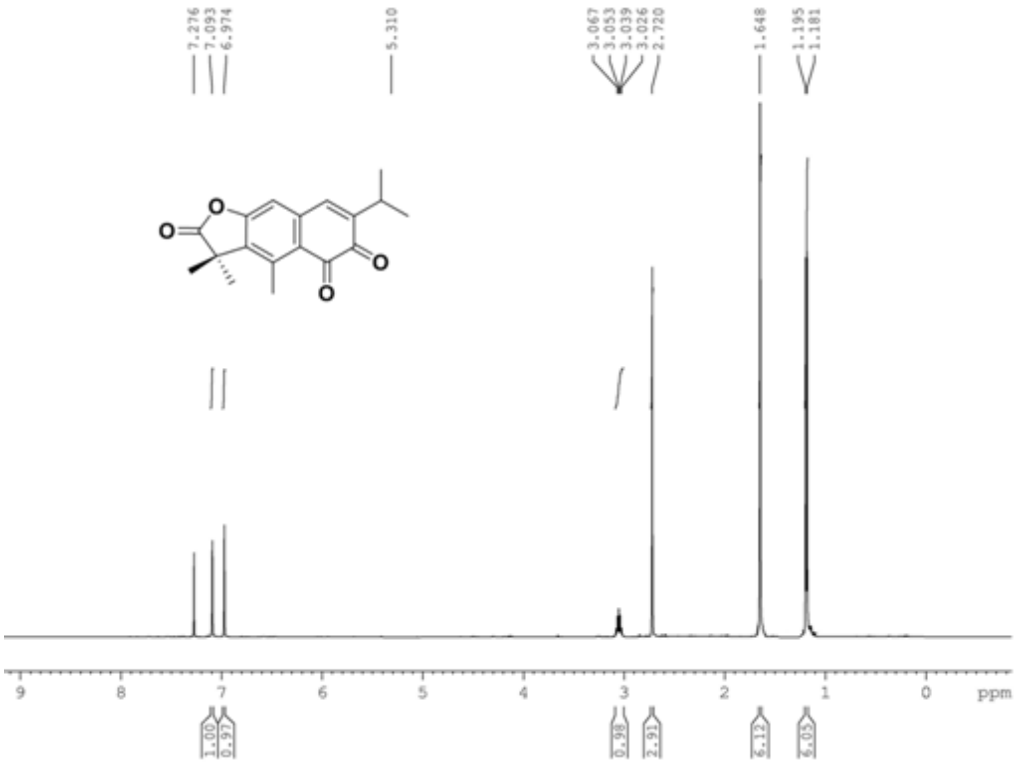


Figure 4.4: ^{1}H NMR spectrum of Bharanginin

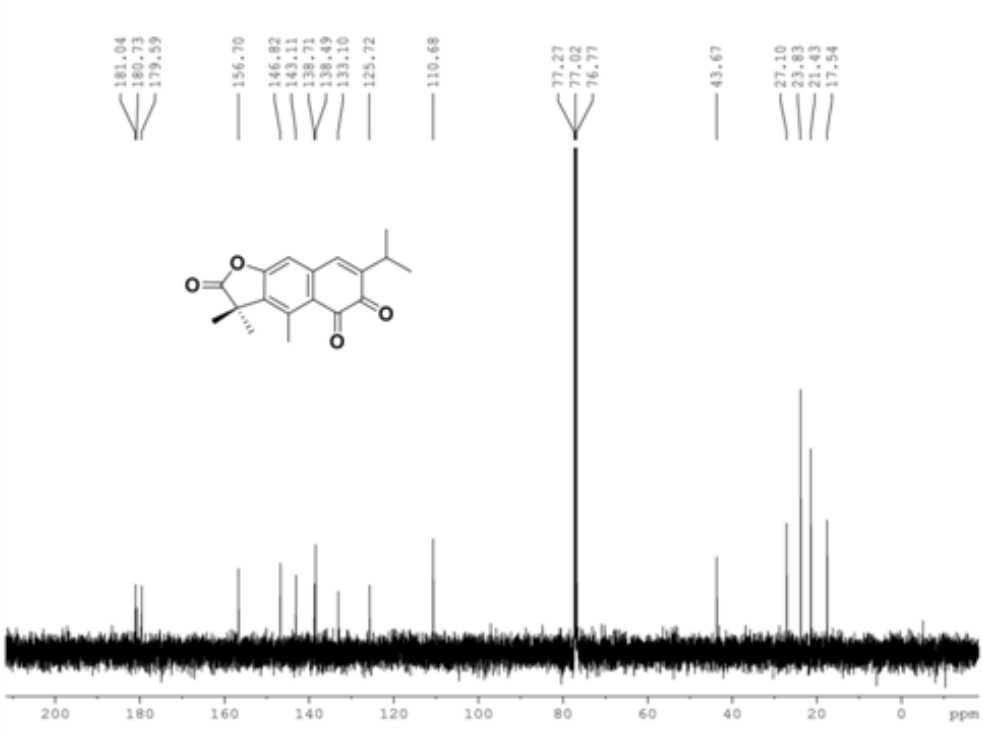
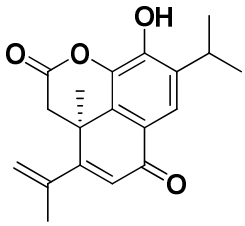


Figure 4.5: ^{13}C NMR spectrum of Bharanginin

Fraction pool 13 (50 mg) was subjected to column chromatography on silica gel using hexane-ethyl acetate (5:1), which yielded compound 46 (9 mg) as colourless crystals and compound 47 (8 mg) as red crystalline solid. IR spectrum of compound 46 showed a broad absorption at 3341 cm^{-1} indicating the presence of hydroxyl group. A strong absorption at 1654 cm^{-1} indicated the presence of enone moiety and a strong absorption at 1775 cm^{-1} suggested the presence of a lactone. A septet at δ 3.36, integrating for one proton and two doublets at 1.29 and 1.31, integrating for six protons in ^{1}H NMR (**Figure 4.6**) spectrum indicated the presence of an isopropyl moiety. Singlet appeared at δ 2.07 integrating for three protons in ^{1}H NMR spectrum, indicated the presence of a methyl group attached to the olefin. Two singlets appeared at δ 5.29 and δ 5.05 each integrating for two protons suggested the presence of olefinic protons. Singlets at δ 1.72 (3H) and 1.59 (3H) suggested the presence of two quaternary methyls. The peaks at δ 183.4 and 165.9 in ^{13}C NMR (**Figure 4.7**) spectrum, confirmed the presence of ketone and a lactone respectively. Peaks in between δ 118.4-162.7 (10C) in ^{13}C NMR spectrum due to alkenyl carbons. The mass spectrum showed that the molecular ion peak

at 313.1442 which is suggesting the $(M+1)^+$ peak. Structure of the compound 46 was unambiguously confirmed by X-ray crystal analysis (**Figure 4.8**) as that shown below and named as pygmaeocinin. To the best of our knowledge, the compound 46 was not reported from any natural source before. Hence the compound was found to be novel molecule of structure shown below.



46

The name, pygmaeocinin was chosen because of its structure was closely related with the chemical structure of pygmaeocin A, which was isolated from *P. herbacea* by Chen *et al* [Chen *et al.*, **1989**].

