Chemoprofiling and Biological Screening of Selected Medicinal Plants

by

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10CC15J39003

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Under the Supervision of

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To My Family and Teachers

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Santhi S.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Å	:	Angstrom
ACN	:	ACN
AR	:	Amalaki Rasayana
Ar	:	Argon
Aq.	:	Aqueous
BM	:	Butea monosperma
CC	:	Column chromatography
CCDC	:	Cambridge crystallographic data center
Calcd	:	Calculated
CDCl3	:	Deuterated chloroform
CD3COCD3	:	Deuterated acetone
CH3CN	:	Acetonitrile
cm	:	Centimetre
cm	:	Centimetre
°C	:	Degree Celsius
d	:	Doublet
1-D	:	One dimensional
2-D	:	Two dimensional
DCM	:	Dichloromethane
dd	:	Doublet of doublets
ddd	:	Doublet of doublets of doublets
DEPT	:	Distortionless enhancement by polarization transfer
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
DOX	:	Doxorubicin
dt	:	Doublet of triplet
EtBr	:	Ethidium bromide
Equiv.	:	Equivalent
ESI	:	Electrospray ionization
EtOAc	:	Ethyl acetate

FT-IR	:	Fourier transform infrared
FDA	:	Food and Drug Administration
Fr	:	Fractions
g	:	Gram
h	:	Hour
HMBC	:	Heterouclear multiple bond correlation
HMQC	:	Heterouclear multiple quantum correlation
HRMS	:	High-resolution mass spectrometry
Hz	:	Hertz
IC_{50}	:	Concentration required for 50 % inhibition
IR	:	Infrared
In (OTf)3	:	Indium (III) triflate
J	:	Coupling constant
LA	:	Lewis acid
m	:	Multiplet
Me	:	Methyl
MeOH	:	Methanol
mg	:	Milligram
min	:	Minute
MTT	:	MTT 3-(4,5-dimethylthiazol-2-yl)- 2,5-iphenyltetrazolium bromide
mL	:	Milliliter
mol	:	Mole
mmol	:	Millimolar
mol %	:	Mole percent
Mp	:	Melting point
MW	:	Molecular weight
NMR	:	Nuclear magnetic resonance
NP	:	Natural Product
N.R	:	No reaction

Nu	:	Nucleophile
o	:	Ortho
p	:	Para
ppm	:	Parts per million
Rf	:	Retention factor
RT	:	Room temperature
Sc(OTf)3	:	Scandium triflate
SD	:	Standard deviation
t	:	Triplet
TLC	:	Thin layer chromatography
TMS	:	Tetramethylsilane
UV	:	Ultraviolet
δ ppm	:	NMR chemical shift in parts per million
α	:	Alpha
β	:	Beta
γ	:	Gamma
δ	:	Delta
λmax	:	The wavelength at which absorbance is maximum
µg	:	Microgram
µL	:	Microlitre
µM	:	Micromolar
µg	:	Microgram

PREFACE

Nature has been a source of therapeutic agents for thousands of years, and an impressive number of modern drugs have been derived from natural sources, based on their use in traditional medicine. Plants produce an enormous variety of natural products with highly diverse structures. Medicinal and aromatic plants, in particular, have been exploited for this biogenic pool of phytochemicals for products such as pharmaceuticals, fragrances, dyes, and insecticides. Natural products will undergo continual use toward meeting the urgent need to develop effective drugs, and they will play a leading role in the discovery of drugs for treating human diseases, especially critical diseases. Accordingly, the present work depicts our efforts on the phytochemical and biological study of eight medicinally important plant species. They are *Calocedrus decurrans* (Cupressaceae family)*, Cedrus deodara* (Pinaceae family), *Bridelia retusa* (Phyllanthaceae family), *Bridelia stipularis* (Phyllanthaceae family), *Butea monosperma* (Fabaceae family), *Curcuma aeruginosa* (Zingiberaceae family), *Curcuma raktakanta* (Zingiberaceae family) and *Hedychium flavescens* (Zingiberaceae family).

Chapter 1 gives a brief introduction to the role of natural products in the modern drug discovery process. Even though it is impossible to furnish a comprehensive review on natural products-based drug discovery, an attempt has been made to exemplify the importance of NPs in modern drug discovery process in a brief manner.

Chapter 2 divided into two parts. Chapter 2A involves the phytochemical investigation of the coniferous plant, *Calocedrus decurrens* and its antiproliferative studies. Chapter 2B involves the phytochemical investigation of the coniferous plant *Cedrus deodara.* Conifers were mainly cultivated for ornamental purposes or lumber industry. Conifers are also renowned for their ability to form an enormous array of natural products, they contain bioactive molecules, which can be a lead compound in drug discovery.

Chapter 3, divided into two parts, represents the phytochemical investigation of two species from the genus *Bridelia, i.e*., *Bridelia retusa*

(chapter 3A) and *Bridelia stipularis* (chapter 3B), belongs to the Phyllanthaceae family. A thorough literature survey indicates that the two species of *Bredelia* – *Bridelia retusa* and *Bridelia stipularis*- used primarily by the tribal people of India have not been subjected to detailed phytochemical investigations. Though some reports are available, no scientific validation of the pharmacological aspects which are responsible for any particular activity of the whole plants or plant parts has been carried out. Hence, we have chosen a thorough reinvestigation of the phytoconstituents present in these two species. The results obtained during antibacterial studies are also presented in this chapter.

Chapter 4 is divided into two parts. A detailed phytochemical investigation of *B. monosperma* depicted in chapter 4A. Considering the importance of traditional knowledge-driven drug development, we focussed our efforts on the scientific validation of an ayurvedic medicine Amalaki Rasayana in chapter 4B. This Rasayana is given to the patients during the Kayakalpa treatment, a rejuvenation therapy in Ayurveda. The preparation of the Rasayana make use of the plants Amla and *Butea monosperma*. So, a comparative evaluation of the anticancer activity of Amalaki Rasayana, Amla and *Butea monosperma* also discussed.

Chapter 5 involves the phytochemical investigation and biological study of three species from the Zingiberaceae family. Zingiberaceous plants are a part of our tradition and culture, used as both medicine and spices. Since the mass screening of plants in search of new drugs are expensive and ineffective, it would be economical and more productive to re-examine the plant remedies described in ancient texts. A phytochemical re-investigation of the medicinally important species *Curcuma aeruginosa* explained in chapter 5A. There are only a few species which extensively studied in this family. So, we have selected two hitherto uninvestigated plant species *Curcuma raktakanta* and *Hedychium flavescens* for our study. Isolation and characterization of bioactives from *C. raktakanta* described in chapter 5B and *H. flavescense* in chapter 5C. The structural modification of germacrone towards the construction of biologically relevant bicyclic system *via* Lewis

acid catalysis also discussed in chapter 5B. The anticancer activity of the compounds from *H. flavescens* is also presented in chapter 5C.

CHAPTER: 1

Role of Natural Products in Drug Discovery

1.1. Introduction

Nature, the master of craftsman of molecules, created almost an endless array of molecular entities. Since prehistoric times, natural products (NPs) have been the backbone of the traditional system of healing throughout the world, and have also been an integral part of history and culture. It is generally accepted that the pharmacological properties of medicinal plants are well-defined by indigenous people of each place in the world. Indian systems of medicine are one of the important systems of medicine among the well-known global traditional systems of medicine. Most of the traditional systems of India, such as Ayurveda, Siddha, Unani, *etc*., have their roots in folk medicine. India is represented by rich natural biodiversity and offers a unique opportunity for drug discovery researchers. Our country is blessed with Eastern Himalayas and the Western Ghats, which are world's 18 hotspots of plant biodiversity and are 7th among the 16 Mega assorted countries, where 70 % of the species occur collectively. Over 7500 plant species have been reported to be used in the traditional Indian systems, including ethno medicines.

Natural products provide an abundant source of potentially attractive scaffolds, which could provide new starting points in drug discovery. The majority of the new drugs have been generated either from natural products (NPs) or natural product–inspired pharmacophores. Just about 200 years ago, in 1803, Friedrich Sertürner isolated the first pharmaceutically active compound, morphine, which is the principal alkaloid from the plant Opium poppy (*Papaver somniferum*). The discovery initiated an era wherein drugs can also be acquired from plants and other natural sources. Subsequently, a myriad of active compounds have been separated from natural sources. The alkaloids such as strychnine (*Strychnos nux vomica*), quinine (*Cinchona officinalis*), colchinne (*Colchicum autumnale*), atropine (*Atropa belladonna*), papaverine (*Papaver somniferum*), *etc.* were also isolated from plants. Aspirin, the most widely used analgesic, was a derivative of salicyline - a glycoside

contained in the bark of willow trees belonging to the family of Salicaceae and the invention of the first antibiotic Penicillin from a microorganism (*Penicillium notatum*), also nourished the new drugs based on NPs.[1] Some natural product-derived medicines that are a hallmark of modern pharmaceutical care includes the antibiotics (penicillin, tetracycline, erythromycin), antimalarials (quinine, artemisinin), antiparasitics (avermectin), lipid control agents (lovastatin and analogs), immuno-suppressants for organ transplants (cyclosporine, rapamycins) and anticancer drugs (taxol, doxorubicin).

An impressive number of modern drugs have been developed from NPs. The review by Newman and Cragg (the best and the comprehensive review so far) provides comprehensive information on new chemical entities that were discovered between 1981- 2014 (**Fig. 1.1**).[2] The review also clearly segregates the classes of new chemical entities as biological macromolecules, botanical drug with defined mixture, unaltered natural products, natural product derivatives, mimics of natural products, natural product pharmacophore-based synthetic products, vaccines and purely synthetic drugs.

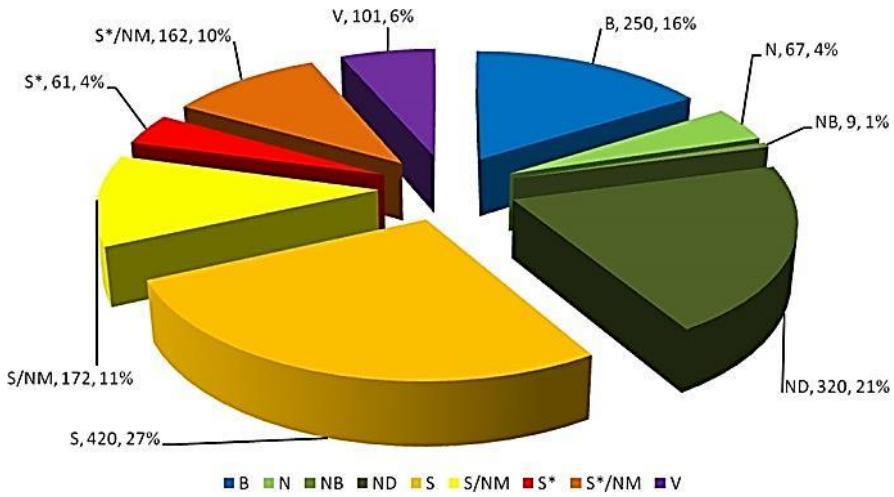


Figure 1.1. All new approved drugs from 1981-2014 (n = 1562). B = Biological macromolecule, N = Unaltered natural product, ND = Natural product derivative, NB = Botanical drug (defined mixture), S = Synthetic drug, S*= Synthetic drug (NP pharmacophore), NM = Mimic of natural product, V = Vaccine.

1.2. Classification of Natural products

There are several ways to classify natural products. The compounds can be grouped into different categories according to their biosynthetic origin, such as alkaloids, phenylpropanoids, terpenes, *etc*. They can also be classified according to their source of origin. The most important natural sources of drugs are higher plants, animals, microbes and marine organisms.

Based on function, NPs are divided into two major classes. (i) Primary metabolites and (ii) Secondary metabolites. Metabolism is the term for a set of chemical reactions that occur in the living organism to sustain life and be classified as primary and secondary metabolism. The products obtained are known as primary and secondary metabolites.[3] It was Albrecht Kossel who classified natural products into these two categories. Primary metabolites are molecules that directly involved in the growth, development and reproduction of the organisms such as carbohydrate, protein, lipids, nucleic acids, hormones, *etc*. Secondary metabolites are chemical products that are produced by the living organism but are not essential for life. They are utilized for chemical communication, chemical defense, *etc*. Alkaloids, flavonoids, steroids, terpenoids, glycosides, *etc.* are examples of secondary metabolites. The biosynthetic pathways involved in the biosynthesis of secondary metabolites (**Fig. 1.2**) include: [4]

- Photosynthesis or gluconeogenesis
- Acetate pathway
- Shikimate pathway
- Mevalonate pathway and methylerythritol phosphate pathway and
- Amino acids pathway

1.2.1. Terpenoids and steroids

The terpenoids form large and structurally diverse natural products derived from C-5 isoprene units joined in a head to tail fashion. Based on the number of isoprene unit attached, they are broadly classified 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-$C_{40}H_{64}$). Terpenoids are essential for plant survival and also possess biological and pharmacological properties that are beneficial to humans.

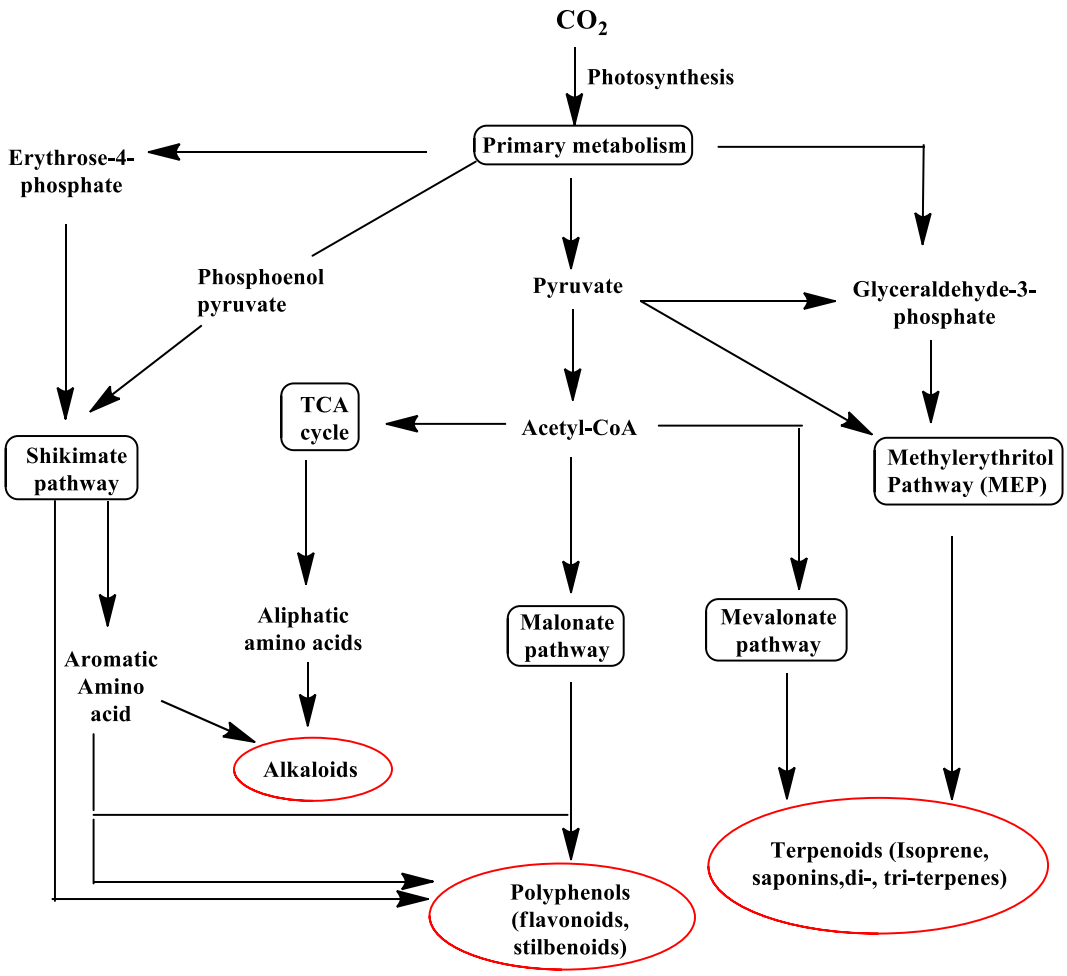


Figure 1.2. The biosynthetic pathway involved in the biosynthesis of secondary metabolites.[5]

The biosynthesis of terpenes starts with the synthesis of the precursor isopentyl diphosphate (IPP) through the mevalonic acid pathway. IPP is utilized in a sequence of elongation reactions to produce a series of prenyl diphosphate homologs, which serve as immediate precursors of the different families of terpenes. Isomerization of IPP by an isomerase produces the allylic isomer dimethylallyl diphosphate (DMAPP), which is considered the first prenyl diphosphate. IPP and DMAPP are combined to form geranyl diphosphate (GPP, a monoterpene). The sequential addition of further IPP units generates farnesyl diphosphate (FPP, sesquiterpenes) and geranylgeranyl diphosphate (GGPP, diterpenes). It is also possible that the head-to-head combination of FPP to give squalene (C30, triterpene) and the C40 intermediate (tetraterpene) (**Fig. 1.3**).[6]

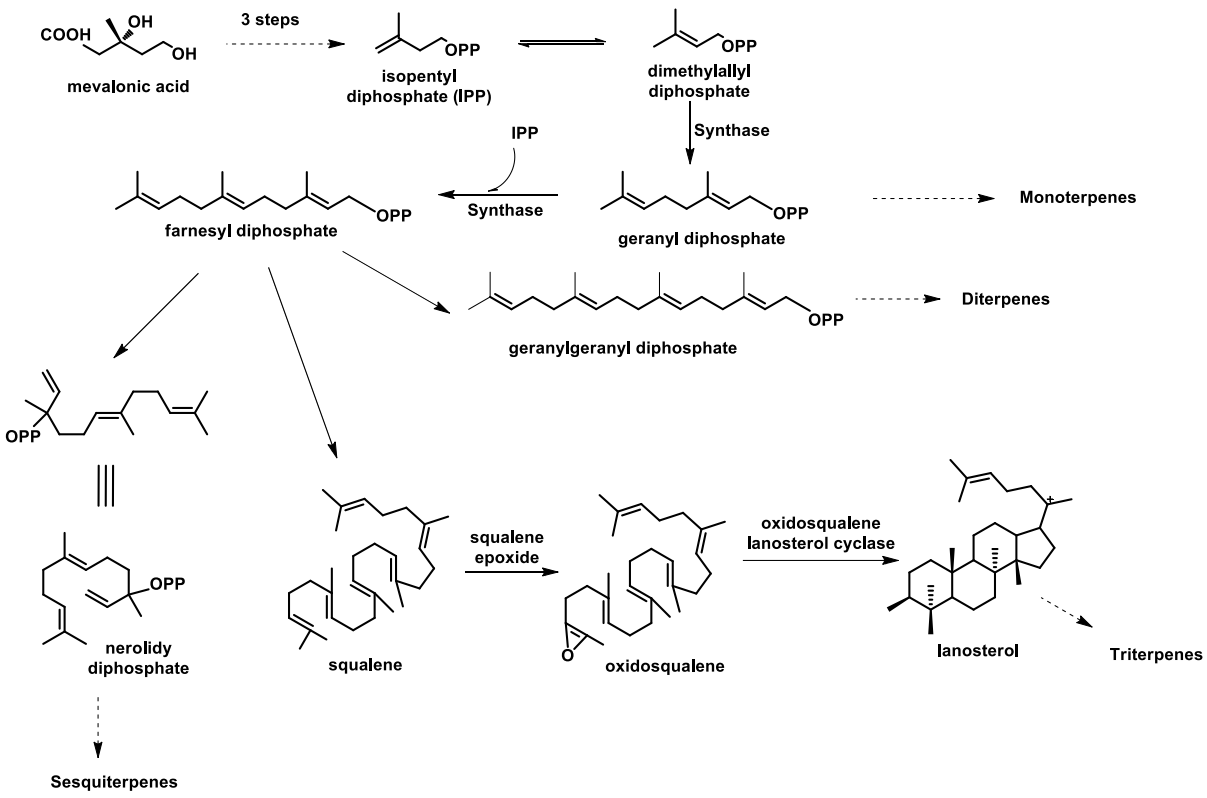


Figure 1.3. Biosynthetic pathway of acyclic terpenes

Monoterpenenoids are significant component of many essential oils and are extensively used for their aromatic qualities. The acyclic compounds include myrcene, geraniol, linalool and cyclic compounds are menthol, camphor, pinene, limonene, *etc.* Myrcene is the active sedating principle of hops and lemongrass, also found in basil and mangos.[7] Geraniol is a common constituent in most of the essential oils that occur in palmarosa oil, rose oil, ninde oil, and citronella oil. Geraniol has a characteristic rose-like odor and exhibits various biochemical and pharmacological properties such as antimicrobial, insect repellent, antitumor activity, *etc*.[8] Menthol is found in the essential oils of the mint family (Mentha spp.), such as peppermint, horsemint and others.[9] Some examples are given in **Figure 1.4**.

Sesquiterpenes are less volatile than monoterpenes. *β*-Caryophyllene is the primary sesquiterpene contributing to the spiciness of black pepper; it is also a major constituent of hops, cloves, rosemary, copaiba, and cannabis. *β*-Caryophyllene has now been shown to be directly beneficial for colitis, osteoarthritis, diabetes, cerebral ischemia, anxiety, depression, liver fibrosis, and Alzheimer's-like diseases. In cancer studies, *β* -caryophyllene stimulates apoptosis and suppresses tumour growth and

also demonstrated synergy with the chemotherapy drug paclitaxel on human tumour cell lines.[7]

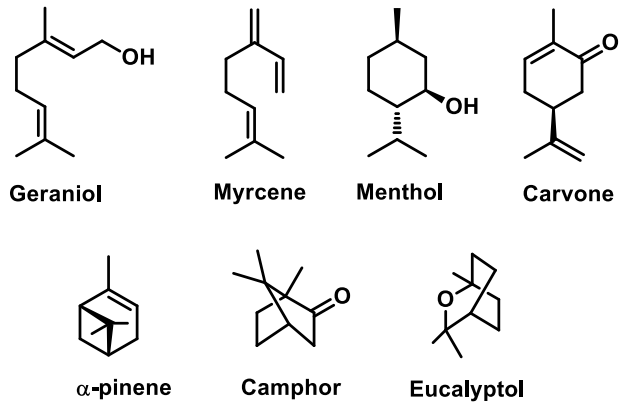


Figure 1.4. Examples for some monoterpenes

Artemisinin is a promising agent against malaria that occurs in *Artemisia annua*. It is a tetracyclic sesquiterpene with a six-membered lactone ring and an unusual 1,2,4-trioxane ring. Parthenolide, a naturally occurring sesquiterpene lactone derived from *Tanacetum parthenium* (Feverfew), exhibits anticancer and anti-inflammatory properties.[10] Avarol, a sesquiterpenoid hydroquinone and its quinone derivative avarone, isolated from the marine sponge *Dysidea avara* exhibit antitumor, antiviral and anti-inflammatory properties.[11] Zerumbone is a monocyclic sesquiterpene from *Zingiber zerumbet* that possess potent anticancer and anti-HIV activity. Some exaples for sesquiterpenes are given in **Figure 1.5**.

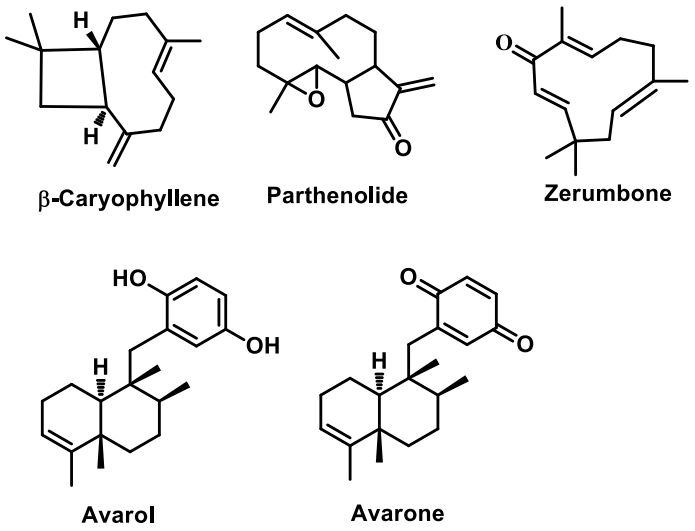


Figure 1.5. Examples for some sesquiterpenes

Diterpenes occur with a fascinating array of structures and biological activities. Paclitaxel and ingenol-3-angelate are examples of important anticancer drugs. In addition, forskolin, triptolide, salvinorin A, ginkgolide B and carnosic acid are prominent compounds showing cardioprotective, analgesic, anti-inflammatory, antioxidant and inhibitor of platelet activating-factor effects.[12] Paclitaxel is a powerful cytostatic agent isolated from the bark of *Taxus brevifolia.* The structure is a diterpene with an *N*-benzoyl phenylisoserinoyl side chain derived from two molecules phenylalanine.

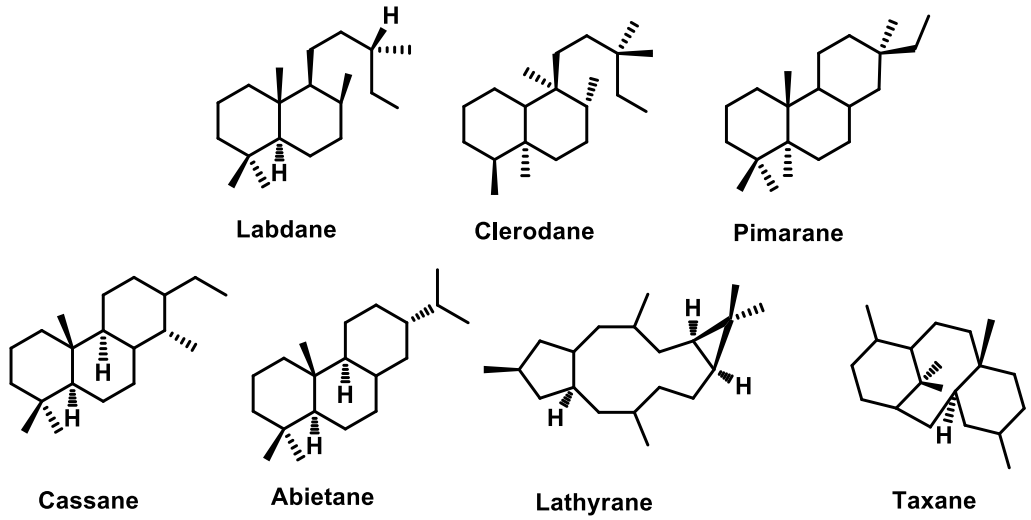


Figure 1.6. Some common diterpene skeleton type

Triterpenes are biosynthetically derived from squalene which include ursolic acid, oleanolic acid, amyrins, *etc*. Licorice root has been used in traditional medicine to alleviate gastritis, bronchitis and jaundice, glycyrrhizin is the major active constituent of these species. Glycyrrhizin is a triterpenoid saponin and the pharmacological actions of Glycyrrhizin includes inhibition of anti-inflammatory and immune regulatory actions, hepatic apoptosis and necrosis, antitumor effects and antiviral effects. [13]

Sterols are organic molecules having a polycyclic framework of common cyclopentanoperhydro- phenanthrene nucleus, a fused four-ring system made up of 17 carbon atoms. The steroids contain a hydroxyl group at C3 (a few ‘has C3-keto) and extra hydroxyl/s at different position/s, a double bond at C5–C6, and often with an aromatized ring. The first member of the steroid family to be discovered was cholesterol by Conradi in 1775 from human gall stones. The three most common

phytosterols are the 4- desmethylsterols, including sitosterol, stigmasterol and campesterol. Digitoxin (**Fig. 1.7**) is a secondary glycoside isolated from *Digitalis purpurea* used to increase cardiac contractility in the treatment of heart failure.[14]

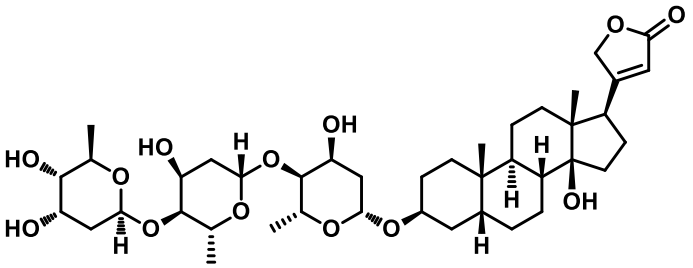
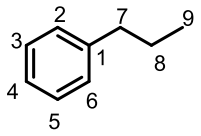


Figure 1.7. Digitoxin

1.2.2. Phenylpropanoids

Phenylpropanoids are parent molecules for all plant polyphenols, the largest class of secondary metabolites produced *via* the shikimic acid pathway. The phenylpropanoids contain a three-carbon side chain attached to a phenol. They are formed as a result of deamination of the amino acid phenylalanine by the enzyme phenyl alanine-ammonia lyase (PAL). The structure of simple phenylpropanoids is C6–C3, the carbon carcass of phenylalanine. Hydroxylation, methylation, and dehydrogenation of cinnamic acid results in the formation of its phenolic derivatives and also simple coumarins.[15] A schematic representation of the biosynthetic pathway is given in **Figure 1.8**.



Phenylpropanoid unit

In the first enzymatic step of the phenylpropanoid pathway, phenylalanine can be deaminated by phenylalanine ammonia-lyase (PAL) to yield cinnamic acid, which is then hydroxylated and transformed to *p*-coumaric acid under the catalysis of cinnamate 4-hydroxylase (C4H) in the second enzymatic step. However, in some monocots, fungi and bacterial species, tyrosine ammonia-lyase (TAL), or bifunctional ammonia-lyase (PTAL) can directly convert tyrosine into *p*-coumaric acid, which bypasses the C4H intermediate. Following this, 4-coumaroyl CoA ligase (4CL)

catalyses the conversion of *p*-coumaric acid into *p*-coumaroyl-CoA, which is an important branch point leading to generate various phenylpropanoid compounds.

Common examples include hydroxycoumarins, phenylpropenes, and lignans. Phenylpropanoids are important components of many essential oils, for example, eugenol (**Fig. 1.9**). It is the main component of clove *Syzygium aromaticum* and also found in soybean (*Glycine max*), beans, coffee, cinnamon (*Cinnamomum verum*), basil (*Ocimum basilicum*), canelinha (*Croton zehntneri*), banana, bay laurel (*Laurus nobilis*), and other foods.

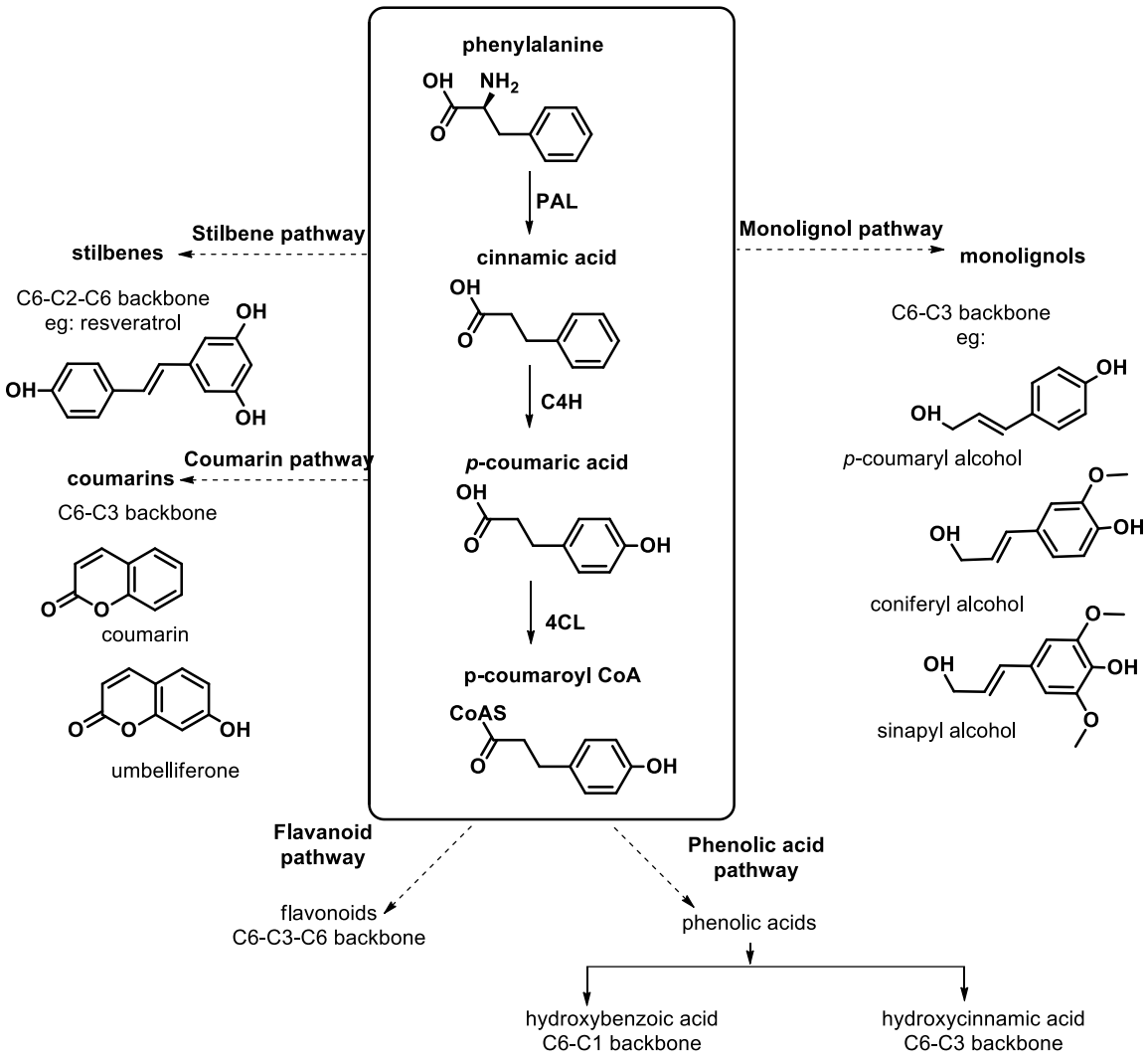


Figure 1.8. A schematic view of the biosynthetic pathway of phenylpropanoids and representative chemical structure.[16]

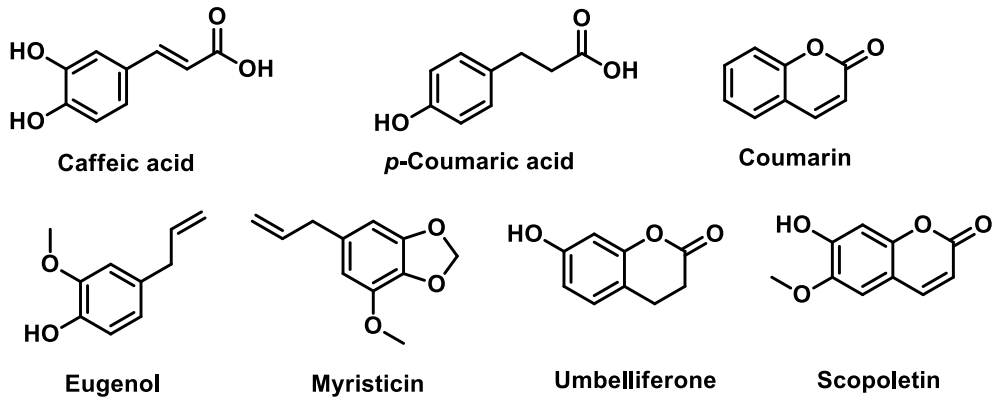
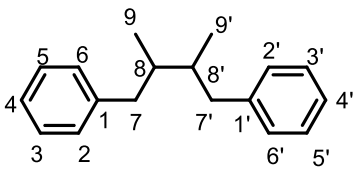


Figure 1.9. Examples for phenylpropanoids

1.2.3. Lignans

The term lignan was first introduced by Haworth (1948) to describe a group of dimeric phenylpropanoids where two C6-C3 are attached by its central carbon C-8. As described earlier, aromatic amino acids *L*-phenylalanine and *L*-tyrosine are produced from the shikimic acid pathway and then converted to a series of cinnamic acid derivatives. The reduction of these acids *via* coenzyme A of related esters and aldehydes forms three alcohols (*p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) that are the main precursors of all lignins and lignans. The peroxidase induces one electron oxidation of the phenol group allowing the delocalization of the unpaired electron through resonance forms. In these hydroxylcinnamyl alcohols, conjugation allows the unpaired electron to be delocalized into the side chain. The radical pairing of these resonance structures originates reactive dimeric systems susceptible to nucleophilic attacks from the hydroxyl group, leading to a wide range of lignans.



Lignan structure

Among these subgroups, the biosynthesis of C9 (9´)-oxygen lignans is the most well-known. This type of lignan is formed through the enantioselective dimerization of two coniferyl alcohol monomeric units (D resonance form of coniferyl alcohol

radical) into pinoresinol *via* intermolecular 8, 8′ oxidative coupling with the aid of dirigent protein. The following steps involve a sequential stereoselective enzymatic reduction of pinoresinol by pinoresinol/lariciresinol reductase to generate lariciresinol and then secoisolariciresinol by secoisolariciresinol dehydrogenase. The main stages of this biosynthetic proposal are depicted in **Figure 1.10.** Secoisolariciresinol gives the presumably common precursor of all dibenzylbutyrolactol lignans and, through the formation of matairesinol and yatein, also forms the aryltetralin lignans. These subclasses of lignans include some important bioactive compounds such as cubebin and podophyllotoxin.[17]

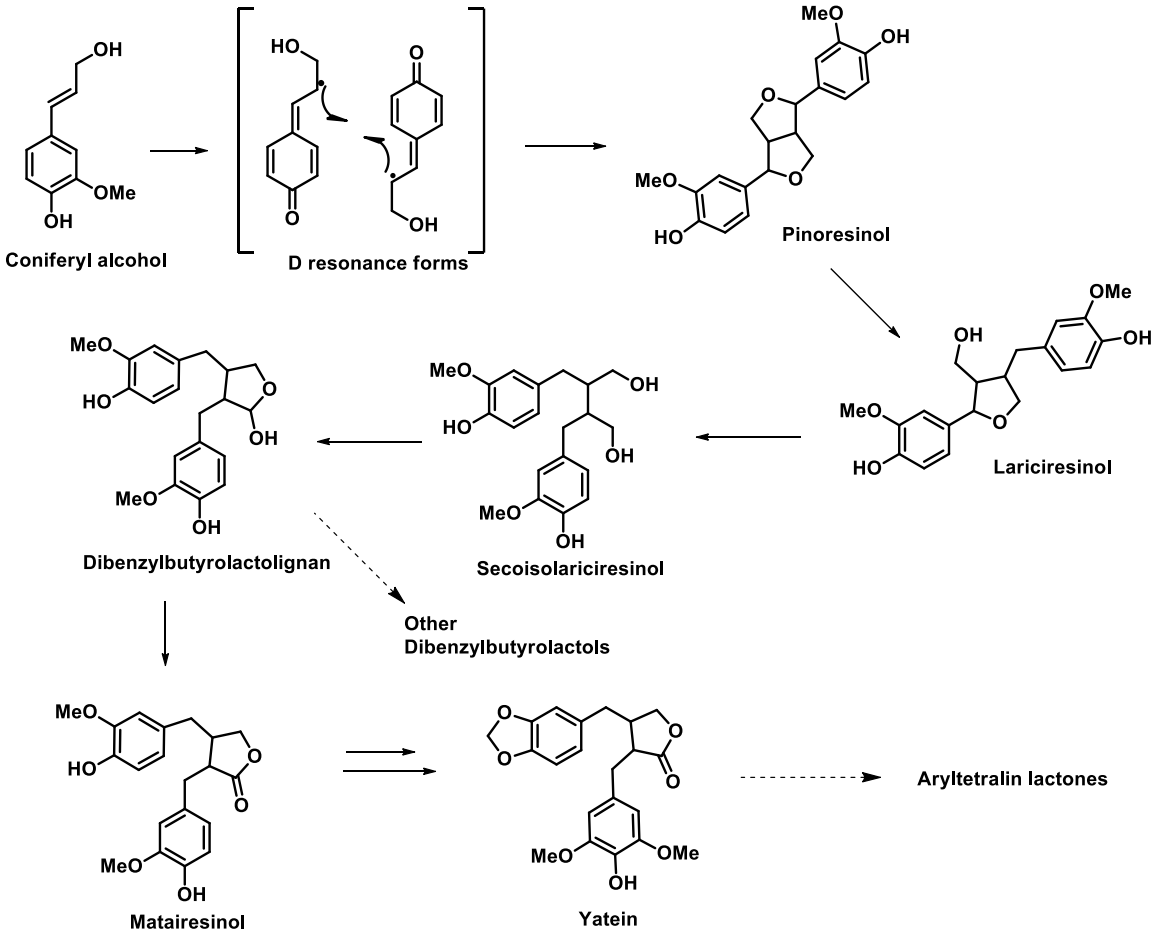


Figure 1.10. Biosynthesis of dibenzylbutyolactoles and aryltetralin lactones

Lignans possess significant pharmacological activities, including antitumor, immunosuppressive, anti-inflammatory, antioxidant, cardiovascular and antiviral activities.[18] Among the various bioactivities of lignans, the anticancer property is the most predominant one. Podophyllotoxin, the aryl tetralin lignan isolated from

Podophyllum peltatum, attracted much attention as an anticancer agent.[19] The derivatives of podophyllotoxin etoposide, teniposide, and etopophos have improved pharmacological profiles over podophyllotoxin. They have been developed and are being used clinically in the treatment of different types of cancer.[20] The main deficiency of these compounds is their cytotoxicity for normal cells and hence side effects derived from their lack of selectivity against tumour cells. Apart from podophyllotoxin, other lignans such as burseran, wikstromol, nordihydroguaiaretic acid, (-)-steganacin, enterolactone, enterodiol, *etc*. are also reported to have very good *in vitro* anticancer activities. [21–23]

1.2.4. Flavonoids

Flavonols are the most extensive spread metabolites in higher plants and predominantly accumulate as glycosides. They are found in fruits, grains, vegetables, bark, roots, flowers, stems, tea and wine. The flavonoids have two benzene rings separated by a propane unit and are derived from flavone. They have a conjugated aromatic system and hence show absorption bands in UV and visible regions. Flavonoids have several subgroups, subgroups depending on the variation of the heterocyclic C ring. The subgroups are chalcones, flavones, flavonols and isoflavones. Quercetin, kaempferol, and quercitrin are common flavonoids present in nearly 70 % of plants.