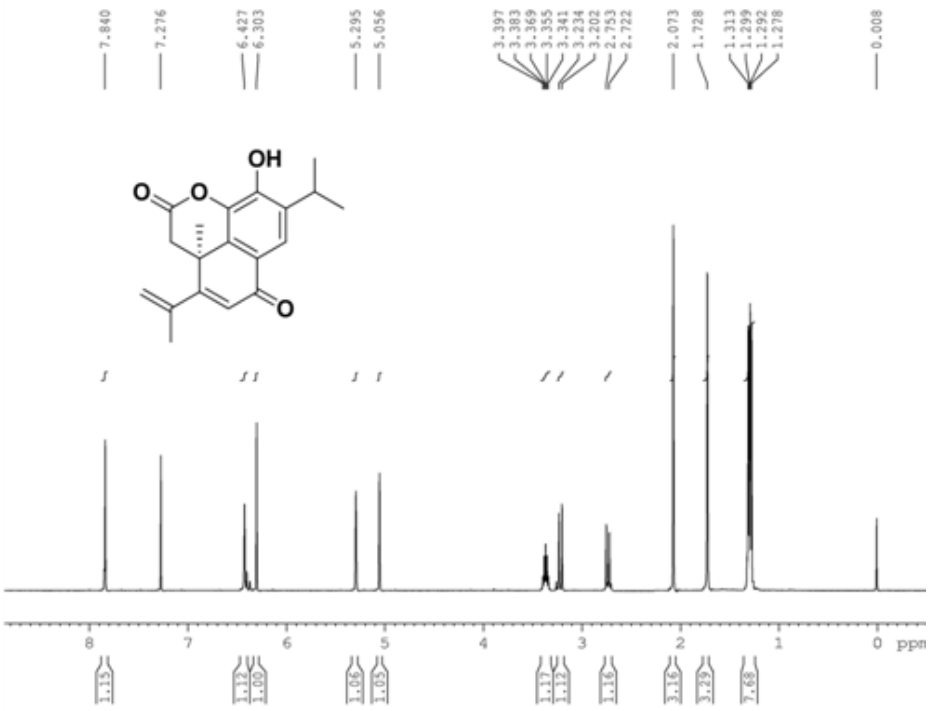


Figure 4.6: ^{1}H NMR spectrum of Pygmaeocinin

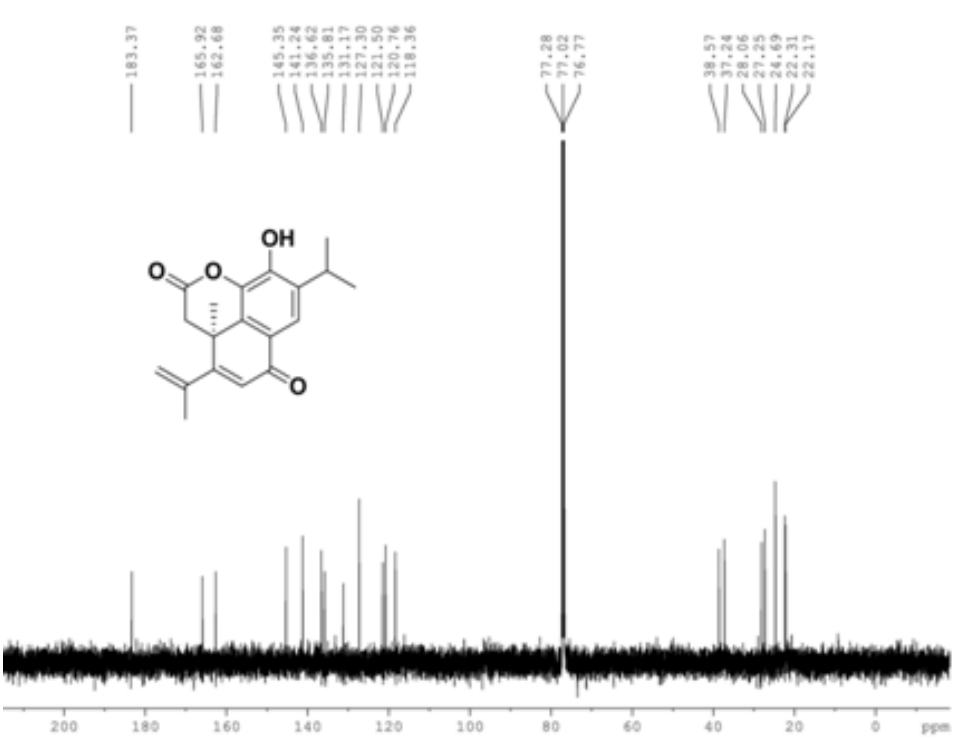


Figure 4.7: ^{13}C NMR spectrum of Pygmaeocinin

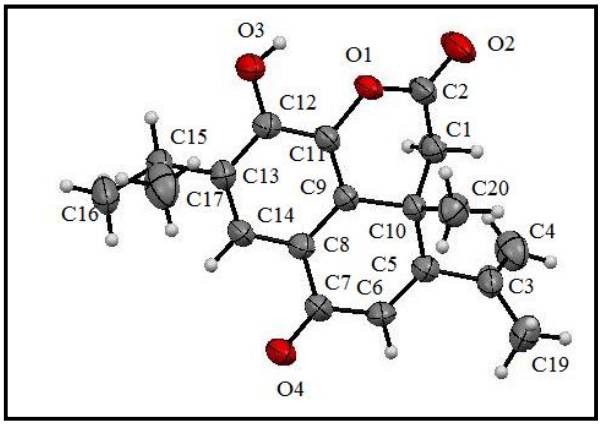
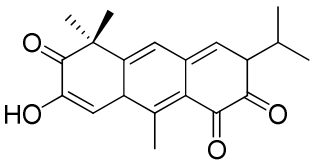


Figure 4.8 : ORTEP diagram of Pygmaeocinin

IR spectrum of compound 47 showed a broad absorption at 3379 cm^{-1} indicating the presence of hydroxyl group. Strong absorptions at 1652 cm^{-1}, 1650 cm^{-1} and 1657 cm^{-1} indicated the presence of three different keto carbonyl groups. A septet at δ 3.06, integrating for one proton and a doublet at δ 1.20, integrating for six protons in the ^{1}H NMR (**Figure 4.9**) spectrum indicated the presence of an isopropyl moiety. Singlets at

δ 1.63 (6H) and 2.78 (3H) suggested the presence of three quaternary methyls. The peaks appearing at δ 198.5, 182.5 and 181.6 in the ^{13}C NMR (**Figure 4.10**) spectrum confirmed the presence of three carbonyl carbons. Peaks in between δ 112.3-150.1 (10C) in the ^{13}C NMR spectrum due to alkenyl carbons were observed. The mass spectrum showed that the molecular ion peak is at 325.1443, which is the $(M+1)^+$ peak. Structure of the compound 47 was unambiguously confirmed by X-ray crystal analysis (**Figure 4.11**) as shown below and named as bharanginione. To the best of our knowledge, compound 47 has not been reported from any natural source before. Hence the compound 47, bharanginione was found to be novel molecule.



47

The name, bharanginione was chosen because of its structure was closely related with the chemical structure of bharanginin.