Flavonoids are synthesized by the phenylpropanoid metabolic pathway in which the amino acid phenylalanine is used to produce 4-coumaroyl-CoA. This combines with malonyl-CoA to yield the true backbone of flavonoids, a group of compounds called chalcones containing two phenyl rings. The first enzyme specific to the flavonoid pathway, chalcone synthase, produce chalcone scaffolds from which all flavonoids derive. Chalcone is subsequently isomerized by the enzyme chalcone flavanone isomerase (CHI) to flavanone. From these central intermediates, the pathway diverges into several side branches, each yielding a different kind of flavonoids. A schematic representation of the biosynthetic pathway is given in **Figure 1.11** and different classes of flavonoids are represented in **Figure 1.12**.[24]

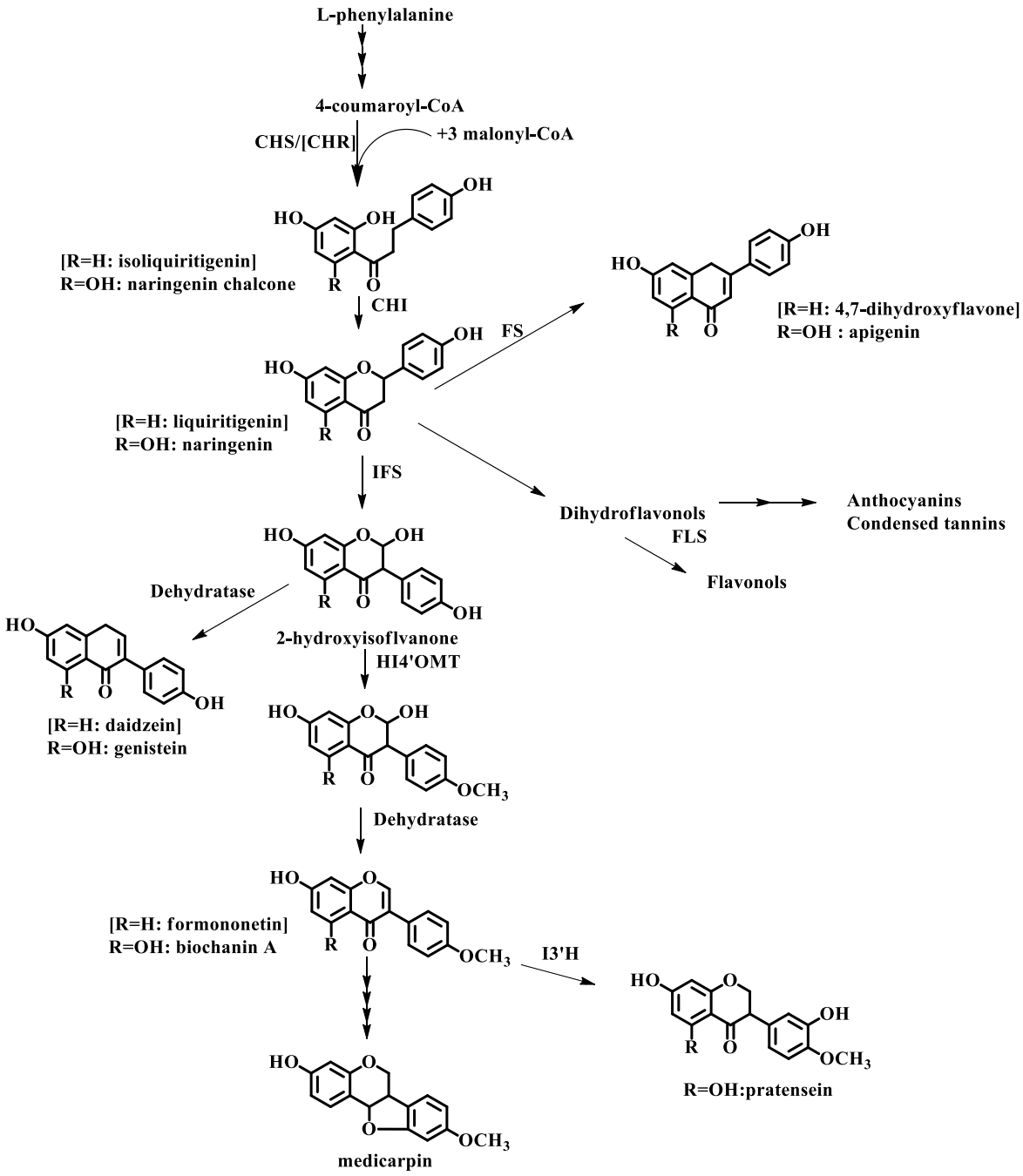


Figure 1.11. Schematic of biosynthetic pathways leading to flavonoid and isoflavonoid natural products. CHI, Chalcone isomerase; CHS, chalcone synthase; CHR, chalcone reductase; DFR, dihydroflavonol 4-reductase; FS, flavone synthase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; HI4'OMT, 2,7,4'-trihydroxyisoflavanone 4'-O-methyltransferase; FLS, flavonol synthase; I3'H, isoflavone 3'-hydroxylase; IFS, 2-hydroxyisoflavanone synthase

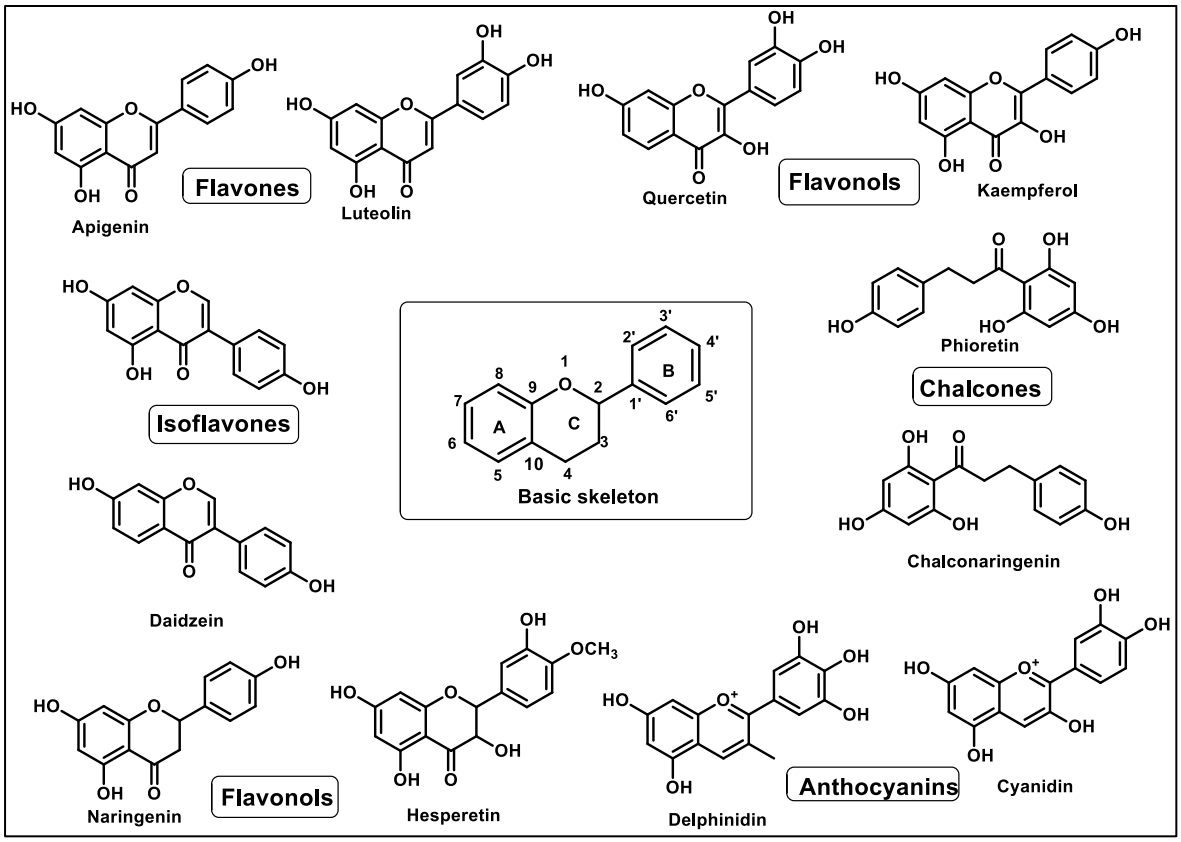


Figure 1.12. Basic skeleton structure of flavonoids and their classes

Flavonoids are enriched with many useful biological activities *viz*, antioxidative, anti-inflammatory, antiallergic, immunomodulatory, antimutagenic and anticarcinogenic activities. Anthocyanins are ubiquitously present in the plant kingdom, responsible for many of the red-orange to blue-violet colours observed in fruits, flowers, and leaves. The red pigment of beet (*Beta vulgaris*) is anthocyanin. Isoflavones are predominantly present in the Fabaceae or Leguminosae family; soybean is a rich source of natural isoflavones. The isoflavones genistein and daidzein are considered as phytoestrogens because of their oestrogenic activity. [25] Genistein acts as a potent anticancer agent that prevents, retards, or blocks carcinogenesis by its pleiotropic mechanisms, including breast, prostate and colon cancer.[26] The isoflavone glabridin, a polyphenolic compound found in *Glycyrrhiza glabra* (Fabaceae), inhibits LDL oxidation *via* a mechanism involving the scavenging of free radicals.[26] Several flavonoids such as catechin, quercetin, apigenin, rutin, naringenin and venoruton are reported for their hepatoprotective activities.[27]

Almost every group of flavonoids can act as antioxidants, especially flavones and catechins. Quercetin, morin, kaempferol, myricetin and rutin exhibited beneficial effects such as anti-inflammatory, antiallergic, antiviral, antioxidants as well as anticancer activity. They have been considered as potential protectors against chronic cardiotoxicity caused by the cytostatic drug doxorubicin. Quercetin has anticancer properties, which include antiproliferative, growth factor suppression, and antioxidant , known to contribute as an apoptosis inductor whereby it decreases the growth of tumours in and brain, liver, colon, and other tissues and inhibits the spread of malignant cells.[28] Higher consumption of phytochemical-rich fruits and vegetables can decrease the risk of cancer.

1.2.5. Alkaloids

Alkaloids are another largest group of secondary metabolites that contain a basic nitrogen atom at any position of the molecule, which does not include nitrogen in an amide or peptide bond. Depending upon their biosynthetic precursor and heterocyclic ring system, alkaloids have been classified into different categories, including indole, tropane, piperidine, purine, imidazole, pyrrolizidine, pyrrolidine, quinolizidine and isoquinoline alkaloids. Biosynthetic pathway of different type of alkaloids are depicted in **Figure 1.13**. & **1.14**.[29]

Even though people were using plants containing alkaloids for medicines, the concept of alkaloids was introduced in the early 19th century. Opium was the first crude drug used as analgesic and narcotics, eventually leads to the isolation of morphine and narcotine.[30] Friedrich Serturner isolated morphine crystals from poppy seed juice and was reported as a sleep-inducing molecule. His discovery leads to the isolation of other alkaloids such as codeine, quinine, strychnine, veratrine and emetine.[31] Strychnine is the extremely poisonous principal alkaloid from the seeds of *Strychnos nux vomica,* inhibits postsynaptic glycine receptors predominantly in the spinal cord, causing involuntary painful skeletal muscle contraction. Caffeine is a purine type alkaloid abundant in coffee (*Coffea arabica* and other *Coffea* species, Rubiaceae), which is a central nervous system stimulant. Since coffee drinkers show a reduced risk for Parkinson's disease, the attention towards caffeine has increased.[32]

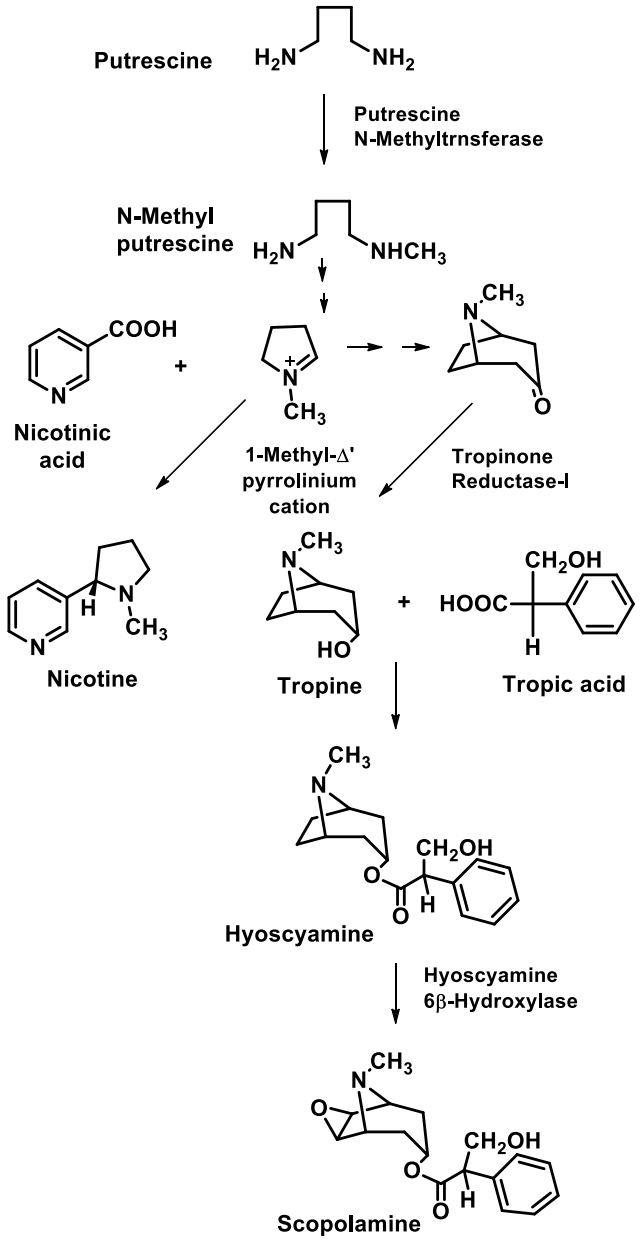
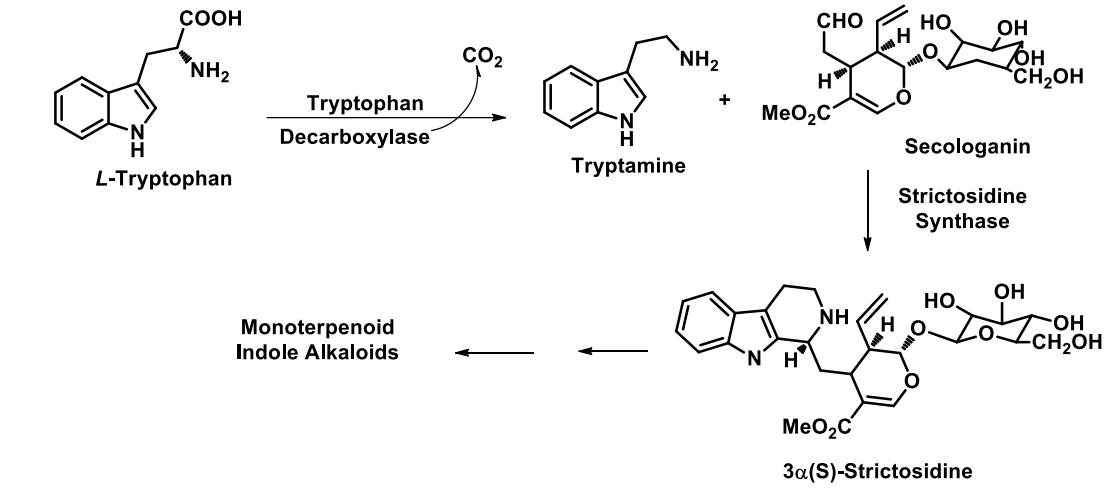
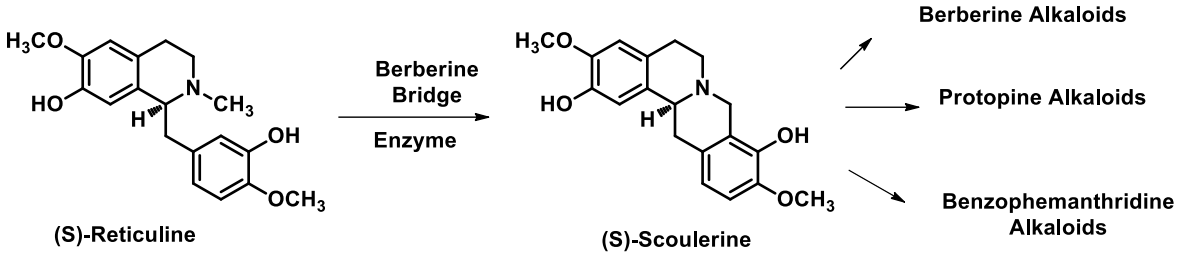


Figure 1.13. Tropane and Nicotine alkaloids

Alkaloids are among the most active components in natural herbs; some of them emerge into chemotherapeutic drugs, such as camptothecin (CPT), a famous topoisomerase I (TopI) inhibitor and vinblastine, which interacts with tubulin.[33] Tropane type alkaloids were reported firstly from the Solanaceae family which include hyoscyamine and scopolamine, used as narcotics. Both alkaloids are extremely poisonous and have hallucinogenic effects. Ergonovine, an alkaloid from the fungus *Claviceps purpurea*, and ephedrine isolated from *Ephedra* species, both act as blood vessel constrictors. Ephedrine is also used in bronchial asthma and to relieve the discomfort of hay fever, sinusitis, and common colds.



(a)



(b)

Figure 1.14. (a) Monoterpenoid Indole alkaloids (b) Benzylisoquinoline alkaloids

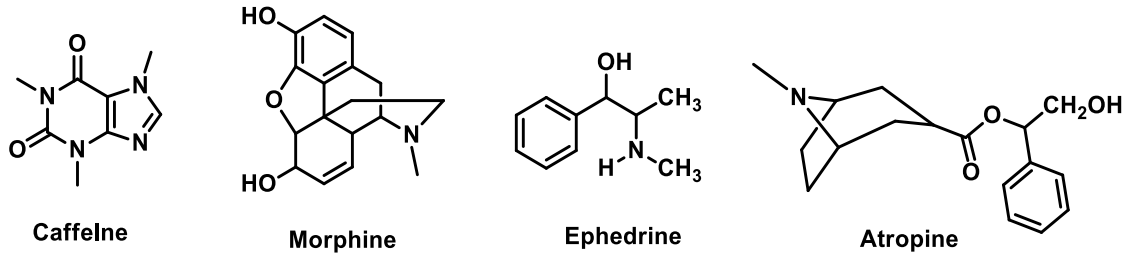


Figure 1.15. Examples of alkaloids

Reserpine is an indole alkaloid isolated from a perennial shrub of the *Rauwolfia* family (*Rauwolfia serpentina*) used as an antipsychotic and antihypertension drug.[34] Belladonna alkaloids have long been used in clinical medicine to reduce gastric acid secretion, as spasmolytics and to treat bradycardia and cholinergic overstimulation, even though they are toxin in

excess amount. Atropine and scopolamine (*Atropa belladonna)* are the two most important belladonna alkaloids.[14]

1.3. Classification based on source/Origin

The American Society of Pharmacognosy defines pharmacognosy as "*the study of natural product molecules (typically secondary metabolites) that are useful for their medicinal, ecological, gustatory, or other functional properties. The natural species that are the source of the compounds under study span all biological kingdoms, most notably marine invertebrates, plants, fungi, and bacteria*".[35] Natural products are diverse; they can be obtained from various sources, based on these they are:

- Plant-derived NPs
- Microbial world derived NPs
- Marine world derived NPs and
- Animal world derived NPs

1.3.1. Plant-derived NPs

The utilization of plants for natural products has a long history since they are having medicinal value and used for the preparation of therapeutics. Ancient authors described medicinal NPs of plant origin and listed approximately 400 different plant species for medicinal purposes. The Ayurvedic database available in classic texts has many applications in identifying new sources of medicine and knowledge about their therapeutic potential. The main classics of Ayurveda are Charaka Samhita, Sushruta Samhita (~400 BC–200 AD) and Ashtanga Hridaya of Vagbhata, give detailed descriptions of over 700 herbs and 6,000 formulations. Approximately 90 % of the Ayurvedic formulations are plant-based. Most of the classical preparations are polyherbal and an ideal combination of ingredients makes excellent results. Some of the commonly used plants in Ayurveda are aswagandha (*Withania somnifera*), curcuma/turmeric, ginger, aloe vera, tulsi, amrut (*Tinospora cordifolia*), pippali (*Piper longum*), haritaki (*Terminalia chebula*), bibhitaki (*Terminalia bellirica*), amla (*Emblica officinalis*) *etc*.

Anticancer drugs such as Taxol (*Taxus brevifolia*), Vinblastine (*Catharanthus roseus*) and antimalarial drugs such as quinine (*Cinchona* spp.) and artemisinin

(*Artemisia annua*) were all discovered from plants and are effective in treating these diseases. Paclitaxel was discovered as the active principle from the Pacific yew (*Taxus brevifolia*) tree (**Fig. 1.16**) and possessed a specific cytotoxic mechanism as an anticancer agent. It is sold under the brand name taxol, used in chemotherapy medication to treat several types of cancer. These include ovarian cancer, lung cancer, breast cancer, cervical cancer, Kaposi sarcoma, and pancreatic cancer.[36]

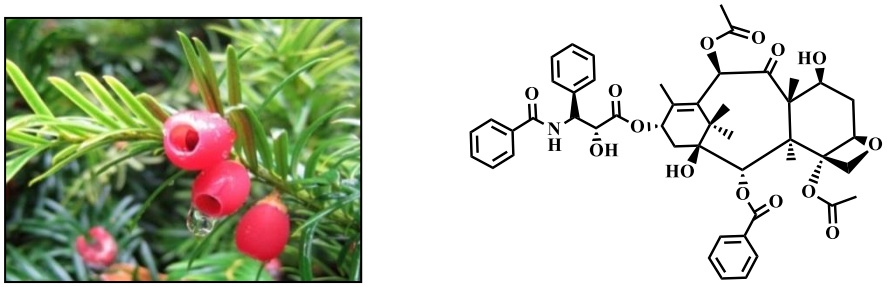


Figure 1.16. *Taxus brevifolia* and paciltaxel

The vinca alkaloids are so famous for being cancer fighters. Vinca alkaloids**:** vinblastine (**Fig. 1.17**), vinorelbine, vincristine and vindesine are the oldest group of the plant alkaloid groups that are used to treat cancer. They were discovered from the plant *Catharanthus roseus* known as Madagascar periwinkle, belongs to the family Apocynaceae. A new synthetic vinca alkaloid, vinflunine that is currently approved in Europe for cancer treatment.[37]

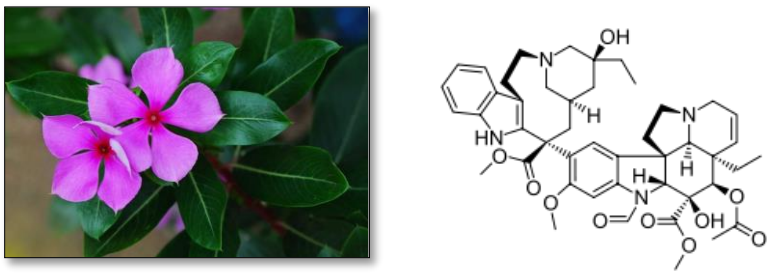


Figure 1.17. *Catharanthus roseus and* vinblastine

The use of semi-synthetic derivatives as drugs developed from plant natural product lead compounds began with medications like aspirin (acetylsalicylic acid). A series of artemisinin-based semi-synthetic antimalarial derivatives, with all of them maintaining the key endoperoxide bridge, such as artemether, arteether, artesunate, and dihydroartemisinin, have been designed to improve the water solubility and the

metabolic stability of artemisinin.[38] Etoposide, teniposide, and etopophos (etoposide phosphate, the prodrug of etoposide) are successful anticancer drugs derived from podophyllotoxin (**Fig. 1.18**).[39] Nitisinone (Orfadin®) is the first drug approved in Europe for the treatment of hereditary tyrosinemia type 1 (HT-1), which is a derivative of leptospermone (**Fig. 1.19**). Leptospermone is an effective herbicide present in the bottlebrush plant *Callistemon citrinus*.[40]

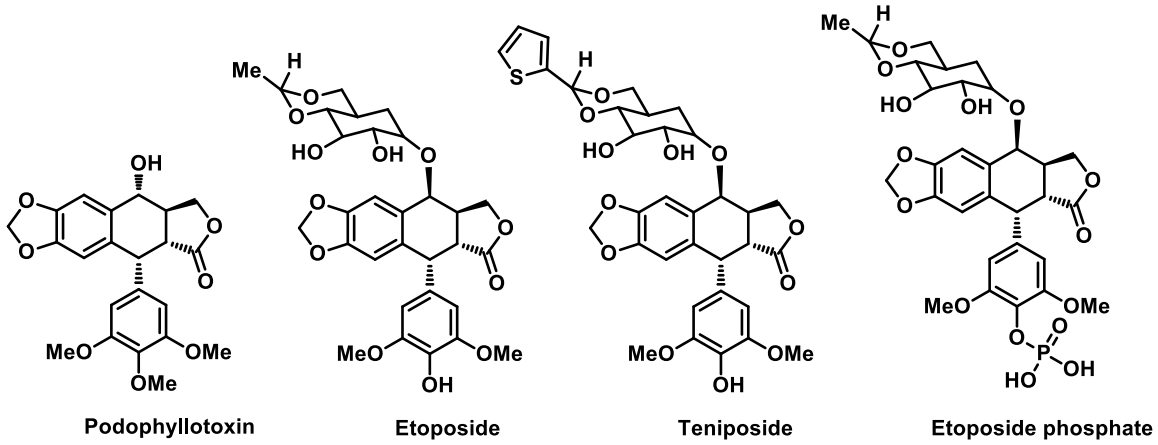


Figure 1.18. Podophyllotoxin and derived drugs

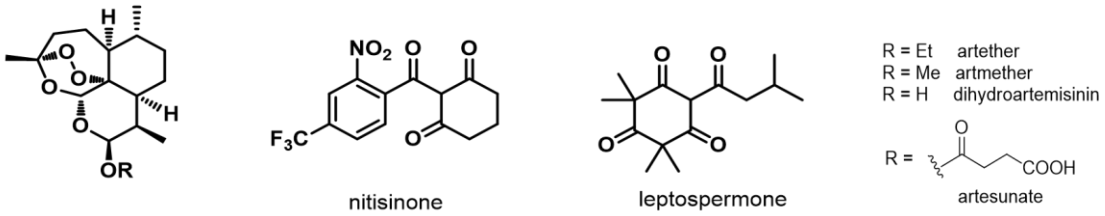


Figure 1.19. Semi-synthetic drugs from Artemisinin and Leptospermone

1.3.2. Microbial world derived NPs

Microbes have made exceptional contributions to medicinal chemistry. The discovery of the antibiotic Penicillin by Alexander Fleming (1928) from the fungus *Penicillium notatum* (**Fig. 1.20**) marked a significant shift from plant to microorganisms as a source of natural products. The massive success of the commercialization of synthetic penicillins prompted researchers to assemble an extensive collection of microbes to explore new antibiotics. Their efforts resulted in the discovery of streptomycin, chloramphenicol, chlortetracycline, cephalosporin C, erythromycin and vancomycin.

Figure 1.20. *Penicillium notatum* and Penicillin

Epothilones are novel class of cytotoxic compounds that function in a similar fashion to paclitaxel and show promise for treating a variety of cancers by inducing microtubule bundling and apoptotic cell death. Epothilones A and B (**Fig. 1.21**) were initially been discovered in 1987 as bioactive components in extracts of the cellulose-degrading myxobacterium *Sorangium cellulosum*.[35] The specific binding of epothilones is different from that of taxanes, which makes epothilones an attractive drug class for treating patients with taxane-resistant malignancies.[41] Hence, significant efforts have gone into the investigation of epothilones for advanced cancer therapy, and these efforts have culminated in the development of five epothilones currently in human clinical trials: patupilone, ixabepilone, fludelone, epothilone D, and BMS-310705.[42]

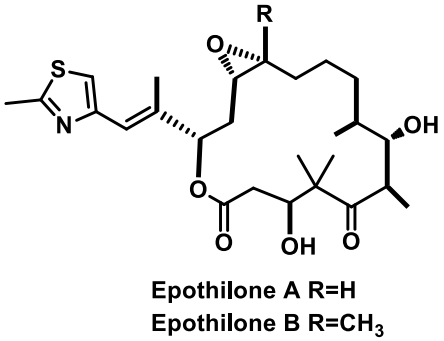


Figure 1.21. Epothilones A and B

Vancomycin is a glycopeptide antibiotic produced by *Streptococcus orientalis*. It is widely used against infections caused by gram-positive bacteria, especially methicillin-resistant *Staphylococcus aureus* and penicillin-resistant *pneumococci.* The

drug is not generally prescribed as the first-choice agent due to its adverse effects such as hypotension, nephrotoxicity, phlebitis, ototoxicity, red man syndrome, hypersensitivity reactions, neutropenia, fever, chills and interstitial nephritis.[43] Nisin has been commercially used as food preservative, produced by *Lactococcus lactis* subsp. Nisin is a thermally stable pentacyclic peptide and class IA antibiotic bacteriocin that is capable of eliminating up to 90 % of Gram-positive beer spoilage bacteria, poses an effective, natural solution. *Lactococcus lactis* has been used for centuries for the fermentation of food, especially in cheese, yogurt, and sauerkraut.[44,45]

1.3.3. Marine world derived NPs

Life has originated from the ocean; the earth covers about 70 % with water, provides significant biodiversity for the exploration of new drugs. The majority of the marine natural products were isolated from sponges, coelenterates (sea whips, sea fans and soft corals), opisthobranch molluscs (nudibranchs, sea hares), echinoderms (starfish, sea cucumber), tunicates and bryozoans. The secondary metabolites from these organisms act like chemical weapons and are highly potent inhibitors of the physiological process in prey, predator or competitor. Over one thousand marine natural products have been reported so far, are undergoing preclinical studies and about 23 of them are in clinical trials between phase I and II. The four approved anticancer drugs include cytarabine (Cytosars), trabectedin (Yondeliss), eribulin mesylate (Halavens) and the conjugated antibody brentuximab vedotin (Acentriss).[46]

The isolation of the arabinonucleosides spongouridine and spongothymidine (**Fig. 1.22**) were the first blockbuster discovery from marine sources. They were isolated from the Carribean sponge *Cryptotethya crypta*.[47] Their structure inspired the researches, leads to the synthesis of analogues of these molecules *i.e.*, Cytarabine (Ara-C) which is widely used in the treatment of various types of leukaemia, such as acute myelogenous leukaemia, acute lymphocytic leukaemia, chronic myelogenous leukaemia, and non-Hodgkin's lymphoma. It is considered as the first commercially available marine-derived drug.[48]

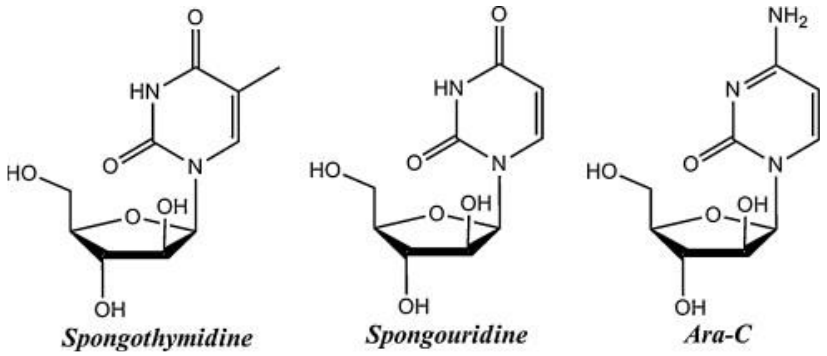


Figure 1.22. Examples for marine derived drugs

Eribulin mesylate (eribulin mesylate is marketed under the trade name halaven) is also a natural product derived antimitotic compound approved by the FDA to treat advanced metastatic breast cancer. This drug is a synthetic derivative of halichondrin B (**Fig. 1.23**), isolated from Japanese marine sponge *Halichondria okadai.*[46,49] Brentuximab vedotin is approved for the treatment of patients with Hodgkin's lymphoma and patients with systemic anaplastic large cell lymphoma (ALCL). It is an antibody-drug conjugate, developed with the active principle monomethylauristatin E, a derivative of dolastatin. Dolastatins was the active component present in the extracts of *Dolabella auricularia* (a gastropod mollusk) found to be having pronounced anticancer activity.[50,51] Sinularin from the soft coral *Sinularia flexibilis,* possess anticancer activities against the human epidermoid carcinoma cell line (KB) and the murine P388 lymphocytic leukemia cell line (PS).[52]

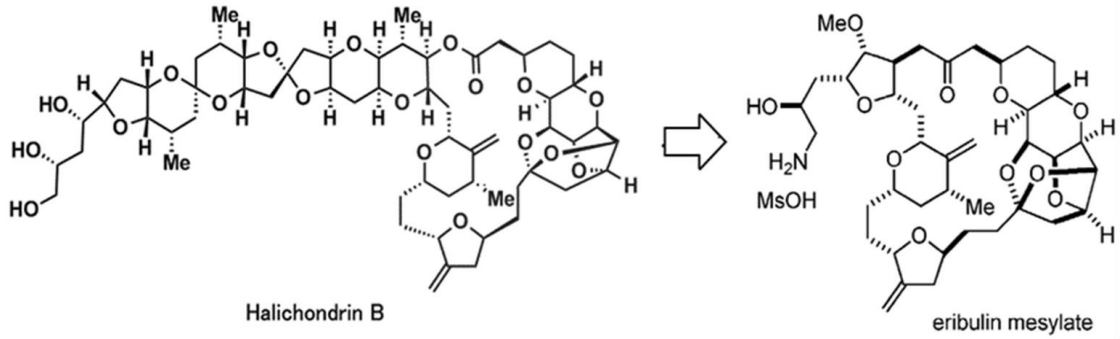


Figure 1.23. Halichondrin B and Eribulin mesylate

Eldoisin (**Fig. 1.24**) is a powerful hypotensive compound obtained from the posterior salivary glands of Eledone species (small octopus) *Eledone moschata*. Cephalosporin is derived from the marine fungus, *Cephalosporium acremonium* has been widely used as an antibiotic drug active against microbes insensitive to penicillin

and ampicillin.[53] Squalamine is the first aminosterol isolated from the dogfish shark *Squalus acanthias* (Squalidae). It has potent antimicrobial activity against *Staphylococcus aureus* and antitumor properties.[54]

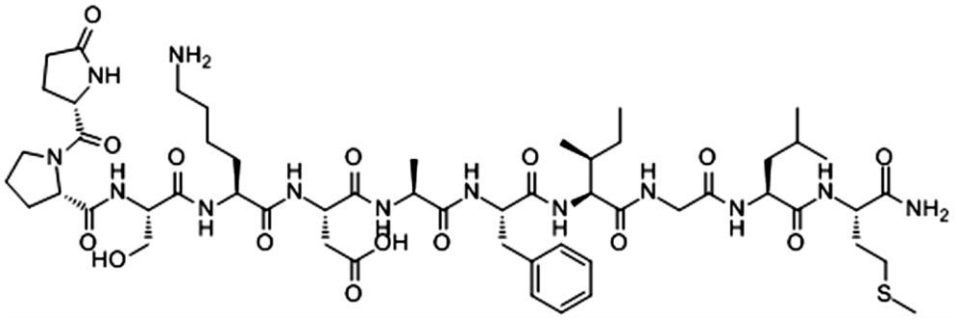


Figure 1.24. Eldoisin

1.3.4. Animal world derived NPs

The literature of natural products is enriched with medications derived from plants and herbs. The drugs reported from animal sources are very few, but the number is ever growing. Heparin, insulin and pituitary hormones were some of the earliest animal-derived therapies. The discovery of insulin has been a milestone in the history of medicine. Insulin has saved the lives of millions of patients with uncontrolled diabetes and ketoacidosis. Banting and Macleod received the Nobel Prize in Medicine in 1923 for the discovery of insulin.[55,56] Heparin is a naturally occurring glycosaminoglycan and it's been more than a century since the invention of heparin isolated from canine liver cells in 1916 by Jay McLean. Heparin is still in use and the main function of it is to inhibit the coagulation of blood.[57]

Specific medications commonly used today are derived from animal venoms, toxins or secretions. Tebanicline (ABT-594) was synthesized from epibatidine derived from the skin of a South American poison dart frog (*Epipedobates tricolor*). It is a less toxic derivative of epibatidine and has potency at least 200 times that of morphine. It was thought to be a very promising analgesic during the initial studies, but Phase II clinical trials demonstrated its toxic nature. Hence further researches are ongoing for a better derivative.[58] TM-601 is a synthetic version of Chlorotoxin, which is a 36-aminoacid disulfide-containing peptide derived from the venom of the deathstalker scorpion (L*eiurus quinquestriatus*).[59] The drug is currently in Phase II clinical trials for the treatment of adult recurrent glioma.[60]

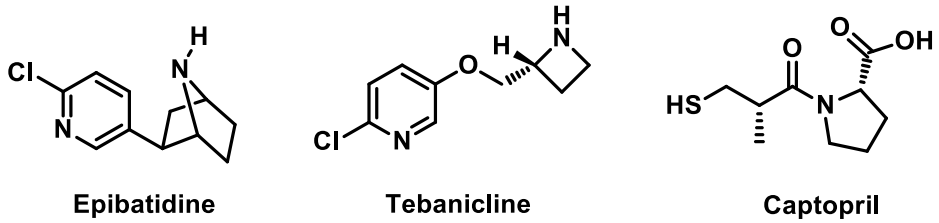


Figure 1.25. Examples of animal-derived drugs

Captopril, an angiotensin-converting enzyme (ACE) inhibitor, used as first-line anti-hypertensive drugs and used their protective properties in congestive heart failure, post-myocardial infarction, and prevention of diabetic nephropathy. It was derived from a peptide in the venom of the Brazilian pit viper (*Bothrops jararaca*) and available under the brand names: Capoten and Captoril.[61] Anti-platelet drugs found in clinics are Integrilin (Eptifibatide) and Aggrastat (Tirofiban). Eptifibatide (Integrilin), a heptapeptide derived from Barbourin, a protein found in the venom of the southeastern pygmy rattlesnake (*Sistrurus miliarius barbouri*). Aggrastat (Tirofiban) is a nonpeptide synthetic molecule based on the structure of Echistatin, and a protein found in the venom of the saw-scaled viper *Echis carinatus*.[62,63]

1.4. Some Important Drug Derived from Natural Products

Natural products can contribute to the search for new drugs in three different ways:

1. By acting as new drugs that can be used in an unmodified state
2. By providing chemical ‘‘building blocks’’ used to synthesize more complex molecules
3. By indicating new modes of pharmacological action that allows the complete synthesis of novel analogues

1.4.1. Antidiabetic drugs from Natural products

Diabetes mellitus is one of the major endocrine disorder affecting nearly 2.5 % of the world population. It is defined as a group of metabolic diseases characterized by high blood sugar level, resulting from defects in insulin secretion, or action, or both. In the indigenous Indian system of medicine, several plants were mentioned for the cure of diabetes. Many conventional drugs have been derived from archetypical molecules in medicinal plants.

Metformin (dimethyl biguanide) was preferred as the first oral blood-glucose level lowering agent for type II diabetics. It was discovered from the plant, *Galega officinalis* Linn., a poisonous herb with the potential to induce hypotension, tracheal frothing, paralysis, and even death. Metformin reduces insulin resistance, lowers gluconeogenesis and thereby relieves the metabolic defects in type 2 diabetes. It has a strong cardioprotective effect, anti-atherothrombotic and anti-inflammatory activity. Studies showed that it reduces the mortality rate in cancer patients treated with metformin. [64,65]

Phloridzin is a dihydrochalcone type compound isolated from the bark of the apple tree, *Malus domestica* belonging to the Rosaceae family. The compound's principal pharmacological action is to lower glucose plasma levels and improves insulin resistance levels through inhibition of sodium-glucose co-transporters (SGLT-2). Since Phloridzin cannot emerge as a lead drug molecule due to its unpleasant adverse drug reactions, it has become the precursor of a new class of antidiabetic drugs-SGLT2 inhibitors *viz*, canagliflozin, empagliflozin, ipragliflozin and dapagliflozin (**Fig. 1.26**).[66,67]

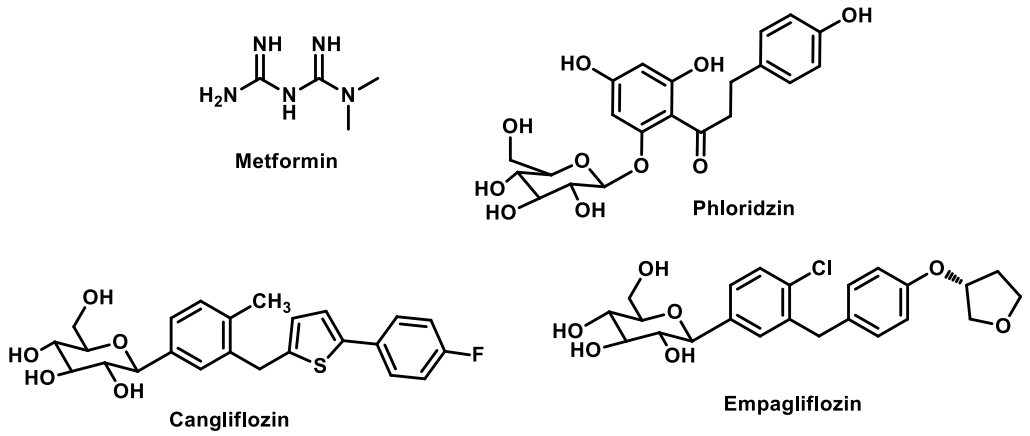


Figure 1.26. Examples of antidiabetic drugs

1.4.2. Antimalarial drugs

Malaria is a mosquito-borne infectious disease. It is naturally transmitted by the bite of a female Anopheles mosquito that is infected by Plasmodium. The disease is mainly caused by infection with five plasmodian protozoan species such as *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium knowlesi*.[68] The two most successful antimalarial drugs, artemisinin

and quinine are derived from medicinal plants. For many years quinine remained as the major antimalarial drug, isolated from *Cinchona* plants. Quinine is an aminoquinoline alkaloid, was largely replaced by synthetic drugs such as chloroquine and primaquine (**Fig. 1.27**). Chloroquine was effective against erythrocytic forms of the Plasmodium parasites.[69] The appearance of drug-resistance *P. falciparum* strains to chloroquine leads to the development of new alternatives to cholroquine.

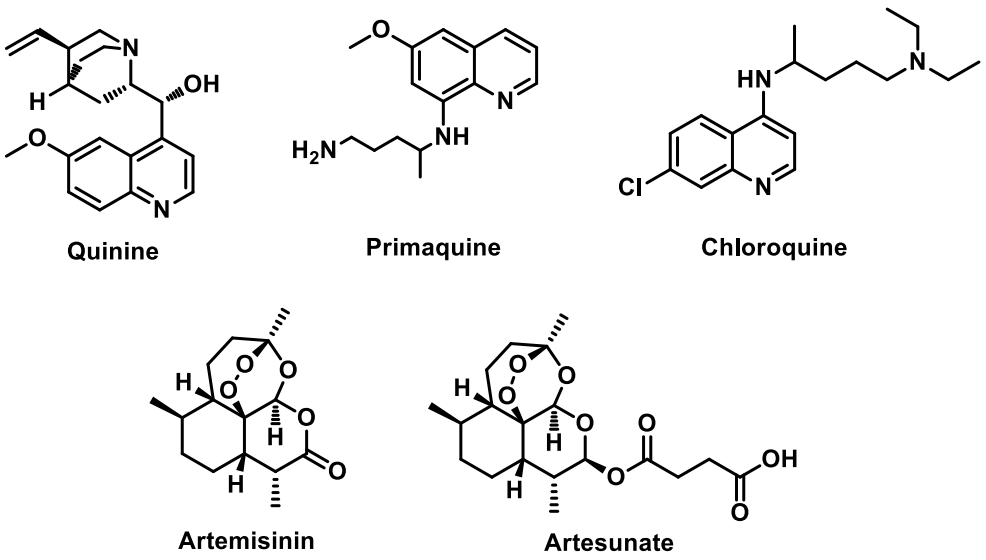


Figure 1.27. Examples of antimalarial drugs

The Chinese traditional treatment for malaria includes the use of the plant extract *Artemisia annua* (Compositae). The bioactive derived from the plant artemisinin, an endoperoxide sesquiterpene lactone, serves as the lead compound for the development of new antimalarials with improved properties. Artemisinin has a higher chemotherapeutic index than chloroquine and is useful in chloroquine-resistant strains of human malaria. At present, the most used artemisinin derivative is the prodrug, dihydroartemisinin, which is metabolized into the pharmacologically active artimisinin (IV) in the body.[69] Another species used is *Dichroea febrifuga* (Saxifragaceae) and its active principle febrifugine has been used clinically against *P. vivax* and *P. ovale*. But its liver toxicity makes it unacceptable to use as an antimalarial drug.[70] Many more natural products possessing various chemical structures, such as alkaloids, chalcones, steroids, flavonoids, terpenes, peptides, xanthones, quinones, coumarins, naphthopyrones, phenols, polyketides, chromenes, lignans, *etc*., have been tested as antimalarial drugs.

1.4.3. Anticancer drugs

Cancer is one of the most life-threatening diseases of the decade. Despite the availability of many anticancer drugs and various chemotherapy options, there is still an acute need for less toxic and more potent cancer drugs and continues to be the concern. Most of the drugs available are not selective to cancer cells and affect the normal cells, leading to severe side effects. Natural therapies, such as the use of plant-derived products in the treatment of cancer, may reduce adverse and toxic side effects of chemotherapy. Nature has blessed us with a plethora of medicinal plants. The discovery of medicinally important herbs and their mechanism of action would provide an alternative and effective therapy towards cancer prevention.

One of the early compounds known for plant-derived anticancer drugs were paclitaxel (taxol) and vinca alkaloids. Paclitaxel was the first compound to be discovered to promote microtubule formation. It has been used to treat several types of cancer, but most commonly for ovarian, breast cancers and non-small cell lung tumors.[71] Vinca alkaloids include vinblastine, vincristine, vindesine, and vinorelbine originally derived from *Catharanthus roseus* (Apocynaceae). They are well-known clinical cytotoxic drugs inhibiting the ability of cancer cells to divide. The mechanism of action of vinca alkaloids is to arrest dividing cells in metaphase by binding tubulin and to prevent its polymerization into microtubules.[72] Vindesine was the first semi-synthetic analogue of vinblastine to enter into the market. The compound is used to treat acute lymphocytic leukaemia, lung carcinomas, breast cancer and chronic myelogenous leukaemia. Vinorelbine (novelbine), another semi-synthetic derivative of vinblastine got FDA approval in 1989 and is used to treat non-small cell lung and advanced breast cancers.[73,74]

Cephalotaxus alkaloids are a multipurpose group of phytochemicals that are used against a wide range of cancer, including A-549 lung cancer, HeLa, SGC-7901 gastric cancer cell lines. Homoharringtonine (**Fig. 1.28**) and isoharringtonine are Cephalotaxus alkaloids isolated from *Cephalotaxus harringtonia*. The alcoholic seed-extract of *Cephalotaxus harringtonia* var drupacea has shown more specific activity against murine leukemic cell lines, which leads to the isolation of these alkaloids.[75] Homoharringtonine (Omacetaxine mepesuccinate) has been approved by the FDA to treat adult patients with chronic myeloid leukaemia (CML).[76]

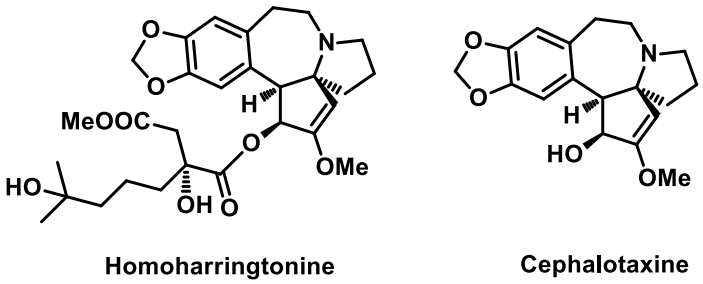


Figure 1.28. Cephalotaxus alkaloids

Colchicine (**Fig. 1.29**), an alkaloid, first isolated from the bulbs and seeds of the autumn crocus (*Colchicum autumnale* L.). The plant extracts were used for gout therapy because of its anti-inflammatory properties.[77] In 2009, the FDA approved colchicine to treat gout and familial Mediterranean fever (FMF). Colchicine as a medicinal drug has limited usage due to its high toxicity and several (-)-colchicine derivatives have been synthesized and tested in terms of their biological activity against many different cancer cell lines in an attempt to reduce unselective cytotoxicity and improve therapeutic activity.[78]

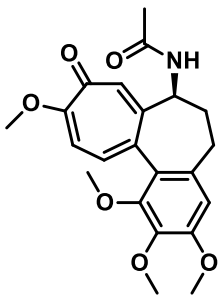


Figure 1.29. Colchicine

The natural lignan podophyllotoxin, an active component of *podophyllum* species, shows cytotoxic activity against a variety of cancer cell lines such as cervical carcinoma, osteosarcoma, nasopharyngeal carcinoma, colon cancer, breast cancer, prostate cancer, small cell lung cancer and testicular carcinoma by inhibiting microtubule assembly. Numerous podophyllotoxin derivatives, namely Etoposide and Teniposide, are used as antineoplastic agents.[79] Camptothecins (**Fig. 1.30**) are alkaloids isolated from the bark eaxtract of *Campthotheca acuminate*, inhibits DNA topoisomerase I. Two analogs of camptothecin have been developed that are clinically active and less toxic than the parent compound: irinotecan and topotecan.[80]

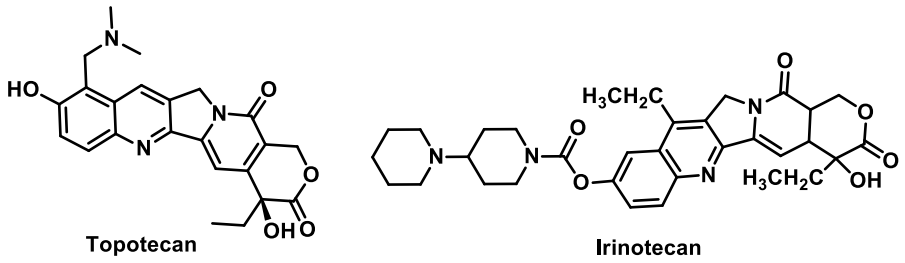


Figure 1.30. Camptothecin alkaloids

1.5. Objectives of the Present Study

The preceding discussions depict NPs are important and valuable resources for drug development. Traditional medicines equally provide useful clues for finding new drugs. Numerous drugs have entered international pharmacopeia through the study of ethnopharmacology and traditional medicine. Since the mass screening of plants in search of new drugs are expensive and ineffective, it would be economical and more productive to re-examine the plant remedies described in ancient texts. But, the knowledge of plants and their medicinal properties that were transmitted from generation to generation is in danger of disappearing. Medicinal plants, both endemic and widespread, their resources and knowledge about their usage must be preserved since these plants could be renewable sources for new drugs.

Considering the importance of traditional knowledge-driven drug development, we focussed our efforts on the phytochemical investigation and pharmacological studies of selected medicinal plants in Ayurveda. The respective studies leading to the identification, isolation and characterization of natural products which constitute an essential part of pharmacognosy. Chapter 1 gives a brief introduction to the role of natural products in the modern drug discovery process.

Chapter 2 involves the phytochemical investigation of two coniferous plants; *Calocedrus deccurrens* (chapter 2A) *and Cedrus deodara* (chapter 2B). As we know, evergreen trees are mainly used in the lumber industry or for ornamental purposes. They also contain bioactive molecules that can be used as a lead in drug discovery. Chapter 2A discusses the phytochemical investigation of *Calocedrus deccurrens* and cytotoxicity studies of selected molecules. Chapter 2B depicts the phytochemical investigation of the stem bark of *Cedrus deodara*.

Chapter 3, divided into two parts, represents the phytochemical investigation of two species from the genus *Bridelia,* i.e., *Bridelia retusa* (chapter 3A) and *Bridelia*

stipularis (chapter 3B). Both the plants are widely used in folklore medicines; hence the study is actually a scientific validation of these species. The results obtained during antibacterial and cytotoxic studies are also presented in this chapter.

Chapter 4 is divided into two parts. A detailed phytochemical investigation of heartwood *Butea monosperma* is depicted in chapter 4A. As we already mentioned that traditional medicines need a scientific validation; in chapter 4B, we tried to analyze the anticancer activity of Amalaki Rasayana. This Rasayana is given to the patients during the Kayakalpa treatment, a rejuvenation therapy in Ayurveda. The preparation of the Rasayana makes use of the plant's Amla and *Butea monosperma*.

Chapter 5 involves the phytochemical investigation and biological study of three species from the Zingiberaceae family. Zingiberaceous plants are a part of our tradition and culture, used as both medicine and spices. A phytochemical re-investigation of the medicinally important species *Curcuma aeruginosa* explained in chapter 5A. There are only a few species which extensively studied in this family. So, we have selected two hitherto uninvestigated plant species *Curcuma raktakanta* and *Hedychium flavescens* for our study. Isolation and characterization of bioactives from *C. raktakanta* and synthetic modification of an abundant natural product germacrone are described in chapter 5B. Phytochemical investigation and the anticancer activity of the compounds isolated from *H. flavescens* are presented in chapter 5C.

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CHAPTER 2

Chapter 2A: Phytochemical Investigation and Anticancer Studies of the Heartwood of *Calocedus decurrens* (Torr.) Florin

2A.1. Introduction

Cupressaceae is the largest conifer family with worldwide distribution. It consists of 32 genera and the members of this family are highly valued for their timber, resin, fruit, and ornamentals.[1,2] The genus *Calocedrus* belongs to this cypress family, is native to eastern Asia and western North America. *Calocedrus* mainly includes four species *viz*., *Calocedrus decurrens*, *Calocedrus macrolepis*, *Calocedrus formosana* and *Calocedrus rupestris.*[3,4] *Calocedrus decurrens* (Torr.) Florin, commonly known as 'Incense cedar' trees, are indigenous to the Pacific region of the United States such as Oregon and California.[5]. The essential oil of the wood of cedar is highly aromatic and contains phenolic terpenes as major constituents. The heartwood was reported to contain carvacrol, thymoquinone, *p*-methoxythymol, *p*-methoxycarvacrol, libocedrol, heyderiol, calocedrol A and calocedrol B.[6–8] Von Rudloff identified the main constituents of leaf essential oil as limonene (31.3 %), Δ-3-carene (21.0 %), α-pinene (9.2 %), myrcene (8.0 %), and α-terpenyl acetate (5.7 %).[9]

(a) **(b)** **(c)**

Figure 2A.1. Calo*cedrus decurrans* (a) tree (b) stem bark (c) leaves and flowers

Veluthoor *et al*., reported that the essential oil of the heartwood contains thymoquinone (35.9 %), carvacrol (29.2%), *p*-methoxythymol (11.0 %) and *p*-methoxycarvacrol (3.2 %) as the major constituents.[10] Garcia *et al*., reported four unusual pinane derivatives: pin-2-en-8-ol, pin-2-en-8-al, pin-2-en-8-yl acetate and methyl pin-2-en-8-oate from the essential oil in young branches of *C. decurrens*.[11] The extracts and essential oil of the heartwood exhibited antifungal, antimicrobial, and biocidal activity, which supports the decay-resistant nature of the wood.[12–14] The most durable and decay-resistant nature of the wood made it unique in the lumber industry, especially for making pencils and general building purposes. Native Americans used cedar leaves decoction for stomach trouble, as a vapor infusion for colds and as a food spice. The steam from the boiled bark is used as a traditional remedy for cold.

2A.1.1. Biosynthesis of Phenyl ethers

The phenomenon of a decrease in decay resistance with the aging of the wood observed in trees of many species, which may involve a variety of chemical changes. The major constituents in cedarwood are the *p*-cymene type phenolic compounds carvacrol, thymoquinone, *p*-methoxythymol and *p*-methoxycarvacrol. It has been established that the oxidative coupling of the phenolic substances forming phenyl ethers plays an essential role in the biosynthesis of other dimeric products. For example, libocedrol may be formed *in situ* by the enzymatic coupling of two molecules of *p*-methoxythymol. The dimer hyderiol is also derived *in situ* by the enzymatic coupling of two different phenols. Similarly, 3-libocedroxythymoquinone formed from thymoquinone and two phenolic monomers (**Fig. 2A.2**). Libocedrol exhibits lesser fungicidal activity than that of its monomer, which is a point fungicide. So as the wood ages, the monomers get converted to the dimeric or trimeric products, which are the cause of the reduction in decay resistance nature of the wood.[13]

2A.2. Literature survey on the phytochemistry and pharmacology of *Calocedrus* species

The genus *Calocedrus* has an eastern Asia and north American disjunct distribution pattern. It mainly includes three species *viz*., *Calocedrus decurrens*, *Calocedrus macrolepis* and *Calocedrus formosana*.[3]

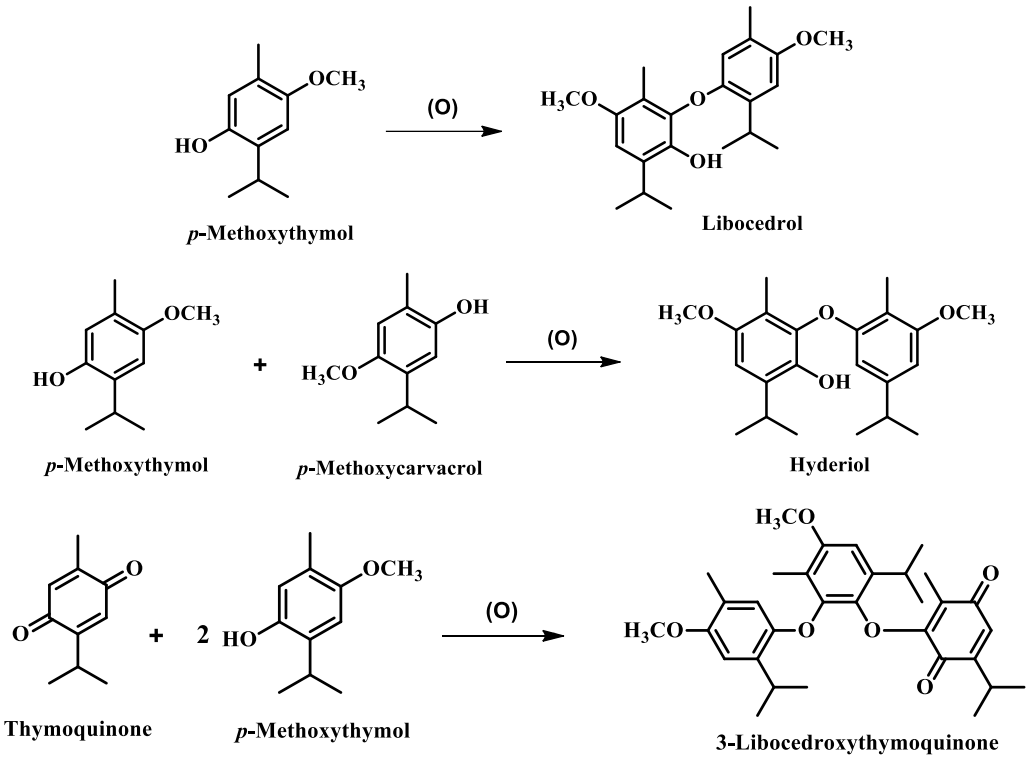


Figure 2A.2. Biosynthetic pathway of phenyl ethers from *C. decurrens*

Calocedrus formosana is commonly called Shonan and indigenous to Taiwan. The lignans hibalactone, (+)-calocedrin and hinokinin were reported from the wood of this plant.[15] Several lignans were isolated from the ethanolic extract of heartwood of *C. formosana viz*., haplomyrfolin, *O*-ethyl-*α*-conidendral, matairesinol, 7,8-dehydro-4-*O*-methyl-thujaplicatin and 5-methoxy-isosalicifoline. Matairesinol and 5-methoxy-isosalicifoline exhibited good antioxidant and antiradical activity.[16] The leaves were reported for more than twenty-seven compounds which includes terpenes, fatty acids, lignans, sesquiterpenes, diterpenoids and phaeophorbides. Some of the isolated compounds from leaves were (+)-sesamine, yatein, shonanin, an epoxy lignan (4,4'-dihydroxy-3,3'-dimethoxy-9,9'-epoxylignan), *trans*-*p*-coumaric acid, tr*ans*-methyl-*p*-coumarate, ariscucurbin-B, *trans*-communal, *trans*-communic acid, agathadiol, agatholal, isocupressic acid, 15-acetylisocupressic acid and lambertianic acid.[17,18] The diterpene sugiol isolated from the bark exhibited potent activity against inflammation. A dose of 30 mM of sugiol was effectively inhibitory for proIL-1*β*, IL-1*β* and TNF-*α* production.[19] Tsai *et al*., studied the immune-modulating property of the plant extract in adult peripheral blood mononuclear cells (hPB-MNCs). By cytomic screening, they have identified sugiol as the compound

with immuno-activating properties from *C. formosana* extracts.[20] A new γ-lactone, calolactone, together with drimane-type sesquiterpene, caloterpene, were reported from the pericarp of *C. formosana*.[21]

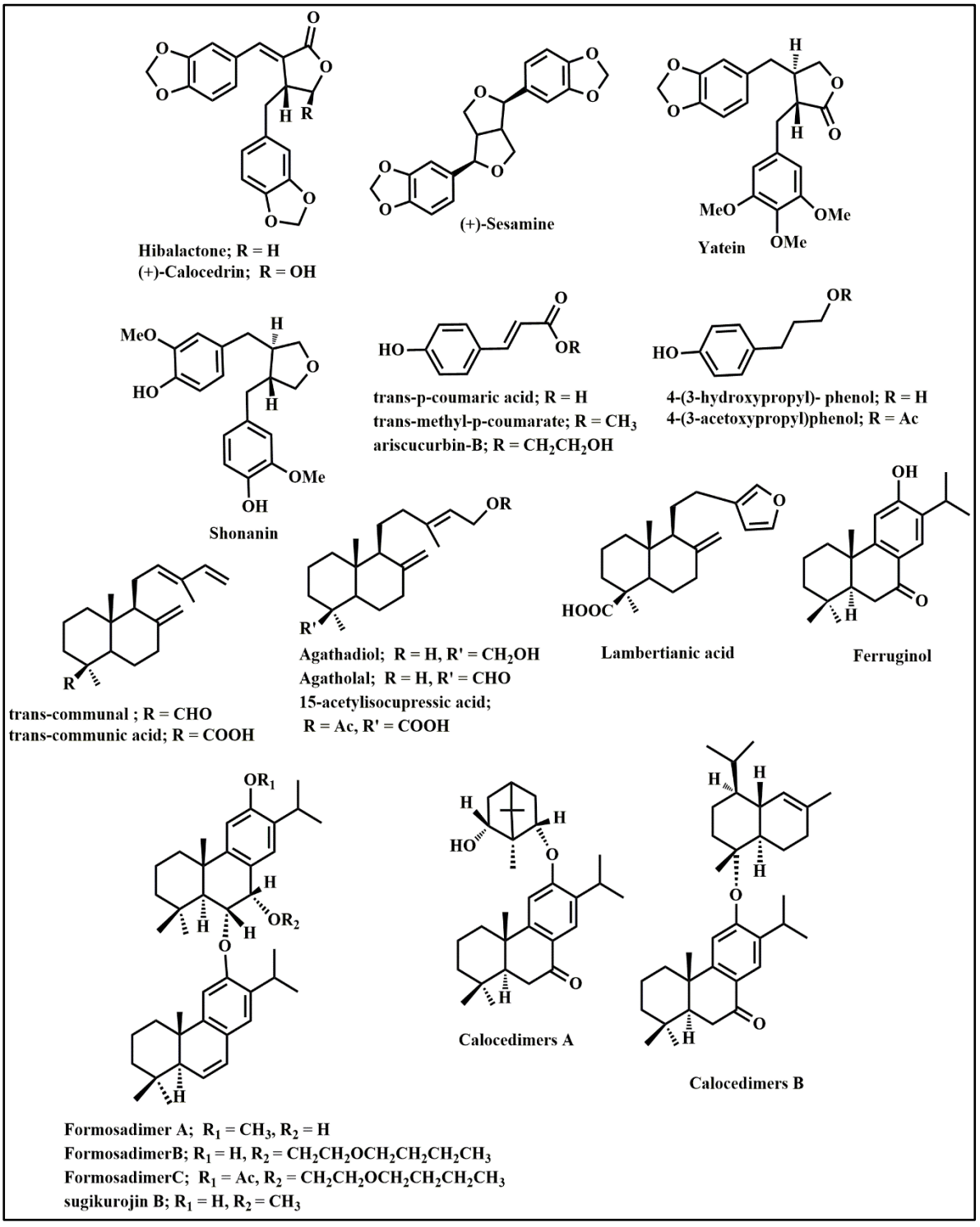


Figure 2A.3. Compounds reported from *Calocedrus formosana*

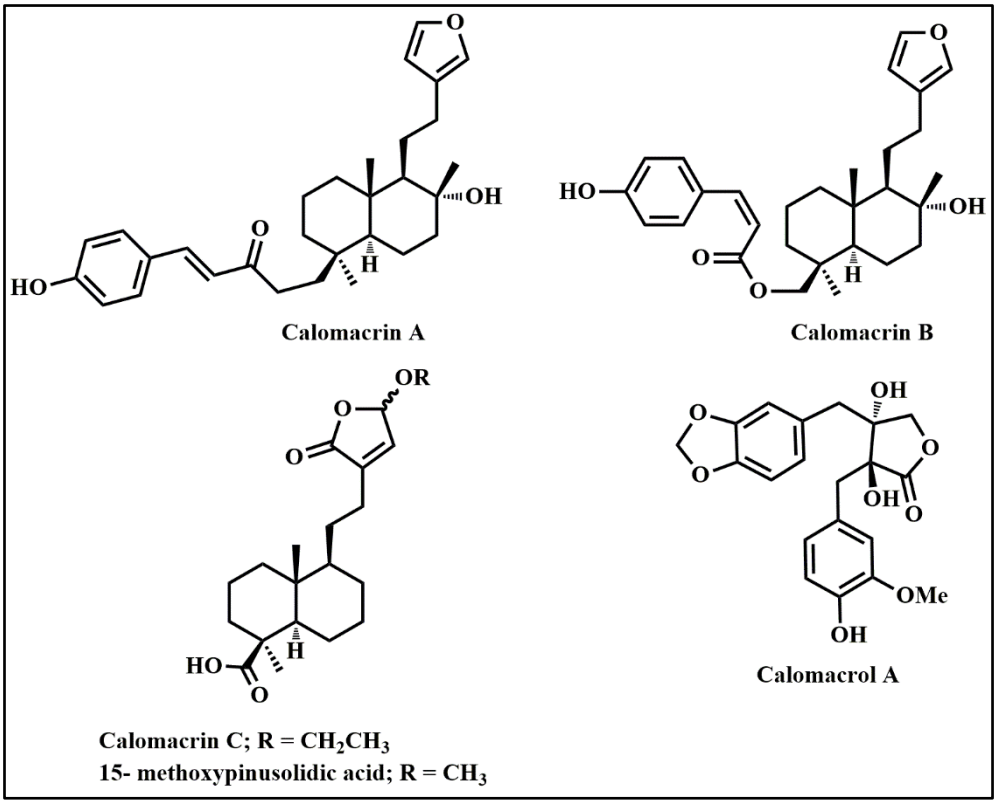


Figure 2A.4. Compounds reported from *Calocedrus macrolepis*

Abietane-*O*-abietane type dimers Formosadimers A, B, and C and sugikurojin B, Calocedimers A-D, labdane caryophyllic acid, secoabietane-type diterpenoids calocetriol, diacetylcalocediol and ferrugimenthenol were also reported from the bark. Ferrugimenthenol exhibited significant cytotoxic activity against human oral epidermoid carcinoma KB cells with an IC_{50} value of 9.0 ± 0.1 μM.[22–25] Labdane diterpenes: Calomacrin A-C, 15-methoxypinusolidic acid, Calomacrol A, matairesinol, 15,16-dihydroxy-8(17),13 (E)-labdadien-19-oic acid, *epi*-pinoresinol, peperoto l,8-hydroxypluviatolide, lambertianicacid and yatein were isolated from the twigs and leaves of *Calocedrus macrolepis*. The compounds were tested for cytotoxicity against A-549, MCF-7, and SW480 cell lines and yatein exhibited good cytotoxicity against all the tested cell lines.[26]

2A.3. Aim and scope of the study

The evergreen coniferous trees were mainly cultivated for ornamental purposes or lumber industry. Conifers are also renowned for their ability to form an enormous array of natural products, they contain bioactive molecules, which can be

lead compounds in drug discovery.[20,25,26] From the literature review, only a few reports are available regarding the phytochemistry and biological property of the species *C. decurrens*. The essential oil of the wood of cedar is highly aromatic and contains phenolic terpenes as major constituents. Our study mainly focused on the phytochemical investigation of the heartwood to identify more constituents present in it, with special emphasis to the anticancer property of the extracts and compounds. To the best of our knowledge, this is the first report on the antiproliferative study of *Calocedrus decurrens*.

2A.4. Extraction, isolation and characterization of compounds from the heartwood of *Calocedrus decurrens*

2A.4.1. Collection and Extraction of plant material

Incense cedar (*Calocedrus decurrens)* trees were collected from a logging and sawmill operations near Warm Springs, Jefferson, Oregon, USA. A voucher specimen was deposited in the Oregon State University Herbarium (Voucher number OSC228848). The heartwood was removed and shavings made and stored in vacuum-sealed containers at -20 °C.

About 1 Kg of the heartwood shavings of the plant material was soaked in hexane (5L * 3 days) and collected the crude extract by removing the solvent in a rotatory evaporator. The procedure was repeated thrice to yield 11 g of the hexane extract. The residue obtained after extraction using hexane was further extracted using acetone (28 g) and finally, with ethanol (17 g). We sequentially carried out the column chromatography of hexane extract followed by acetone extract. A total of eight compounds were isolated and successfully characterized. The total procedure is given in **Figure 2A.5**.

2A.4.2. Preliminary cytotoxic studies of the extracts

The crude extracts were screened for cytotoxic analysis against human lung adenocarcinoma (A549) using MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) assay. Doxorubicin (DOX) was used as the standard drug. From the results, hexane extract exhibited potent cytotoxicity (< 5 µg/mL) compared doxorubicin (1.63 µg/mL) at 24 h. Acetone and ethanol extracts showed

an IC_{50} value of 6.09 and 37.16 µg/mL at 48 h (**Table 2A.1**). The effect of various concentrations are presented in **Figure 2A.6**.

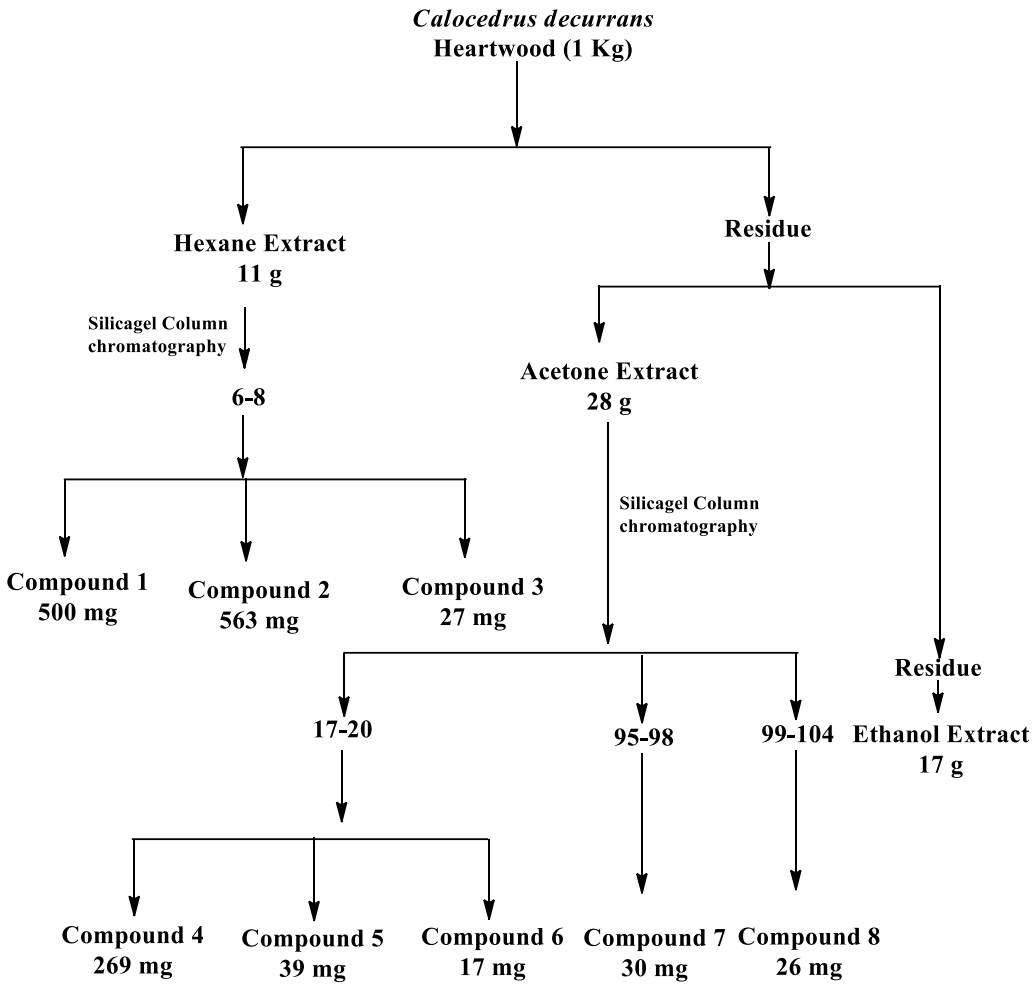


Figure 2A.5. Extraction process

2A.4.3. Isolation and characterization of compounds

The hexane extract was subjected to column chromatographic (CC) purification using silica gel (100-200 mesh). Column elution started with 100 % hexane and polarity subsequently increased by increasing the amount of ethyl acetate. Fractions collected were pooled into three fraction pools after analyzing the TLC. Fraction pool 2 (Fr.6-8) obtained by eluting the column with 5 % ethyl acetate-hexane polarity showed excellent UV active spots. So, we selected this fraction for further purification using silica gel CC. We have successfully isolated two compounds designated as compound **1** and compound **2**. The remaining crude was again subjected to Sephadex LH- 20 column to afford compound **3**.

Table 2A.1. IC_{50} values of extracts in A549 cell lines

Extracts	IC_{50} of extracts in µg/mL	
	A549	
	24 h	48 h
Hexane	<5	<5
Acetone	11.26 ± 0.01[b]	6.09 ± 0.005[b]
Ethanol	> 100[c]	37.16 ± 0.08[b]
DOX[a]	1.63 ± 0.22[b]	1.18 ± 0.16[b]

[a]Standard drug;[b] Statistical significance - DOX *vs*Extracts p<0.001; [c]IC_{50} not acheived; A549- human lung adenocarcinoma

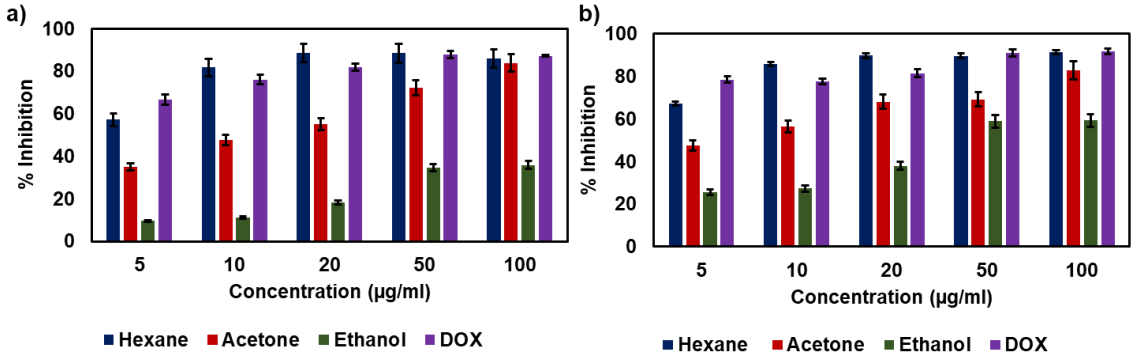
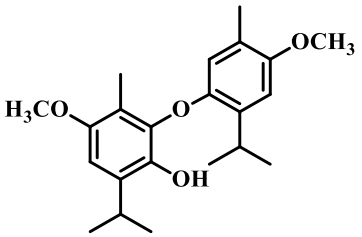


Figure 2A.6. MTT assay of extracts on A549 cells (a) at 24 h and (b) at 48 h

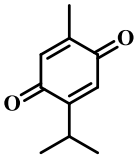
Compound **1** was obtained as colorless amorphous solid (500 mg). The ^{1}H NMR spectrum (**Fig. 2A.7**) of the compound suggested the presence of three aromatic protons (δ 6.77, 6.62 & 6.21 ppm), one hydroxyl group (δ 5.02), two methoxy groups (δ 3.82 & 3.81), two isopropyl groups [δ 3.54 (hpt, J = 7.0 Hz, 1H), 3.32 (hpt, J = 7.0 Hz, 1H), 1.34 (d, J = 7.0 Hz, 6H) & 1.27 (d, J = 6.5 Hz, 6H)] and two methyl groups [δ 2.04 (s, 3H) & 1.91 (s, 3H)], respectively. The ^{13}C NMR spectrum (**Fig. 2A.8**) revealed the presence of 22 carbons and the HRMS analysis showed a molecular ion peak at m/z 381.2024, which is the $(M+Na)^+$ peak. The mass 358 indicates that there should be an ether linkage between the two aromatic rings. So, combining the spectral data and HRMS analysis, the molecular formula of the compound was assigned as $C_{22}H_{30}O_4$. The proton attachment to various carbons was assigned based on HMQC and HMBC correlations. The methoxy groups at δ 3.82 and

3.81 correlate with carbon at δ 151.4 and 153.0, leading to the conclusion that the methoxy groups were attached at the C-6 & C-6' carbons, respectively. The hydroxyl group at δ 5.02 showed a strong correlation with carbons at δ 132.2 (C-4) & 140.4 (C-3 and C-2), we can conclude that the hydroxyl group is attached to the C-3 carbon and the ether linkage is at C-2. From the spectral data and on comparison with literature reports, the compound was identified as a diterpene phenol, composed of two *p*-cymene units linked through an ether bridge, named **Libocedrol**.[8]



Compound **1- Libocedrol**

Compound **2** was isolated as a yellow crystalline solid (563 mg). The ^{1}H NMR spectrum (**Fig. 2A.9**) displayed two aromatic protons (δ 6.59 & 6.52), one methyl group (δ 2.03) and one isopropyl group [δ 3.02 (hpt, J = 6.5 Hz, H-8) & 1.13 (d, J = 7.0 Hz, 3H)]. The ^{13}C NMR spectrum (**Fig. 2A.10**) revealed ten carbon atoms, which includes two aromatic carbonyl groups at δ 188.6 and 187.6 ppm. The HRMS analysis showed a molecular ion peak at 163.0389, which is the $(M-H)^{+}$ peak. Therefore, combining the data the molecular formula of the compound is assigned as $C_{10}H_{12}O_2$. From the spectral data and on comparison with the literature reports, compound **2** was identified as **Thymoquinone**.[27] Thymoquinone is the major component present in *Nigella sativa* and reported to possess potent anticancer activity.[28] Thymoquinone exert anti-inflammatory, anti-oxidant and anti-neoplastic effects both *in vitro* and *in vivo*.[29]



Compound **2- Thymoquinone**

Compound **3** was isolated as a reddish crystalline solid (27 mg). The ^{1}H NMR spectrum (**Fig. 2A.12**) was similar to that of compound **1**, but one methoxy group and hydroxyl group were absent. The ^{13}C NMR spectrum (**Fig. 2A.13**) showed the presence of 21 carbon signals with two carbonyl groups at δ 188.5 and 181.7 ppm.