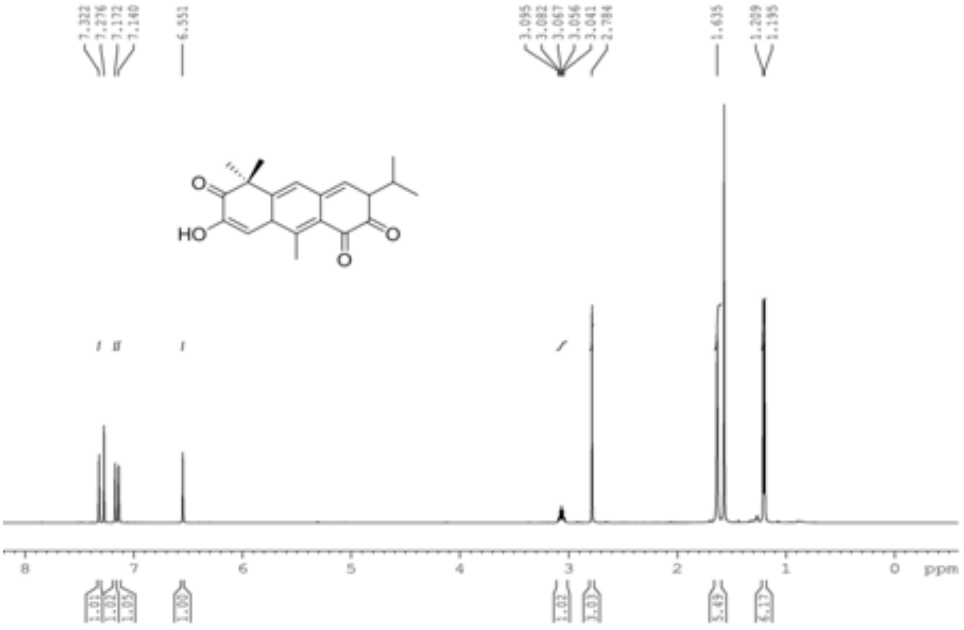


Figure 4.9: ^{1}H NMR spectrum of Bharanginione

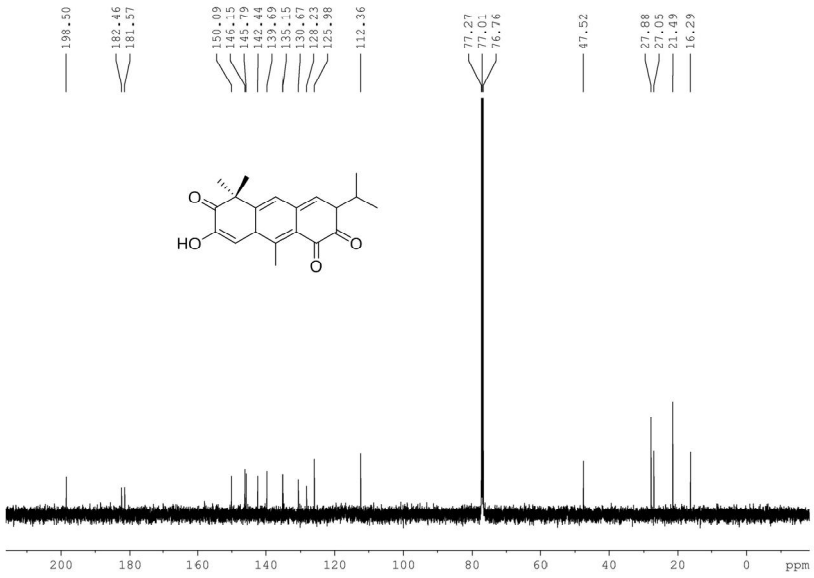


Figure 4.10: ^{13}C NMR spectrum of Bharanginione

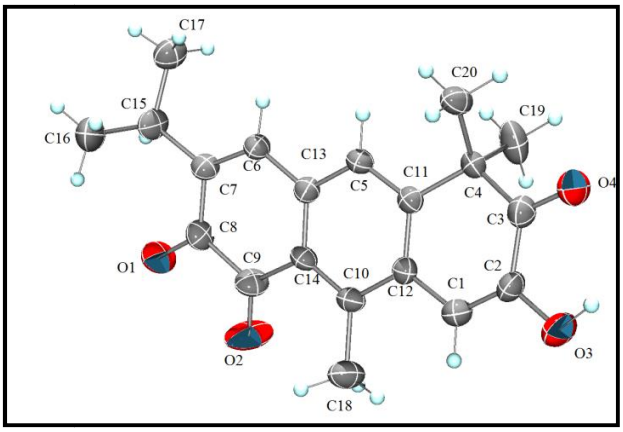
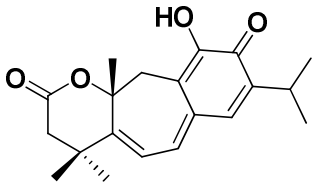


Figure 4.11 : ORTEP diagram of Bharanginione

Fraction pool 15 (1.2 g) was subjected to crystallization in dichloromethane-hexane, which afforded compound 48 (500 mg) as orange crystals. IR spectrum of the compound 48 showed a broad absorption at 3343 cm^{-1} indicating the presence of hydroxyl group. Strong absorptions at 1600 cm^{-1} and 1745 cm^{-1} indicated the presence of a keto carbonyl group and a lactone. A septet at δ 3.09, integrating for one proton and two doublets at 1.16 and 1.18, integrating for six protons in ^{1}H NMR (**Figure 4.12**)

spectrum indicated the presence of an isopropyl moiety. Singlets at δ 1.28 (3H), 1.34 (3H) and 1.43 (3H) suggested the presence of three quaternary methyls. The peaks appearing at δ 179.1 and 170.4 in ^{13}C NMR (**Figure 4.13**) spectrum, confirmed the presence of ketone and lactone functionalities respectively. Peaks in between δ 113.9-159.3 (8C) in the ^{13}C NMR spectrum suggested the presence of alkenyl carbons. The mass spectrum showed the molecular ion peak at 329.50, which is the $(M+1)^+$ peak. Further, by comparison of the spectroscopic data obtained with that reported in earlier, it was confirmed that the compound 48 was bharangin [Sankaram *et al.*, **1988**] and structure is shown below.



48

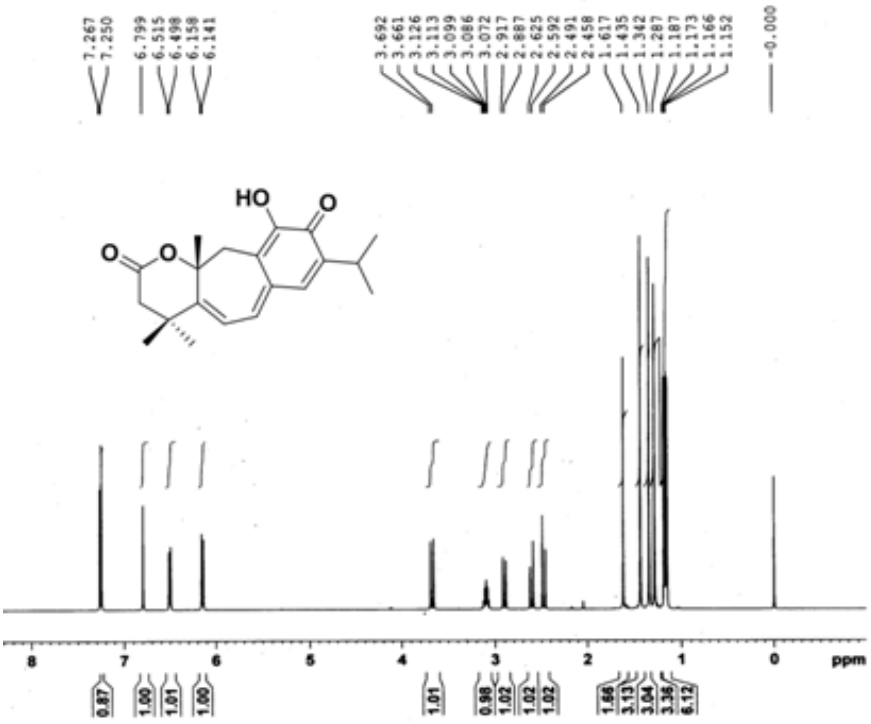


Figure 4.12: ^{1}H NMR spectrum of Bharangin

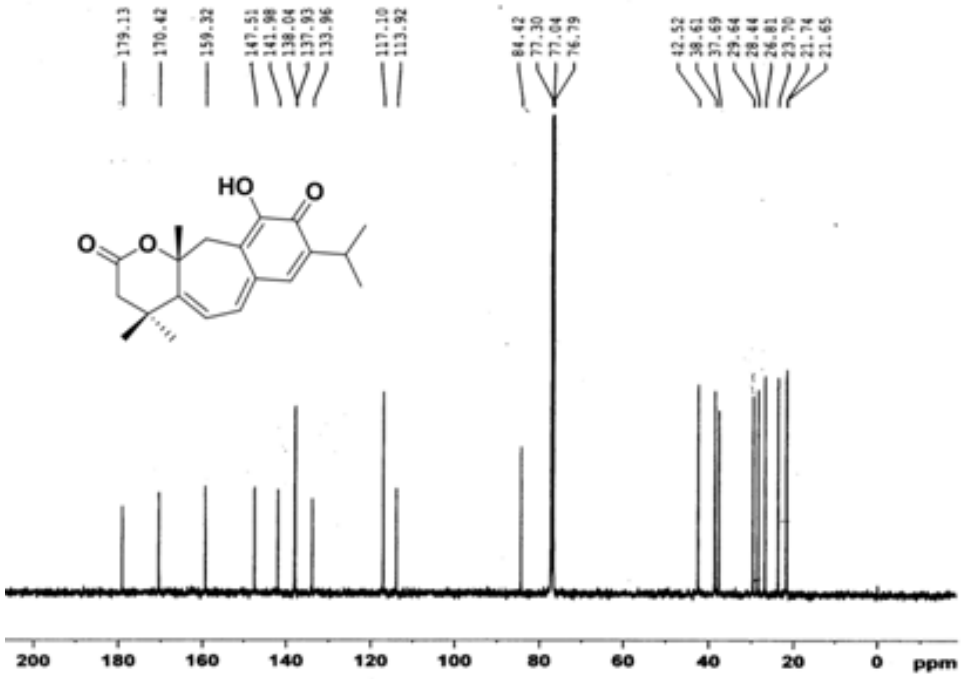
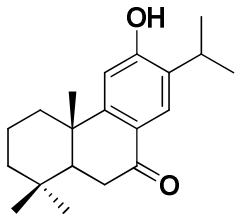


Figure 4.13: ^{13}C NMR spectrum of Bharangin

Fraction pool 16 (60 mg) was subjected to crystallization in dichloromethane in hexane, which afforded compound 49 (8 mg) as white solid. IR spectrum of the compound 49 showed a broad absorption at 3340 cm^{-1} indicating the presence of hydroxyl group and a strong absorption at 1710 cm^{-1} indicated the presence of a keto carbonyl group. A septet at δ 3.12, integrating for one proton and two doublets at 1.24 and 1.26, integrating for six protons in ^{1}H NMR (**Figure 4.14**) spectrum indicated the presence of an isopropyl moiety. Singlets at δ 0.92 (3H), 0.98 (3H) and 1.22 (3H) suggested the presence of three quaternary methyls. Peak appearing at δ 198.6 in the ^{13}C NMR spectrum confirmed the presence of a ketone moiety. Peaks in between δ 109.9-158.2 (6C) in ^{13}C NMR (**Figure 4.15**) spectrum as well as the presence of the protons at δ 7.91 and δ 6.69 suggested aromatic system. The mass spectrum showed the molecular ion peak at 301.21, which is the $(M+1)^{+}$ peak. Detailed analysis of the spectroscopic data of this compound obtained earlier from *Calocedrus formosana* suggested that the compound 49 was sugiol, structure is shown below. The literature on sugiol [Chao *et al.*, **2004**] further confirmed the structure.