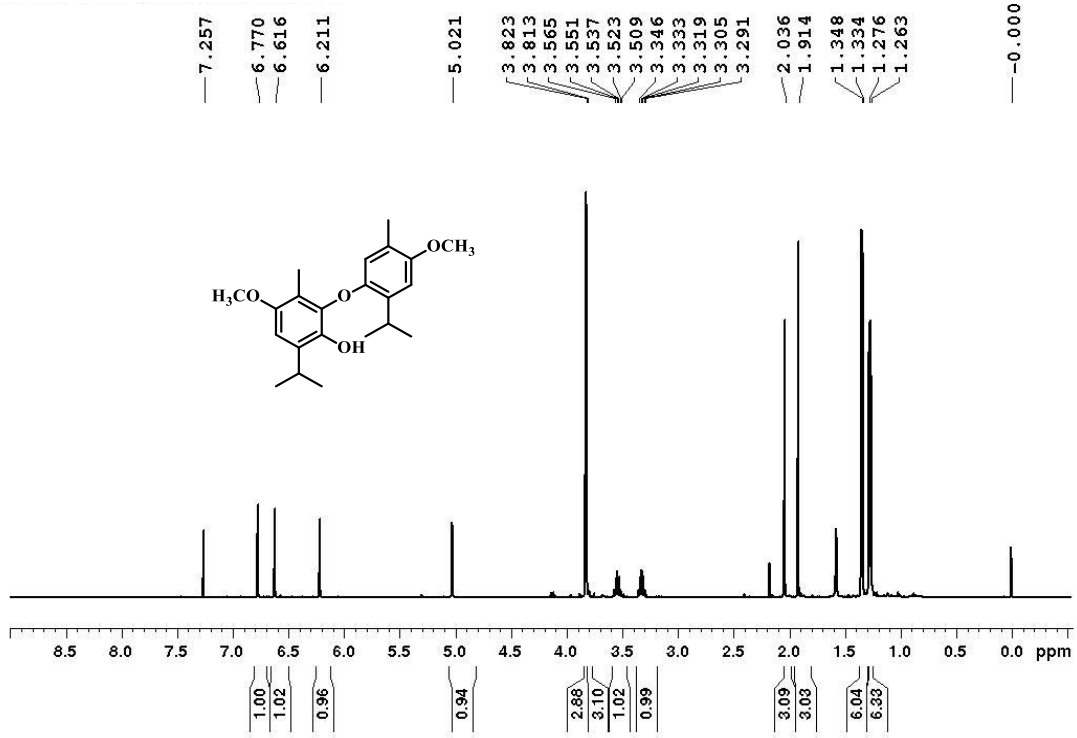


Figure 2A.7. ^{1}H NMR spectrum of compound **1** in $CDCl_3$

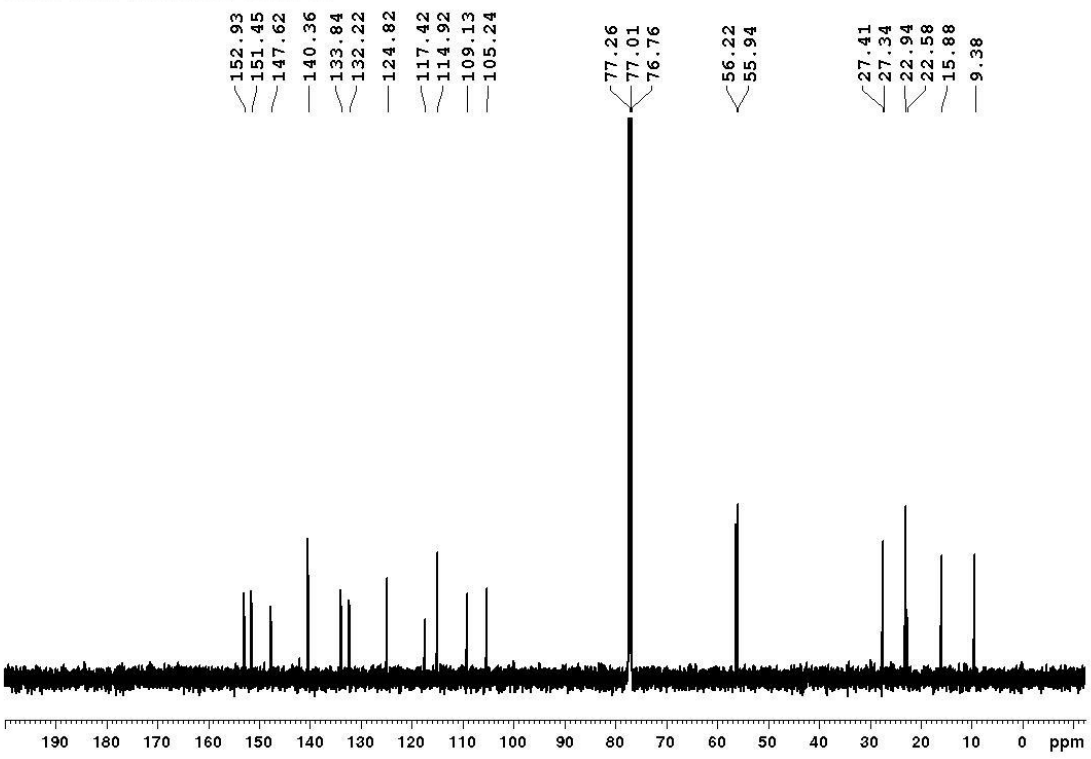


Figure 2A.8. ^{13}C NMR spectrum of compound **1** in $CDCl_3$

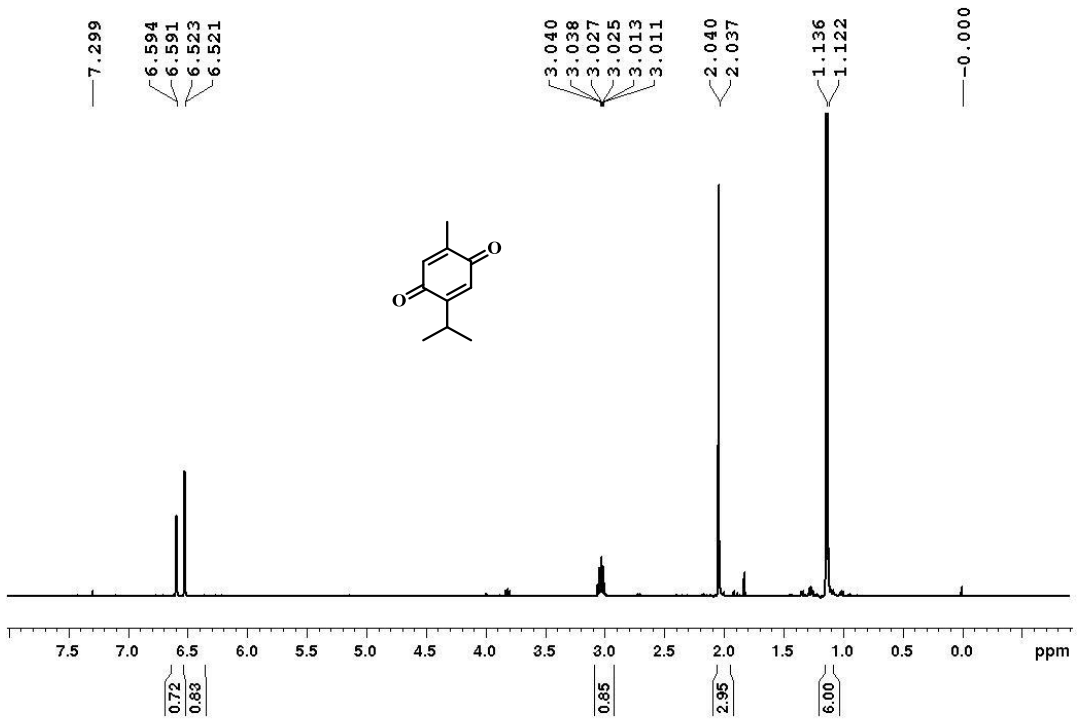


Figure 2A.9. ^{1}H NMR spectrum of compound **2** in $CDCl_3$

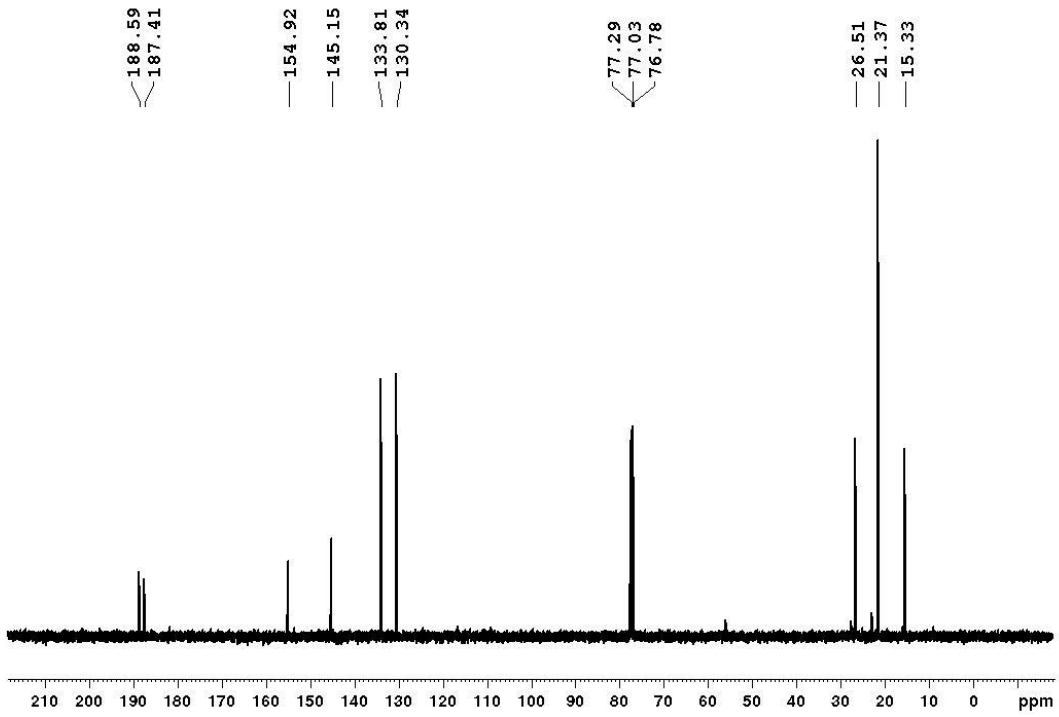
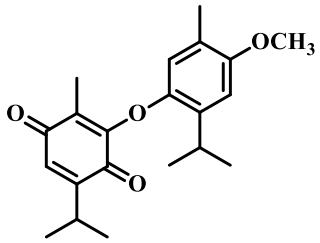


Figure 2A.10. ^{13}C NMR spectrum of compound **2** in $CDCl_3$

The HRESIMS analysis showed a molecular ion peak at 365.1725, which is the $(M+Na)^+$ peak. Combining the mass and spectral data, the molecular formula of the compound was assigned as $C_{21}H_{26}O_4$. The protons and carbons were assigned based on COSY, HMQC and HMBC analysis. In the HMBC spectrum, the methoxy proton at δ 3.82 showed a correlation with carbon at δ 153.5, concluding that the methoxy group is attached to the C-6' carbon. The carbonyl peaks at δ 188.5 and 181.7 correlate with corresponding protons at δ 1.99 (H-7) and 6.54 (H-3) confirming the position of the carbonyl groups as C-5 and C-2. Finally, the structure was confirmed with single-crystal XRD (**Fig. 2A.11**). Crystal data for **3** (CCDC 2086733): $C_{21}H_{26}O_4$. M= 342.42, Triclinic, space group P-1, a= 8.666(5)Å, b=9.634(6) (3) Å, c= 13.266(8)Å, α= 108.260(10)°, β= 90.060(4)°, γ=110.285(10)°, cell formula units Z=2, crystal density=1.162 mg/m3, T=302(2), 3503 reflections collected, [1505 reflections with I > 2\s(I)] R factor gt 0.0872. The single crystal X-ray structure of the compound is reported for the first time. Hence compound **3** was confirmed as **Libocedroquinone**, a *p*-cymene type quinone.[8]



Compound **3 – Libocedroquinone**

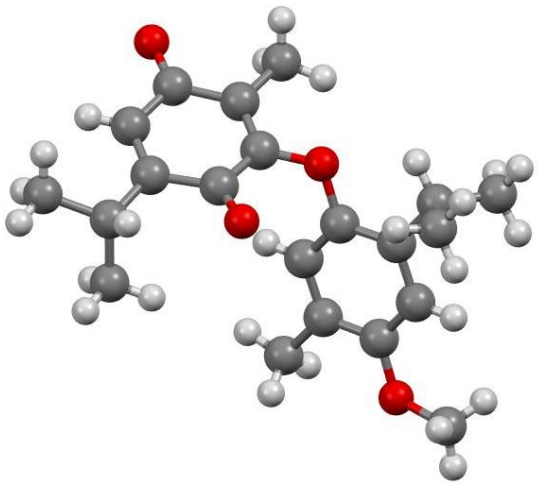


Figure. 2A.11.ORTEP diagrum of Libocedroquinone

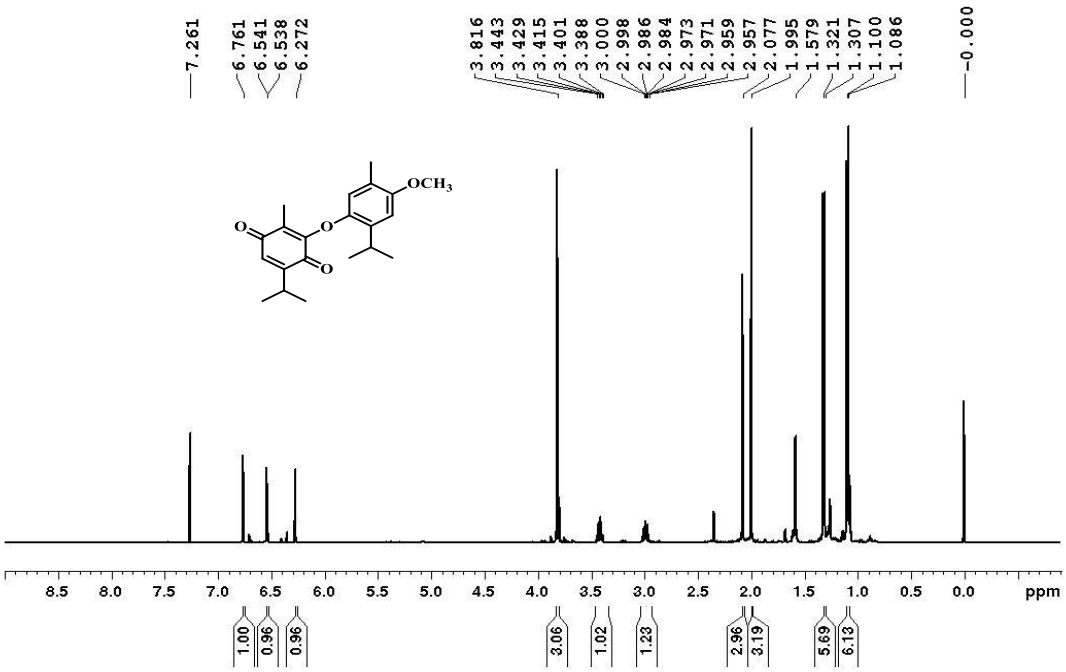


Figure 2A.12. ^{1}H NMR spectrum of compound **3** in $CDCl_3$

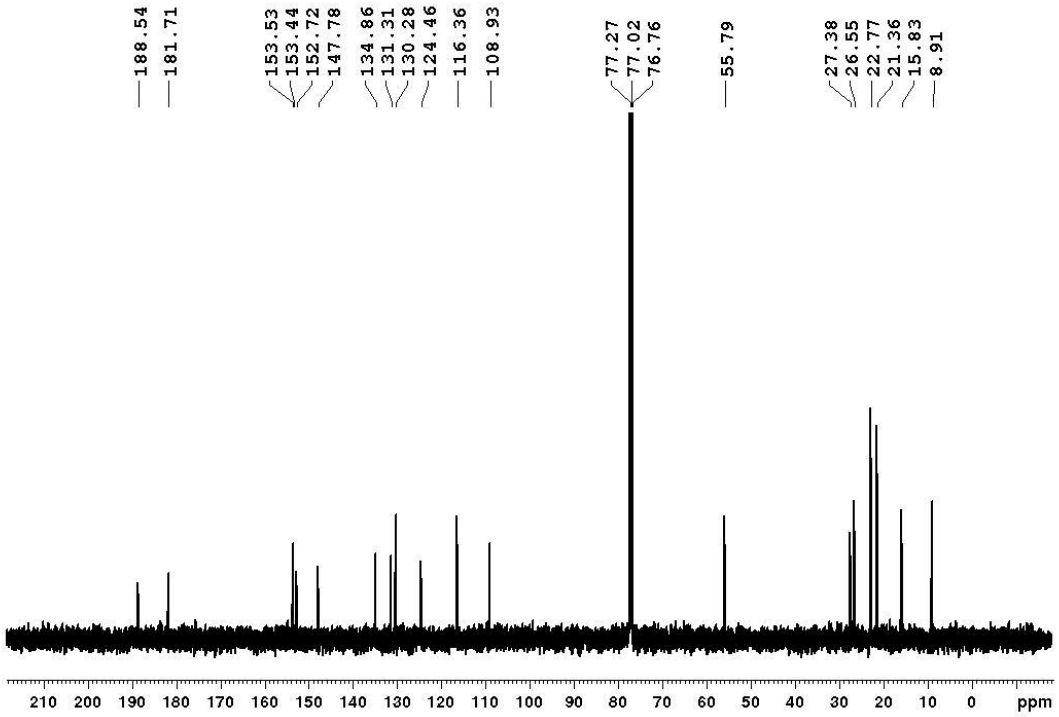
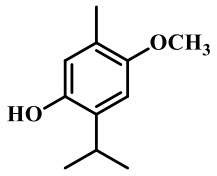


Figure 2A.13. ^{13}C NMR spectrum of compound **3** in $CDCl_3$

Next, we carried out the column chromatographic separation of the acetone extract. About 28 g of the acetone extract was subjected to silica gel CC (100-200 mesh) afforded four compounds along with the compounds previously isolated from the hexane extract. Fraction 17-20 obtained by eluting the column with 5 % polarity of ethyl acetate in hexane yielded three compounds. Compound **4** (269 mg) and **5** (39 mg) were obtained as an oily mixture, which was further purified. The ^{1}H NMR spectra of the two compounds were similar, having a *p*-cymene type skeleton with two aromatic protons, one methyl group, one isopropyl group, one methoxy group and a hydroxyl group, but a shift in the peak values were noticed.

The mass spectral analysis of compound **4** showed a molecular ion peak at *m/z* 181.0497, which could be the $(M+H)^+$ peak. The ^{13}C NMR spectrum (**Fig. 2A.15**) established 11 carbon signals, with a methoxy group resonating at *δ* 56.4 ppm. Combining all the data, the molecular formula of the compound was assigned as $C_{11}H_{16}O_2$. The ^{1}H NMR spectrum (**Fig. 2A.14**) showed two aromatic protons at *δ* 6.68 and 6.53 ppm. The presence of an isopropyl group was confirmed with signals at *δ* 3.19 (hpt, *J* = 7.0 Hz, 1H) & 1.23 (d, *J* = 7.0 Hz, 6 H) ppm. In the COSY spectrum, the proton at *δ* 6.68 (H-5) correlates with the methoxy group at *δ* 3.78 and methine proton at *δ* 3.19 (H-8) ppm confirmed the attachment of methoxy in the C-6 carbon. Similarly, the singlet at *δ* 6.53 (H-2) correlates with the methyl group at *δ* 2.13 (H-7) ppm. Further the position of carbons and protons were assigned based on HMQC and HMBC analysis and the structure was confirmed as ***p*-Methoxythymol**.[6]



Compound **4 - *p*-Methoxythymol**

Similarly, the ^{1}H NMR spectrum (**Fig. 2A.16**) of compound **5** contains two aromatic protons (*δ* 6.62 and 6.65), one methoxy group (*δ* 3.77), one isopropyl group [*δ* 3.24 (hpt, *J* = 7.0 Hz, 1H) & 1.16 (d, *J* = 7.0 Hz, 6H)] and one hydroxyl group (*δ* 4.83). The peak at *δ* 6.62 (H-2) correlates with the methoxy protons at *δ* 3.77 and *δ* 6.65 (H-5) connects with the septet of isopropyl group at δ 3.24 (H-8) in the COSY spectrum. The molecular ion peak was observed at *m/z* 179.1068, which is the $(M-H)^+$ peak.

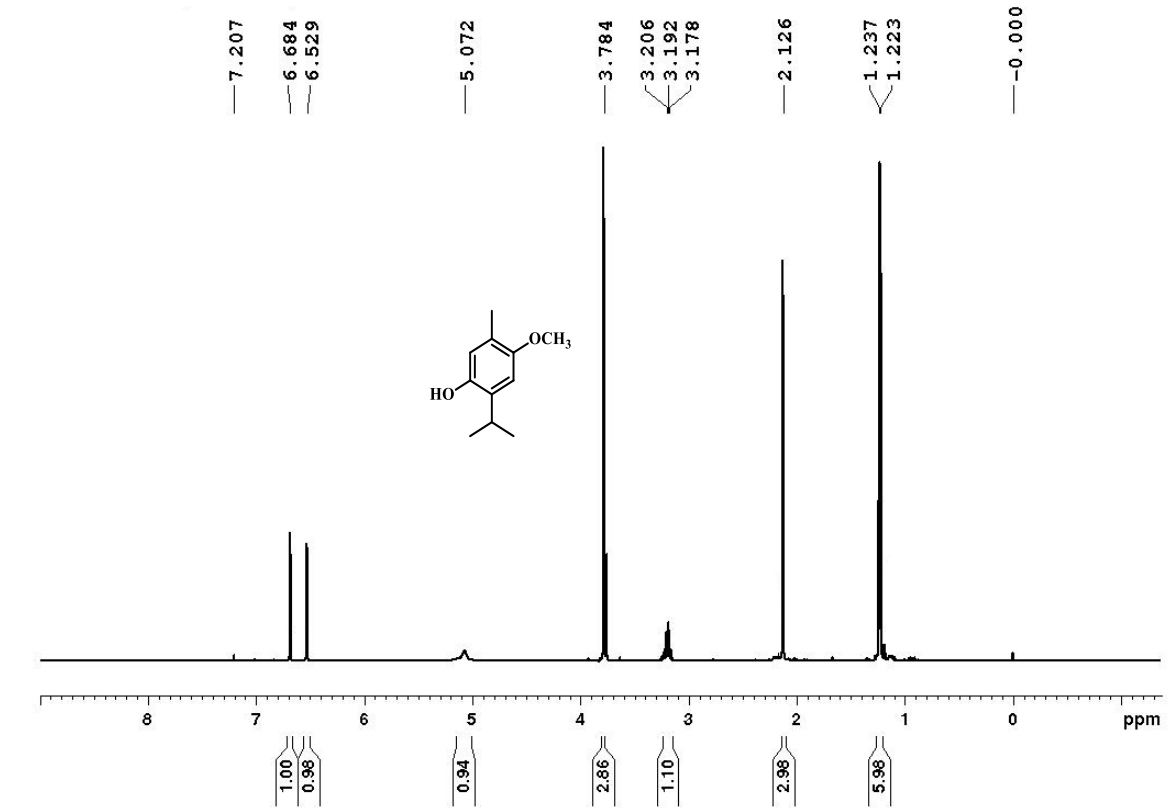


Figure 2A.14. ^{1}H NMR spectrum of compound **4** in $CDCl_3$

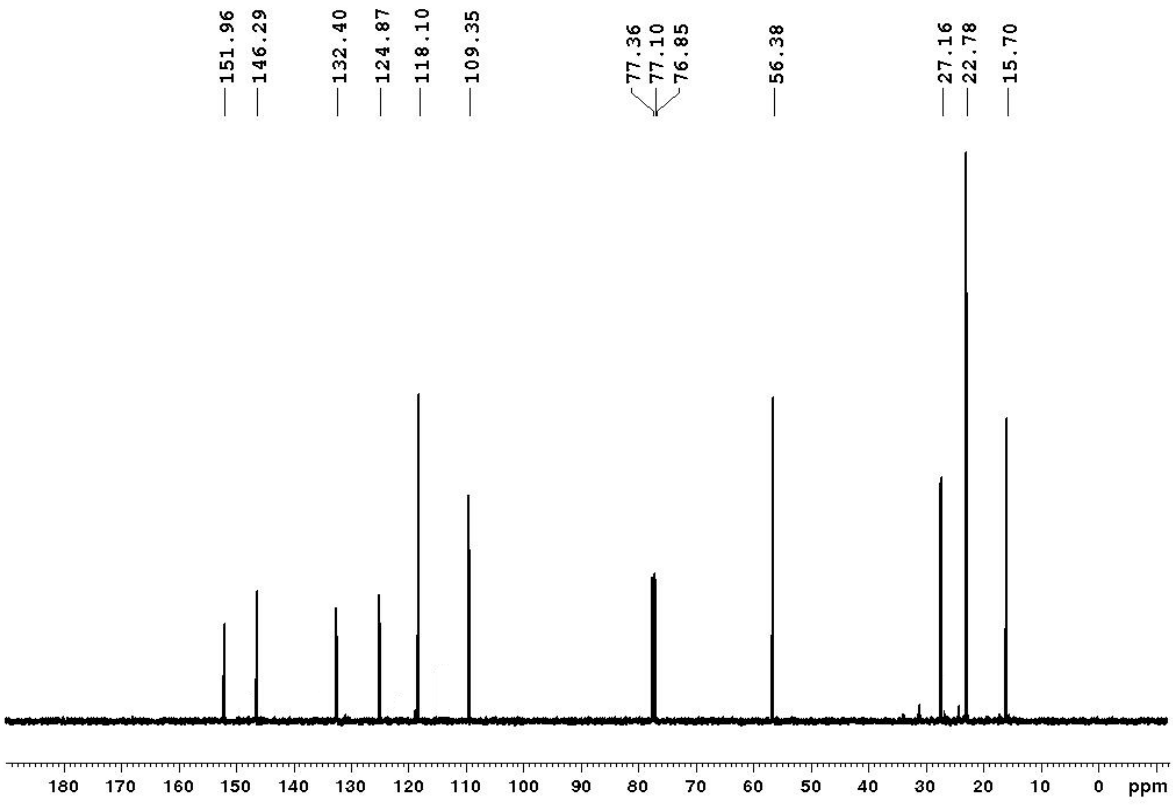


Figure 2A.15. ^{13}C NMR spectrum of compound **4** in $CDCl_3$

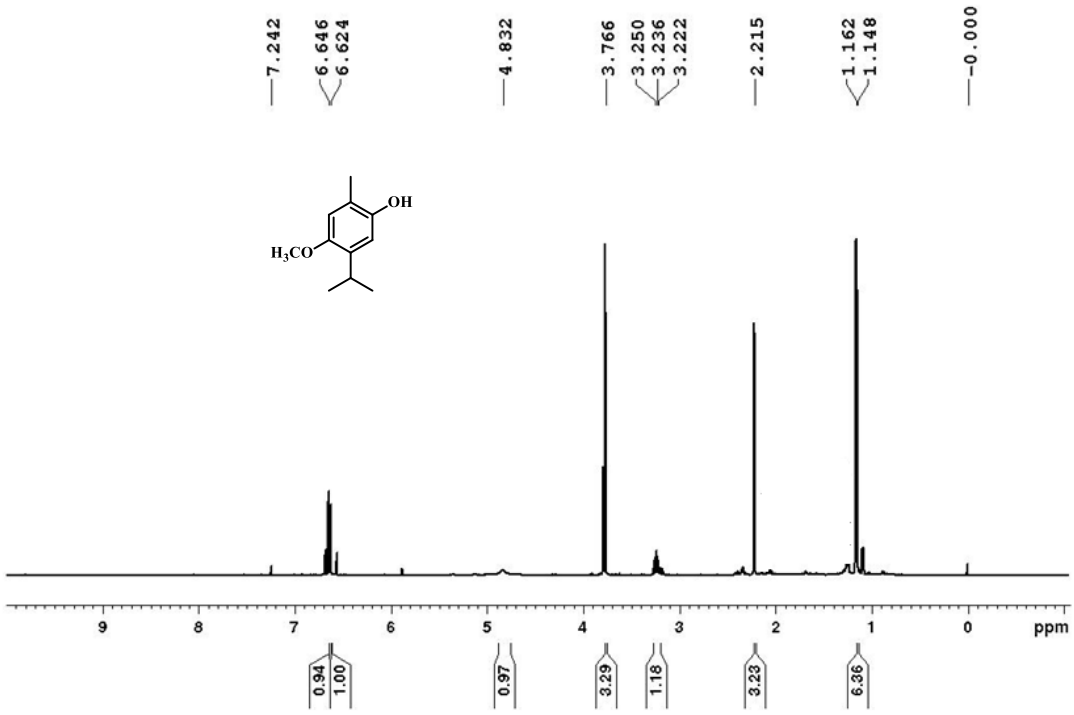


Figure 2A.16. ^{1}H NMR spectrum of compound **5** in $CDCl_3$

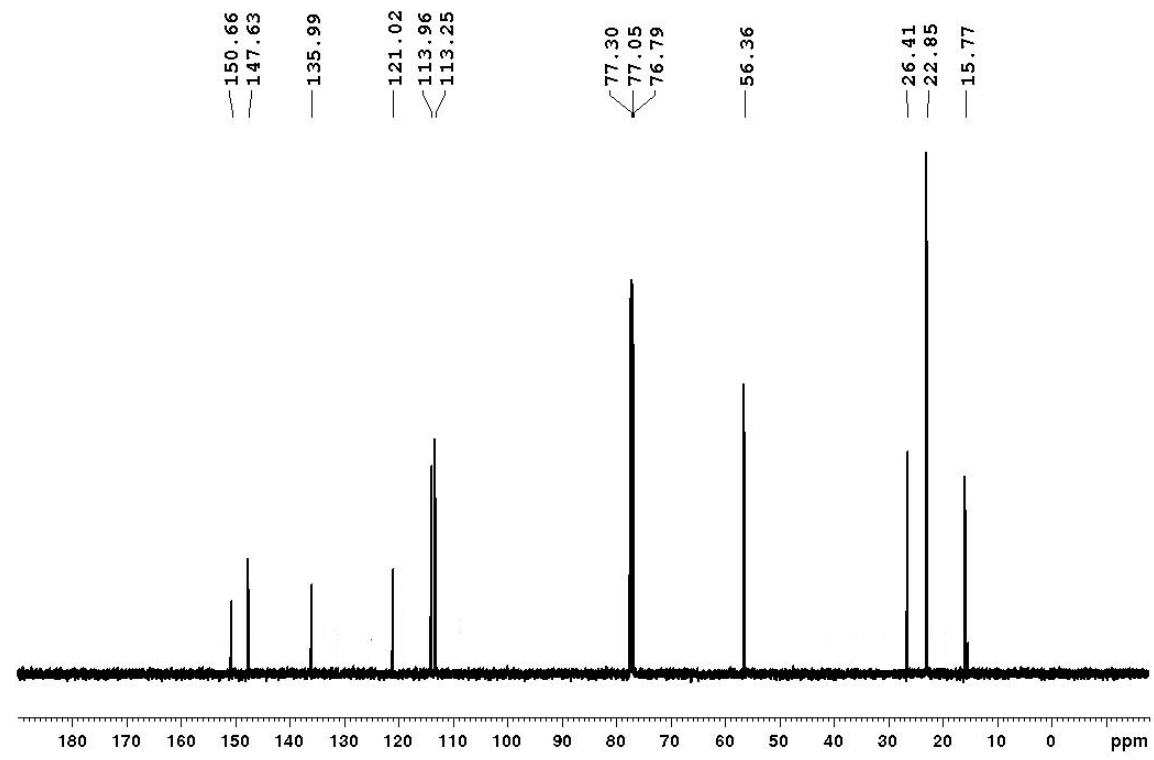
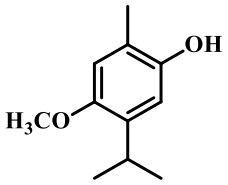


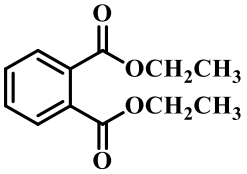
Figure 2A.17. ^{13}C NMR spectrum of compound **5** in $CDCl_3$

Hence, the compound is identified an isomer of the previous one and characterized as ***p*-Methoxycarvacrol**.[6]



Compound **5 - *p*-Methoxycarvacrol**

Compound **6** was obtained from the same fraction as a colourless oily substance of 17 mg. The ^{1}H NMR spectrum(**Fig. 2A.18**) revealed the presence of two aromatic proton signals each integrating for two [δ 7.73 (dd, J = 3.5, 5.5 Hz, 2H), 7.53 (dd, J = 3.0, 5.5 Hz, 2H)]. A methylene [δ 4.37 (q, J = 7.0 Hz, 4H)] and methyl [1.37 (t, J = 7.5 Hz, 6H)] groups were also observed. The ^{13}C NMR spectrum (**Fig. 2A.19**) exhibited six-carbon signals with one carbonyl group at δ 167.7 ppm. The data showed the structure of the compound was a symmetric one. The HRMS data with molecular ion peak at *m/z* 245.0792 $(M+Na)^{+}$, supports the molecular formula of the compound as $C_{12}H_{14}O_4$. From the spectral data and literature reports, the compound was identified as **Diethyl phthalate**.[30] It is reported for the first time from this species.



Compound **6 - Diethyl phthalate**

Compound **7** was isolated from fraction 95-98 as a white crystalline solid. The molecular formula was found to be $C_{10}H_{20}O_3$ on the basis of HRMS analysis with a parent ion peak at *m/z* 211.1307 $(M+Na)^{+}$. The ^{1}H NMR spectrum (**Fig. 2A.20**) exhibited a multiplet at δ 3.36 indicated the presence of an oxygenated proton and the corresponding carbon peak observed at δ 75.5 ppm. Three methyl groups resonated at δ 1.09 as singlet and δ 0.76 (6H) as a doublet of doublets. The peaks at δ 4.52, 3.84 and 3.15 ppm corresponded to three hydroxyl groups. The ^{13}C NMR spectrum (**Fig. 2A.21**) showed the presence of 10 carbons (two are merged with the acetone peaks) including, two oxygenated quaternary carbon signals (δ 74.7 & 71.2),

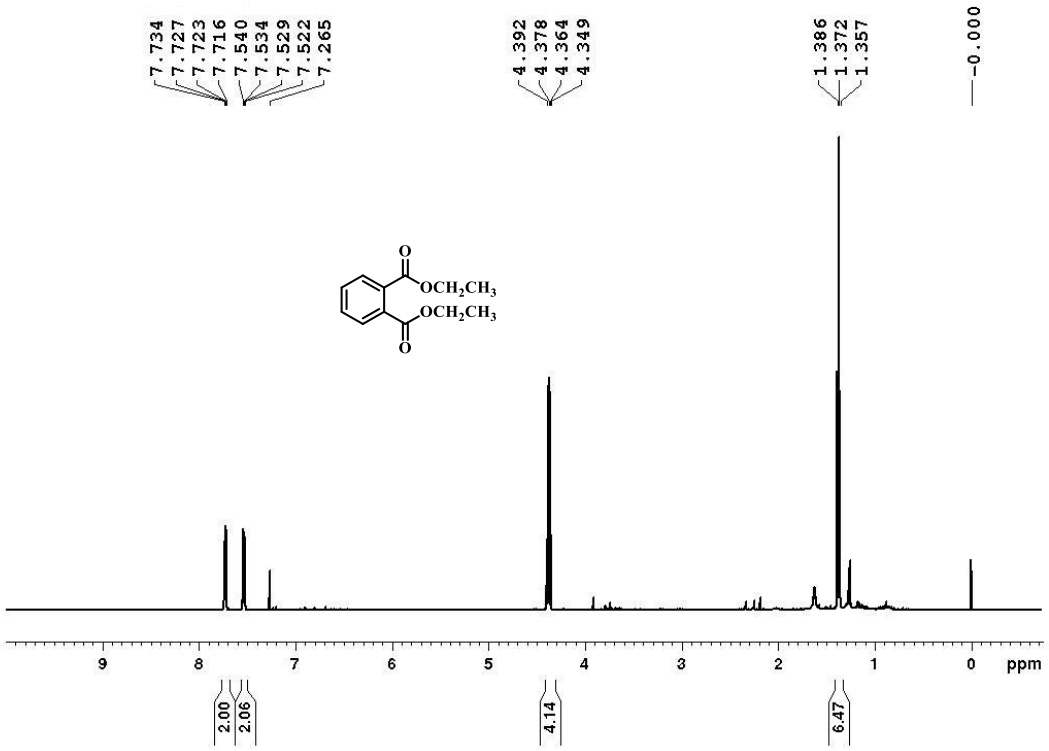


Figure 2A.18. ^{1}H NMR spectrum of compound **6** in $CDCl_3$

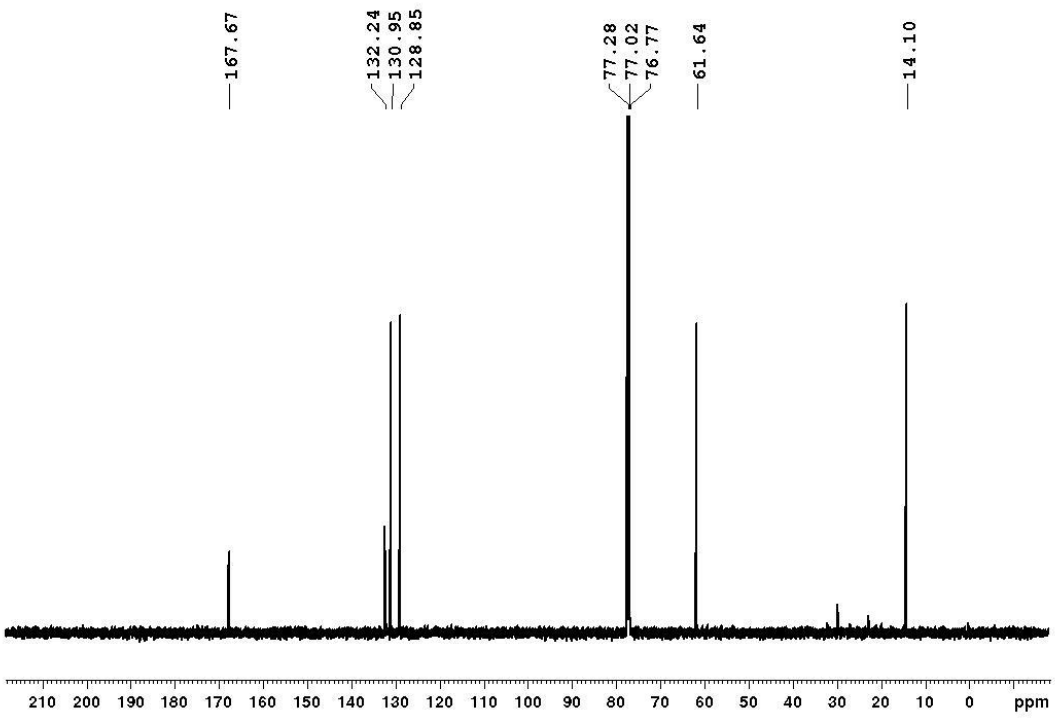
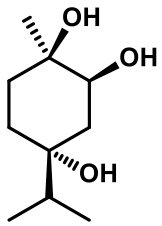


Figure 2A.19. ^{13}C NMR spectrum of compound **6** in $CDCl_3$

three methylene (δ 34.6, 30.3 & 30.2), two methines (δ 75.5 & 39.1) and three methyl (δ 27.8 & 17.2) carbons. Thus, based on the spectral data and comparison with literature reports the compound was assigned as a *p*-menthane-1,2,4 triol. The position of the hydroxyl group assigned based on COSY analysis. The peaks at δ 4.52 (d, J = 7.0 Hz) showed a correlation with proton at δ 3.36 (H-2) and δ 3.84 correlates with δ 1.83 (H-3). Remaining protons were assigned based on HMBC and HMQC analysis. Hence, compound **7** was identified as **(1R,2R,4R)-p-menthane-1,2,4-triol**. To the best of our knowledge, this compound is reported for the first time from *Calocedrus* genus. It was previously reported from *Artemisia suksdorfi* (Asteraceae family) and *Daucus carota* (Apiaceae family). [31,32]



Compound **7- (1R,2R,4R)-p-menthane-1,2,4-triol**

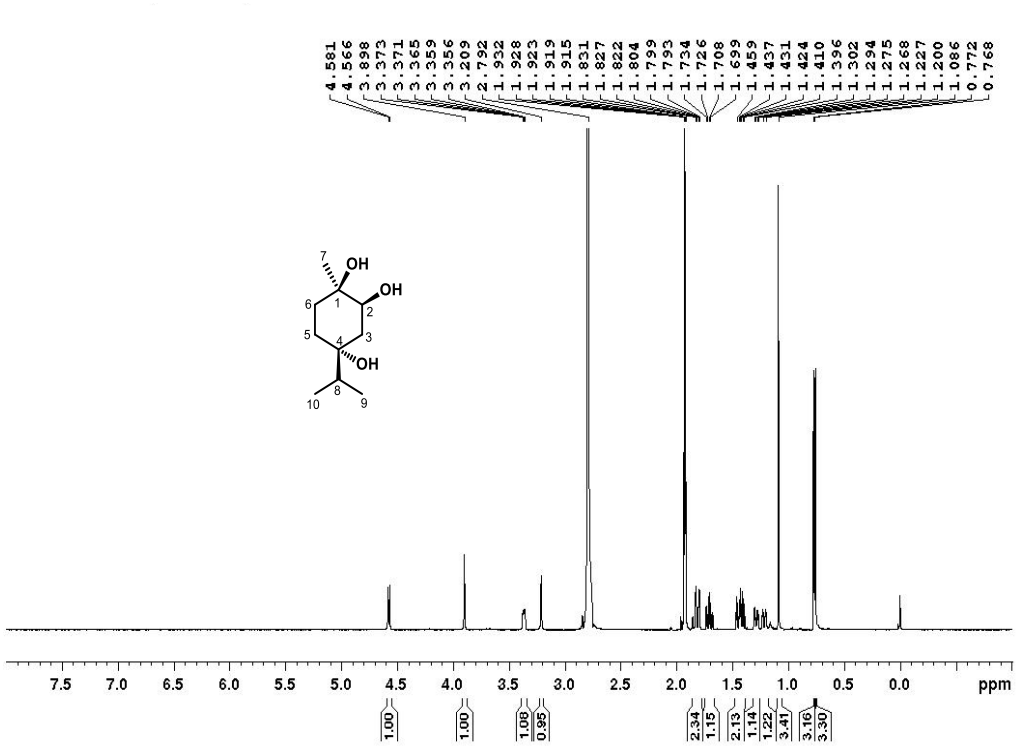


Figure 2A.20. ^{1}H NMR spectrum of compound **7** in Acetone-d6

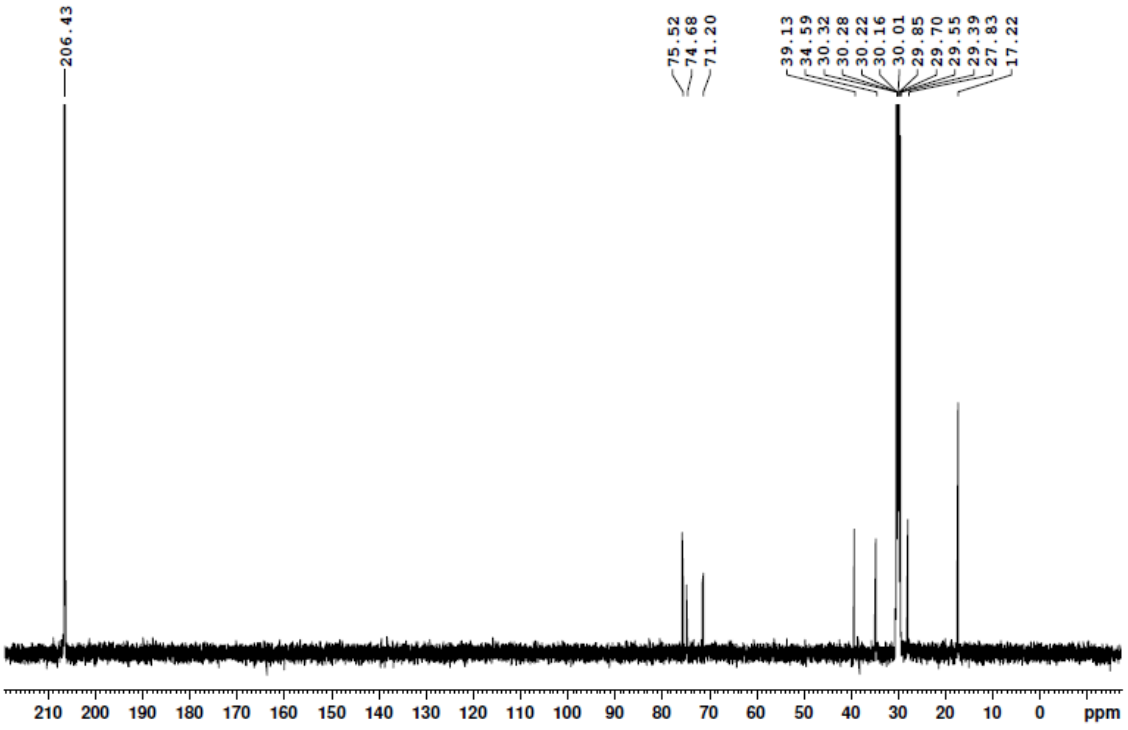
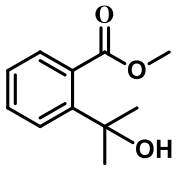


Figure 2A.21. ^{13}C NMR spectrum of compound **7** in Acetone-d6

Fractions 99-104 were subjected to CC separation to obtain compound **8** as a reddish oily substance (26 mg). The HRESIMS analysis indicated a molecular formula $C_{11}H_{14}O_3$ from the $(M+H)^+$ peak at *m/z* 195.1016. The ^{1}H NMR spectrum of compound **8** has resonances due to two *ortho-meta* coupled aromatic protons [δ 7.43 (dd, *J* = 2.0, 12.5 Hz, 1H) & 7.29 (dd, *J* = 2.0, 10.5 Hz, 1H)], two *ortho*-coupled aromatic protons [δ 6.97 (d, *J* = 12.0 Hz, 1H)] & 6.77 (d, *J* = 10.0 Hz, 1H)], one methoxy group (δ 3.72) and two methyl group [δ 1.38 (s, 6H)]. The ^{13}C and DEPT-135 spectra exhibited 11 carbon signals, which includes a carbonyl group (δ 180.1), three quaternary carbons (δ 165.0 149.7 & 72.9), four methines (δ 136.9, 136.2, 127.4, & 113.4), one methoxy (δ 56.2) and two methyl group (δ 31.4). The ^{1}H and ^{13}C were assigned based on COSY, HMBC and HMQC spectra. The correlation between the methyl group (δ 1.38, H-8 &-H9) with –OH (δ 4.22) in the COSY spectrum confirms the hydroxyl group is attached to the C-7 carbon. The structure of the compound was identified as **methyl 2-(2-hydroxypropan-2-yl)benzoate** and the assigned structure is given below. To the best of our knowledge, so far there is no reports on this compound and considered as a new skeleton. The 1D and 2D spectra are shown in **Figure 2A.22-25**.