49

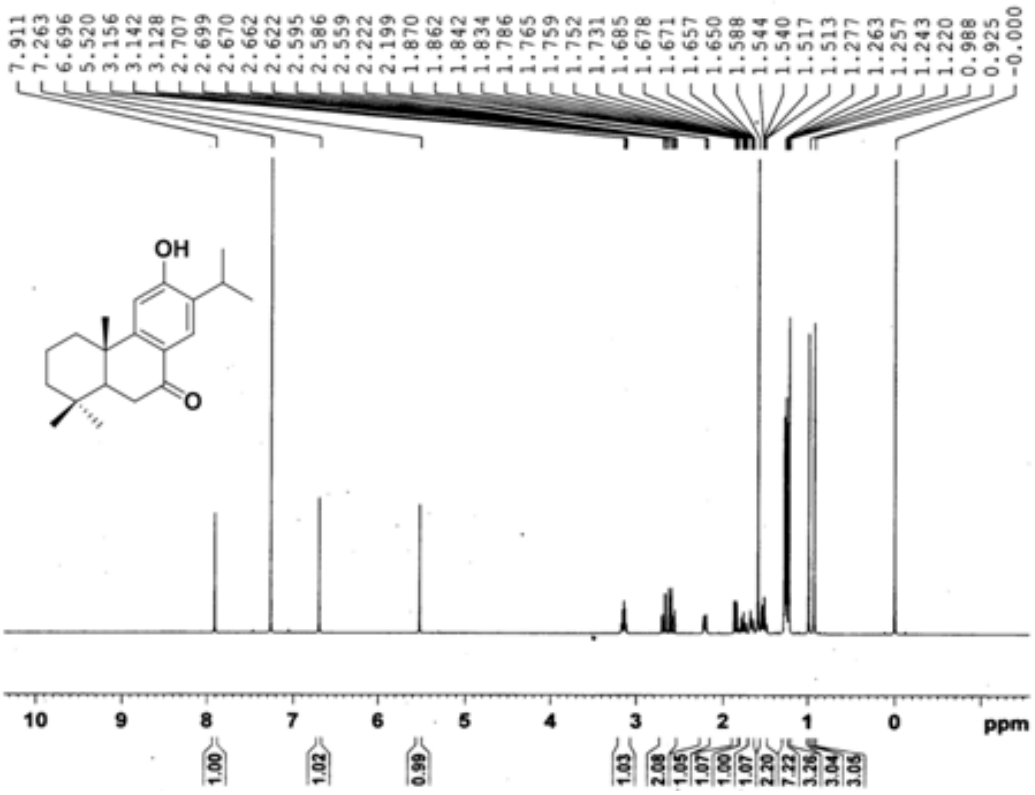


Figure 4.14: ^{1}H NMR spectrum of Sugiol

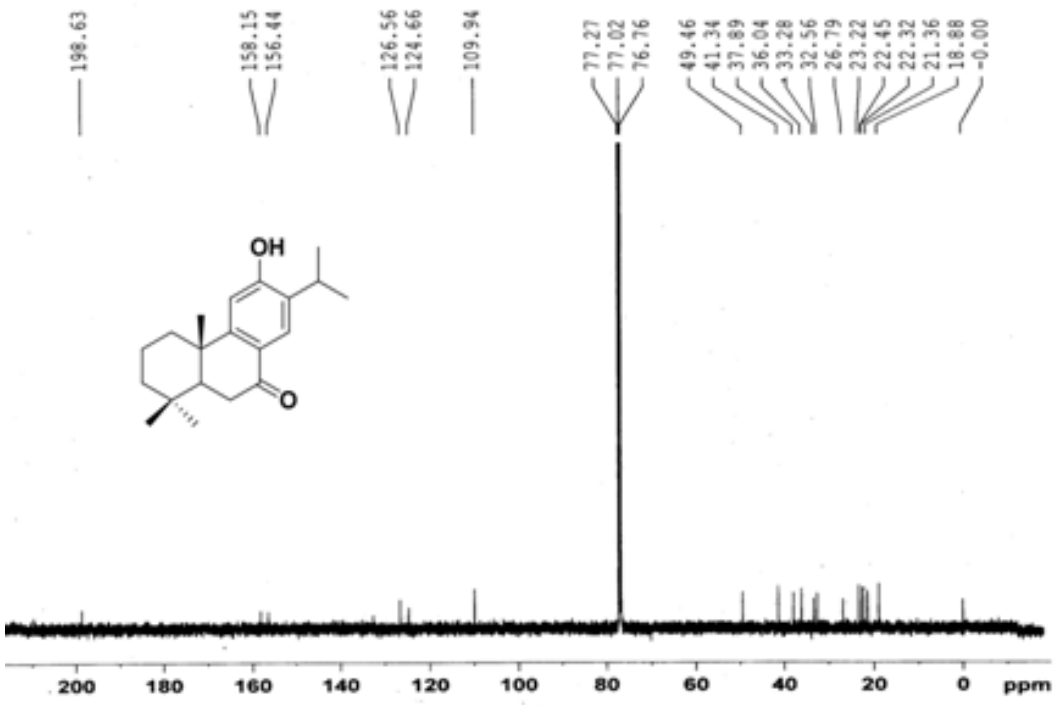


Figure 4.15: ^{13}C NMR spectrum of Sugiol

4.5.3. Anticancer activity studies of bharangin isolated from *P. herbacea* roots

Premna herbacea/ Pygmacopremna herbacea extract has exhibited anticancer activity *in vitro* and in tumor-bearing mouse models [Dhamija *et al*., **2013**]. Investigators have isolated a number of diterpenoids from the roots of this plant, one of which is bharangin [Sankaram *et al*., **1988**; Sathish *et al*., **2009**]. Literature reports on bharangin, a diterpenoid quinonemethide, indicates that it exhibites strong antimicrobial activity and reversed a drug-resistant phenotype of *Escherichia coli* cells carrying multidrug-resistant plasmids [Murthy *et al*., **2006**]. Extracts from the root nodules of *P.herbacea* exhibit anticancer and anti-inflammatory effects [Narayanan *et al.*, **2000**]. As bharangin is the major compound in *P. herbacea* roots, it was of interest to study whether the anticancer activity was due to it. Therefore a detailed anticancer activity study of bharangin was undertaken in collaboration with Dr. Bharath B. Aggarwal, university of Texas.

NF- κB is a pleiotropic transcription factor found in nearly all animal cell types. It plays a key role in regulating the immune response to infection. Aberrant regulation of NF-κB and the signaling pathways that control its activity is involved in inflammation,

drug resistance, radioresistance, tumor survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells. In resting cells, NF-κB stays in an inactive state in the cytoplasm as a heterotrimer consisting of the subunits p50, p65, and the inhibitory subunit IκBα. In response to a variety of inducers such as cytokines and growth factors, IκB kinase (IKK) is rapidly activated, which in turn phosphorylates IκBα at Ser^{32} and Ser^{36}. Phosphorylated IκBα then undergoes polyubiquitination and subsequent proteolytic degradation. After phosphorylation, p65 is released from the cytoplasm and translocated to the nucleus, where it binds to a specific DNA sequence and activates the transcription of more than 500 genes [Gupta *et al.*, **2010 a,b**]. Therefore, agents that can down-modulate this pathway have potential in cancer therapy. We postulated that bharangin is one such agent.

The study was aimed at determining whether bharangin has anticancer potential and, if so, via what mechanism. Several assays like electrophoretic mobility shift assay to examine NF-κB activation in leukemia and myeloma cells, western blot analysis of cytoplasmic, nuclear, and whole-cell extracts of untreated and treated cells, invasion assay to determine the effect of bharangin on TNF- α induced invasion, kinase assay to examine the effect of bharangin on IKK and TGF- α -activated kinase 1 (TAK1) activity, immunocytochemistry for NF-κB p65 localization to determine the effect of bharangin on nuclear translocation of p65, NF-κB-dependent reporter gene expression to find the effect of bharangin on induction of NF-κB-dependent reporter gene transcription by TNF- α, TNF receptor (TNFR) 1, TNFR-associated death domain (TRADD), TNFR-associated factor 2 (TRAF2), NF-κB–inducing kinase (NIK), TAK1/TAB1, IKK-β, and p65 were carried out. Assessment of apoptosis using a Live/Dead assay to assess the plasma membrane integrity and intracellular esterase activity, analysis of apoptosis using a phosphatidylserine externalization assay were also carried out. Molecular docking of bharangin into the NF- κB p65 to generate the energy-minimized three-dimensional structure of bharangin were also done.

The various finding are as follows. Bharangin suppresses NF-κB activation in tumor cells, inhibits NF-κB activation induced by carcinogens, tumor promoters, and other inflammatory agents. It was found that bharangin-induced inhibition of NF-κB

activation is not cell-type–specific, it inhibits constitutive NF-κB activation in myeloma cells, bharangin inhibits IκBα degradation, suppresses the expression of proteins involved in tumor cell proliferation, invasion, and angiogenesis, suppresses the proliferation of tumor cells, potentiates apoptosis induced by TNF-α and chemotherapeutic agents in tumor cells.

To summarize bharangin, a diterpenoid quinonemethide that can suppress pro-inflammatory pathways specifically. We found that bharangin suppresses nuclear factor (NF)-κB activation induced by pro-inflammatory cytokine, tumor promoter, cigarette smoke, and endotoxin. Inhibition of NF-κB activation was mediated through the suppression of phosphorylation and degradation of inhibitor of nuclear factor-κB (IκBα); inhibition of IκBα kinase activation; and suppression of p65 nuclear translocation, and phosphorylation. The diterpenoid inhibited binding of p65 to DNA. A reducing agent reversed the inhibitory effect, and mutation of the Cys38 of p65 to serine abrogated the effect of bharangin on p65-DNA binding. Molecular docking revealed strong interaction of the ligand with the p65 via two hydrogen bonds one with Lys37 and another with Cys38.

The inhibitory effect of bharangin on NF-κB activation was specific, in as much as binding of activator protein-1 and octameric transcription factor 1 to DNA was not affected. Suppression of NF-κB activation by this diterpenoid caused the downregulation of the expression of proteins involved in tumor cell survival, proliferation, invasion, and angiogenesis, leading to potentiation of apoptosis, suppression of proliferation, and invasion of tumor cells. Furthermore, the genetic deletion of p65 and mutation of p65 Cys^{38} residue to Ser abolished the affect of bharangin. Overall, the results demonstrate that bharangin specifically inhibits the NF-κB activation pathway by modifying p65 and inhibiting IkBα kinase activation and potentiates apoptosis in tumor cells.

4.6. Experimental

General experimental details are given in Chapter 2.

4.6.1. Extraction

P. herbacea seeds (2 kg) were purchased from registered Ayurvedic vendor in Trivandrum. Seeds were cleaned and dried in an air oven maintained at 50^{o}C. It was the coarsely powdered. The powdered material was subjected to extraction using acetone (2.5

L X 3) at room temperature. Acetone extract obtained (20 g) was subjected to column chromatography on silica gel (100-200 mesh) by eluting with ethyl acetate in hexane. The pictorial representation of the isolation procedure of compounds is depicted in **Figure 4.16**.

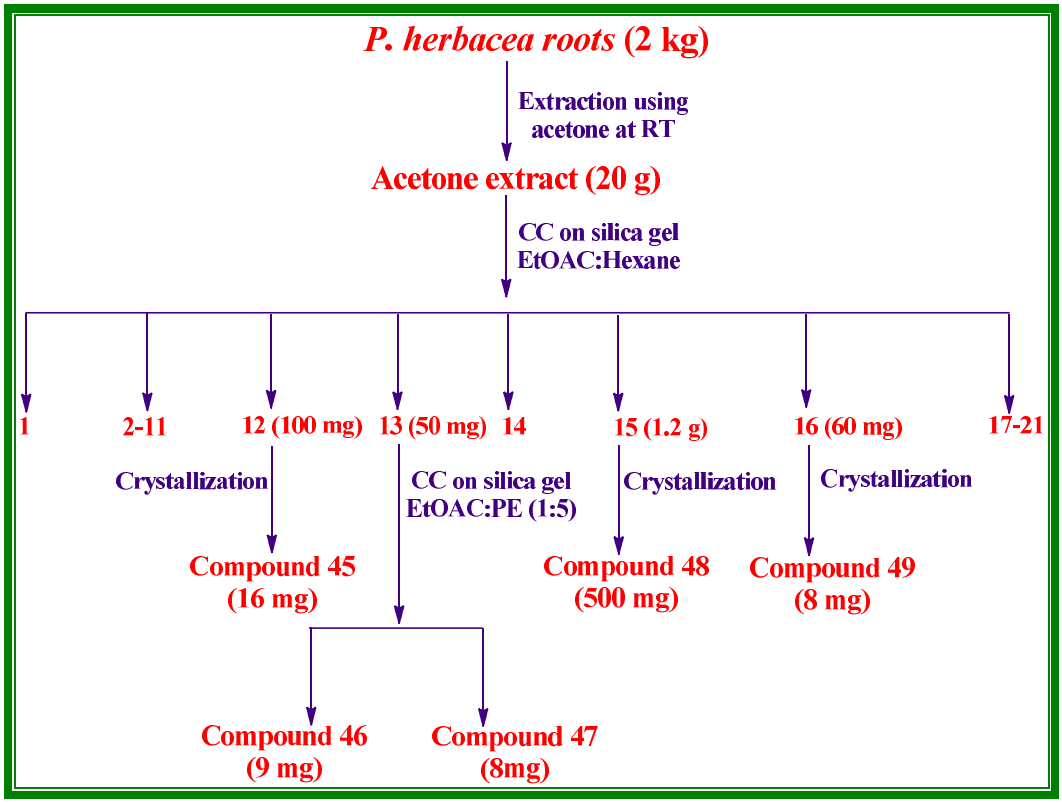


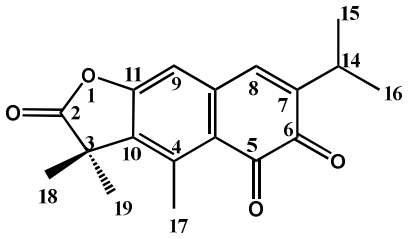
Figure 4. 16 : Pictorial representation of isolation of chemical compounds from *P. herbacea* roots

4.6.2. Isolation of compound 45

Twelfth fraction pool contained a UV active spot. The fraction pool was subjected to crystallization in dichloromethane-hexane mixture which yielded compound 45. The compound 45 was identified as bharanginin by comparing the IR, ^{1}H NMR and ^{13}C NMR spectral details shown below with those reported in literature.

FT-IR(NaCl $\nu_{max,}$ cm^{-1})	:	2925, 1810 s (C=O), 1679 s (C=O), 1660 s (C=O), 1594, 1464, 1191, 1005

^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 7.09 (1H, *s*, H-8), 6.97 (1H, *s*, H-9), 3.03 (1H, *m*, H-14), 2.72 (3H, *s*, H-17), 1.64 (6H, s, H-18 & H-19), 1.18 (6H, *d*, *J* = 7 Hz , H-15 & H-16)
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 179.6 (C-2), 43.7 (C-3), 146.8 (C-4), 181.0 (C-5), 180.7 (C-6), 143.1 (C-7), 138.5 (C-8), 110.7 (C-9), 138.7 (C-10), 156.7 (C-11), 133.1 (C-12), 125.7 (C-13), 27.1 (C-14), 21.4 (C-15 & C-16), 17.5 (C-17), 23.8 (C-18 & C-19) (C assignments were made as per literature)
FAB MS	:	299.11 $[M+H]^+$ (calcd for $C_{18}H_{19}O_4$, 299.12).
Melting point	:	135-137^{o}C

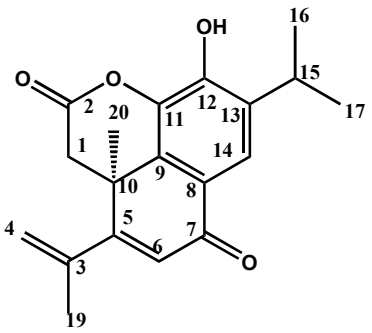


4.6.3. Isolation of compound 46

Thirteenth fraction pool showed two major UV active compounds. The fraction pool was subjected to purification using column chromatography on silica gel by eluting with 20% ethyl acetate in hexane, compound 46 was obtained as colourless crystals and compound 47 as red crystalline solid. The compound 46 was identified as pygmaeocinin. Structure of the compound 46 was unambiguously confirmed from single crystal X-ray analysis. To the best of our knowledge the compound was not reported from any natural sources before. Hence the compound was identified as novel.