Compound **8 -Methyl 2-(2-hydroxypropan-2-yl)benzoate**

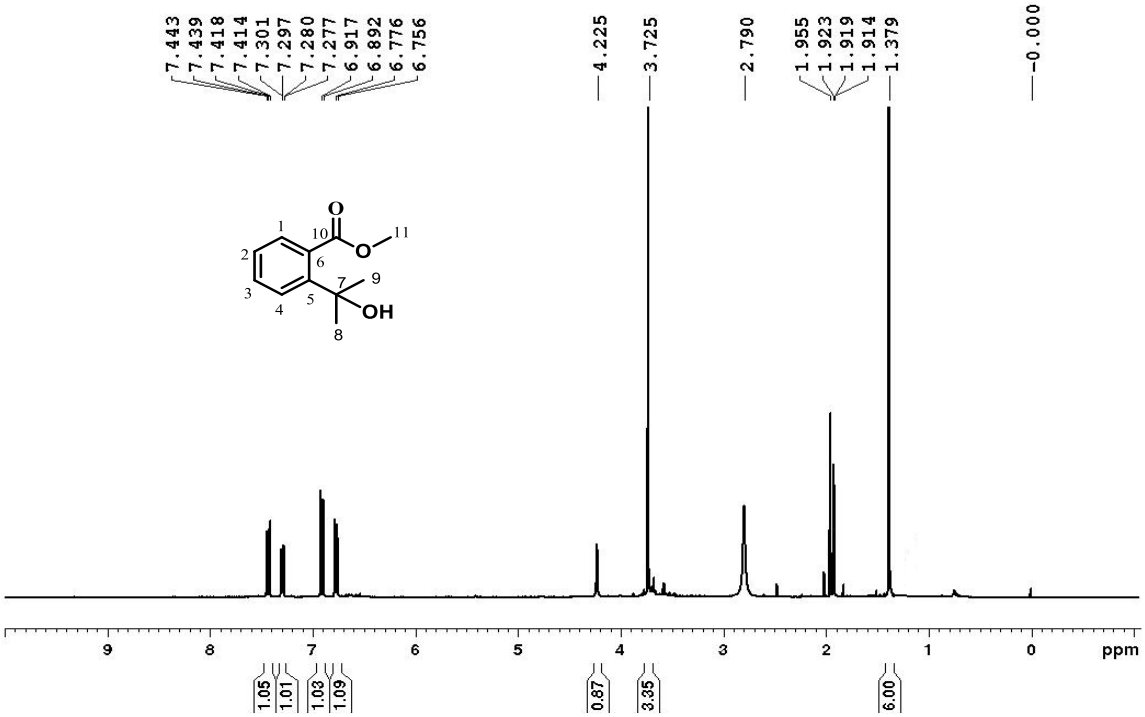


Figure 2A.22. ^{1}H NMR spectrum of compound **8** in Acetone-d6

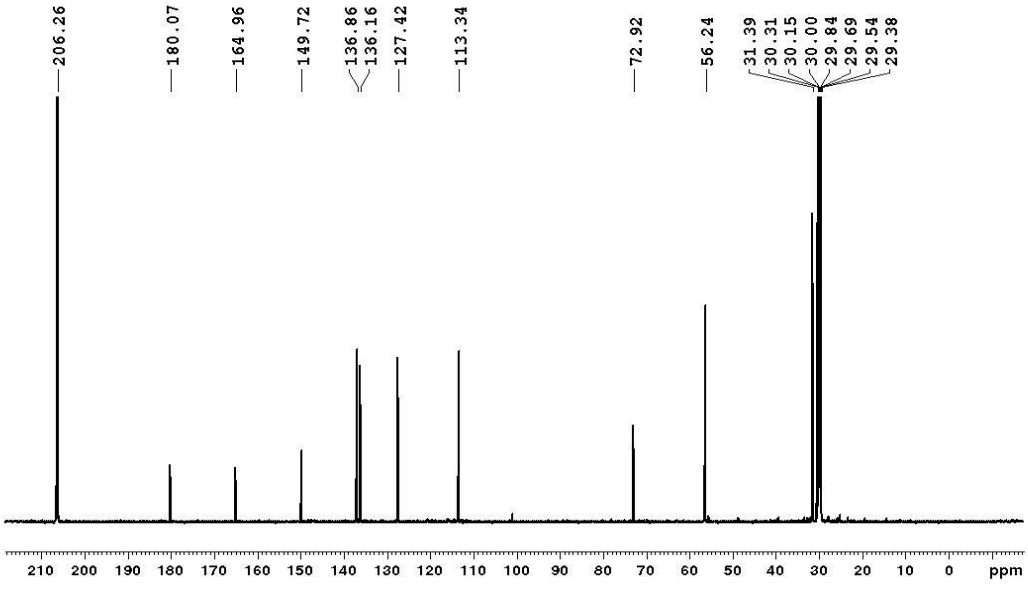


Figure 2A.23. ^{13}C NMR spectrum of compound **8** in Acetone-d6

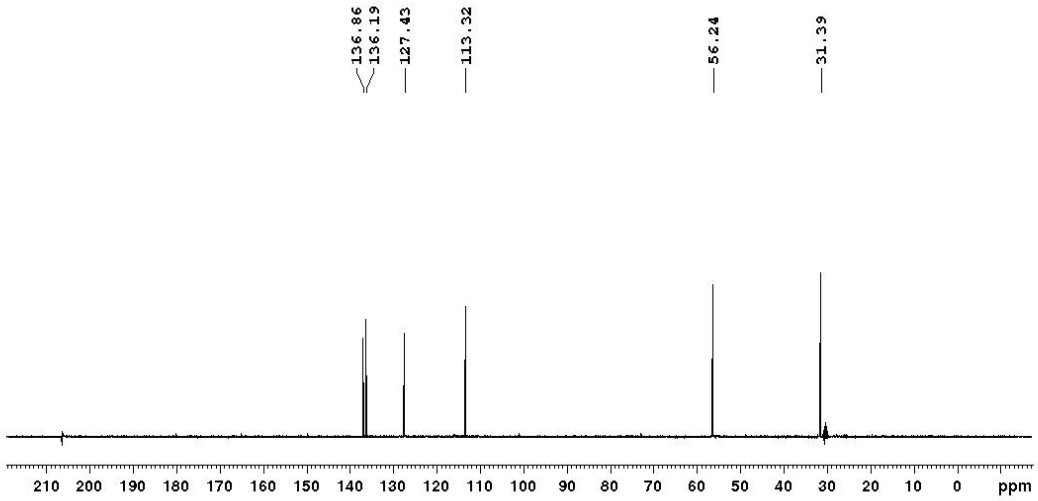


Figure 2A.24. DEPT-135 of compound **8**

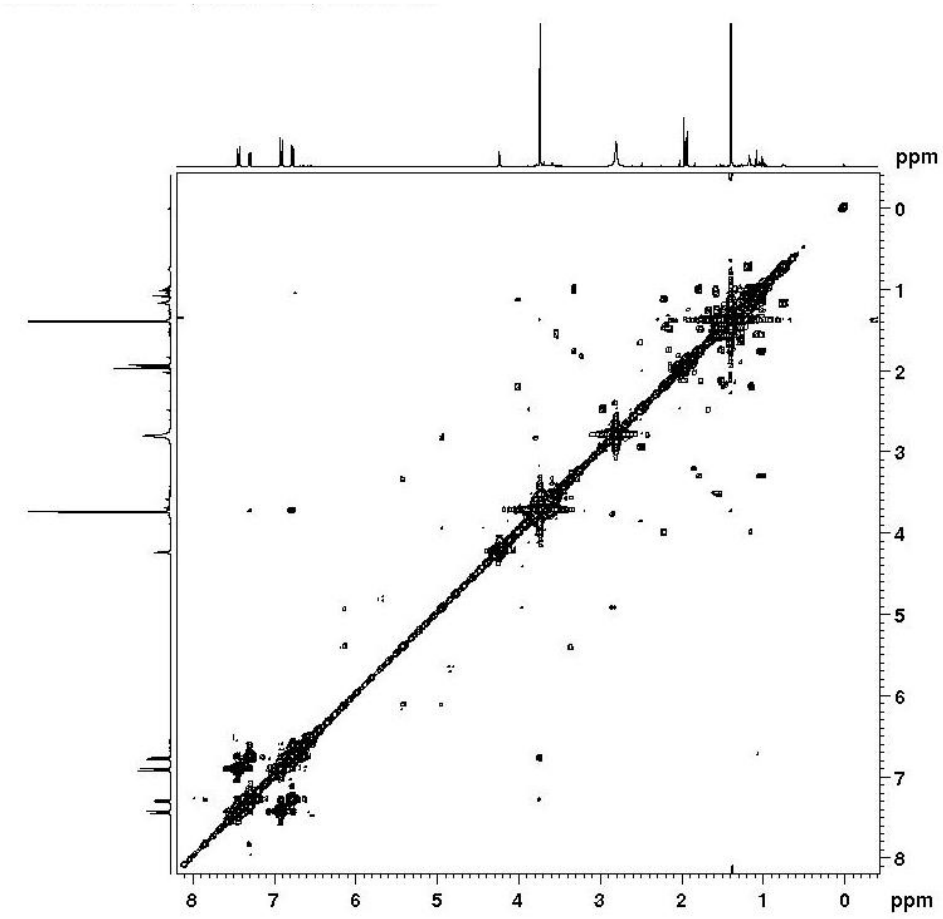


Figure 2A.25. COSY spectrum of compound **8**

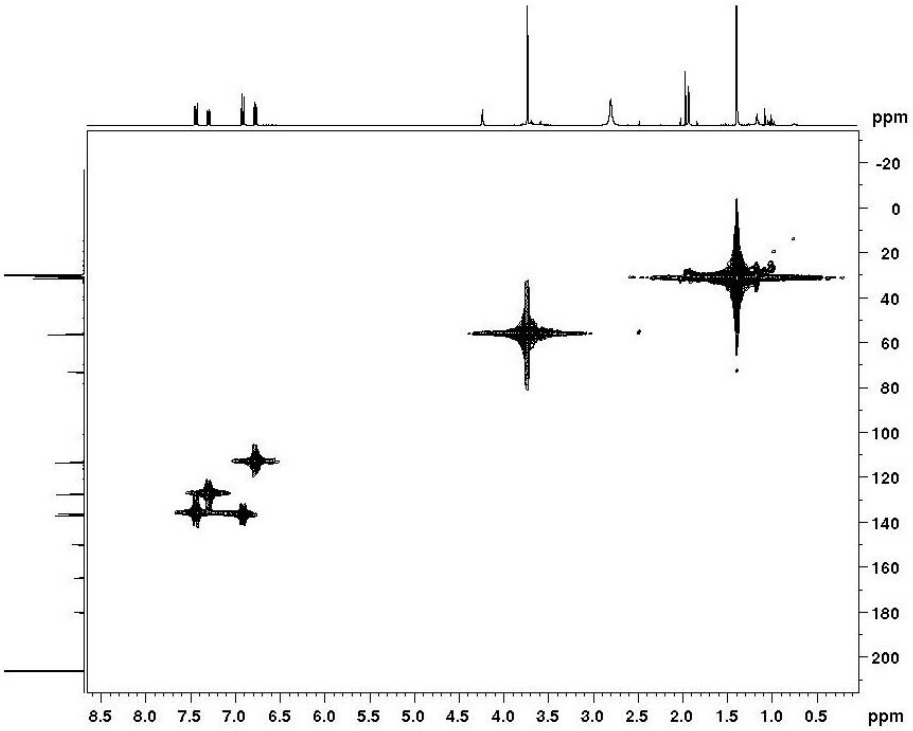


Figure 2A.26. HMQC spectrum of compound **8**

2A.5. Biological studies

2A.5.1. *In vitro* anticancer activity

As per the preliminary cytotoxic studies of the extracts, hexane was exhibiting promising activity against A549 cell line; further studies were concentrated on the compounds isolated from the hexane extract. The isolated compounds were examined for their growth inhibitory properties against A549 and WI-38 cell lines. The standard drug used was Doxorubicin (DOX) with similar concentrations as the compounds. Treatment of each compound resulted in a concentration-dependent reduction of cell viability. The cytotoxicity results in terms of IC_{50} values is depicted in **Table 2A.2** and the effect of various concentrations is represented in **Figure 2A.27**. Compound **1** and **3** showed an IC_{50} value of 7 µM and 4.6 µM for 24 h and 7.2 µM and 3 for 48 h respectively. Compound **2** did not show significant cytotoxicity up to 20 µM at 24 h whereas an IC_{50} value of 16.5 µM was obtained in 48 h. DOX showed IC_{50} at 1.8 µM in 24 h and 0.8 µM in 48 h. Compound **1** and **3** exhibited similar cytotoxic effects of doxorubicin upon 48 h of treatment ($P > 0.05$, non-significant, when compared to DOX, compound **1** and **3**. Whereas in terms of IC_{50} value, compound **3** is found to be more efficient when compared to other compounds.

Table 2A.2. IC_{50} values of different compounds in A549 and WI-38 cell lines at 24 h and 48 h. Average of more than three independent experiments.

Compounds	IC_{50} of Compounds in µM			
	A549		WI-38	
	24 h	48 h	24 h	48 h
1	7 ± 1.5[b]	7.2 ± 0.3[b]	16.2 ± 1.6	11.4 ± 2.6[b]
2	>20[c]	16.5 ±1.2[b]	>20[c]	14.4 ± 1.7[b]
3	4.6 ± 0.9[b]	3 ± 0.2[b,d]	>20[c]	5.6 ± 1.2[d]
DOX[1]	1.8 ± 0.2	0.8 ± 0.03	16.5 ± 0.5	4.2 ± 0.8

[a]Standard drug; [b]Statistical significance: DOX vs. Compounds p<0.001; c IC-50 not achieved; [d]Compound 1 vs. Compound 3 p<0.001, A549 - human lung adenocarcinoma; WI-38 - normal lung fibroblast

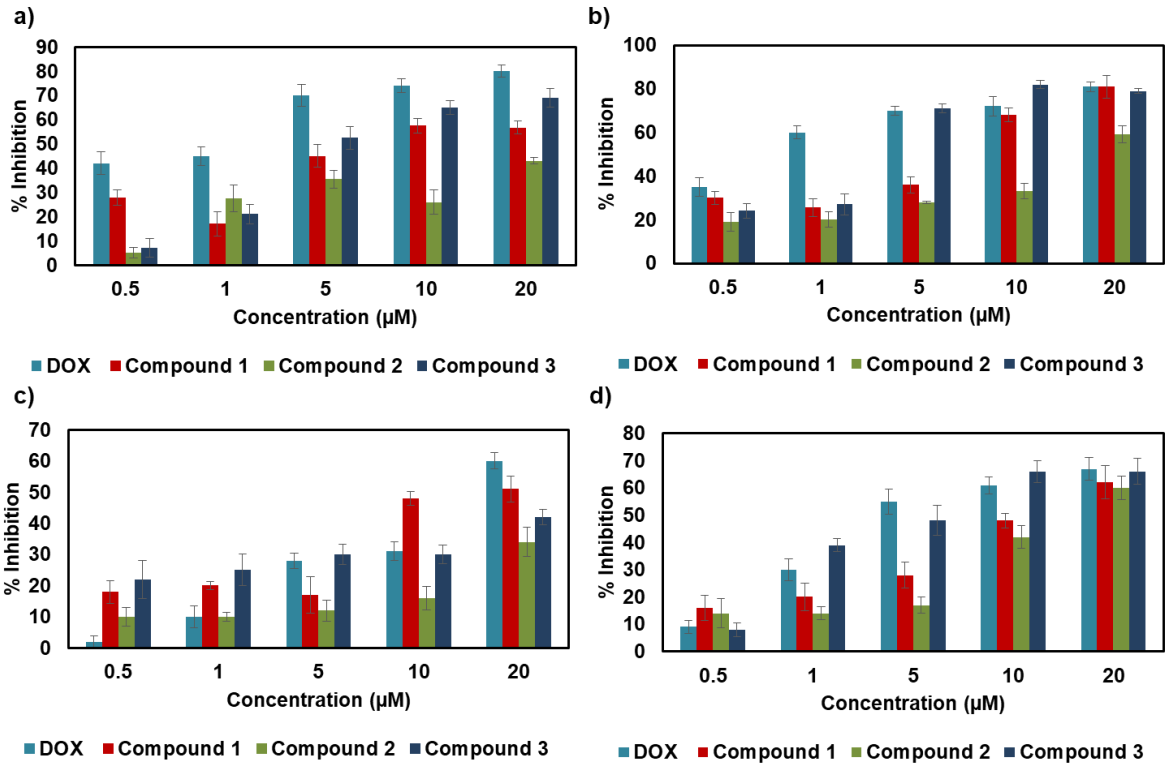


Figure 2A.27. Evaluation of cytotoxicity of compounds by MTT assay on A549 (a) at 24 h (b) at 48 h and on WI-38 (c) at 24 h and (d) at 48 h.

Upon 24 h treatment, IC_{50} value of compound **3** in A549 cell line was 4.6 ± 0.9 µM whereas for WI-38 the IC_{50} value was not achieved even at a concentration of 20 µM emphasizing its minimal cytotoxic effects in normal cells. Even though these

compounds are not as effective as doxorubicin against human lung adenocarcinoma cell line, they exhibited low toxicity against normal cell line which was comparable with doxorubicin (16.5 μM) at 24 h.

2A.5.2. Live dead assay (Acridine orange/ethidium bromide staining)

In acridine orange-ethidium bromide dual staining procedure, acridine orange is a nonfluorescent membrane-permeant dye which will be turned into fluorescent upon conversion by cellular esterase enzymes; thus, all the viable cells will be appeared green in colour with FITC filter. Whereas, ethidium bromide being impermeant to cell membrane, enters only into dead cells with compromised cell membranes and gives red fluorescence upon binding with DNA. Fluorescent images of the live-dead assay showed a reduced number of cells in the compound **3** treated group with most of the cells exhibiting yellow/red flourescence when compared to the untreated cells (**Fig. 2A.28**). Thus, the results indicated the apoptosis-inducing ability of compound **3** even at a concentration of 4.6 μM. Out of the selected compounds, compound **3** was exhibiting appreciable apoptotic effects towards the lung adenocarcinoma cells (A549), but found to be least toxic to normal lung fibroblast cells (WI-38) at the specified concentration.

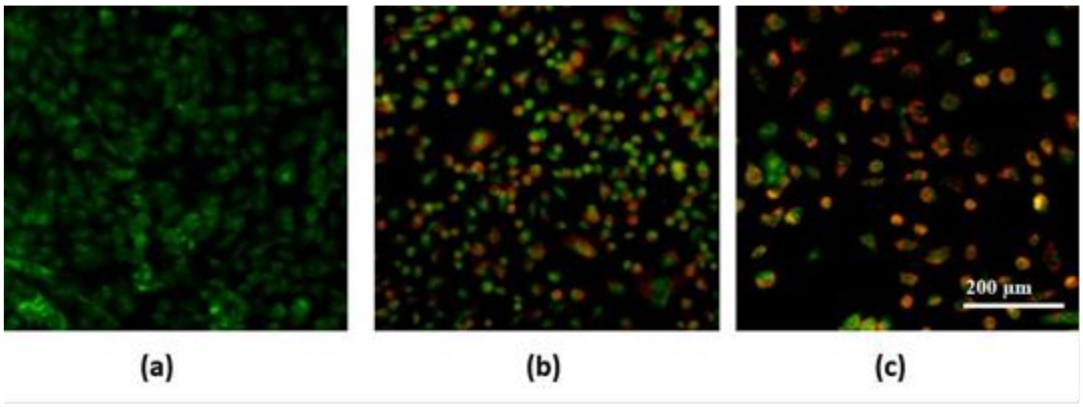


Figure 2A.28. Induction of apoptosis by live-dead assay (a) control (b) A549 cells treated with compound **3** at 4.6 μM (c) doxorubicin at 2.16 μM. Scale bar corresponds to 200 μm.

According to literature reports, thymoquinone (**2,** TQ) was reported to exhibit significant anticancer activity against human pancreatic adenocarcinoma, uterine sarcoma and leukemic cell lines.[28,33] Previous studies showed that TQ promoted apoptosis in A549 lung cancer cells *via* the activation of p53 and caspase cascade-dependent pathways.[34,35] From the results libocedrol and libocedroquinone

exhibited significant anticancer potency compared to TQ. Libocedroquinone is biosynthetically derived from the oxidative coupling of thymoquinone and *p*-methoxythymol.[13] As a structural derivative of thymoquinone, it exhibits excellent activity than thymoquinone towards cancer cells and less toxic to normal cells. Our findings suggested that libocedroquinone could be developed as an effective chemotherapeutic agent for lung cancer. More studies are needed to uncover the exact molecular targets and mechanism of the action of libocedroquinone.

2A.6. Conclusion

In summary, we have isolated eight compounds from the hexane and acetone extracts of *C. deccurrens,* which includes a new compound. During cytotoxicity studies, hexane extract showed excellent activity compared to doxorubicin and other extracts; further studies were carried out with the compounds isolated from hexane extract *i.e.*, compound **1**-**3**. Compound **3** (Libocedroquinone), exhibited good cytotoxicity comparing other compounds against A549 cell line with an IC_{50} of 4.8 µM at 24 h. The compound was found to be less toxic with the normal lung fibroblast cell line WI-38. The results indicated that libocedroquinone could be a potential candidate as an antiproliferative agent against lung cancer. Even though *C. decurrens* is an ornamental tree, it holds great potential to be as a chemopreventive agent and further investigations are necessary to validate its activity.

2A.7. Experimental section

Different analytical techniques were used for the characterization of the compounds. The IR spectra were recorded with a Bruker FT-IR spectrometer. The nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AMX 500 spectrometer ($CDCl_3$ & acetone-d6 as solvents). The chemical shift for NMR spectra is reported as δ in units of parts per million (ppm) downfield from tetramethylsilane (δ 0.0 ppm) and relative to the signal of the solvent. Mass spectra were measured using Thermo Scientific Exactive mass spectrometer under ESI/HRMS at 60,000 resolutions. The melting points were performed on a Buchi melting point apparatus. The diffraction data of single crystals were collected on a Rigaku Saturn 724+ diffractometer using graphite monochromated Mo-Kα radiation.

2A.7.1. Cell lines and Cultural conditions

Human lung adeno-carcinoma cell (A549) and human lung fibroblast cell (WI-38) were procured from National Centre for Cell Science (NCCS), Pune, India. Short tandem repeat profiling (STR) was carried out to confirm the genetic identity of cell lines and the cells were cultured in DMEM medium supplemented with 10 % heat-inactivated FBS, 100 U/mL penicillin and 100 µg/ mL streptomycin in an incubator at 37˚ C in a humidified atmosphere of 5 % CO_2.

2A.7.2. Cell proliferation assay

The cell growth inhibitory effect of isolated compounds and the extract was analysed using 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) assay as previously reported.[36] In this assay, absorbance of formazan crystals formed by the enzymatic conversion of tetrazolium salt by mitochondrial dehydrogenase is measured at 570 nm using a microplate spectrophotometer (BioTek synergy/H1 microplate reader). The rate of inhibitory effect on cell proliferation was calculated using the formula: Proliferation rate (PR) % = [Abs sample/Abs control] x 100; Inhibitory rate (IR) % = 100 – PR. For this, A549 and WI-38 cells were seeded at a density of 8000 cells/well in 96 well plate and incubated for 24 h. Various concentrations of the test materials ranging from 0.5 µM to 20 µM were added to the cells for 24 and 48 h. After the completion of the incubation period, 0.5 mg/mL MTT in HBSS was added to the cells and again kept for 2.5 h and the so formed crystals were dissolved in DMSO and the absorbance were read at 570 nm. Concentrations giving 50 % inhibition on cell growth (IC_{50}) were also calculated.

2A.7.3. Live dead assay to determine apoptotic cells

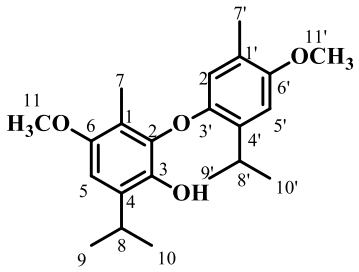
From MTT assay, the most potent compound with the least IC_{50} value at 24 hours was selected for further screening. Dual staining with acridine orange and ethidium bromide is one of the most commonly employed methods to detect apoptotic cells, where the differential uptake of two fluorescent dyes by viable and non-viable cells are monitored.[37] To perform the assay, A549 cells were seeded at a density of 5000 cells/ well and treated with or without compound **3**. Dual staining reagent was prepared by adding 1 µL each of acridine orange (from 5 mg/ mL stock) and ethidium bromide (from 3 mg/ mL stock) to 1ml PBS and 100 µL from this was added to compound **3** treated and untreated cells. After 5 minutes of incubation,

cells were washed with PBS and observed under the FITC filter of the fluorescence microscope (Nikon Eclipse TS 100, Japan).

2A.8. Spectra

Compound 1 (Libocedrol)

Compound **1** was obtained as a colourless amorphous solid (500 mg) from fractions 6-8, obtained by eluting the column with 5 % ethyl acetate-hexane polarity. The compound was characterized as **Libocedrol** based on the spectral data obtained, as shown below.



Molecular formula: $C_{22}H_{30}O_4$

FT-IR (Neat, υ_{max} cm^{-1}): 2962, 1500, 1460, 1427, 1195, 1124 cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 6.77 (s, 1H, H-5'), 6.62 (s, 1H, H-5), 6.21 (s, 1H, H-2'), 5.02 (C3-OH), 3.82 (s, 3H, H-11), 3.81 (s, 3H, H-11'), 3.54 (hpt, *J* = 7.0 Hz, 1H, H-8'), 3.32 (hpt, *J* = 7.0 Hz, 1H, H-8), 2.04 (s, 3H, H-7'), 1.91 (s, 3H, H-7), 1.34 (d, *J* = 7.0 Hz, 6H, H- 9' & 10'), 1.27 (d, *J* = 6.5 Hz, 6H, H-9 & 10) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 152.9 (C-6'), 151.4 (C-6), 147.6 (C-3'), 140.4 (C-3 & 2), 133.8 (C-4'), 132.2 (C-4), 124.8 (C-1'), 117.4 (C-1), 114.9 (C-2'), 109.1 (C-5'), 105.2 (C-5), 56.2 (C-11), 55.9 (C-11'), 27.4 (C-8'), 27.3 (C-8), 22.9 (C- 9 & 10), 22.6 (C- 9 & 10), 15.9 (C-7'), 9.4 (C-7) ppm.

HRMS (ESI): *m/z* calcd for $C_{22}H_{30}O_4$ is 358.2144. Found 381.2024 $(M+Na)^+$.

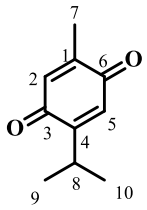
Compound 2 (Thymoquinone)

Compound **2** was obtained from the subcolumn of fraction 6-8 as yellow crystalline solid (563 mg). The compound was confirmed as **Thymoquinone** based on spectral data obtained, as shown below.

Molecular formula: $C_{10}H_{12}O_2$

FT-IR (Neat, υ_{max} cm^{-1}): 2966, 1657, 1614,1249 cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 6.59 (d, *J* = 1.5 Hz,



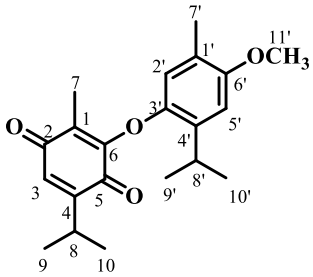
1H, H-2), 6.52 (d, *J* = 1.0 Hz, 1H, H-5), 3.02 (hpt, *J* = 6.5 Hz, H-8), 2.03 (s, 3H, H-7), 1.13(d, *J* = 7.0 Hz, H-9 & 10) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 188.6 (C-3), 187.6 (C-6), 154.9 (C-4), 145.2 (C-1), 133.8 (C-2), 130.3 (C5-), 26.5 (C-8), 21.4 (C-9 & 10), 15.3 (C-7) ppm.

HRMS (ESI): *m/z* calcld for $C_{10}H_{12}O_2$ is 164.0837. Found 163.0389 $(M-H)^+$.

Compound 3 (Libocedroquinone)

Compound **3** was obtained as a reddish crystalline solid (27 mg) from fractions 6-8. The structure was confirmed as **Libocedroquinone** based on spectral data and finally with single-crystal XRD.



Molecular formula: $C_{21}H_{26}O_4$

FT-IR (Neat, υ_{max} cm^{-1}): 2965, 2931, 1658, 1612, 1458, 1196, 1088 cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 6.76 (s, 1H, H-5'), 6.54 (s, 1H, H-3), 6.27 (s, 1H, H-2'), 3.82 (s, 3H, H-11'), 3.42 (hpt, *J* = 7.0 Hz, 1H, H-8'), 2.99 (hpt, *J* = 6.5 Hz, 1H, H-8), 2.08 (s, 3H, H-7'), 1.99 (s, 3H, H-7), 1.31 (d, *J* = 7.0 Hz, 6H, H-9' & 10'), 1.09 (d, *J* = 7.0 Hz, H-9 & 10) ppm.

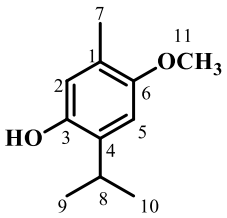
^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 188.5 (C-2), 181.7 (C-5), 153.5 (C-6'), 153.4 (C-4), 152.7 (C-6), 147.8 (C-3'), 134.9 (C-4'), 131.3 (C-1), 130.3 (C-3), 124.5 (C-1'), 116.4 (C-2'), 108.9 (C-5'), 55.8 (C-11'), 27.4 (C-8'), 26.6 (C-8), 22.8 (C-9' & 10'), 21.4 (C- 9 & 10), 15.8 (C-7'), 8.9 (C-7) ppm.

HRMS (ESI): *m/z* calcld for $C_{21}H_{26}O_4$ 342.1831. Found 365.1725 $(M+Na)^+$.

Compound 4 (*p*- Methoxythymol)

Fraction 17-20 obtained by eluting the column with 5 % ethyl acetate-hexane polarity, on further purification yielded three compounds. Compound **4** & **5** obtained as a reddish oily mixture, which was again purified and separated.

Compound **4** (269 mg) was characterized as ***p*-methoxythymol** based on spectral data and on comparison with literature reports.



Molecular formula: $C_{11}H_{16}O_2$

FT-IR (Neat, υ_{max} cm^{-1}): 2927, 2857, 1720, 1502, 1200 cm^{-1}.

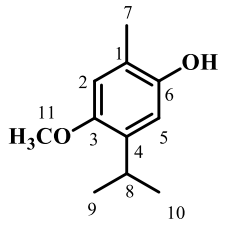
^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 6.68 (s, 1H, H-5), 6.53 (s, 1H, H-2), 5.07 (br s, 1H, -OH), 3.78 (s, 3H, H-11), 3.19 (hpt, *J* = 7.0 Hz, H-8), 2.13 (s, 3H, H-7), 1.23 (d, 6H, *J* = 7.0 Hz, H-9 & 10) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 152.0 (C-6), 146.3 (C-3), 132.4 (C-4), 124.9 (C-1), 118.01 (C-2), 109.3 (C-5), 56.4 (C-11), 27.2 (C-8), 22.8 (C-9 & 10), 15.7 (C-7) ppm.

HRMS (ESI): *m/z* calcld for $C_{11}H_{16}O_2$ is 180.1150. Found 181.0497 $(M+H)^+$.

Compound 5 (*p*- Methoxycarvacrol)

Compound **5** was characterized as ***p*- Methoxycarvacrol**, an isomer of compound **4** obtained as a reddish oily compound (39 mg). The spectral data were assigned from HMBC and HMQC correlations.



Molecular formula: $C_{15}H_{22}O_2$

FT-IR (Neat, υ_{max} cm^{-1}): 2961, 1651, 1510, 1459, 1199, 1010 cm^{-1}.

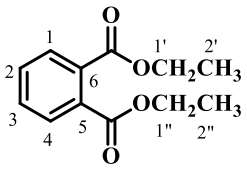
^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 6.65 (s, 1H, H-5), 6.62 (s, 1H, H-2), 4.83 (br s, 1H, -OH), 3.77 (s, 3H, H-11), 3.24 (hpt, *J* = 7.0 Hz, 1H, H-8), 2.22 (s, 3H, H-7), 1.16 (d, *J* = 7.0 Hz, 6H, H-9 & 10) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 150.7 (C-6), 147.6 (C-3), 136.0 (C-4), 121.0 (C-1), 114.0 (C-2), 113.3 (C-5), 56.4 (C-11), 26.4 (C-8), 22.8 (C-9 &10), 15.8 (C-7) ppm.

HRMS (ESI): *m/z* calcld for $C_{15}H_{22}O_2$ is 180.1150. Found 179.1068 $(M-H)^+$.

Compound 6 (Diethyl phthalate)

Compound **6** was also obtained from the same fraction 17-20 as colourless oily substance (17 mg). Based on the spectral data and on comparison with literature reports, the structure of the compound was identified as **Diethyl phthalate**.



Molecular formula: $C_{12}H_{14}O_4$

FT-IR (Neat, υ_{max} cm^{-1}): 1719, 1367, 1272, 1120, 1071, 743 cm^{-1}.

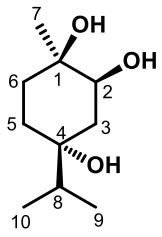
^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 7.73 (dd, *J* = 3.5, 5.5 Hz, 2H, H-1 & 4), 7.53 (dd, *J* = 3.0, 5.5 Hz, 2H, H-2 & H-3), 4.37 (q, *J* = 7.0 Hz, 4H, H-1'& 1"), 1.37 (t, *J* = 7.5 Hz, 6H, H-2 & 2") ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): *δ* 167.7 (C=O), 132.2 (C-5 & 6), 130.9 (C-1 & 4), 128.8 (C-2 & 3), 61.6 (C-1'& 1"), 14.1 (C-2'& 2") ppm.

HRMS (ESI): *m/z* calcld for $C_{12}H_{14}O_4$ is 222.0892. Found 245.0792 $(M+Na)^+$.

Compound 7 ((1R,2R,4R)-p-menthane-1,2,4-triol)

Compound **7** was isolated from fraction 95-98 as a white crystalline solid (30 mg). The compound was characterized as **(1R,2R,4R)-p-menthane-1,2,4-triol** based on spectral data obtained and on comparison with literature reports.



Molecular formula: $C_{10}H_{20}O_3$

FT-IR (Neat, υ_{max} cm^{-1}): 3345, 2910, 2400 cm^{-1}.

^{1}H NMR (500 MHz, Acetone-d6, TMS): *δ* 4.57 (d, *J* = 7.5 Hz, 1H, C2-OH), 3.90 (s, 1H, C4-OH), 3.36 (m, 1H, H-2), 3.21 (s, 1H, C1-OH), 1.83-1.80 (m, 2H, H-3, H-5), 1.71 (ddd, *J* = 13.0, 13.5, 4.0 Hz, 1H, H-5), 1.45 (m, 1H, H-3), 1.41 (m, 1H, H-8), 1.28 (m, 1H, H-6), 1.22 (m, 1H, H-6), 1.09 (s, 3H, H-7), 0.76 (dd, *J* = 7.0, 2.0 Hz, 6H, H-9 &10) ppm.

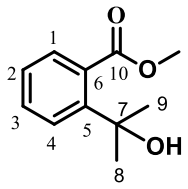
^{13}C NMR (125 MHz, Acetone-d6, TMS): *δ* 75.5 (C-2), 74.7 (C-4), 71.2 (C-1), 39.1 (C-8), 34.6 (C-3), 30.3 (C-

6), 30.2 (C-5), 27.8 (C-7), 17.2 (C-9 & 10) ppm.

HRMS (ESI): *m/z* calcld for $C_{10}H_{20}O_3$ is 188.1412. Found 211.1307 $(M+Na)^+$.

Compound 8

A reddish oily substance (26 mg) was isolated from fractions 99-104 labeled as compound **7**. Based on the detailed spectral analysis, the compound was identified as **methyl 2-(2-hydroxypropan-2-yl)benzoate**. To the best of our knowledge, the compound is reported for the first time and considered new skeleton.



Molecular formula: $C_{11}H_{14}O_3$

^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 7.43 (dd, J = 2.0, 12.5 Hz, 1H, H-3), 7.29 (dd, J = 2.0, 10.5 Hz, 1H, H-2), 6.97 (d, J = 12.0 Hz, 1H, H-4), 6.77 (d, J = 10.0 Hz, 1H, H-1), 4.22 (s, 1H, -OH), 3.72 (s, 3H, $-OCH_3$), 1.38 (s, 6H, H-8 & 9) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 180.1 (C-10), 165.0 (C-6), 149.7 (C-5), 136.9 (C-4), 136.2 (C-3), 127.4 (C-2), 113.4 (H-1), 72.9 (C-7), 56.2 (C-11), 31.4 (C- 8 & 9) ppm.

HRMS (ESI): *m/z* calcld for $C_{11}H_{14}O_3$ is 194.0942. Found 195.1016 $(M+H)^+$.

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Chapter 2B: Isolation of Chemical Markers from the Stem Bark of *Cedrus deodara* (Roxb.) Loud

2B.1. Introduction

India has a rich repository of medicinal plants and nearly about 70 % of the medicinal plants are found in tropical forests in Eastern and Western Ghats, Chota Nagpur plateau, Aravalis, Vindhyas and the Himalayas.[1] The Himalayan region is one of the great reservoirs of aromatic and medicinal plants. *Cedrus deodara* is a valuable medicinal plant in the Himalayan region with high therapeutic potential. *Cedrus deodara* is an important species in the cedar family, native to the western Himalayas, eastern Afghanistan, south-western Tibet, northern Pakistan and western Nepal.[2] In India, deodar forests are distributed in Himachal Pradesh, Uttar Pradesh and Jammu & Kashmir. It belongs to the Pinaceae family and is widely grown as an ornamental tree and timber. The wood has an aromatic smell and decay-resistant in nature. The name "deodar" is derived from modern Indian language derivatives of the Sanskrit name "devdar," meaning "timber of the gods." *C. deodara* plant has a long history of ayurvedic and folklore applications in diverse cultures. In Ayurveda, devadaru is an ingredient in many of the ayurvedic preparations such as dhanwanthararishtam, ashtapathradi keram, balaguloochuadi keram, kottamchukkadi choornam, dasamoolarishtam, dhanwantharam kuzhambu, devdarvadi kashaya, *etc*. Various extracts or constituents from different parts of the plant has been reported for several medicinal properties *viz,* spasmolytic, immunomodulatory, anticancer, antifungal, disinfectant, anti-arthritic, antioxidant, antifilarial, antiallergic, antiurolithiatic, molluscicidal, antidiabetic, antitubercular, antisecretory and antiulcer activities.[3]

2B.2. Ethnopharmacology

Cedrus deodara is extensively used among the local people of the Himalayan region. The plant is used for the treatment of fever, diabetes, intestinal parasite, sinusitis, hoof and skin diseases, external parasites, scabies and broken horn.[4,5]

Wood is carminative, diaphoretic and diuretic and is used for fever, flatulence, heart palpitation, paralysis, pulmonary troubles and urinary diseases.[6] Wood oil is applied externally for arthritis, rheumatic pains, ulcers and body ache.[7–9]

(a) **(b)** **(c)**

Figure 2B.1. *Cedrus deodara* (a) tree (b) stem bark (c) pine cone

Wood oil also acts as an antibacterial agent when applied to infected wounds as it destroys bacteria and relieves the wounds faster.[10] Gujjar communities of Bangus valley of Kashmir apply the wood oil to their legs and hands before venturing into the paddy fields for work. It protects them from waterborne irresistible itching and allergy locally called as "Kheaez".[11] The extract of heartwood is used for the treatment of piles.[12] Wood oil is used for skin diseases and respiratory troubles and also applied as anti-leech. The dried bark decoction is used for fever, diarrhoea & dysentery. Leaf extract is massaged to get relief from body pain.[13] The bark oil is used to cure stomach worm and itching. Oil is mixed with *Rubus paniculatus* Sm. leaf powder and used externally for dermatitis.[14] Bark is astringent, useful in fevers, diuretic, carminative, ant flatulent, useful in pulmonary and urinary disorders.[15] Jaunsari tribe of Uttarakhand use the decoction of the bark for the management of diabetes, it is taken twice after the meal.[16] Bark powder is used for the treatment of abdominal problems. Leaves and resin paste applied in boils, cuts and wounds. Resin also applied for the treatment of cracked feet.[17] Oil extracted from stem and bark is massaged on the body and head to relieve arthralgia and headache.[18] Oil is applied against the foot and mouth illnesses in cattle and utilized as a repellent of lice and ticks.[19] Extract from the stem is applied externally to kill the external pests on sheep and goat and also in severe skin infections in humans.[20] The fumes of wood and bark are used as snake repellent.[21] Storage bins made up of Deodar wood are extensively used for storage of paddy and maize grains,

since such containers are safe against insect-pest and disease infestation because of the insecticidal activity of Deodar wood oil. Deodar branches and leaves are used as weedicides in paddy crop. Maize seed is treated with Deodar oil before sowing it in the field to prevent insect pest infestation.[22]

2B.3. Phytochemistry

Wood: Bisarya *et al*., reported the sesquiterpenes himachalol, allohimachalol, (+)-longiborneol from the essential oil of *C. deodara*.[23–25] Agarwal *et al.,* reported taxifolin, cedeodarin, cedrin, dihydromyricetin, cedrinoside, himasecolone, isopimaricacid, taxifolin-3'-glucoside, dihydrodehydrodiconiferyl-alcohol and it's 4'-glucoside, cedrusin and it's 4'-glucoside, cedrusinin, isolariciresinol and lariciresinol from cedarwood.[26–29] Joseph *et al*., reported two major sesquiterpenes *β*-himachalene and *α*-himachalene from the essential oil.[30] Krishnappa *et al.,* reported limonenecarboxylic acid and deodardione from the essential oil.[31] Kulshreshtha *et al.,* reported the sesquiterpene alcohols himadarol, centdarol and isocentdarol from the wood.[32,33] Pande *et al.,* reported the *cis*- and *trans*- form of atlantones.[34] Shankaranarayan *et al*., reported atlantolone, deodarone, isohimachalone, and oxidohimachalene. [35–38]

Bhan *et al.,* reported 7-hydroxytodomatuic acid, Δ^{10}-dehydroepitodomatuic acid, Δ^{7}-dehydrotodomatuic acid, minor amounts of limonene-8-carboxylic acid, geronic acid, 4-acetylcyclohex-1-ene-carboxylic acid from the wood.[39] (E)-(2S,3S,6S)-atlantone-2,3,6-triol, (E)-(2S,3S,6R)-atlantone-2,3-diol, (E)-*α*-atlantone, were isolated from the saw dust.[40] (-)-Wikstromol, (-)-matairesinol, dibenzylbutyrolactone, himaphenolone, 3,4-bis(3,4-dimethoxyphenyl)-furan-2,5-dione, dewardiol and dewarenol were also identified from cedarwood.[41–44] Thirty four compounds were identified from the essential oil of wood and the major components were *β*-himachalene (38.3 %), *α*-himachalene (17.1 %) and *γ*-himachalene (12.6 %).[45]

Pine needles: Zhang *et al.,* reported 10-nonacosanol, dibutylphthalate, phthalic acid bis-(2-ethylhexyl) ester, 9-hydroxy-dodecanoic acid, protocatechuic acid, (E)-1-O-*p*-coumaroyl-*β*-D-glucopyranoside, 5-*p*-trans-coumaroylguinic acid, ethyl laurate, 3*β*-hydroxy-oleanolic acid methyl ester, ethyl stearate, shikimic acid, ferulic acid *β*-

glucoside, methylconiferin, 1-[3-(4-hydroxyphenyl)-2-propenoate]-*α*-D-glucopyranoside and (+)-(6S,9R)-9-O-*β*-D-glucopyranosyloxy-6-hydroxy-3-oxo-*α*-ionol.[46–48] Liu *et al*., reported cedrusone A, quercetin, 2R,3R-dihydroquercetin, myricetin-3-O-(6”-O-E-*p*-coumaroyl)-*α*-D-glucocopyranoside, hyperoside, kaempferol, 2R,3R-dihydromyricetin, myricetin, 3’,5’-di-O-methylmyricetin-3-O-(6’’-O-acetyl)-*α*-D-glucopyranoside, myricetin-3-O-*β*-D-glucopyranoside, 3',4'-dimethoxy myricetin-3-O-*β*-glucopyranoside, isorhamnetin-3-O-β-D-glucopyranoside, kaempferol-3-O-*β*-D-glucopyranoside, kaempferol-3-O-(6''-O-E-coumaroyl)-*β*-D-glucopyranoside, cedrin, kaempferol-3-O-(6''-O-E-feruloyl)-*β*-D-glucopyranoside and shikimic acid Bu ester.[49–51]