FT-IR (NaCl $\nu_{max,}$ cm^{-1})	:	3341 br (-OH), 2923 1775 s (C=O), 1654 s (C=O), 1495, 1338, 1228, 1164

^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 7.84 (1H, *s*, H-14), 6.42 (1H, *br. s*. -OH), 6.30 (1H, *s*, H-6), 5.29 (1H, *s*, H-4), 5.05 (1H, *s*, H-4'), 3.35 (1H, *m*, H-15), δ 3.20 (1H, *d*, *J* = 16 Hz, H-1), 2.72 (1H, *d*, *J* = 16 Hz, H-1') , 2.07 (3H, *s*, H-19), 1.72, (3H, *s*, H-20), 1.29 (6H, *d*, H-16 & H-17)
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 38.6 (C-1), δ 165.9 (C-2), 141.2 (C-3), 118.4 (C-4), 145.4 (C-5), 120.8 (C-6), 183.4 (C-7), 121.5 (C-8), 162.7 (C-9), 37.2 (C-10), 136.6 (C-11), 141.2 (C-12), 135.8 (C-13), 127.3 (C-14), 28.1 (C-15), 24.7 (C-16 & C-17), 22.2 (C-19), 27.3 (C-20)
HR-ESIMS	:	313.1442 $[M+H]^+$ (calcd for $C_{19}H_{21}O_4$, 313.1440).
Melting point	:	160-162^{o}C



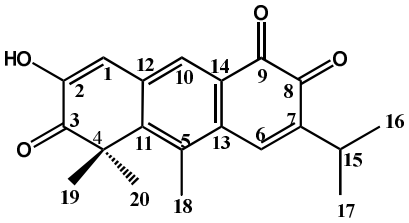
4.6.4. Isolation of compound 47

After the isolation of pygmaeocinin, compound 47 was obtained as red crystalline solid on further elution with 20% ethyl acetate in petroleum ether. The compound 47 was identified as bharanginione. Structure of the compound 47 was unambiguously confirmed from single crystal X-ray analysis. To the best of our knowledge the compound was not reported from any natural sources before. Hence the compound was identified as novel.

FT-IR (NaCl $\nu_{max,}$ cm^{-1})	:	3379 br. (-OH), 2926, 1657 s (C=O), 1652 s (C=O), 1650 s

(C=O), 1460, 1379, 1216, 1058

^{1}H NMR (500 MHz, $CDCl_3$) : δ 7.32 (1H, *s*, H-1), 7.17 (1H, *s*, H-6) 7.14 (1H, *s*, H-10), 6.55 (1H, *s*, -OH), 3.06 (1H, *m*, H-15), 2.78 (3H, *s*, H-18), 1.63 (6H, *s*, H-19 & H-20), 1.20 (6H, *d*, *J* = 7 Hz, H-16 & H-17).



^{13}C NMR (125 MHz, $CDCl_3$) : δ 112.4 (C-1), δ 150.1 (C-2), 198.5 (C-3), 47.5 (C-4), 135.1 (C-5), 139.7 (C-6), 142.4 (C-7), 181.6 (C-8), 182.5 (C-9), 126.0 (C-10), 128.2 (C-11), 145.8 (C-12), 146.2 (C-13), 130.7 (C-14), 27.9 (C-15), 27.1 (C-16 & C-17), 16.3 (C-18), 21.5 (C-19 & C-20)

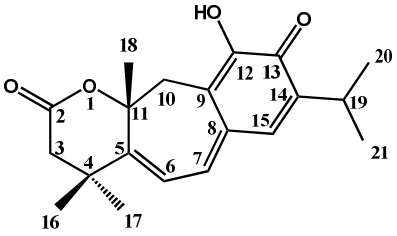
HR-ESIMS : 347.1262 $[M+Na]^+$ (calcd for $C_{20}H_{20}O_4Na$, 347.1259).

Melting point : 202-203^{o}C

4.6.5. Isolation of compound 48

Fraction pool 15 was subjected to crystallization in dichloromethane-hexane mixtures to afford compound 48 as red orange crystals. The compound was identified as bharangin using various spectroscopic techniques and literature.

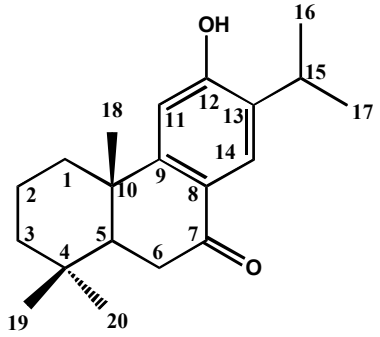
FT-IR(NaCl ν_{max}, cm^{-1}) : 3343 br. (-OH), 2965, 1745 s (C=O), 1600 s (C=O), 1527, 1348, 1222, 1169, 1069



^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 7.25 (1H, *s*, H-15), 6.80 (1H, *s*, -OH), 6.51 (1H, *d*, *J* = 8.5 Hz, H-7), 6.16 (1H, *d*, *J* = 8.5 Hz, H-6), 3.69 (1H, *d*, *J* = 15.5 Hz, H-3a), 3.11 (1H, *m*, H-19), 2.91 (1H, *d*, *J* = 15.5 Hz, H-3b), 2.62 (1H, *d*, *J* = 16 Hz, H-10a), 2.49 (1H, *d*, *J* = 16 Hz, H-10b), 1.43 (3H, *s*, H-18), 1.34 (3H, *s*, H-16), 1.28 (3H, *s* H-17), 1.17 (6H, *d*, H-20 & H-21)
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 179.1 (C-2), 42.5 (C-3), 37.7 (C-4), 159.3 (C-5), 117.1 (C-6), 137.9 (C-7), 134.0 (C-8), 113.9 (C-9), 38.6 (C-10), 84.4 (C-11), 147.5 (C-12), 170.4 (C-13), 142.0 (C-14), 138.0 (C-15), 29.6 & 28.4 (C-16 & C-17), 26.8 (C-18), 23.7 (C-19), 21.7 & 21.7 (C-20 & C-21) (C assignments were made as per literature)
FAB MS	:	329.50 $[M+H]^+$ (calcd for $C_{20}H_{25}O_4$, 329.17).
Melting point	:	213-214^{o}C

4.6.6. Isolation of compound 49

Sixteenth fraction pool contain single UV active compound. The fraction pool was subjected to crystallization in dichloromethane-hexane mixture which was yielded compound 49. The compound 49 was identified as sugiol by comparing the IR, ^{1}H NMR and ^{13}C NMR spectral details shown below with those reported in literature.



FT-IR (NaCl $\nu_{max,}$ cm^{-1}) : 3340 br. (-OH), 2963, 1710 s (C=O), 1529, 1248, 1222, 1078, 1069

^{1}H NMR (500 MHz, $CDCl_3$) : δ 7.91 (1H, *s*, H-14), 6.69 (1H, *s*, H-11), 5.52 (1H, s, -OH), 3.14 (1H, *m*, H-15), 2.67, (1H, *dd*, J_1 = 4.7 Hz, J_2 = 1.8 Hz, H-6a), 2.19, (1H, *dd*, J_1 = 13 Hz , J_2 = 1.8 Hz, H-6b), 2.55-δ 1.49 (7H, H-1-H-3 & H-5), 1.27 (6H, *d*, H-16 & H-17), 1.22 (3H, *s*, H-18), 0.98 (3H, *s*, H-19), 0.92 (3H, *s*, H-20)

^{13}C NMR (125 MHz, $CDCl_3$) : δ 37.9 (C-1), 18.9 (C-2), 41.3 (C-3), 33.3 (C-4), 49.5 (C-5), 36.0 (C-6), 198.6 (C-7), 124.7 (C-8), 156.4 (C-9), 41.3 (C-10), 109.9 (C-11), 158. 2 (C-12), 131.3 (C-13), 126.6 (C-14), 26.8 (C-15), 22.5 & 22.3 (C-16 & C-17), 26.8 (C-18), 21.4 & 18.9 (C-19 & C-20) (C assignments were made as

per literature)

FAB MS : 301.21 $[M+H]^+$ (calcd for $C_{20}H_{29}O_2$, 301.21).

Melting point : 290-292^oC

4.7. Conclusion

Three known compounds and two novel compounds were isolated and identified from the roots of *P herbacea*. Bharangin, bharanginin and sugiol are the reported compounds isolated from this plant. Two novel molecules named as, pygmaeocinin and bharanginione were also isolated and the structures were unambiguously confirmed by single crystal X-ray analysis. Anticancer activity of bharangin and its mode action has also been evaluated.

Summary and conclusion

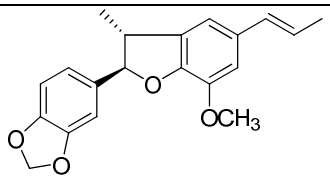
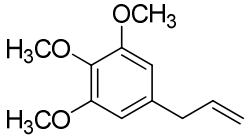
Medicinal plants and spices play an important role in the daily life of human beings. Medicinal plants are rich sources of biologically active compounds and have served as leads for the development of novel drugs used in modern systems of medicine. It has been estimated that more than 50% of world population still rely on crude plant drug preparations. Traditional systems of medicines like Ayurveda, Siddha and Unani which use many medicinal plants are especially popular in India. As a large number of these medicinal plants are unexplored phytochemically, the scope of research in this area is very high. This thesis involves the phytochemical study of one of the most important spices viz., *Myristica fragrans* as well as two important medicinal plants, viz., *Azadirachta indica* and *Pygmacopremna herbacea.*

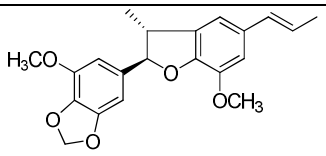
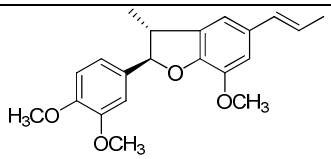
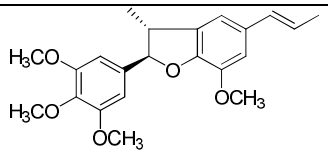
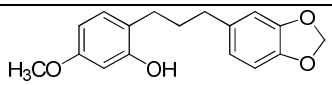
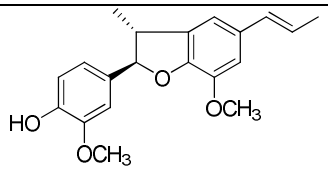
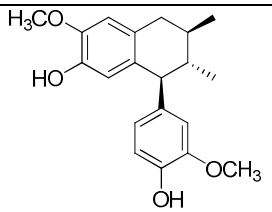
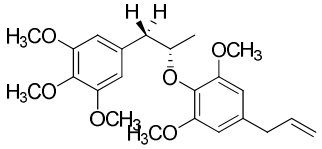
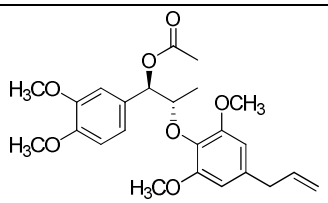
Chapter 1 gives an overview of natural products with special reference to the chemical diversity of secondary metabolites and their classification. A brief description of drugs from natural products especially for cancer as well as the biological significance of reactive oxygen species also given. The use of spices as antioxidants is also outlined in this chapter.

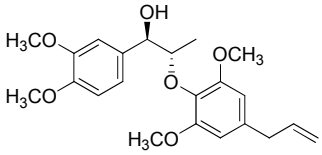
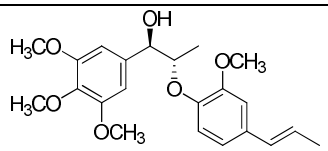
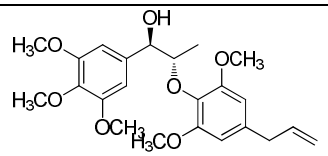
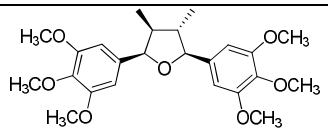
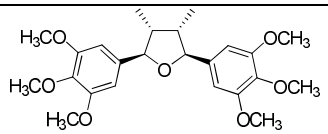
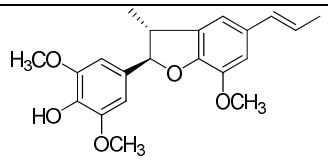
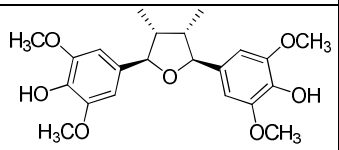
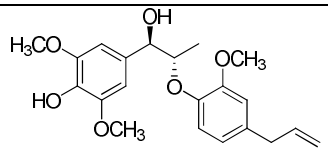
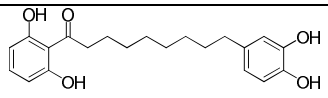
Chapter 2 deals with the phytochemical investigation on the spice plant *Myristica fragrans*. Here, a brief outline of the genus *Myristica* is given along with the detailed survey of literature on the phytochemistry of *M. fragrans*. The Chapter 2 is divided into two; part A and part B. In first part of the chapter, the isolation of chemical constituents from various parts of *M. fragrans* fruit is described. The pericarp, seed and mace from the fruit were separated and used for detailed studies. Ten compounds have been isolated from the fruit pericarp of *M. fragrans*, viz., acuminatin, licarin A, elemicin, 7-methoxy-3-methyl-5-((*E*)-prop-1-enyl)-2-(3,4,5-trimethoxyphenyl)-2,3-dihydrobenzofuran, licarin B, guaiacin, *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-acetoxy- 3,4,3',5'- tetramethoxy-8-*O*-4'-neolignan, *erythro*-(*7S,8R*) -$\Delta^{8'}$-7-hydroxy -3,4,3',5'- tetramethoxy- 8-*O*-4'-neolignan, virolane and surinamensin. Of these, the latter two are being isolated for the first time from *M. fragrans*. Similarly, *M. fragrans* mace and seed were subjected to phytochemical investigations. Twelve known compounds viz., licarin B, elemicin, acuminatin, (*2S,3R*) -7-methoxy-3-methyl-5-((*E*)-

prop-1-enyl)-2-(3,4,5-trimethoxyphenyl)-2,3-dihydrobenzofuran, licarin A, guaiacin, (*8R*)-$\Delta^{8'}$-3,4,5,3', 5'-pentamethoxy-8-*O*-4'-neolignan, *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-acetoxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan, *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-hydroxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan, raphidecursinol B, *erythro*-(*7S,8R*)-$\Delta^{8'}$-4,7-dihydroxy-3,5,3'-trimethoxy-8-*O*-4'-neolignan, *erythro* surinamensin were obtained from mace. Seven compounds namely, trimyristin, licarin A, guaiacin, *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-acetoxy-3,4,3', 5'-tetramethoxy-8-*O*-4'-neolignan, *erythro*-(*7S,8R*)-$\Delta^{8'}$-7-hydroxy-3,4,3', 5'-tetramethoxy-8-*O*-4'-neolignan, raphidecursinol B and malabaricone C were isolated from seed kernels.

Part B of the second chapter outlines the isolation of chemical constituents from the *M. fragrans* stem bark. This is the first study being reported on the bark of *M. fragrans* which has resulted in the isolation and characterization of the compounds viz., licarin B, elemicin, (*2S,3R*)-7-methoxy-3-methyl-5-((*E*)-prop-1-enyl)-2-(5-methoxy,3,4-methylenedioxyphenyl)-2,3-dihydrobenzofuran, licarin A, fragransin B_1, odoratisol A, 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'-tetramethoxy-8-*O*-4'-neolignan and surinamensin. In addition, grandisin and *(7S,8S, 7'R,8'R)*-3,3',4,4',5,5'-hexamethoxy-7,7', 8,8'-lignan have also been isolated from *M. fragrans* for the first time. The structures of the compounds isolated from different parts of *M. fragrans* are given below.