Li *et al*., reported 1-(4'-hydroxy-3'-methoxyphenyl)-2-[4"-(3-hydroxypropyl)-2"-methoxyphenoxy]-1,3-propanediol, (7S,8R)-9,9'-dihydroxy-3,3'-dimethoxy-7,8-dihydro-benzofuran-1'-propanol base neolignan-4-O-*β*-D-glucoside, (7R,8R)-3',9,9'-trihydroxy-3-methoxy-7,8-dihydro-benzofuran-1'-propanol base neolignans-9-O-*α*-L-rhamnoside, (6R,9R)-6-hydroxy-3-oxo-α-ionol-9-O-*β*-D-glucopyranoside, (6R,9R)-3-oxo-α-ionol-9-O-β-D-glucopyranoside, shikimic acid Bu ester and quinic acid Bu ester.[52] Bai *et al*., reported ferulic acid, osthole, beta-phenylacrylic acid, paeonol, magnolol and honokiol.[53] 3-*p*-trans-coumaroyl-2-hydroxyquinic acid, massonianoside B, trans-ferulic acid-4-O-*β*-D-glucopyranoside, (6S,9R)-roseoside. isorhamnetin, stigmasterol, oleanolic acid, parahydroxybenzaldehyde, β-sitosterol, syringaresinol, daucosterol, *p*-hydroxybenzoic acid, gallicin, gallic acid, secoxyloganin and pelargonidin-3-O-glucoside were also reported from pine needles.[54–57] 26 compounds were identified from the essential oil of pine needles and the main components are; α-terpineol (30.2 %), linalool (24.47 %), limonene (17.01 %), anethole (14.57 %), caryophyllene (3.14 %), and eugenol (2.14 %).[58]

Pollen grains: Ohmoto *et al*., reported dehydroabieticacid, 15-hydroxydehydroabietic acid, 7*α*,18-dihydroxydehydroabietanol, naringenin, *β*-sitosteryl-*β*-D-glucoside, 7*β*,15-dihydroxydehydroabietic acid, hexadecane-1,16-diol 7-caffeoyl ester from pollen grains of *C. deodara*.[59]

Stem bark: *α*-himachalene, *β*-himachalene, *γ*-himachalene, *δ*-himachalene, ethyl-23-methyl-pentacosanoate, deodarin and tannins were reported from the stem bark.[60–63]

Root: An abietane type diterpene, centdaroic acid was reported from the roots of deodar.[64]

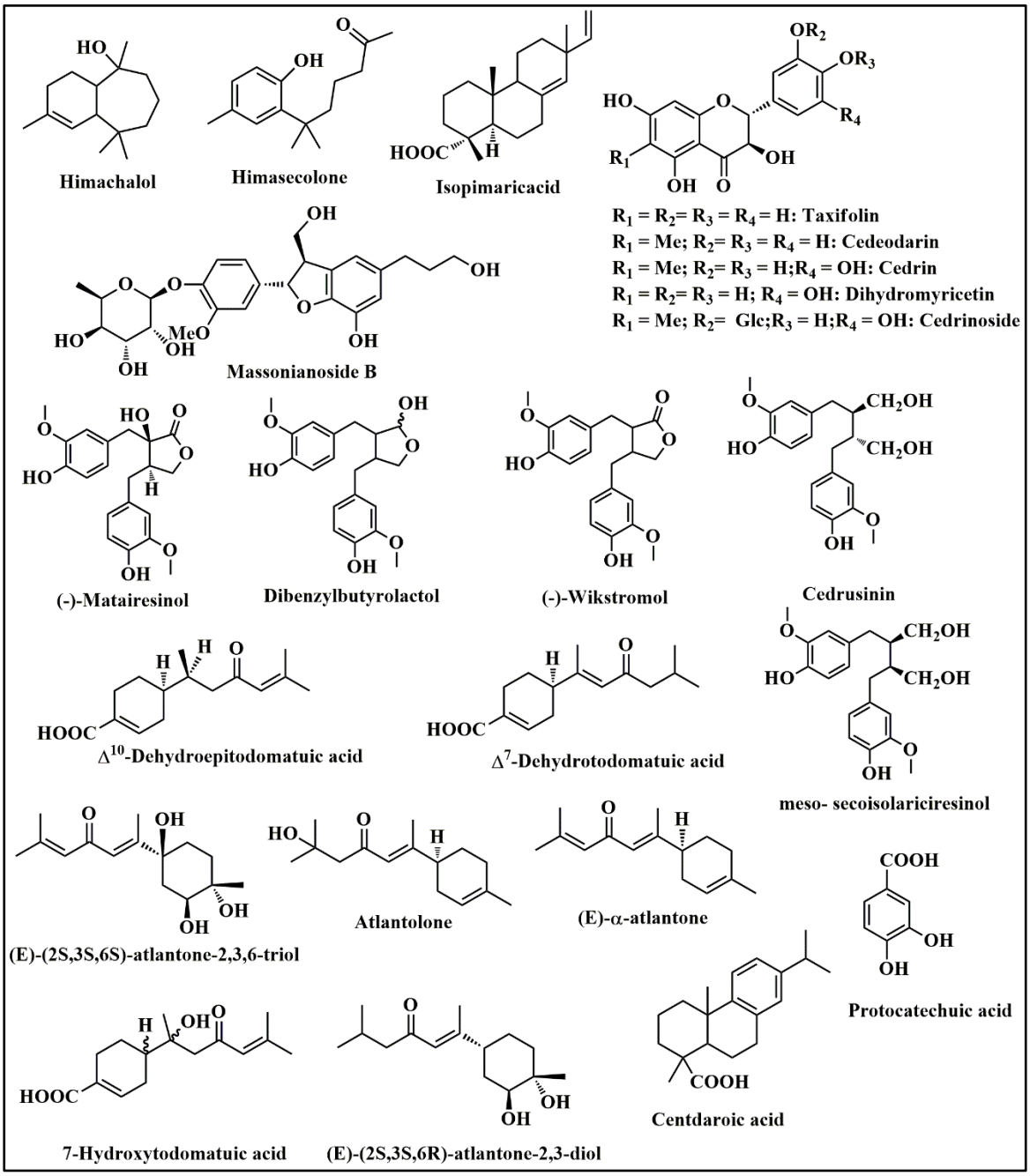


Figure 2B.2. Chemical structure of some compounds reported from *C. deodara*

2B.4. Pharmacology

In the year 2000, Rao *et al.,* patented the use of (-)-matairesinol and (-)-wikstromol together with or associated with therapeutically acceptable additives as antioxidants and hepatoprotective agents. The antioxidant and free radical scavenging activity of matairesinol and wikstromol were patented in the same year and also reported the use of these compounds for the treatment of arteriosclerosis.[65] The synergistic composition of lignans (AP9-cd) exhibiting anticancer activity against various human cancer cell lines from cervix, breast, colon, neuroblastoma, liver, mouth, lung, ovary and prostate tissues was patented by Rao *et al*., in the year 2003.[66] Shashi *et al.,* reported that AP9-cd induces apoptosis in Molt-4 and HL-60 leukemia cells. AP9-cd mediated early NO formation leads to caspases activation, peroxide generation, and mitochondrial depolarization, which may be responsible for mitochondrial-dependent and -independent apoptotic pathways involved in the killing of leukemia cells by AP9-cd.[67]

In vitro cytotoxicity studies of CD lignan mixture showed significant dose-dependent effects against several cancer cell lines from different tissues such as breast, neuroblastoma, cervix, liver, colon and prostrate with IC_{50} values ranges of 16.4 ng/mL to 116.03 mg/mL depending on the cell line. *In vivo* anticancer activity of CD lignan mixture was studied using Ehrlich ascites carcinoma and colon carcinoma (CA-51) models in mice and the effect was comparable with 5-fluorouracil.[68] The flavonoids myricetin, quercetin, kaempferol and isorhamnetin from pine needles were capable of inhibiting the tumour proliferation through the regulation of cell cycle and apoptosis.[55] Zhao *et al.,* reported the neuroprotective effects of cedrin from deodara on PC12 cells against the neurotoxicity induced by amyloid β_{1-42}. The results showed the viability of PC12 cells improved by amyloid β_{1-42} can be enhanced by cedrin.[69]

Cedar oil exhibited excellent antibacterial activity.[70] The essential oil of pine needles showed significant inhibitory and sterilizing activity against typical food-borne microorganisms *via* the induction of cytoplasmic outflow and plasmolysis.[71] Wu *et al*., reported the antibacterial activity and mode of action of 2R,3R-dihydromyricetin (DMY) against *S. aureus*. DMY effectively kills *S. aureus* by

damaging the bacterial membrane and binding to intracellular DNA.[72] 3-*p*-trans-coumaroyl-2-hydroxyquinic acid evaluated for antibacterial activity against food-born pathogens and proved to be a good candidate for the development of natural food preservative.[73] Bai *et al*., reported the antibacterial mechanism of shikimic acid against *S. aureus* by examining membrane permeability, membrane integrity, membrane potential, TEM, membrane fluidity and membrane protein binding assays. Shikimic acid interacts with *S. aureus* membrane proteins and lipids, resulting in causing cell membrane dysfunction and bacterial damage or even death.[74]

Cedarwood oil is toxic against house-hold insects *viz*., Indian mosquito, *Anopheles stephensi* and red cotton bug, *Dysdercus Koenigii*.[75,76] Makhaik *et al*., reported the antimosquito property of essential oil of seven plants, on the adults of two mosquito species*, Culex quinquefasciatus* and *Aedes aegypti*. Adults of *A. aegypti* were insensitive towards the oil of *C. deodara* under the treated concentration range and in the case *C. quinquefasciatus*, oil showed an LC_{50} 2.48 %, indicating low activity.[77] Himachalol and *β*-himachalene isolated from the cedarwood oil exhibited insecticidal activity against the pulse beetle (*Callosobruchus analis* F.) and the housefly (*Musca domestica* L.).[78] Nisha *et al*., screened 20 medicinal plants for *in vitro* macrofilaricidal activity against adult *Setaria digitata* (cattle filarial worm) by worm motility assay and MTT reduction assay. The methanolic extract of cedarwood exhibited 47.97 and 86.56 % inhibition in formazan formation at 1 mg/ml at an exposure period of 1 and 4 h, indicating the maximum activity at the minimum concentration.[79] The essential oils of *C. deodara* exhibits strong fungicidal activity. Dikshit *et al*., investigated the effect of the oil for the control of the fungal deterioration of seeds of coriander, *Coriandrum sativum* L., and fennel, *Foeniculum vulgare* Mill. On mycofloral analysis, the oil had checked the appearance of ten fungi on the seeds of *C. sativum*, and seven fungi on the seeds of *F. vulgare.* Cedrus oil was strongly fungitoxic and did not show any harmful effect on the germination and seedling growth of the two species.[80]

The ethanolic extract of stem wood of *C. deodara* exhibited antihyperglycaemic activity in Streptozotocin-induced diabetic rats, showed significant lowering of blood glucose level.[81] Similarly, Devmurari *et al.,* reported

the antihyperglycaemic activity of the ethanolic extract in alloxan-induced hyperglycaemic rats. Oral administration of the extracts at doses of 50 mg/kg and 100 mg/kg, resulted a marked decline in blood glucose level and the effect was comparable to the standard drug glibenclamide.[82] The aqueous extract of the heartwood of *C. deodara* at a dose of 500 mg/kg exhibited antidiabetic activity in alloxan-induced diabetic rats. Treatment with the extract significantly decreased serum glucose level in hyperglycaemic animals and the result was observed from the 5th day onwards.[83] The petroleum ether extract of heartwood was reported to have hypoglycaemic and antidiabetic activity in alloxan-induced diabetic rats. The extract at all doses (100, 200 and 400 mg/kg) produced a hypoglycaemic effect in fasting rats and the effect by 400 mg/kg dose was comparable to glibenclamide-treated group which was used as standard drug.[84] The essential oil of cones showed inhibitory activity against *α*-amylase and longipinene was found to be the potent inhibitor present in it. The *α*-amylase inhibitory activity of longipinene increased remarkably with the increase in concentration.[85]

Rathor *et al*., reported the anti-inflammatory activity of aqueous extract of stem bark in adult albino rats. The extract was found to inhibit carrageenin induced oedema, granuloma formation and formation of granulation tissue.[86] Similarly, the methanolic extract of the stem bark exhibited significant anti-inflammatory activity at a dose of 100 mg/kg *i.e.,* 43.47 % inhibition in carrageenan-induced hind paw oedema.[87] Tandan *et al*., reported the anti-inflammatory activity of the wood essential oil of *C. deodara* in rats and mice. The oil effectively inhibited carrageenan-induced hind paw oedema at doses of 100, 300 and 1000 mg/kg.[88] Shinde *et al.,* extensively studied the anti-inflammatory activity of *C. deodara* wood oil against rat paw edema and possible mechanism of action. *C. deodara* wood oil has potent anti-inflammatory activity against carrageenan-induced rat paw edema at doses of 50 and 100 mg/kg body weight. The inhibitory effect on inflammation in rats ascribed to the inhibition of the enzyme cyclooxygenase leading to inhibition of prostaglandin synthesis.[89] Moreover, the wood oil was effective against compound 48/80- and nystatin-induced paw edema in rats. The anti-inflammatory activity was attributed to the membrane stabilizing activity of *C. deodara* wood oil.[90]

Ramesh *et al*., reported the diuretic and anti- urolithiatic activity of the heart wood of *C. deodara* and concluded that the plant has great potential to inhibit stone formation.[91] A novel polysaccharide obtained from the pine needles possessed antioxidant activity.[92] Study on the histopathological changes in the intestinal tissues of albino rats exhibited some adverse effects such as oedema on sub-mucosal and mucosal layers, erosion of the epithelium, congestion of blood vessels as well as the presence of inflammatory cells on esophagus, ileum and stomach. Hence the results indicated that *C. deodara* root oil is not very safe at doses of 0.5 ml/kg and 2.5 ml/kg because of causing damage to GIT organs.[93]

2B.5. Aim and scope of the study

Over the past few years, the need of scientific validation of ayurvedic drugs become essential for the development of potent therapeutic leads. *Cedrus deodara* is a part of number formulations in Ayurveda for various ailments. From the literature reports, phytochemical investigation regarding the stem bark of *Cedrus deodara* is limited. Keeping this in view, our work focussed at the chemoprofiling of the stem bark, hoping our results can uphold the traditional claims.

2B.6. Extraction, isolation and characterization of compounds from the stembark of *Cedrus deodara*

2B.6.1. Collection and Extraction of plant material

The plant material of *Cedrus deodara* was collected from the Himalayan valleys of Jammu. About 5 Kg of the stem bark was collected, dried and powdered. From this, 900 g was subjected to extraction with hexane (3L*3 days) using a mechanical stirrer for three times. The extract was concentrated under reduced pressure was yielded 5 g of the hexane extract. Similarly, extraction was carried out with acetone (74 g) and ethanol (30 g).

2B.6.2. Preliminary cytotoxic studies of the extracts

The crude extracts were screened for cytotoxic analysis against cervical carcinoma (HeLa) and breast cancer (MDA-MB-231) cell lines using MTT assay. Doxorubicin (DOX) was used as the standard drug. From the results, hexane extract

exhibited potent cytotoxicity (43.3 and 64.41 µg/mL) in both cell lines compared to other extracts at 24 h. Acetone and ethanol extract showed an IC_{50} value of 50.36 and 67.57 µg/mL at 48 h in MDA-MB-231 cell line (**Table 2B.1**). The effect of various concentrations is shown in **Figure 2B.3**.

Table 2B.1. IC_{50} values of extracts in HeLa and MDA-MB-231 cell lines

Extract	Cytotoxicity (IC_{50}) in µg/mL			
	HeLa		MDA-MB-231	
	24 h	48 h	24 h	48 h
Hexane	78.12 ± 0.02	43. 3± 0.03	64.41 ± 0.02	22.57 ± 0.01
Acetone	98.54 ± 0.05	79.3 ± 0.05	96.06 ± 0.05	50.36 ± 0.09
Ethanol	>100	48.5 ± 0.03	>100	67.57 ± 0.02

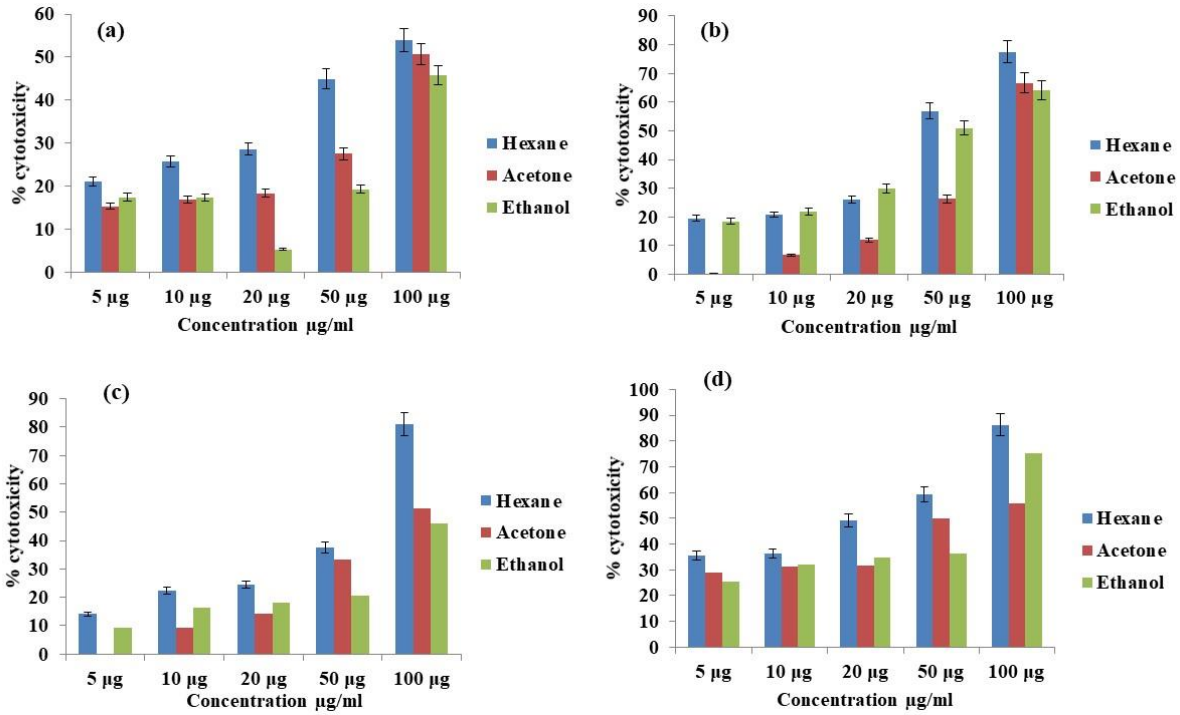


Figure 2B.3. MTT assay of extracts (a) on HeLa cells at 24 h (b) on HeLa cells at 48 h (c) on MDA-MB-231 cells at 24 h (d) on MDA-MB-231 cells at 48 h.

2B.6.3. Isolation and characterization of compounds

About 70 g of the acetone extract was subjected to column chromatography on 100-200 mesh silica gel packed in hexane. The polarity was increased by adding an aliquot of ethyl acetate and finally with 20 % ethyl acetate- methanol polarity to give a total of 64 fractions. Each fraction was collected in a 500 ml conical flask and pooled into eight fraction pools. We could successfully isolate six compounds from the fraction pools. The extraction process is illustrated in the chart given below (**Fig. 2B.4**).

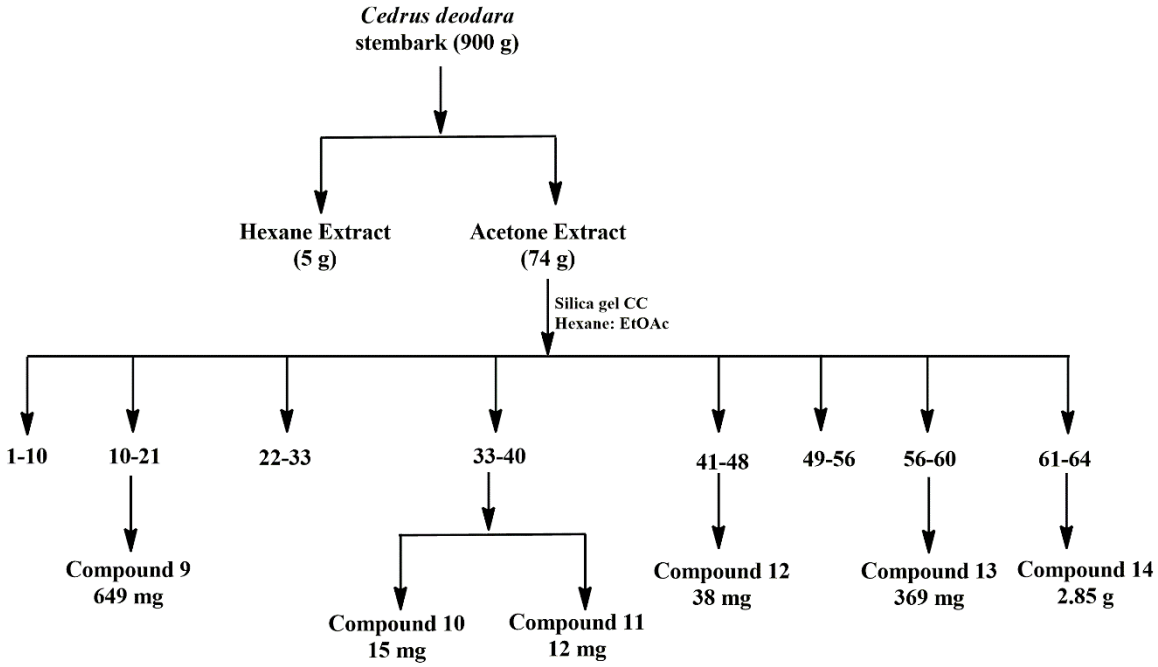
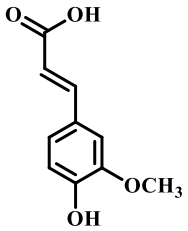


Figure 2B.4. Extraction process

Fraction pool 2 (Fr.10-21) on silica gel CC separation with 10 % ethyl acetate in hexane polarity yielded a white amorphous solid (649 mg) as compound **9**. The IR spectrum of the compound showed absorption peaks corresponding to carboxylic acid OH-stretching (3379 cm^{-1}) and carboxylic acid C= O stretching (1716 cm^{-1}). The molecular formula of the compound was assigned as $C_{10}H_{10}O_4$ which was supported by a molecular ion peak at *m/z* 195.0530 $(M+H)^+$ in the HRMS analysis. The ^1H NMR spectrum (**Fig. 2B.5**) displayed three aromatic peaks [(δ 7.07 (dd, *J* = 6.5, 1.5 Hz, 1H), 7.03 (d, *J* = 1.5 Hz, 1H) & 6.91 (d, *J* = 8 Hz, 1H) ppm] and a characteristic signal for a methoxy proton (δ 3.93 ppm). The presence of two protons with couplin constant *J* = 16.0 Hz corresponded to two olefinic protons attached to an aromatic

ring. The ^{13}C NMR spectrum (**Fig. 2B.6**) displayed ten carbon signals; the acid carbonyl carbon appeared at δ 167.4, olefinic carbon signal at δ 144.6 & 114.7, methoxy carbon at δ 56.0 and aromatic carbons resonated at δ 147.9, 146.8, 127.1, 123.0, 115.7 & 109.3 ppm, respectively. From the spectral data and on comparison with literature reports compound **9** was identified as **Ferulic acid**.[94] Ferulic acid is an important phenolic acid that is commonly present in the leaves, fruits, and seeds of most plants. It exhibits several biological activities such as anti-oxidant, anti-inflammatory, anti-microbial, hepatoprotective, anti-allergic, anti-carcinogenic, anti-thrombotic and antiviral. It is reported to increases the sperm viability and also shown to have vasodilatory actions. Reports are available for its metal chelation, activation of transcriptional factors, gene expression, modulation of enzyme activity, and signal transduction properties.[95]



Compound **9** - **Ferulic acid**

Fraction pool 4 (Fr. 33-40) on silica gel CC with 30 % ethyl acetate in hexane polarity yielded two compounds designated as compound **10** and **11**. Compound **10** (15 mg) was found to be a mixture of three compounds. The ^{1}H NMR spectrum (**Fig. 2B.7**) was similar to that of lignans. It was previously reported that the heartwood of *Cedrus deodara* contained a lignan mixture known as CD lignan mixture.[41,66] The mixture consisted of three lignans *viz*; (-)- matairesinol, (-)-wikstromol and dibenzylbutyrolactol lignan. The mixture was reported to exert a synergetic effect against cancer as compared to the individual compounds. To confirm the compounds, we have taken up the HRMS analysis of the mixture. The mass spectrum contained three prominent peaks. The peak values obtained were 397.1265 $(M+Na)^+$, 383.1471 $(M+Na)^+$ and 357.0586 $(M-H)^+$ corresponded to (-)-matairesinol, dibenzylbutyrolactol and (-)-wikstromol, respectively (**Fig. 2B.8**). Therefore, the mixture was identified as CD lignan mixture and we kept the mixture as such for further studies.

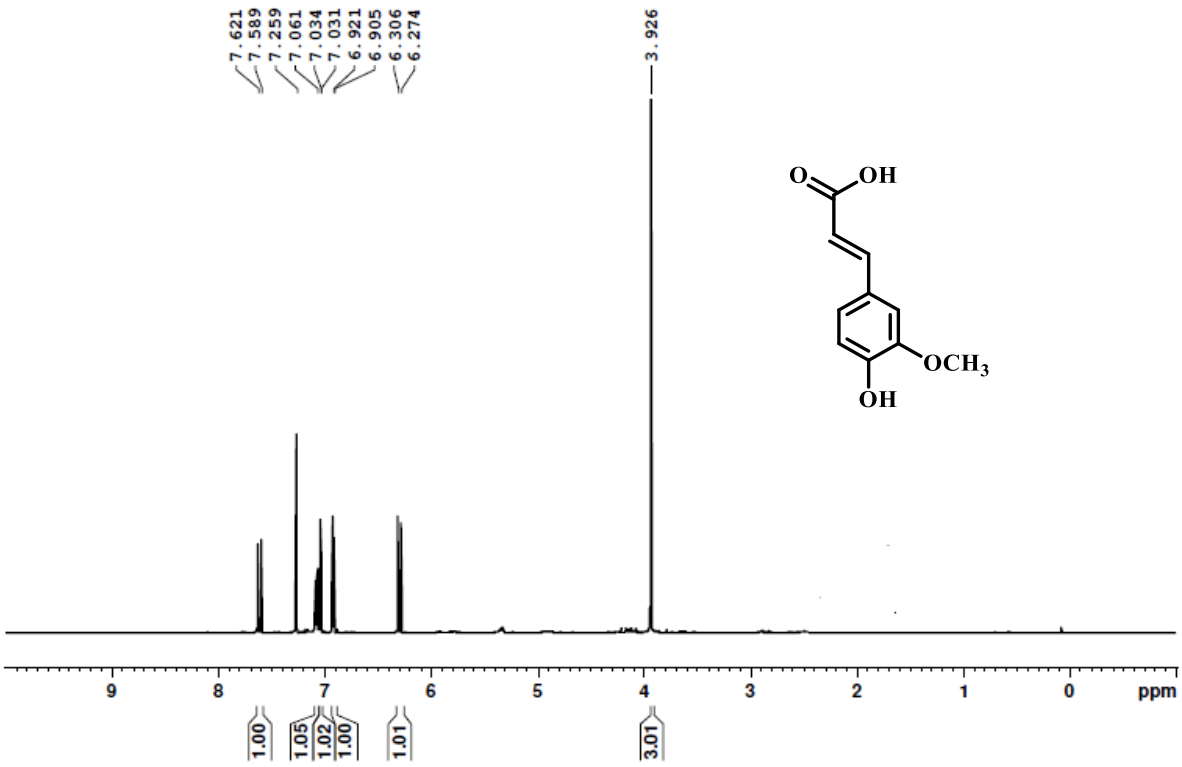


Figure 2B.5. ^{1}H NMR spectrum of compound **9** in $CDCl_3$

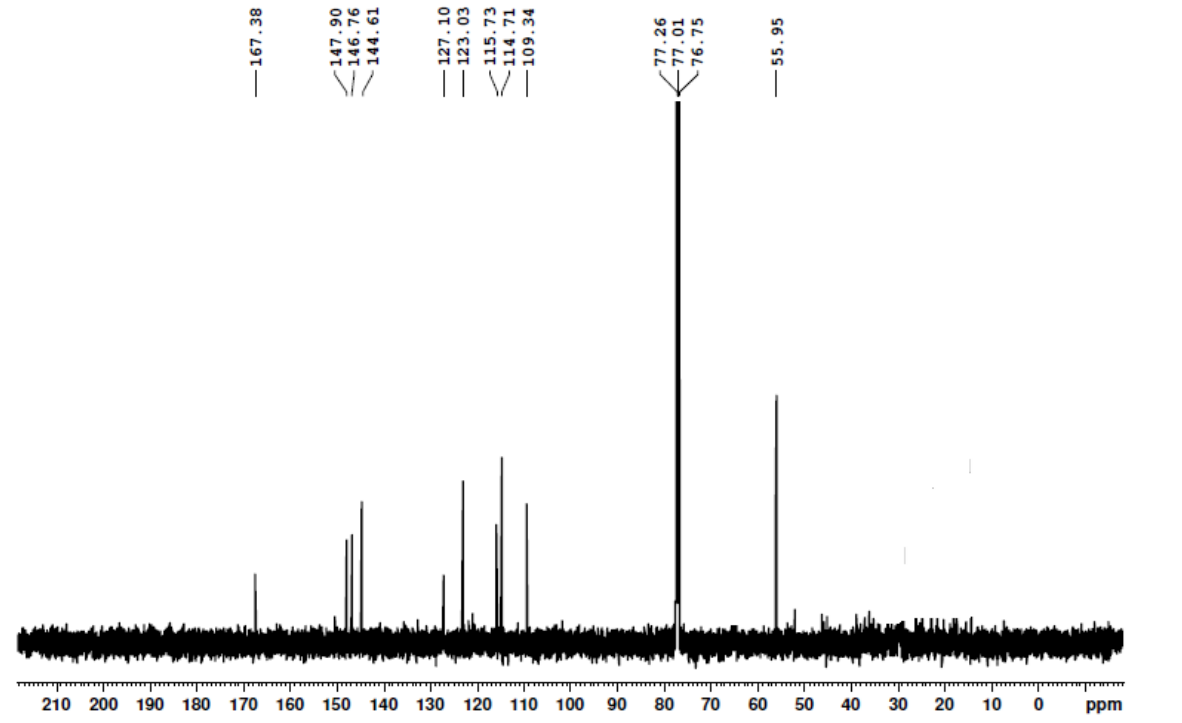
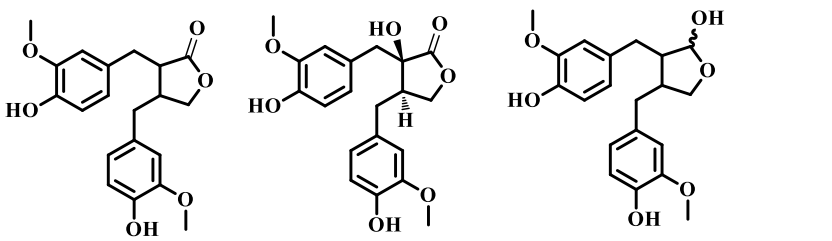


Figure 2B.6. ^{13}C NMR spectrum of compound **9** in $CDCl_3$



Compound **10 – CD lignan mixture**

Compound **11** was isolated from fraction pool 4 as a pale yellow amorphous solid (12 mg). The IR spectrum revealed the presence of a hydroxyl (3372 cm^{-1}) and an ether group (1113 & 1033 cm^{-1}). The ^{1}H NMR spectrum (**Fig. 2B.9**) indicated the presence of six aromatic protons (2H multiplets at δ 6.86, 6.81 and 6.69 ppm) suggests two 1,3,4- substituted benzene ring in the molecule. The broad singlet at δ 5.66, integrated for two protons, can be attributed to the presence of hydroxyl groups in the aromatic ring. Doublets at δ 4.79 integrating for one proton suggested the presence of a deshielding group such hydroxy or ether linkage. Two singlets at δ 3.87 & 3.86 ppm integrating for three protons were indicative of two aromatic methoxy groups. Doublet of doublets at δ 2.90 & 2.54 corresponded to the benzylic methylene groups. The ^{13}C NMR spectrum (**Fig. 2B.10**) showed the presence of 20 carbon atoms. The presence of two methoxy groups was confirmed by the peak at δ 55.9 ppm. DEPT-135 spectra showed the presence of three methylene carbons and three methine carbons. The –OH attached carbon appeared at δ 72.9 ppm. The protons and carbons were assigned based on COSY, HMBC and HMQC correlations. The correlation between the protons at δ 3.90 (H-9') & 2.40 (H-8') in the COSY spectrum and the HMBC correlation between the carbon at δ 60.9 (C-9') with a proton at δ 2.40 confirms that the methyl group at C-9' attached to the C-8' carbon. The mass spectrum of the compound gave molecular ion peak at *m/z* 382.4406, which is the $(M-H+Na)^{+}$ peak. From all the spectral data and on comparison with literature reports, the compound was identified as **(+)-Lariciresinol**.[96] Lariciresinol is an enterolignan precursor and displays potent antifungal and antibacterial activity.[97,98]

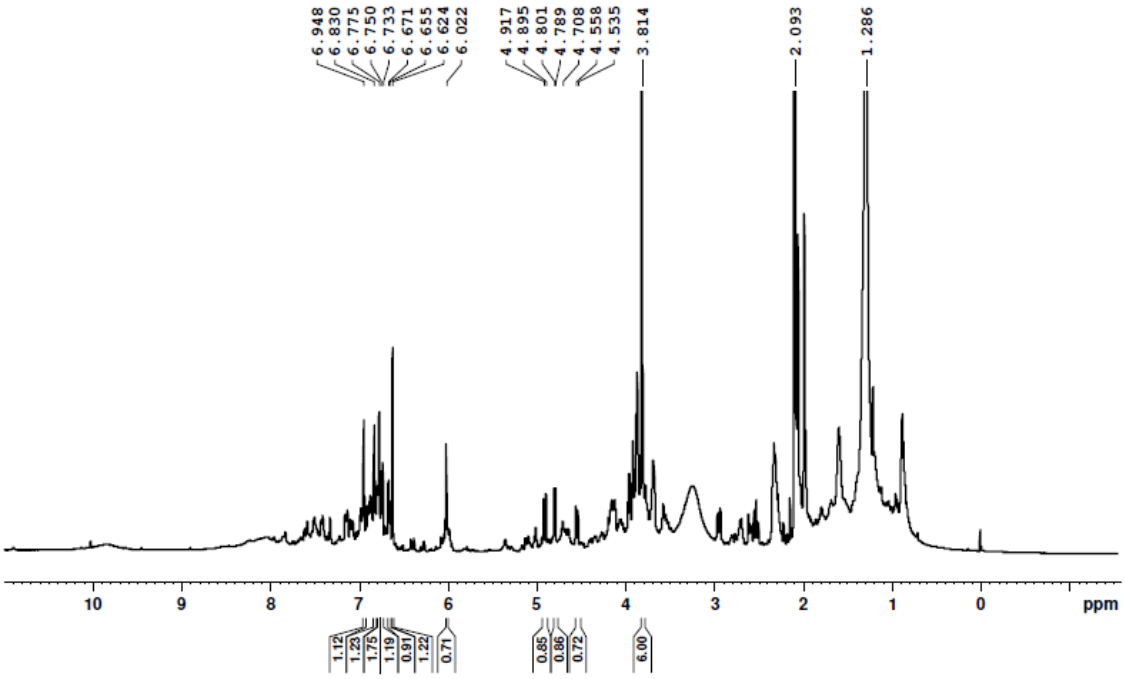


Figure 2B.7. ^{1}H NMR spectrum of CD lignan mixture (**10**) in $CDCl_3$

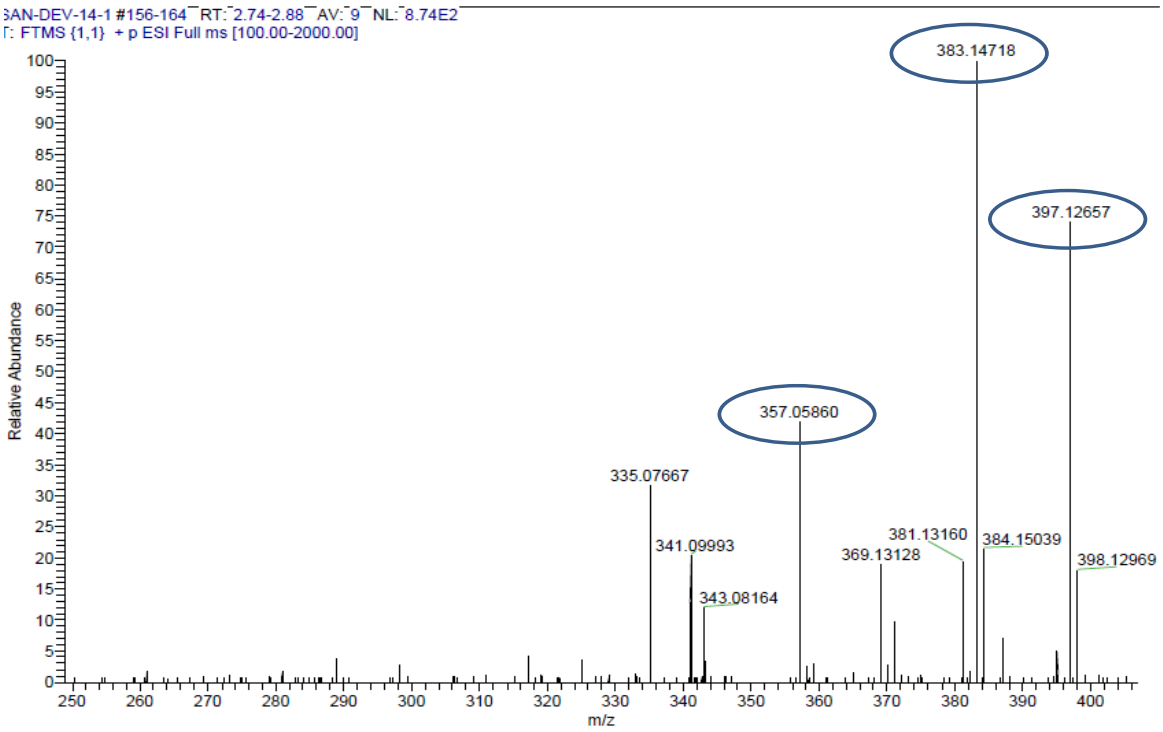


Figure 2B.8. HRMS spectrum of CD lignan mixture (**10**)

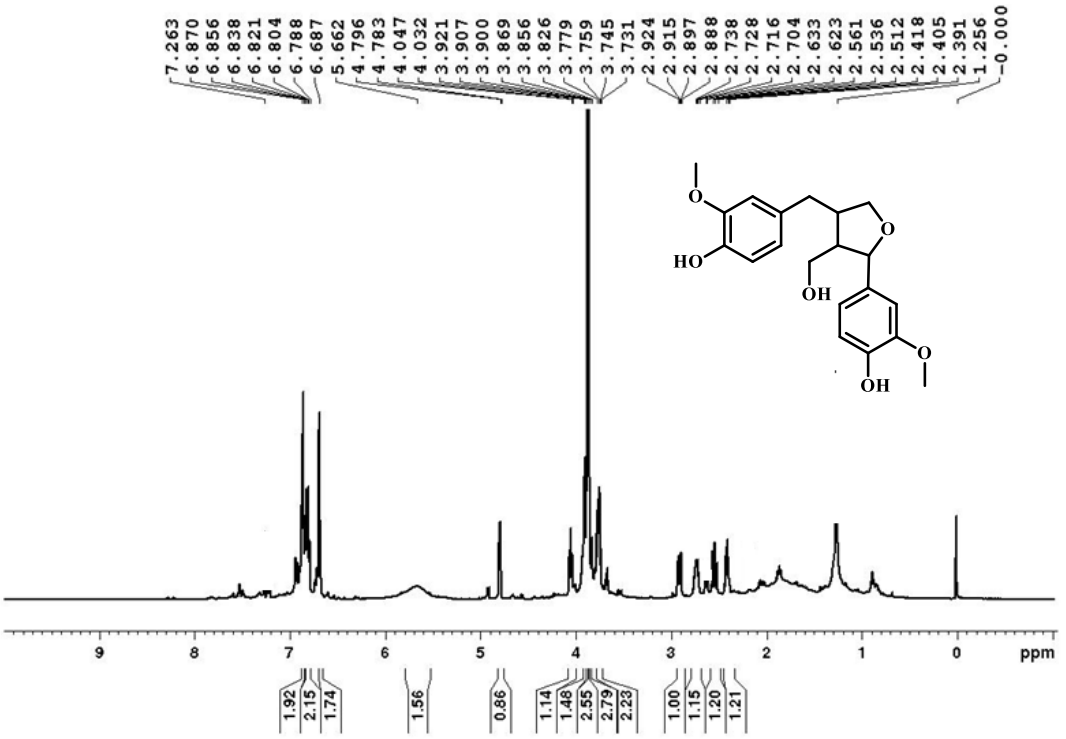


Figure 2B.9. ^{1}H NMR spectrum of compound **11** in $CDCl_3$

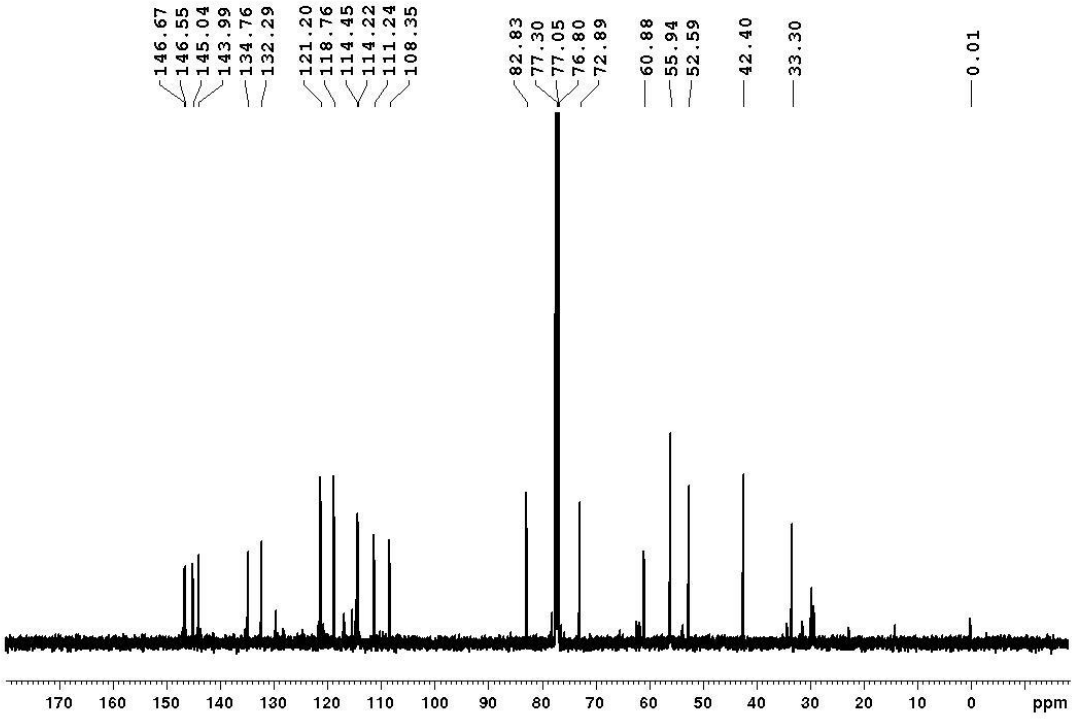
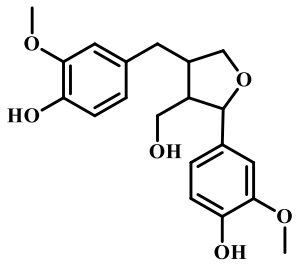
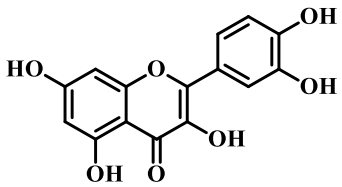