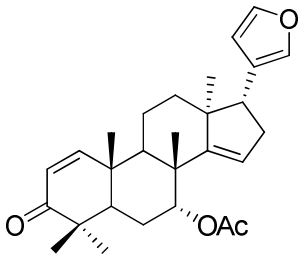
Compound	Structure	Pericarp	Seed kernel	Mace	Bark
Licarin B		✓		✓	✓
Elemicin		✓		✓	✓

(*2S, 3R*)-7-methoxy-3-methyl-5-((*E*)-prop-1-enyl)-2-(5-methoxy,3,4-methylenedioxyphenyl)-2,3-dihydrobenzofuran				✓	✓
Acuminatin		✓		✓	
7-methoxy-3-methyl-5-((*E*)-prop-1-enyl)-2-(3,4,5-trimethoxyphenyl)-2,3-dihydrobenzofuran		✓		✓	
Virolane		✓			
Licarin A		✓	✓	✓	✓
Guaiacin		✓	✓	✓	
(*8R*)-$\Delta^{8'}$-3,4,5,3', 5'-pentamethoxy-8-*O*-4'-neolignan				✓	
Erythro-(*7S,8R*)-$\Delta^{8'}$-7-acetoxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan		✓	✓	✓	

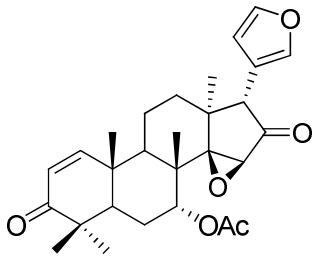
Erythro-(*7S,8R*)-$\Delta^{8'}$-7-hydroxy-3,4,3',5'-tetramethoxy-8-*O*-4'-neolignan		✓	✓	✓	✓
Erythro surinamensin		✓		✓	✓
Raphidecursinol B			✓	✓	✓
Grandisin					✓
(*7S,8S,7′R,8′R*)-3,3',4,4',5,5'-hexamethoxy-7,7', 8,8'-lignan					✓
Odoratisol A					✓
Fragransin B_1					✓
Erythro-(*7S,8R*)-$\Delta^{8'}$-4,7-dihydroxy-3,5,3'-trimethoxy-8-*O*-4'-neolignan				✓	✓
Malabaricone-C			✓		

Chapter 3 describes the isolation and anticancer activities of major phytochemicals from *Azadirachta indica* seeds and cake. The phytochemicals isolated

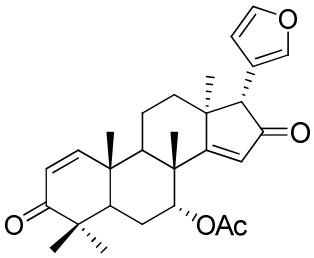
are (i) azadirone, (ii) epoxyazadiradione, (iii) azadiradione and (iv) 17β-hydroxyazadiradione, structures of which are given below.



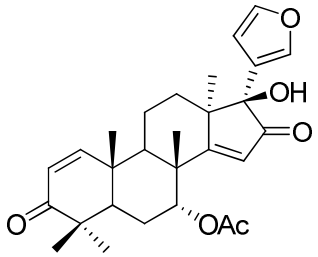
Azadirone



Epoxyazadiradione



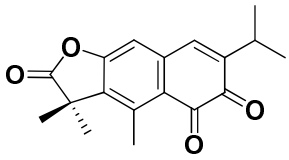
Azadiradione



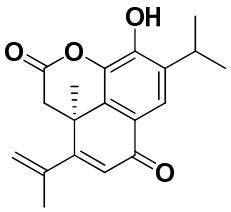
17β-hydroxyazadiradione

Anticancer activity and mechanism of action of azadirone has been studied in detail. The results indicate that azadirone can sensitize cancer cells to TRAIL through ROSERK-CHOP–mediated up-regulation of DR5 and DR4 signaling, down-regulation of cell survival proteins, and up-regulation of proapoptotic proteins.

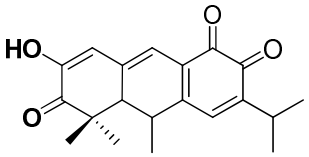
Chapter 4 deals with the phytochemical investigation of the medicinal plant *Pygmacopremna herbacea* and anticancer activity of the compound bharangin isolated from *P. herbacea* roots. *Pygmacopremna herbacea* belonging to the genus *Premna* is used in several Ayurvedic preparations. Here, a brief outline of the genus *Premna* is portrayed along with a detailed literature survey of *P. herbacea*. Phytochemical investigation of the roots of *P. herbacea* led to the identification of five compounds including two novel compounds. The compounds isolated are bharangininin, pygmaeocinin, bharanginione, bharangin and sugiol. Of these, pygmaeocinin and bharanginione are novel compounds. Structures of isolated compounds are shown below.



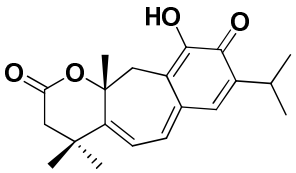
Bharanginin



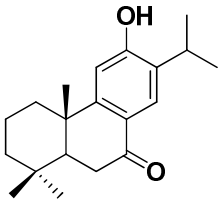
Pygmaeocinin



Bharanginione



Bharangin



Sugiol

In conclusion, we have carried out detailed phytochemical investigations of fruit and bark of the important spice plant *Myristica fragrans* and two medicinal plants viz., *Azadirachta indica* and *Pygmacopremna herbacea*. A total of 19 compounds were isolated from *M. fragrans* of which virolane, *erythro* surinamensin, grandisin and *(7S,8S, 7'R,8'R)*-3,3',4,4',5,5'-hexamethoxy-7,7', 8,8'-lignan are reported for the first time from *M. fragrans*. Azadirone isolated from *A. indica* seeds and bharangin from *P. herbacea* has been found to have potent anticancer activity. Furthermore, two novel compounds viz., , pygmaeocinin and bharanginione were also isolated from *P. herbacea*.

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LIST OF PUBLICATIONS

1. Gupta, S. C.; Kannappan, R.; Kim, J.; Rahman, G. M.; **Francis, S. K.;** Raveendran, R.; Nair, M. S.; Das, J.; Aggarwal, B. B. "Bharangin, a diterpenoid quinonemethide, bolishes constitutive and inducible nuclear factor-κB (NF-κB) activation by modifying p65 on cysteine 38 residue and reducing inhibitor of nuclear factor-κB α kinase activation, leading to suppression of NF-κB-regulated gene expression and sensitization of tumor cells to chemotherapeutic agents. *Mol. Pharmacol*. **2011**, *80*, 769-81.
2. Gupta, S. C.; **Francis, S. K.;** Nair, M. S.; Mo, Y. Y.; Aggarwal, B. B. "Azadirone, a limonoid tetranortriterpene, induces death receptors and sensitizes human cancer cells to Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) through a p53 Protein-independent Mechanism". *J. Biol. Chem.* **2013**, *288*, 32343-32356.
3. **Francis, S. K**.; Suresh, E.; Nair, M. S. "Chemical constituents from *Myristica fragrans* fruit". *Nat. Prod. Res*. **2014**, *28*, 1664-1668.
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5. **Francis, S. K.;** Sunil, V.; Nair, M. S. "Novel diterpenes from *Pygmacopremna herbacae* roots". **2016** (To be Communicated to *Phytochemistry letters*).

PAPERS PRESENTED AT CONFERENCES

1. **Sajin Francis. K**, Rajeev K. K and Mangalam S. Nair. Poster No. PP-48,"Isolation of antioxidant compounds from the spice plant *Myristica fragrans*". International symposium on phytochemistry held at Kerala State Science and Technology, Thiruvananthapuram on April 25, 2015.
2. **Sajin Francis. K** and Mangalam S. Nair. Poster No. PP- 416,"Phytochemical nvestigation and bioactivity studies on the medicinal plant *Azadirachta indica*" CRSI-NSC held at IITB Mumbai on February 7-9, 2014.
3. **Sajin Francis. K** and Mangalam S. Poster No. PP- 241, "Isolation, characterization and antioxidant studies of the medicinal plant *Myristica*

fragrans". 5th International Symposium on Current Trend in Drug Discovery and Research (CTDDR-2013) held at CDRI Lucknow on February 26-28,2013.

4. **Sajin Francis. K**, Dhanya. S. R and Mangalam S. Nair. "Isolation of heterocyclic compounds and bioactivity studies on the medicinal plant *Azadirachta indica*". Poster No. POS- 133, 3rd International Conference on Heterocyclic Chemistry, held at Department of Chemistry, University of Rajasthan, Jaipur on December 10-13, 2011.
5. **Sajin Francis. K** and Mangalam S. Nair. " Isolation, characterization and biological studies of Bharangin, a diterpene quinonnmethide". Poster No. PP-02, National Seminar on Recent Trends in Chemical Sciences: Frontiers and Challenges (RTCSFC-2011), held at Department of Chemistry, University of Kerala, Trivandrum on August 25-26, 2011.