Figure 2B.10. ^{13}C NMR spectrum of compound **11** in $CDCl_3$



Compound **11 – (+)-Lariciresinol**

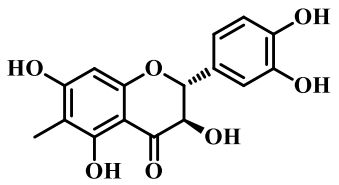
Fraction pool 5 (Fr. 41-48) on silica gel CC afforded a yellow solid of 38 mg as compound **12**. The ^{1}H NMR spectrum (**Fig. 2B.11**) consisted of five aromatic protons that resonated from *δ* 7.84 to 6.28 ppm. The ^{13}C NMR spectrum (**Fig. 2B.12**) showed 15 carbon signals in which the carbonyl carbon appeared at δ 175.7 ppm. The spectral data were similar to that of the common flavonol **Quercetin**. It was confirmed with mass spectral analysis with a molecular ion peak at *m/z* 303.0511 $(M+H)^{+}$.[99] Quercetin is a natural flavonoid found abundantly in vegetables and fruits and exerts numerous pharmacological properties such as anti-amyloidogenic, anticancer, anti-inflammatory, antioxidant and antiviral activities.[100]



Compound **12 – Quercetin**

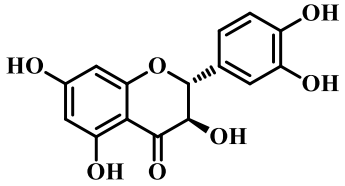
Fraction pool 7 (Fr. 56-60) was further purified by silica gel CC and yielded 369 mg of yellow solid of compound **13**. The UV_{max} at 286 and 333 nm were very closely related to dihyroflavonol. The IR spectra revealed the absorptions correspnding to hydroxyl (3375 cm^{-1}) and carbonyl stretching (1638 cm^{-1}). The ^{1}H NMR spectrum (**Fig. 2B.13**) indicated a pair of doublets at *δ* 4.99 (d, *J* = 11.0 Hz, 1H, H-2) and *δ* 4.59 (dd, *J* = 4.0, 11.5 Hz, 1H, H-3), typical of the AB system of vicinal protons at the C-2 and C-3 position of 3-hydroxyflavanone. It also consisted of a singlet at *δ* 1.99 ppm corresponding to an aryl methyl and a singlet at *δ* 6.02 suggested that this aromatic proton can be either attach to C-6 or C-8. The HMBC correlation of the methyl proton (*δ* 1.99 ppm) with carbons *δ* 105.1 (C-6), 165.5 (C-5), & 162.1 (C-7) suggested that the methyl group is attached to the 6th position. The

^{13}C NMR spectrum (**Fig. 2B.14**) indicated that the compound contains 16 carbon atoms. The signal at δ 198.3 ppm confirmed the presence of a carbonyl group. The peaks at δ 73.3 and 84.5 ppm could be attributed to the C-3 and C-2 carbons. The mass spectrum of the compound showed a molecular ion peak at *m/z* 341.0640, which is the $(M+Na)^+$ peak. From all the above spectral details and on comparison with literature reports the compound was identified as **Cedeodarin**.[26]



Compound **13 – Cedeodarin**

Fraction pool 8 (Fr. 61-64) obtained by eluting the column at 60 % ethyl acetate in hexane polarity on further CC yielded a yellow coloured crystalline solid termed as compound **14** (2.85 g). The molecular formula of the compound was depicted as $C_{15}H_{12}O_7$ based on the HRMS analysis in which the molecular ion peak observed at *m/z* 303.05119 $(M-H)^+$. The IR spectrum depicted the absorptions correspnding to hydroxyl (3428 cm^{-1}) and carbonyl stretching (1636 cm^{-1}). The UV spectrum of the compound showed a lower intensity peak at 327 nm, appearing as a shoulder to the peak at 290 nm. This type of UV spectrum are characteristic of flavanone or flavanonol (usually in flavanone or flavanonol, the lower intensity band I appears as a shoulder to band II). The ^1H NMR spectrum (**Fig. 2B.15**) was similar to that of the previous one except for the absence methyl group. The singlet at C-6 appeared at δ 5.95 ppm. Hence from the spectral data and on comparison with literature reports compound **14** was identified as **Taxifolin**.[101] Taxifolin is abundantly found in olive oil, grapes, citrus fruits and onions and exerts different biological activities such as anti-oxidative, anti-inflammatory, anti-proliferative and anti-coagulative effects.[102]



Compound **14 -Taxifolin**

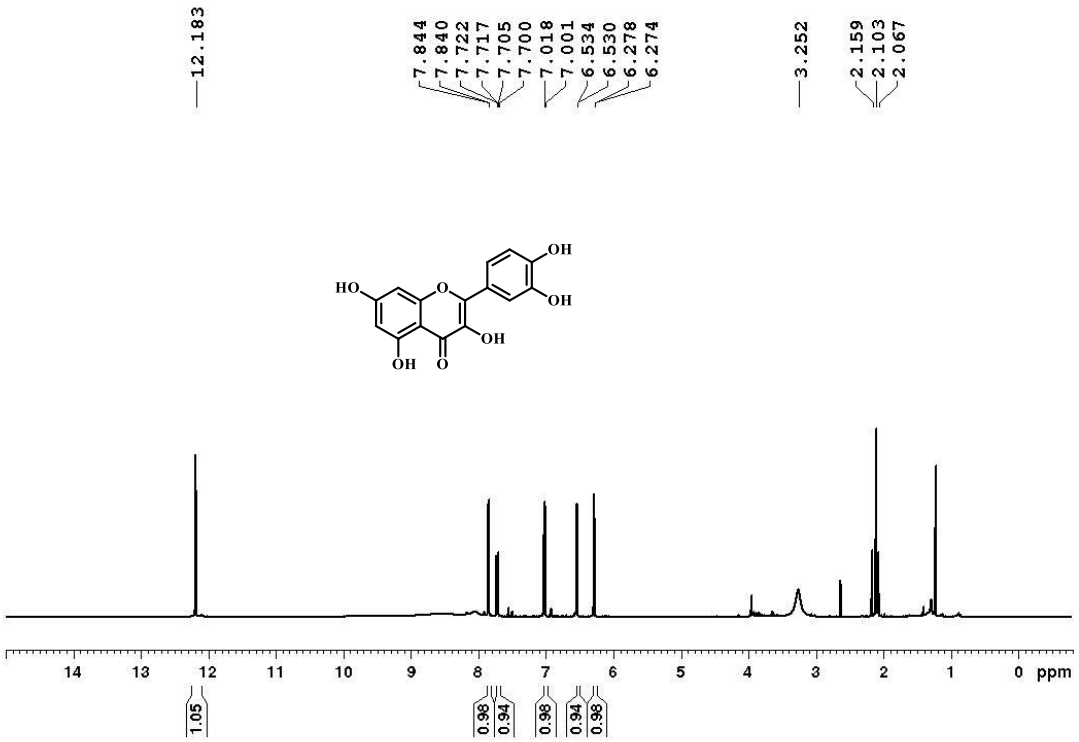


Figure 2B.11. ^{1}H NMR spectrum of compound **12** in Acetone-d6

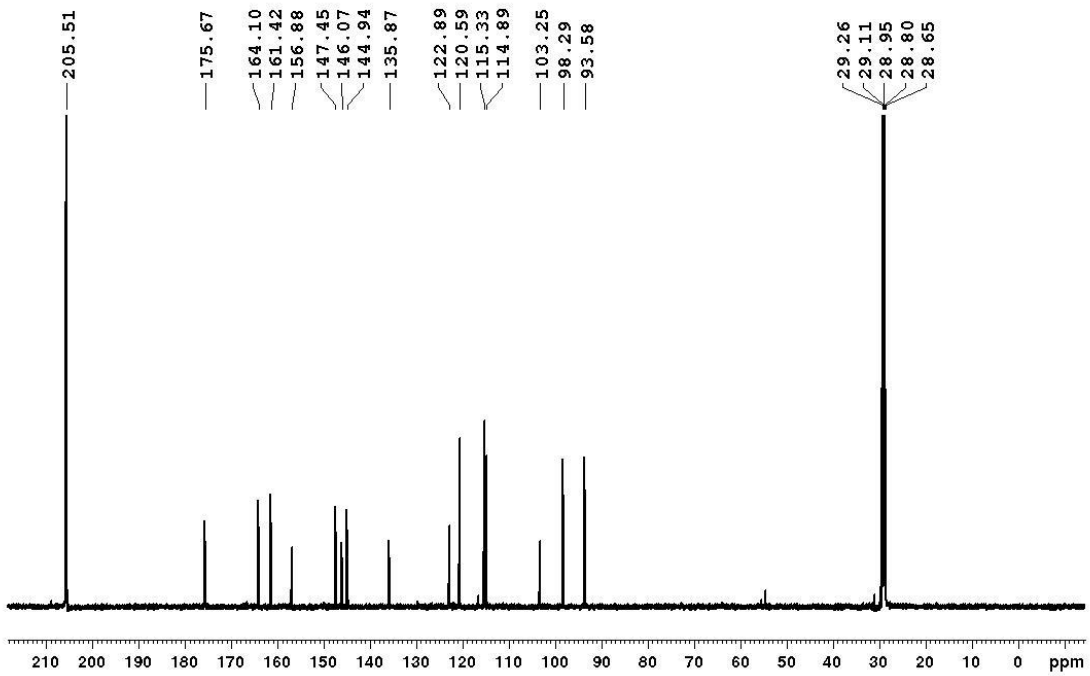


Figure 2B.12. ^{13}C NMR spectrum of compound **12** in Acetone-d6

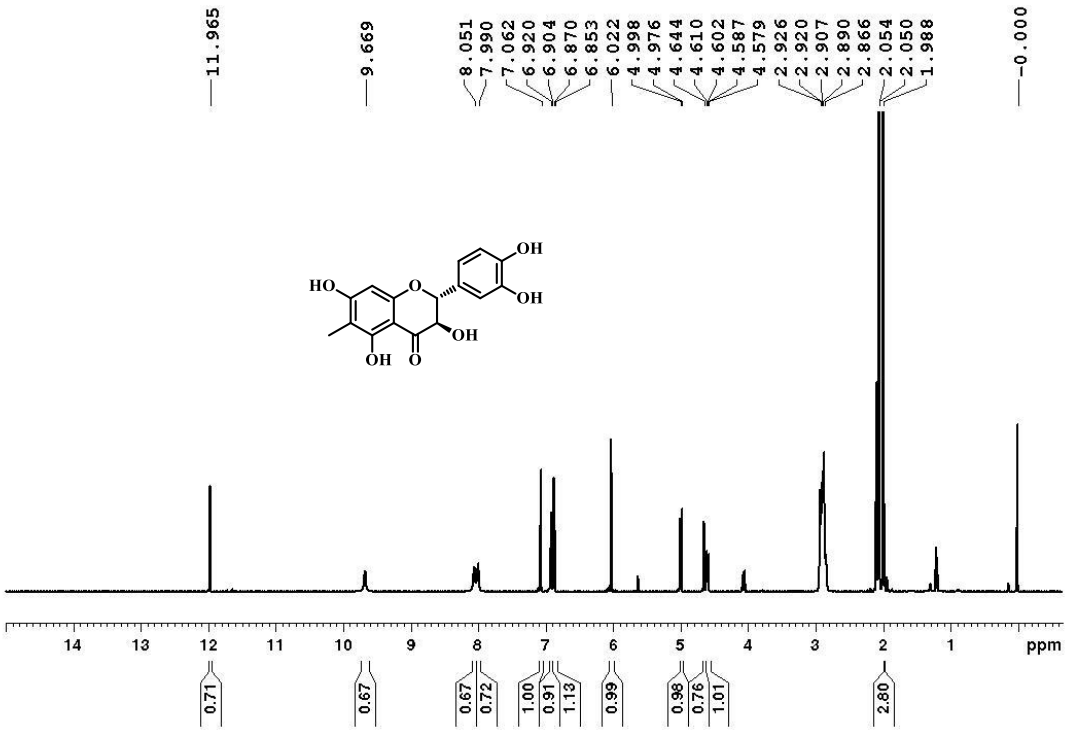


Figure 2B.13. ^{1}H NMR spectrum of compound **13** in Acetone-d6

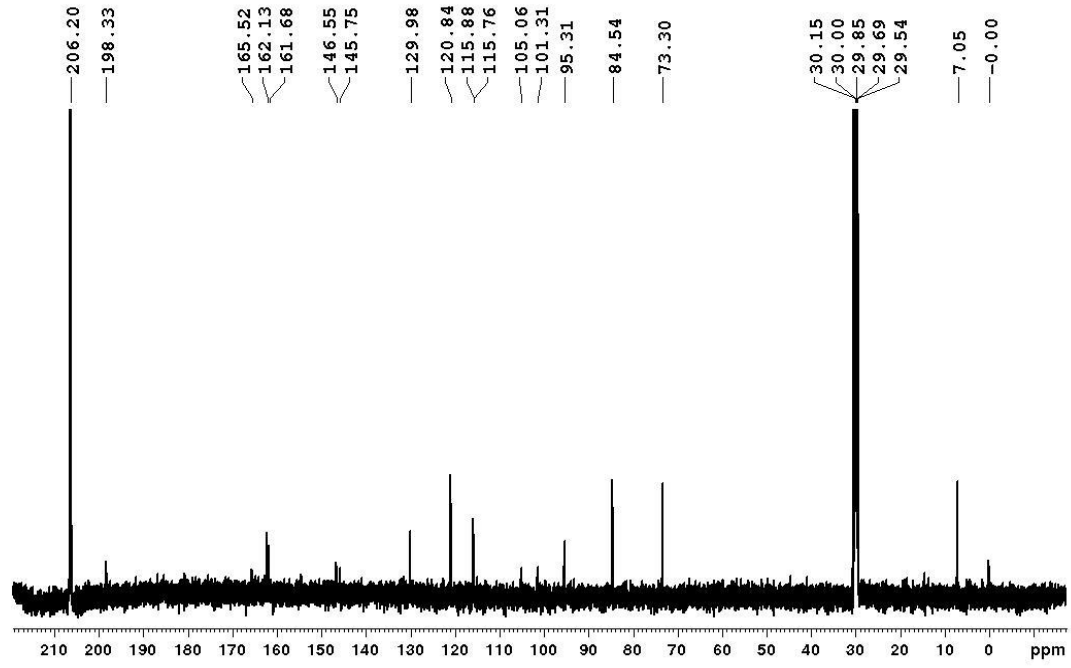


Figure 2B.14. ^{13}C NMR spectrum of compound **13** in Acetone-d6

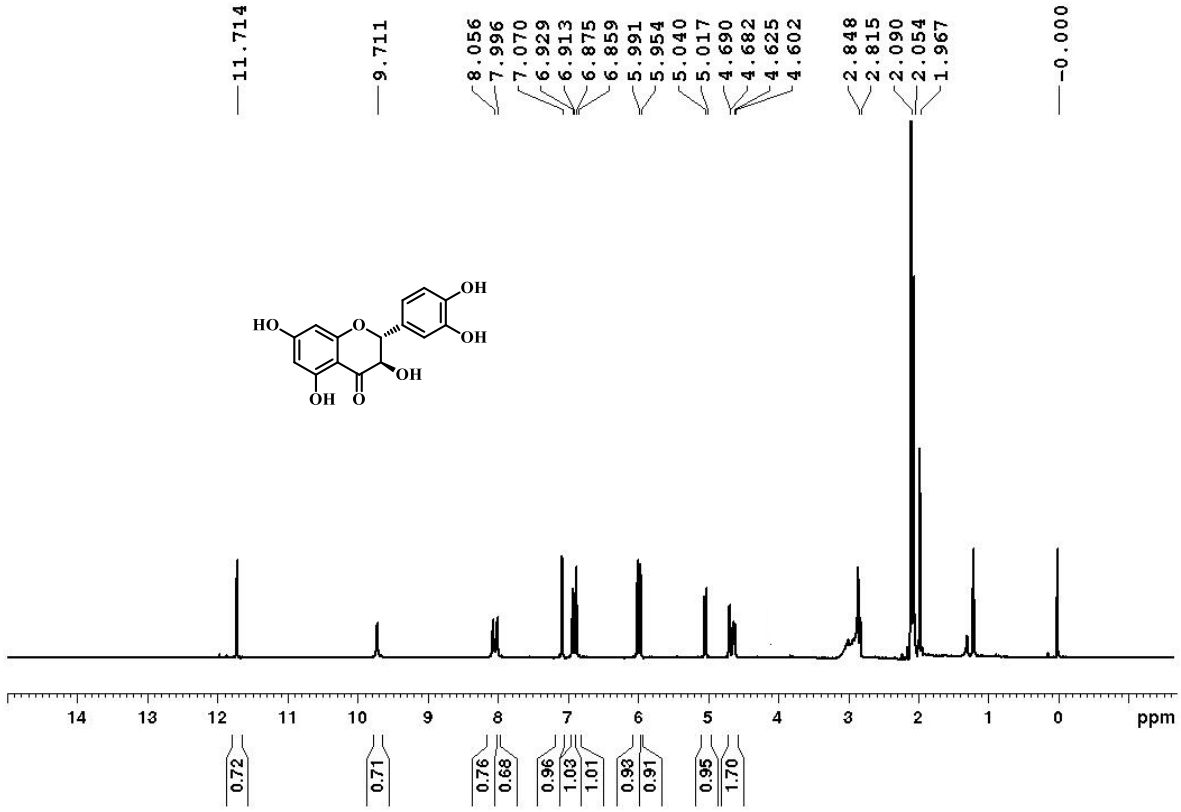


Figure 2B.15. ^{1}H NMR spectrum of compound **14** in Acetone-d6

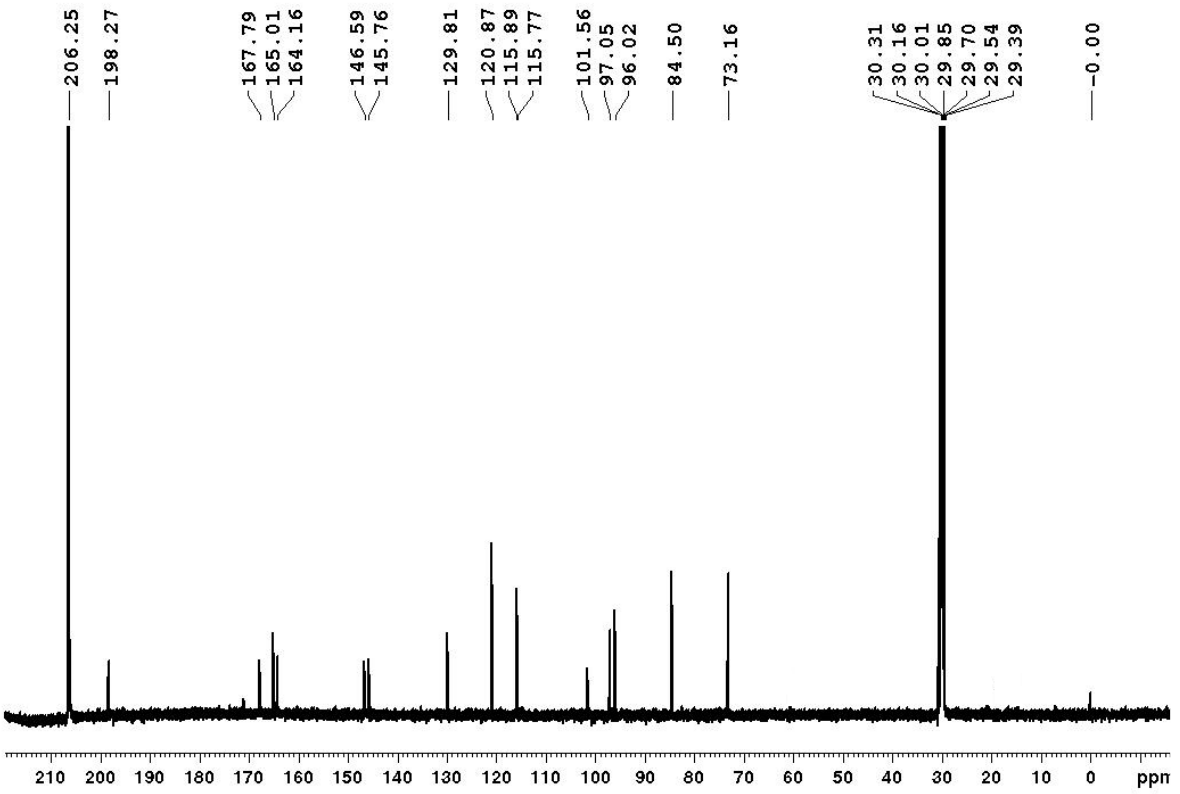


Figure 2B.16. ^{13}C NMR spectrum of compound **14** in Acetone-d6

2B.7. Preliminary cytotoxic studies of the extracts

The isolated compounds were screened for cytotoxic analysis against breast cancer (MDA-MB-231) cell line using MTT assay. Doxorubicin (DOX) was used as the standard drug and the results are shown in **Table 2B.2**. From the results, cedeodarin (**13**) exhibited potent cytotoxicity with an IC_{50} 59.09 µM followed by quercetin (IC_{50} 73.9 µM) at 48 h. Compound 9 and 14 did not exhibited cytotoxicity up to 100 µM (**Fig. 2B.19.).**

Table 2B.2. IC_{50} values of compounds in MDA-MB-231 cell lines

Compound	**IC_{50} of compounds in µM**	
	MDA-MB-231	
	24 h	48 h
9	> 100	>100
12	> 100	73.9 ± 0.05
13	> 100	59.09 ± 0.02
14	> 100	> 100

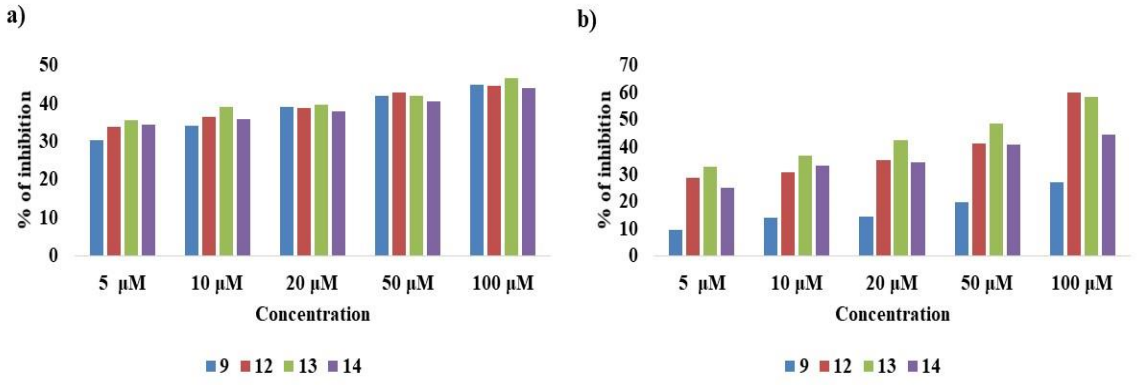


Figure 2B.17. MTT assay of compounds on MDA-MB-231 cells (a) at 24 h (b) at 48 h

2B.8. Conclusion

In summary, we have isolated and characterized six molecules from the acetone extract of *Cedrus deodara*. The compounds are ferrulic acid, (+)-lariciresinol, CD-lignan mixture, taxifolin, cedeodarin and quercetin. All the compounds were

previously reported from the heartwood of *C. deodara*, but it is reported for the first time from the stem bark. Preliminary cytotoxicity studies revealed hexane extract exhibited good anticancer activity against cervical and breast carcinoma cell lines.

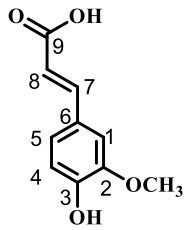
2B.9. Experimental Section

The general experimental procedure, as described in section 2A.6. and preliminary cytotoxicity analysis as in section 2A.7. in Chapter 2A.

2B.10. Spectral Data

Compound 9 (Ferulic acid)

Compound **9** was isolated from fraction pool 2 as white amorphous solid (649 mg). The spectral studies of the compound matched with that of **Ferulic acid** and the structure is as shown below.



Molecular formula: $C_{10}H_{10}O_4$

FT-IR (Neat, υ_{max} cm^{-1}): 3379, 2970, 2900, 2742, 1716, 1265, 1539, 1620 cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 7.60 (d, J = 16.0 Hz, 1H, H-7), 7.07 (dd, J = 6.5, 1.5 Hz, 1H, H-5), 7.03 (d, J = 1.5 Hz, 1H, H-1), 6.91 (d, J = 8.0 Hz, 1H, H-4), 6.29 (d, J = 16.0 Hz, 1H, H-8), 3.93 ppm.

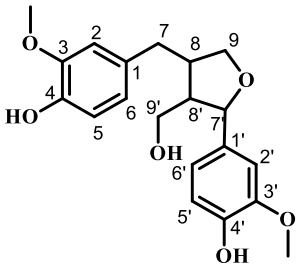
^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 167.4 (C-9), 147.9 (C-3), 146.8 (C-4), 144.6 (C-7), 127.1 (C-6), 123.0 (C-1), 115.7 (C-2), 114.7 (C-8), 109.3 (C-5), 56.0 (-OMe) ppm.

HRMS (ESI): *m/z* calcld for $C_{10}H_{10}O_4$ is 194.0579. Found 195.0530 $(M+H)^+$.

Compound 11 (Lariciresinol)

Compound **11** obtained from fraction pool 4 as pale yellow amorphous solid (63 mg). The structure was confirmed as **Lariciresinol** based on the spectral details and literature reports.

Molecular formula: $C_{20}H_{24}O_6$



FT-IR (Neat, υ_{max} cm^{-1}): 3372, 2937, 2885, 1606, 1515, 1449, 1154, 1113, 1033 cm^{-1}.

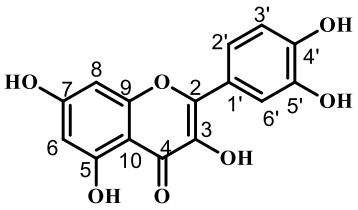
^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 6.86 (m, 2H, H-5', H-2), 6.81 (m, 2H, H-5, H-6'), 6.69 (m, 2H, H-6, H-2'), 5.66 (-OH), 4.79 (d, *J* = 6.5 Hz, 1H, H-7'), 4.05 (t, *J* = 8.0 Hz, 1H, H-9), 3.90 (m, 1H, H-9'), 3.87 (s, 3H, -OCH_3), 3.86 (s, 3H, -OCH_3), 3.78-3.73 (m, 2H, H-9, H-9'), 2.90 (dd, *J* = 9.0, 4.5 Hz, 1H, H-7), 2.73 (m, 1H, H-8), 2.54 (t, *J* = 12.5 Hz, 1H, H-7), 2.40 (t, *J* = 6.5 Hz, 1H, H-8') ppm .

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 146.7 (C-3), 146.6 (C-3'), 145.0 (C-4'), 144.0 (C-4), 134.8 (C-1'), 132.3 (C-1), 121.2 (C-6), 118.8 (C-6'), 114.4 (C-5), 114.2 (C-5'), 111.2 (C-2), 108.4 (C-2'), 82.8 (C-7'), 72.9 (C-9), 60.9 (C-9'), 55.9 (-OCH_3), 52.6 (C-8'), 42.4 (C-8), 33.3 (C-7) ppm.

HRMS (ESI): *m/z* calcld for $C_{20}H_{24}O_6$ is 360.1572. Found 382.4406 $(M- H+Na)^+$.

Compound 12 (Quercetin)

Fraction pool 5 on CC separation afforded a yellow coloured solid (38 mg) as compound **12**. The IR, ^{1}H and ^{13}C NMR was closely related to the common flavonol **Quercetin**. The spectral details are given below.



Molecular formula: $C_{15}H_{10}O_7$

Melting point: 202 ± 2° C

FT-IR (Neat, υ_{max} cm^{-1}): 3392, 3369, 1654, 1609, 1558, 1508, 1458, 1429 cm^{-1}.

^{1}H NMR (500 MHz, Acetone-d6, TMS): δ 7.84 (d, *J* = 2.0 Hz, 1H), 7.71 (dd, *J* = 8.5, 2.5 Hz, 1H), 7.01 (d, *J* = 8.5 Hz, 1H), 6.53 (d, *J* = 2.0 Hz, 1H), 6.28 (d, *J* = 2.0 Hz, 1H), 12.18 (-OH) ppm.

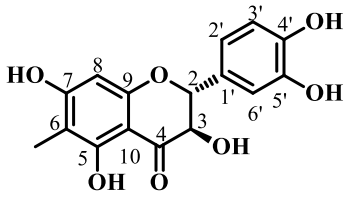
^{13}C NMR (125 MHz, Acetone-d6, TMS): δ 175.7 (C-4),

164.1 (C-7), 161.4 (C-5), 156.9 (C-9), 147.4 (C-2), 146.1 (C-4'), 144.9 (C-3'), 135.9 (C-3), 122.9 (C-1', 120.6 (C-6'), 115.3 (C-5'), 114.9 (C-2'), 103.2 (C-10), 98.3 (C-6), 93.6 (C-8) ppm.

HRMS (ESI): *m/z* calcld for $C_{15}H_{10}O_7$ 302.0426. Found 303.0511 $(M+H)^+$.

Compound 13 (Cedeodarin)

Compound **13** was isolate from fraction pool 7 (Fr. 56-60) as yellow solid (369 mg). The compound was successfully characterized as a dihydroflavanol, **Cedeodarin** based on the spectral details obtained and on comparison with the literature reports.



Molecular formula: $C_{16}H_{14}O_7$

Melting point: 202 ± 2° C

UV (MeOH, λ_{max}, nm): 286, 333 nm.

FT-IR (Neat, υ_{max} cm^{-1}): 3375, 2926, 2857, 1638, 1499, 1452, 1288, 990 cm^{-1}.

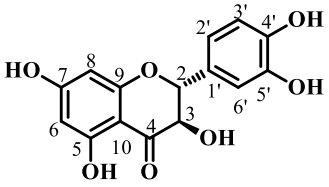
^{1}H NMR (500 MHz, Acetone-d6, TMS): δ 7.06 (s, 1H, H-2'), 6.91 (d , *J* = 8.0 Hz, H-6'), 6.86 (d, *J* = 8.5 Hz, H-5'), 6.02 (s, 1H, H-6), 4.99 (d, *J* = 11.0 Hz, 1H, H-2), 4.64 (br s, 1H, C3-OH), 4.59 (dd, *J* = 4.0, 11.5 Hz, 1H, H-3), 1.99 (s, 3H, $-CH_3$), 7.99 (br s, 1H, C4'-OH), 8.05 (br s, 1H, C3'-OH), 9.67 (br s, 1H, -OH), 11.96 (s, 1H, C5-OH) ppm.

^{13}C NMR (125 MHz, Acetone-d6, TMS): δ 198.3 (C-4), 165.5 (C-5), 162.1 (C-7), 161.7 (C-9), 146.6 (C-4'), 145.8 (C-3'), 130.0 (C-1'), 120.8 (C-6'), 115.9 (C-2'), 115.8 (C-5'), 105.1 (C-6), 101.3 (C-10), 95.3 (C-8), 84.6 (C-2), 73.3(C-3), 7.0 ($-CH_3$) ppm.

HRMS (ESI): *m/z* calcld for $C_{16}H_{14}O_7$ is 318.0739. Found 341.0640 $(M+Na)^+$.

Compound 14 (Taxifolin)

Compound **14** was isolate from fraction pool 8 (Fr. 61-64) as yellow solid (2.85 g). The compound was identified as the dihydroflavanol, **Taxifolin** based on the spectral data obtained.



Molecular formula: $C_{15}H_{12}O_7$

Melting point: 224 ± 2° C

UV (MeOH, λ_{max}, nm): 290, 327 nm.

FT-IR (Neat, υ_{max} cm^{-1}): 3428, 2953, 2833, 1636, 1610, 1510, 1473, 1415, 970, 775 cm^{-1}.

^{1}H NMR (500 MHz, Acetone-d6, TMS): *δ* 7.07 (s, 1H, H-2'), 6.92 (d, *J* = 8.0 Hz, H-6'), 6.87 (d, *J* = 8.0 Hz, H-5'), 5.99 (s, 1H, H-6), 5.95 (s, 1H, H-6), 5.02 (d, *J* = 11.5 Hz, 1H, H-2), 4.72 (br s, 1H, C3-OH), 4.62 (d, *J* = 11.5 Hz, 1H, H-3), 7.99 (br s, 1H, C4'-OH), 8.06 (br s, 1H, C3'-OH), 11.73 (s, 1H, C5-OH) ppm.

^{13}C NMR (125 MHz, Acetone-d6, TMS): *δ* 198.3 (C-4), 167.8 (C-5), 165.0 (C-7), 164.2 (C-9), 146.6 (C-4'), 145.8 (C-3'), 129.8 (C-1'), 120.9 (C-6'), 115.9 (C-2'), 115.8 (C-5'), 101.6 (C-10), 97.0 (C-6), 96.0 (C-8), 84.5 (C-2), 73.2 (C-3) ppm.

HRMS (ESI): *m/z* calcld for $C_{15}H_{12}O_7$ is 304.0583. Found 303.05119 $(M-H)^+$.

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CHAPTER: 3

Comparative Evaluation of the Antibacterial & Anticancer Activity of *Bridelia retusa* and *Bridelia stipularis*

3.1. Introduction

Phyllanthaceae is the family of flowering plants in the eudicot order Malpighiales. The recent classification of angiosperms distinguishes five lineages of Euphorbiaceae *sensu lato* at family rank, namely, Euphorbiaceae *sensu stricto*, Pandaceae, Phyllanthaceae, Picrodendraceae and Putranjivaceae following molecular work on Euphorbiaceae *s.l.*[1] The name Phyllanthaceae was first validly published in a Russian book entitled Tekhno- botanico Slovar by Martynov in 1820. For convenience, the obsolete, older concept of Euphorbiaceae, known as Euphorbiaceae *s. l.* is sometimes still used instead of Phyllanthaeae describing certain plants of the family.

Phyllanthaceae comprises of about 2000 species in 59 genera. It includes two subfamilies, Phyllanthoideae and Antidesmatoideae.[2] The subfamily Phyllanthoideae sensu stricto covers four tribes: Poranthereae, Wielandieae, Bridelieae, and Phyllanthoideae. Members of Phyllanthaceae are pantropical and include trees, shrubs, semi–succulents, phyllocladous taxa, annual herbs and even a free-floating species. Vegetatively, most Phyllanthaceae are uniform with pinnate venation, entire margins, and simple indumentum. Flowers are small and actinomorphic but display great diversity in shape, size, and number of floral organs. Some of the genera have recently been sunk into others, while other genera have recently been divided. The family Phyllanthaceae has a predominantly pantropical distribution. Of its several genera, *Bridelia Willd.* is of special interest because it has disjunct equally distributed species in Africa and tropical Asia, *i.e.*, 18–20 species in Africa, Madagascar (all endemic) and 18 species in tropical Asia (some shared with Australia).[3]

3.2. *Bridelia*

The generic name *Bridelia* was named in honor of Samuel Elisée Bridel-Brideri by the German botanist Carl Ludwig Willdenow.[4] *Bridelia* consists of about 60 species scattered throughout Asia, Africa, and Australia. The chemical constituents of *Bridelia* genus plants have been reported to contain triterpenoids, benzenoids, flavonoids, tannins, green pigments, *etc*. Several *Bridelia* species are traditionally used, throughout Africa and Asia, for treating several ailments including sexual diseases (*B. atroviridis*), anemia (*B. cathartica*), bronchitis (*B. balansae*), intestine disorders and painful joints (*B. michranta*), fever, diabetes and diarrhoea (*B. ndellensis*), dental caries (*B. grandis*), rheumatism (*B. retusa*), fever (*B. tomentosa*), rheumatism, abdominal pain and arthritis (*B. sclereoneura*).[5]

3.3. Literature survey on the Phytochemistry and Pharmacological uses of *Bridelia* species

3.3.1. *Bridelia atroviridis* Muell. Arg.

Bridelia atroviridis is a bush in the Phyllanthaceae family, present in several countries of equatorial Africa. African women use the aqueous extract of its leaves to facilitate difficult labor or produce abortion. Like various other herbal medicines, *B. atroviridis* is taken empirically and might not be safe for female health. Corallo *et al*., studied the effect of the aqueous extract of the leaves on the mechanical activity of rat uterus. The study showed that the aqueous extract of *B. atroviridis* is a potent spasmogenic drug in the rat uterus. It induces contraction, the amplitude of which is in the same range as that induced by oxytocin, one of the most potent uterotonic agents in the last stages of pregnancy. It was the first detailed investigation of the uterotonic action of *Bridelia*.[6] Corallo *et al*., also investigated *in vivo* and *in vitro* cardiovascular effects of the leaves of *B. atroviridis* in the rat cardiovascular system. The *in vivo* study of the lyophilized decoction of the leaves caused a decrease in arterial pressure and heart rate in an anesthetized rat and *in vitro* studies of the extract induced dose-dependent negative inotropic and chronotropic effects in isolated rat heart. Hence *B. atroviridis* seemed to have a direct impact in rat heart.[7]

3.3.2. *Bridelia balansae* Tutch

Bridelia balansae Tutch is a small tree distributed in Indo-China, southern China, Ryukyus and Taiwan. Its leaves are used as an antitussive to treat bronchitis in China. The leaves of *B. balansae* contains two long-chain esters balansenate I (6,8,11-trimethyldodecanoic acid (2E)-3-methylhexadec-2-enyl ester) and balansenate II (10,12,15-trimethylhexadedecanoic acid (2E)-3-methylhexadec-2-enyl ester), the eburicane-like triterpenoid bridelone (hexadecahydro-4,4,10,13,14-pentamethyl-17-(5-methyl-1,4-dimethylenehexyl)-3H-cyclopenta[a]phenanthren-3-one), the de-imino xanthine, bridelonine (5-(3-methylbut-2-enyl)pyrrolo[3,4-d]imidazole-4,6(1H,5H)-dione) and the two adenine analogues 9-(3-methylbut-2-enyl)adenine and 1-(3-methylbut-2-enyl)adenine along with N^6 -(3-methylbut-2-enyl)adenine, 3-(3-methylbut-2-enyl)adenine, and adenine (**Fig. 3.1**.). [8]

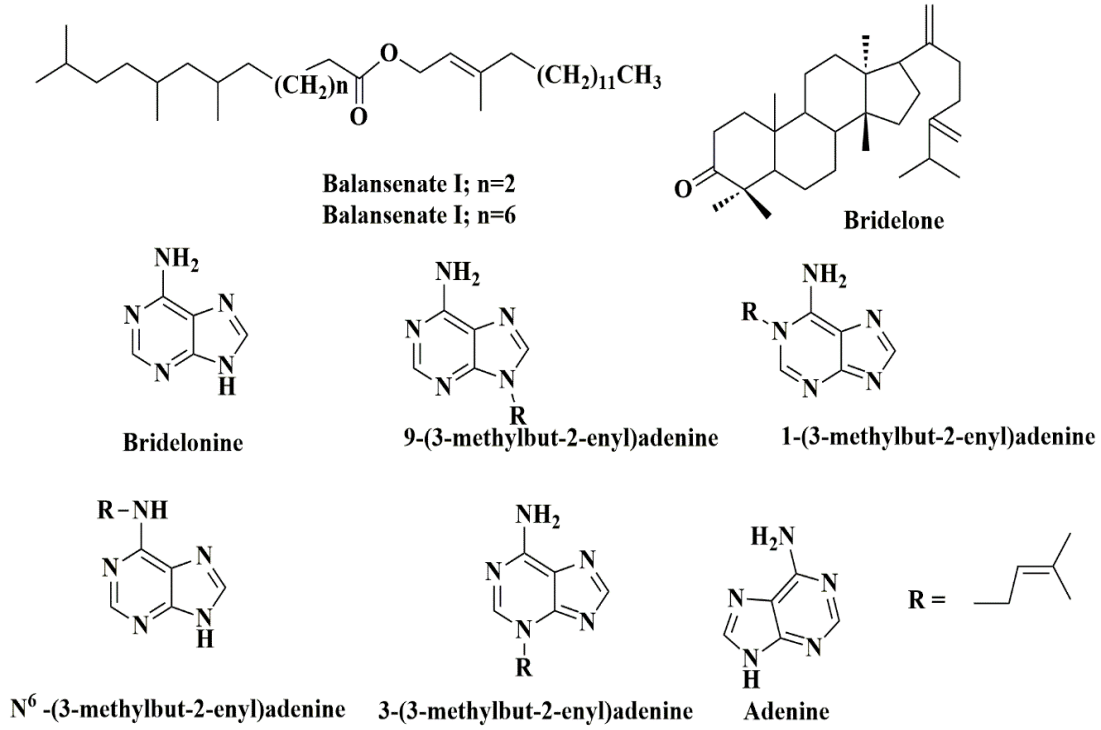

Figure 3.1. Compounds reported from *Bridelia balansae* Tutch

3.3.3. *Bridelia cathartica* Bertol. f.

Bridelia cathartica is a scrambling shrub or small tree with long, somewhat zig-zag branches and leaves held in one plane. *B. cathartica* grows in the sandy soils of Maputaland and the Zululand coastline and also occurs in Mpumalanga, Limpopo,

Swaziland and Mozambique, extending into tropical Africa. The roots of *B. cathartica* decoction is used to treat malaria and fever, mainly from Southern African regions.[9] The laypeople in a rural community in northern Maputaland use the roots and leaves to treat gynecological and obstetric disorders and Sexual Transmitted Infections. The rural inhabitants in this area, mostly women, prefer to use traditional medicine over allopathic medicine to treat STIs.[10] According to Chhabra *et al.,* the roots can be used to treat cardiac pains. The root decoction mixed with milk is taken to treat liver diseases. A qualitative analysis of the root bark indicated the presence of anthocyanins, emodins, anthracene glycosides, steroids/triterpenoids, flavonoids, tannins and volatile oils.[11]

3.3.4. *Bridelia crenulata* Roxb.

Bridelia crenulata is a medium-sized deciduous tree. The branchlets and leaves (beneath) are densely brownish tomentose. These trees are found in India, especially in Karnataka, Orissa and Tamil Nadu. The women of the Paliyan tribes in Tirunelveli district of Tamil Nadu consume the stem bark extract to cure menorrhagia and the inhabitants of Mayurbhanj district in Orissa use stem bark liquor of *B. crenulata* to prevent pregnancy. The liquor is given after menstruation. Significantly less information is available on this plant regarding its pharmacognosy, pharmacology or phytochemistry in common medicinal plant literature. Ramesh *et al*. examined the phytochemical and antibacterial studies of the bark's aqueous and methanolic extract. Phytoconstituents reported to be isolated from the stem bark of *B. crenulata* are *n*-octacosanol, *α*-amyrin, *β*-sitosterol, friedelin, *epi*-friedelinol, *β*-sitosterol-3-*β*-D-glucopyranoside and luteoferol (**Fig. 3.2.).**[12] Luteoferol was reported to have good antibacterial activity.[13]

3.3.5. *Bridelia ferruginea* Benth.

Bridelia ferruginea, the most studied species in the genus *Bridelia* is an indigenous medicinal plant in Nigeria. It is usually a gnarled shrub that sometimes reaches the size of a tree in a suitable condition. The common names in Nigeria include Kirni, Kizni (Hausa), Iralodan (Yoruba), Maren (Fulani) and Ola (Igbo). It is commonly found in the savannah, especially in the moist regions of Guinea to Zaire and Angola. The bark is dark grey, rough and often marked scaly.[14]

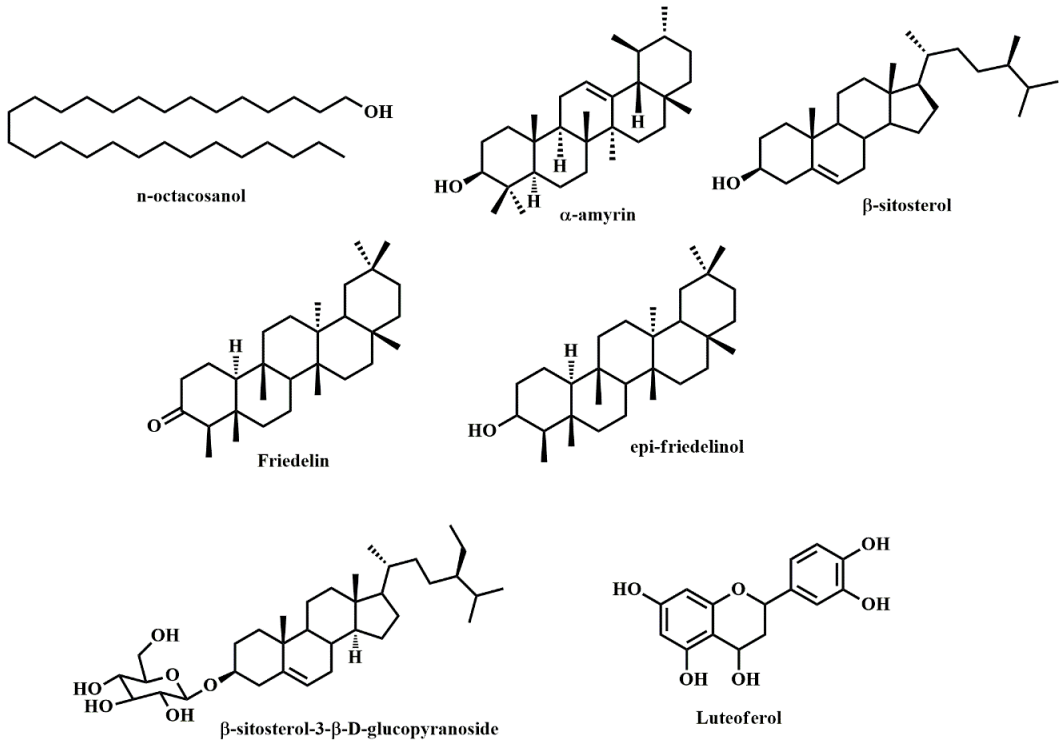

Figure 3.2. Compounds reported from *Bridelia crenulata* Roxb

B. ferruginea Benth is used extensively for a variety of ailments and a source of local dyestuffs. Crude extracts of this plant have been reported to lower the fasting blood sugar level in albino rats and humans. A decoction of the leaves has been used to treat diabetes. Local men use the root decoction for the treatment of gonorrhoea infections. It is traditionally used in Nigeria, as a mouthwash and as a remedy for candidal oral thrush. In Minna, Northern Nigeria, the bark is used to treat infections due to poisoned-arrow wounds. Irobi *et al*., reported the phytochemical and antimicrobial properties of the plant bark. The water and ethanol extracts exhibited promising antimicrobial activity against *S. aureus, C. albicans, E. coli, S. epidermidis, S. lactis. P. mirabilis. P. vulgaris, S. pyogenes* and *Klebsiella sp.*.[15] The aqueous extract of the stem bark of *B. ferruginea* was reported to be effective in the acute and chronic phases of the inflammatory process. The anti-inflammatory activity was studied using carrageenan-induced paw oedema in rats and mice and the cotton pellet granuloma method. The extract (10 to 80 mg/kg p.o.) significantly inhibited the carrageenan-induced rat paw oedema, with an ID_{50} value of 36 mg/kg.[16] In addition to this, the stem bark extract also showed potent analgesic and antipyretic effect, justifying the folkloric usage in the relief of pain and fever. The methanol extract of the fruit exhibited promising antimicrobial activity.[17] The antimicrobial activity of the crude

methanol extract of the different parts of the plant were carried out *in vitro,* exhibited a wide range of activity on *S. typhi, P. mirabilis, E. coli, C. albicans* and *S. aureus*. The root extracts inhibited the growth of *E. coli, S. aureus, S. typhi, P. mirabilis* and *C. albicans* at concentrations of 40-100 mg/ml, respectively, while the stem bark had minimum inhibitory concentration (MIC) of 60 mg/ml on *S. typhi* and 10 mg/ml on *C. albicans*.[18]

The ulcer protective and anti-diarrhoeal activity of aqueous stem bark extract examined in rats and mice showed significant activity, hence justifying the plant's use by the local population.[19] Bakoma *et al.,* investigated the acute toxicological studies of the hydroethanolic extract of the root bark. The results showed that the extract did not cause any death or toxicity of the main organs in rodents.[20] The examination of the acetone and ethyl acetate extract of the *B. ferruginea* root in fructose drinking mice showed that the plant extract could prevent the development of insulin resistance, hyperglycaemia and hyperlipidaemia as well as reduce glucose intolerance.[21] Ikechukwu *et al*., investigated the effect of aqueous extract of stem bark of *B. ferruginea* and butachlor on some kidney markers of *Clarias gariepinus* to determine the consequence of the two toxicants on the kidney of *Clarias gariepinus*. There was a significant increase ($p<0.05$) in serum concentrations of creatinine, urea blood nitrogen, sodium and potassium in the catfish exposed to both test toxicants. But the effect was more pronounced in butachlor-exposed fish than the plant extract.[22]

Several studies were carried out to examine the use of *B. ferrugineae* against diabetes. Traditionally, the root of *Bridelia ferruginea* is used for type 2 diabetes. Ameyaw *et al.,* reported the use of *B. ferruginea* in the management of diabetes mellitus.[23] Another study was also carried out to see the influence of *B. ferruginea* extracts on pregnancy-induced impaired glucose tolerance in rats. *B. ferruginea* caused a reduction in glycaemic response to glucose challenge and an increased glucose tolerance in rats that had pregnancy-induced glucose intolerance.[24] Aja *et al.,* reported the antidiabetic and liver enzymes activities of aqueous extracts of *B. ferruginea* leaves in alloxan-induced diabetic albino rats.[25] Over the past 20 years, the Centre for Plant Medicine Research (CPMR), Mampong-Akwapem, uses the leaves of *B. ferruginea* as an antidiabetic product under the trade name Bridelia Tea. People with diabetes who visit the clinic are counseled to prepare an aqueous infusion from

the dried leaves taken at a dose of 100 ml three times daily. The dry weight of the *B. ferruginea* in this infusion is approximately 0.97 (± 0.20) g. Thomford *et al.*, undertook a study to analyse retrospective clinical data on the product Bridelia tea.[26]

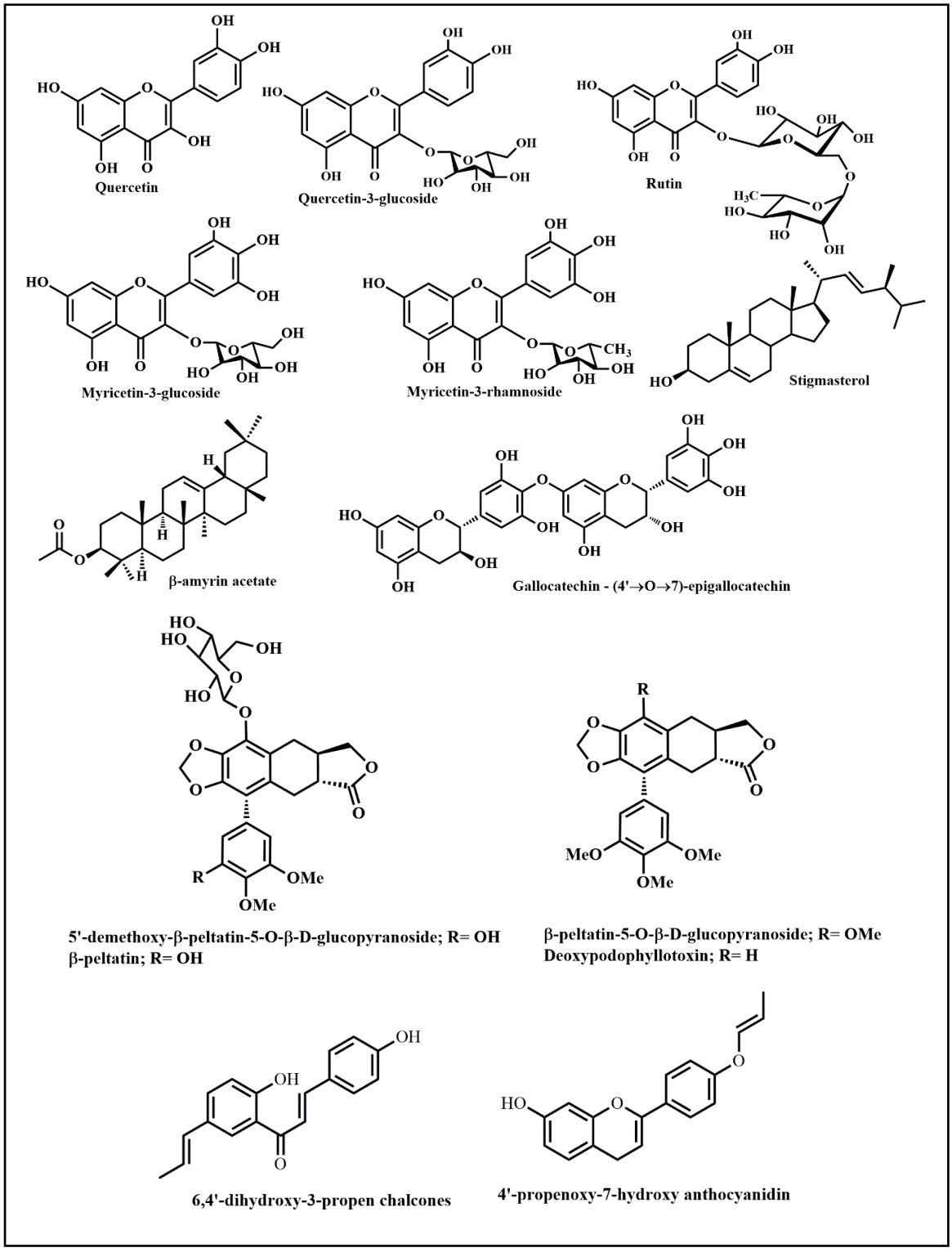

Figure 3.3. Compounds reported from *Bridelia ferruginea* Benth.

The aqueous methanol extract of the leaves of *B. ferrruginea* is rich in flavonoids such as quercetin, quercetin-3-glucoside, rutin, myricetin-3-glucoside and myricetin-3-rhamnoside. The petroleum extract of the roots yielded the terpenoids friedo-oleanan-3-*β*-ol, friedo-oleanan-3-one, *β*-sitosterol and stigmasterol.[27] Fabiyi *et al*., identified the triterpene *β*-amyrin acetate (12-Oleanen-3yl acetate) for the first time from the leaves of *B. ferruginea* and its cytotoxicity studies were also carried out.[28] The stem bark reported to contain the biflavonoid gallocatechin-(4'→O→7)-epigallocatechin.[29] Cytotoxic lignans were reported from the roots of the plant. Podophyllotoxin derivatives such as 5'-demethoxy-*β*-peltatin-5-*O*-*β*-D-gluco pyranoside, *β*-peltatin, *β*-peltatin-5-*O*-*β*-D-glucopyranoside and deoxy-podophyllotoxin. 5'-demethoxy-β-peltatin-5-*O*-*β*-D-glucopyranoside was reported for the first time from the genus. All compounds exhibited antitumor activity.[30] Pettit *et al*., reported the SAR studies of the synthetically derived podophyllotoxin derivatives, focused on 4-aza-podophyllotoxin structural modifications. Such structural changes with alkyl and 4-fluorobenzyl substituents at the 4-aza position provided the most potent cancer cell growth inhibitory activity (GI50 0.1 to <0.03 μg/mL) against a panel of six human cancer cell lines and one murine cancer cell line.[31] Chalcones and anthocyanidins represent essential groups of natural products that possess a wide range of pharmacological activities such as antibacterial, anticancer, antioxidant, antitubercular, antitumor, anti-inflammatory and anti-malarial properties. Two flavonoids compound 6,4′-dihydroxy-3-propen chalcones and 4′-propenoxy-7–hydroxy anthocyanidin were reported from the leaves.[32]

3.3.6. *Bridelia glauca* Bl. f. balansae Tucht.

Bridelia glauca Bl. F. *b*alansae (Tucht) is an evergreen tree distributed in Okinawa, Taiwan, Southern China, Indochina and Philippines. The chemical investigation of leaves of *B. glauca* afforded six megastigmane glucosides, named bridelionosides A–F, along with seven known megastigmane glucosides.: 3-oxo-a-ionol glucoside, inamoside, roseoside, megastigman-4-en-3-on-9,13-diol 9-O-*β*-glucopyranoside, ampelopsisionoside, alangionoside, and actinidioionoside.[33] A lignan glycoside, bridelioside, has been isolated from *B. glauca f. balansae* named (+)-isolariciresinol 2a-*O*-*α*-L- arabinopyranoside (**Fig. 3.4**.).[34]

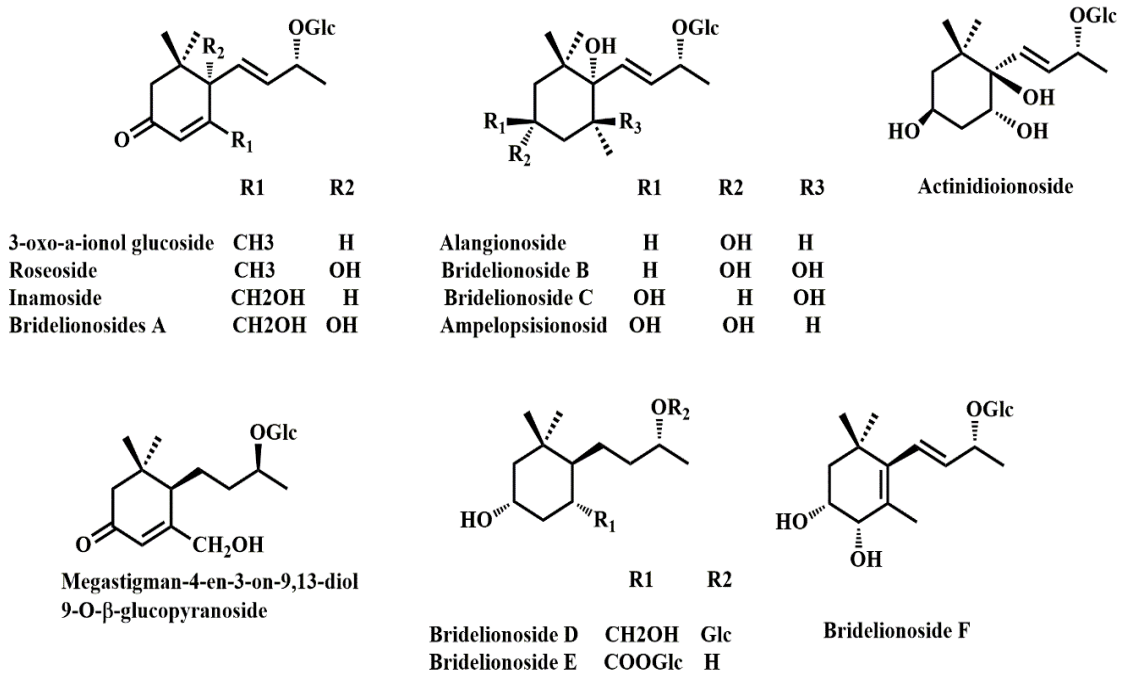

Figure 3.4. Compounds reported from *Bridelia glauca Bl. f. balansae Tucht.*

3.3.7. *Bridelia grandis (*Pierre ex Hutch).

Bridelia grandis (Pierre ex Hutch) is a medicinal plant traditionally used in Cameroon by pygmies Baka. The pygmies Baka are famous as traditional healers; their traditional medicine is an empirical knowledge based on the use of forest plants for therapeutic applications. In the Pygmies Traditional Medicine, the stem bark of this plant is considered as the essential constituent. They use the decoction of *B. grandis* stem bark as a remedy for oral cavity infection. To validate the use of the plant Ngueyem *et al*., investigated the preliminary *in vitro* antimicrobial properties as well as their phytochemical constituents. All the extracts showed significant antimicrobial activity, including a decoction in water prepared following the pygmies' original procedure. Water, methanol and methanol-water mixture extracts exhibited antibacterial activity with MIC between 0.5 and 2 mg/ml, justifying the traditional use of *B. grandis* stem bark for oral cavity affection.[35] Further studies showed that the antibacterial activity showed by water and methanol extracts of *B. grandis* stem bark correlated to the high content in polyphenols, especially condensed tannins. Moreover, the antioxidant power together with the antibacterial activity make this plant a potential natural preservative for food and cosmetic products.[36]

B. grandis is used in most parts of tropical Africa for the treatment of diabetes. Njamen *et al.,* studied the antidiabetic properties of the methanolic extract of stem bark in ob/ob (obese diabetic) and db/db mice. The plant extract induced hypoglycaemic effects in type 2 diabetic ob/ob and db/db mice, thus confirming its antidiabetic properties.[37] Capelli *et al*., reported that *B. grandis* water extract can interfere with human cell adhesion. They investigated the impact of a water extract of stem bark of *B. grandis* on human cells *in vitro*, analyzing the structural changes with light and electron microscopy. The preliminary results highlight an intense antiproliferative effect of the water extract.[38]

3.3.8. *Bridelia micrantha* (Hochst) Baill

Bridelia micrantha (Hochst) Baill, the Mitzeeri or the Coastal Golden-leaf, is recorded in several countries of tropical Africa and the Island of Réunion in the Indian Ocean. The plant is a semi-deciduous to a deciduous tree in the Phyllanthaceae family. The use of medicinal plants for AIDS-related conditions is common in South Africa. Bessong *et al*., screened fractions of the methanol extracts of Venda medicinal plants for activity against HIV-1 reverse transcriptase (RT) and integrase (IN). The *n*-butanol fraction obtained from the crude methanol extracts of the roots of *B. micrantha* was observed to be the most active inhibiting the RNA-dependent-DNA polymerization (RDDP) activity of HIV-1 RT with an IC_{50} of 7.3 µg/ml. [39]

Nwaehujor *et al.,* studied the hepatoprotective and antioxidant activities of the methanolic leaf extract of *B. micrantha* on paracetamol-induced liver damage in Wistar rats. From their observation, the absence of signs of toxicity and death in rats treated with the extract showed that it is relatively safe and explains why this plant has wide acceptance in traditional medicine practice. The experiment also revealed that ethyl acetate extracts of *B. micrantha* have a better hepatoprotective capacity.[40] Okeleye *et al*., reported the antimicrobial activity of the stem bark of *B. micrantha* on *Helicobactor pylori* (which is a major risk factor for gastritis, ulcers and gastric cancer). The acetone (100 %) and ethyl acetate (93.5 %) extracts were observed to be the most active against all the *H. pylori* strain tested, compared with the other solvents used.[41]

Green *et al.,* reported the *in vitro* antimycobacterial activity of *n*-hexane sub-fraction from *B. micrantha* against MTB H37Ra and a clinical isolate resistant to all five first-line antituberculosis drugs. The *n*-hexane fraction showed 20 % inhibition of MTB H37Ra and almost 35 % inhibition of an MTB isolate resistant to all first-line drugs at 10 μg/mL indicates that the *n*-hexane fraction of *B. micrantha* has antimycobacterial activity. GC/MS analysis of the fraction resulted in the identification of twenty four constituents representing 60.5 % of the fraction. Some of the 24 compounds detected included Benzene, 1.3-bis (3-phenoxyphenoxy (13.51 %), 2-pinen-4-one (10.03 %), N(b)-benzyl-14-(carboxymethyl) (6.35 %) and the least detected compound was linalool (0.2 %).[42] Mburu *et al*., investigated the phytochemical properties of *B. micrantha* used to treat malaria among the Digo community in Kenya. The crude extracts of the whole plant were obtained using hexane, ethyl acetate and methanol were analyzed through various quantitative and qualitative analyses. A variety of phytochemicals such as alkaloids, saponins, flavonoids, phenolic compounds and tannins were found to be present in the extracts and could be responsible for the anti-malarial activity exhibited by the plant, *B. micrantha*.[43]

Ngo Bum *et al.,* reported the anticonvulsant and sedative effects of the decoction of *B. micrantha* by using *in vivo* mice model (maximal electroshock, strychnine, pentylenetetrazol, picrotoxin, isonicotinic hydrazide acid)-induced convulsions. The decoctions of *B. micrantha* protected 100 % of mice against the convulsions induced by PIC (Picrotoxin) and 80 % of mice from STR (Strychnine) - induced convulsions. The inhibition of STR-induced seizures suggested the existence of anticonvulsant properties and the involvement of glycine receptors. These results allow understanding at least in part of the use of these decoctions in traditional medicine in Africa and particularly in Cameroon, to treat insomnia and epilepsy.[44] Adika *et al.,* studied the antioxidant and antidiabetic activity of the methanolic extracts of leaves. The crude extracts showed significant activity comparable to that of glibenclamide, an oral sulfonylurea and validated its use in Nigerian traditional medicine to treat diabetes mellitus.[45]

Chinaka *et al*., evaluated the anti-inflammatory activities of the methanol leaf extract of *B. micrantha* using acute, sub-acute and chronic models of inflammation in

Wistar rats. The results indicated that although well-established anti-inflammatory drugs are known to alleviate oedema and pain conditions, the extract of *B. micrantha* exhibited a more potent anti-inflammatory effect in laboratory animals.[46] The phytochemicals so far reported from the plants are taraxerone, friedelin, epifriedelinol, gallic acid, taraxerol and ellagic acid have been isolated from *B. micrantha* wood. Pegel *et al.,* reported leucodelphinidin and caffeic acid was from the leaves.[47]

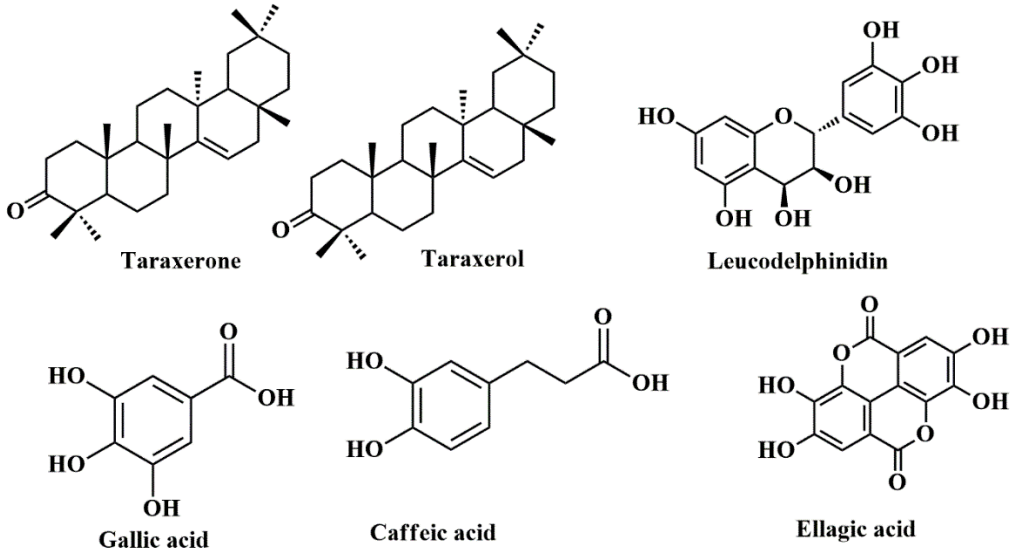

Figure 3.5. Compounds reported from *Bridelia micrantha* (Hochst) Baill

3.3.9. *Bridelia Montana* var. montana (Roxb.)

Bridlia montana var. montana (Roxb.) Willd is a monoecious branched shrub, branchlets glabrous. This species has restricted global distribution occurring only in India and Burma. Within India, it has been distributed in the Sub-Himalayan tract from the Jhelum eastwards to Assam up to an altitude of 1800 m, in Bihar, in the dry forests of the Eastern Ghats in Orissa and Andhra Pradesh. The plant is used in Ayurveda for the preparation of the ayurvedic drug Pashanabheda.[48] Pashanbheda is given for various ailments but mainly as a diuretic and lithotriptic. It is said to have the property of breaking and disintegrating the kidney stones. The bark is having astringent, anthelmintic properties and also useful in dissolving kidney stones.[49] The leaves of *B. montana* have been used in the western part of the country as an astringent and are claimed to possess anthelmintic property. Yadav *et al.,* reported the sterols *β*–sitosterol and its glycoside from the leaves.[50] The flavanone naringin

was also reported from the plant.[51] *B. montana* is one of the neglected species because of forest habitat, but it contains flavanone naringin. Naringin is a flavanone-7-O-glycoside between the flavanone naringenin and the disaccharide neohesperidose.

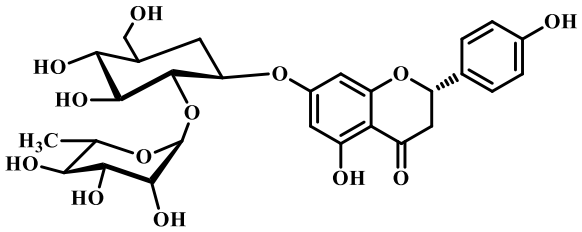

Figure 3.6. Naringin

3.4. Aim and scope of the present study

Several species of *Bridelia* are widely used in traditional and folklore medicine. Our strategy for selecting a plant for the study is mainly focused on the ethnopharmacology of the plant species. A thorough literature survey indicates that the two species of *Bredelia* – *Bridelia retusa* and *Bridelia stipularis*- used primarily by the tribal people of India have not been subjected to detailed phytochemical investigations. Though some reports are available, no scientific validation of the pharmacological aspects which are responsible for any particular activity of the whole plants or plant parts has been carried out. Hence, we have chosen a thorough reinvestigation of the phytoconstituents present in these two species.

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Chapter 3A: Phytochemical Investigation of *Bridelia retusa* (L.) Sprengel.

3A.1. Introduction

Bridelia retusa commonly called "Spinous Kino Tree" is widely distributed in Sri Lanka, India, Nepal, SE Asia mainland, Sumatra, Malay Peninsula, Java, N Borneo, Philippines (unknown from Mindanao), Lesser Sunda Islands (Bali, Lombok, Sumba, Flores, Alor, Timor). It is also found to be present throughout hotter parts of Asia, in India along the base of the Himalaya from Kashmir to Mishmi and outwards to Ceylon, throughout the presidency in deciduous forests. It is a small or moderate-sized deciduous tree, armed with long conical thorns and having dark brown bark armed with long conical spines when young. The fruits are greenish-yellow in color.

Figure 3A.1. Leaf, seed and sem bark of *Bridelia retusa*

3A.2. Ethnopharmacology

B. retusa has been used in Indian folk medicine to treat a broad spectrum of diseases such as liver diseases, for removal of urinary concretion, inflammatory diseases, *etc*. It is being used in the indigenous systems of medicine for the treatment of rheumatism and also as astringent. The drug part used is the greyish-brown roots of this plant. The species is also well known in ayurvedic medicine for treating kidney stones. The bark of *B. retusa* is used with gingelly oil for painful

conditions of musculoskeletal systems. Bark extract possesses antiviral, hypotensive and anticancer activities. The decoction of stem bark with country liquor is used to prevent pregnancy and also for diarrhoea and earaches. A paste of the stem bark is applied to wounds and bark juice taken for snake bite. The *Kani* tribals in Kouthalai of Tirunelveli hills, Tamil Nadu utilise the paste of leaves of *B. retusa* and *Curculigo orchioides* and the oils of castor, coconut and gingelly is mixed and applied externally to cure wounds.[1] The people of Nawarangpur district, Odisha, uses the bark grounded with black peppers for treating urinary congestion.[2]

3A.3. Phytochemistry

Phytochemical screening and antimicrobial activity of *B. retusa* leaves were examined in different extracts such as n-hexane, diethyl ether, chloroform, acetone and ethanol. Ethanol extract showed maximum phytoconstituent concentration and significant antimicrobial activity.[3] Phytochemical studies on *B. retusa* disclosed major chemical constituents including steroids, triterpenoids, tannins and flavonoids. GC and GC-MS analysis of the essential oils obtained from the fruits and leaves of *B. retusa* were carried out and the major constituents of the leaf essential oil were identified as phytol (33.4 %), phthalic acid (5.2 %), 6,13-dimethoxy-2,3,9,10-tetramethylpentacene-1,4,8,11-tetrone (3.4 %), heptacosane (2.3 %) and nonacosane (1.2 %). The major components of the fruit essential oil were identified as dibutyl sebacate (25.6 %), phytol isomer (4.8 %), diacetin (4.3 %), tricosane (3.9 %), isophytol (2.7 %), phthalic acid (1.9 %), hexadecanoic acid (1.5 %) and eicosane (1.2 %).[4]

Jayasinghe *et al.,* reported the isolation of eight compounds from the stem bark of *B. retusa* and antifungal studies of the isolated compounds. The compounds are bisabolane sesquiterpenes, (E)-4-(1,5-dimethyl-3-oxo-1-hexenyl) benzoic acid, (E)-4-(1,5-dimethyl-3-oxo-1,4-hexadienyl) benzoic acid, (R)-4-(1,5-dimethyl-3-oxo-4-hexenyl) benzoicacid, (R)-4-(1,5-dimethyl-3-oxohexyl) benzoic acid (ar-todomatuic acid), (-)-isochaminic acid, 5-allyl-1,2,3-trimethoxybenzene (elemicin), 4-isopropylbenzoic acid (cumic acid) and (+)-sesamin (**Fig. 3A.2**). All these compounds showed fungicidal activity on TLC bioautography method at very low concentrations except elemicin.[5] A long chain fatty acid with formula $C_{77}H_{152}O$ was reported from leaves of *B. retusa.*[6]

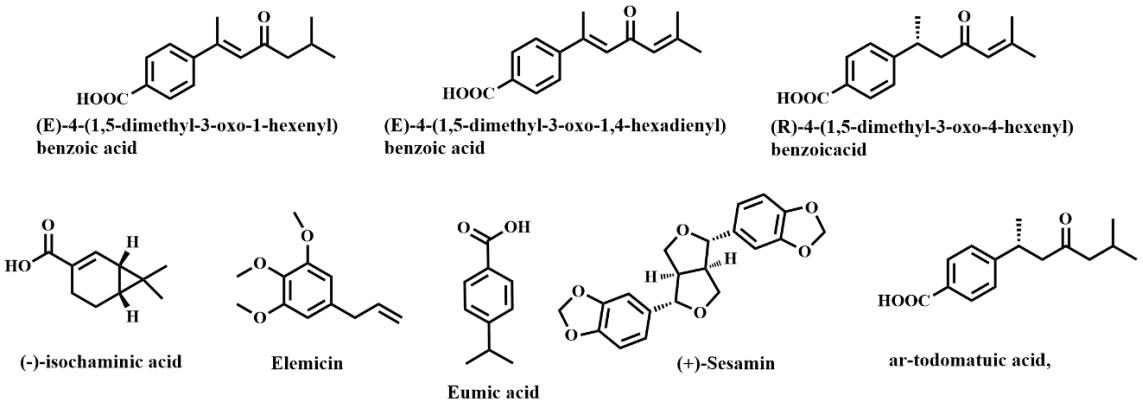


Figure 3A.2. Compounds reported from *Bridelia retusa*

3A.4. Pharmacology

Tatiya *et al*., reported the hypoglycaemic potential of *B. retusa* bark on normal, oral glucose tolerance study and alloxan-induced diabetic rats. The *n*-butanol extract of the bark showed a significant fall of blood sugar level in alloxan-induced diabetic rats, but it did not affect the blood glucose level on normal rats.[7] Cordeiro *et al.,* reported the antihelmintic activity of petroleum ether, chloroform, ethanol and aqueous extract of the stem bark of *B. retusa* on adult African nightcrawlers (*Eudrilus euginae*) earthworms. The antihelmintic activity was maximum in chloroform extract, which was better than that of the standard diclofenac. The ethanol extract showed similar effects to that of the standard, while the rest of the extracts showed poor results.[8] Mehre *et al*., evaluated the anti-inflammatory activity of the aqueous and alcoholic extract of the bark in carrageenan-induced rat paw oedema. Both extracts exhibited significant dose-dependent inhibition of rat paw oedema at a dose of 50 mg/kg and 100 mg/kg orally compared to the control group. The anti-inflammatory activity was reported for the first time from this plant and is comparable to standard drug diclofenac sodium (15 mg/kg orally).[9]

Paula *et al.,* reported the cytotoxicity and anti-proliferating property of the plant extracts using Calu-6 and HL-60 cells; the studies showed significant results in the case of Calu-6 cells.[10] Kumar *et al*., studied the antinociceptive and anti-inflammatory potentials of methanolic extract of *B. retusa* fruit (BRME) against different animal models in rodents. The extract exhibited significant antinociceptive and anti-inflammatory properties, providing a scientific basis for its ethnobotanical

uses for treating various ailments.[11] The antioxidant, brine shrimp lethality and antimicrobial activities of *B. retusa* fruit were studied using *n*-hexane, ethyl acetate, and methanol extracts. Methanolic extracts showed good DPPH free radical scavenging activity with IC_{50} of 168.757 µg/mL. Ethyl acetate extract showed the highest reducing capacity in the CUPRAC test. Methanolic extracts showed good cytotoxic activity with an IC_{50} value of 20 µg/ml in brine shrimp lethality bioassay.[12]

3A.5. Extraction, isolation and characterization of compounds from various parts of *Bridelia retusa*

3A.5.1. Collection of plant material and extraction

The plant material of *Bridelia retusa* was collected form CSIR-NIIST campus Trivandrum. The plant material was authenticated by the taxonomist of M. S. Swaminathan Research Foundation (MSSRF), Kerala and a voucher specimen (M.S.S.H. 807) was deposited in the herbarium repository of the institute. We have carried out the phytochemical investigation of seeds, leaves and stem bark of *B. retusa*. About 1 Kg of the dried, powdered seed was extracted with various solvents in the order of increasing polarity. The seeds were first soaked in 5 L of hexane at room temperature for three days and the solvent was removed under reduced pressure to get the crude extract.

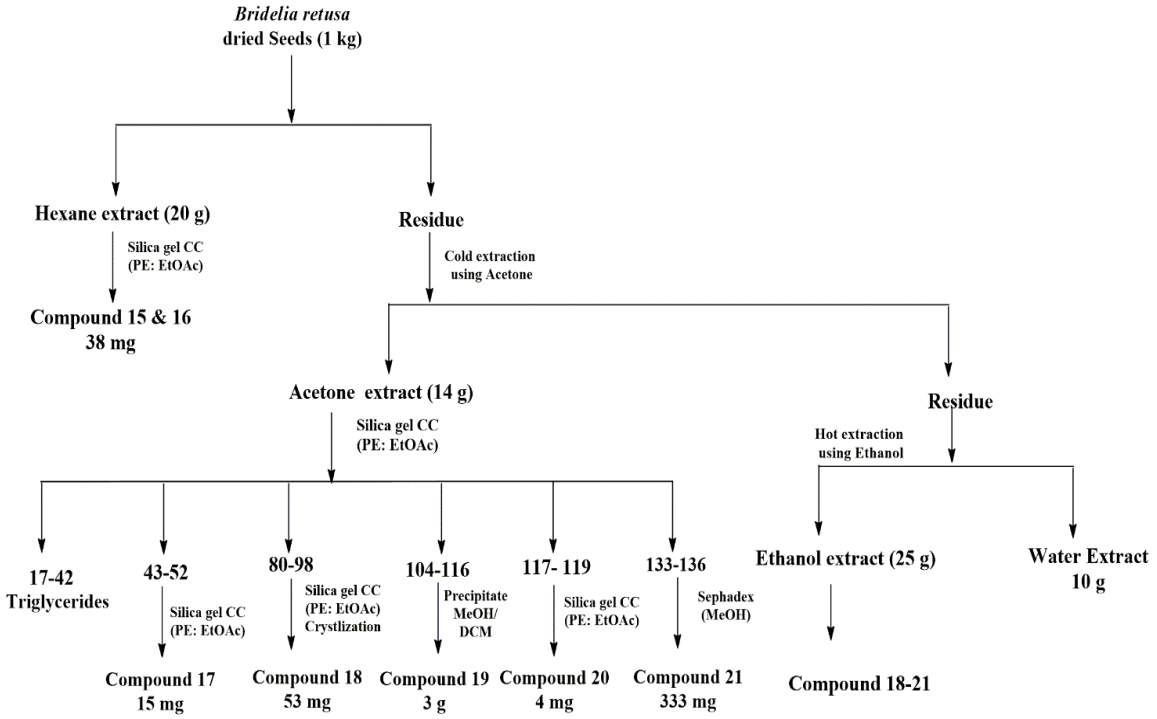


Figure 3A.3. Extraction Process

The procedure was repeated for three more days to obtain 20 g of the hexane extract. The process was repeated with acetone and yielded 12 g of the acetone extract and finally, hot extraction with ethanol was carried out in a heating mantle. The extraction yielded about 25 g of ethanol extract and water extract was also collected. The extraction process is shown in **Figure 3A.3**.

3A.5.2. Preliminary antibacterial studies of seeds of *B. retusa*

All the extracts were tested for their antibacterial activity against ESKAP pathogen panel. Minimum inhibitory concentration (MIC) was determined by performing the antibiotic susceptibility testing on the extracts in accordance with the standard CLSI guidelines.[13] Levofloxacin was used as the reference compounds. The MIC of the compounds ranges from 32-64 µg/mL. Acetone and ethanol extracted exhibited good activity against *S. aureus* with MIC value 32 µg/mL. The results are given in **Table 3A.1**.

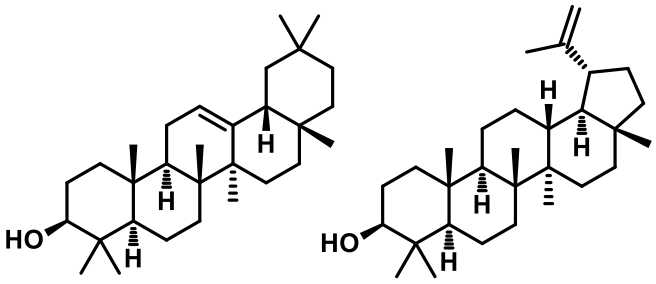
Table 3A.1. MIC values (µg/mL) of the extracts against ESKAP panel of bacteria

Extract	*E. coli* ATCC 25922	*S. aureus* ATCC 29213	*K. pneumoniae* BAA 1705	*A. baumannii* BAA 1605	*P. aeruginosa* ATCC 27853
Acetone	>64	**32**	>64	>64	>64
Ethanol	>64	**32**	>64	>64	>64
Water	>64	>64	>64	>64	>64
Levofloxacin	0.0156	0.25	64	8	1

3A.5.3. Isolation and characterization of major compounds from *Bridelia retusa* seeds

After examining the TLC, all the extracts were sequentially subjected to silica gel CC eluted with hexane-ethyl acetate mixtures and ethyl acetate-methanol mixture of increasing polarities. We could isolate a colourless solid (38 mg) compound from the hexane extract, which showed only a single spot in the TLC. The nature of the ^{1}H NMR spectrum (**Fig. 3A.4**) was that of a triterpene, but it showed around 100 protons and in ^{13}C NMR spectrum (**Fig. 3A.5**), 60 carbons were observed. The mass spectral data suggested that the compound has a molecular

formula $C_{30}H_{50}O$, as the parent peak was observed at *m/z* 427.3943 which could be the $(M+H)^+$ peak. So, it is clearly indicated that it's a mixture of two triterpenes. The ^1H NMR spectrum showed three olefinic protons, one at δ 5.16 and two olefinic protons at δ 4.69 and 4.57 ppm representing an exocyclic double bond in the system. In addition, a typical CH-O was observed at δ 3.18 ppm. In the ^{13}C NMR spectrum, the olefinic carbon appeared at δ 151.0, 145.2, 121.7 & 109.3 ppm and carbon bonded to the hydroxyl group at the C-3 position observed at δ 78.9 ppm. On a detailed analysis of the spectrum, we found that the compound is a mixture of **Lupeol** and ***β*- Amyrin** designated as compound **15** and **16**. The proton and carbons were assigned based on COSY, HMQC and HMBC analysis and was in good agreement with the literature reports.[14] The peaks at δ 5.16, 145.2 and 121.7 ppm were characteristic peaks for β-amyrin and δ 4.69, 4.57, 151.0 and 109.3 ppm were that of lupeol.[14] These compounds are reported for the first time from this species.



Compound **15- *β*- Amyrin** Compound **16- Lupeol**

13 g of the acetone extract was subjected to column chromatographic purification using silica gel (100-200 mesh). Column elution started with hexane and gradually polarity was increased by increasing the amount of ethyl acetate in hexane-ethyl acetate mixture. Final elution was carried out using 50 % ethyl acetate-methanol mixture. A total of 136 fractions of approximately 150 ml each were collected. According to the similarity in TLC, fractions were pooled into nine fraction pools.

Fraction pool 3 (Fr. 43-52) obtained by eluting the column with 30 % ethyl acetate-hexane showed the presence of a UV active compound. The fraction on CC in 230-400 silica gel afforded a white solid with some minor impurities. It was further purified by a pencil column yielded compound **17** (15 mg). The UV spectrum showed absorption maxima at 236 and 286 nm, which corresponded to a furofuran

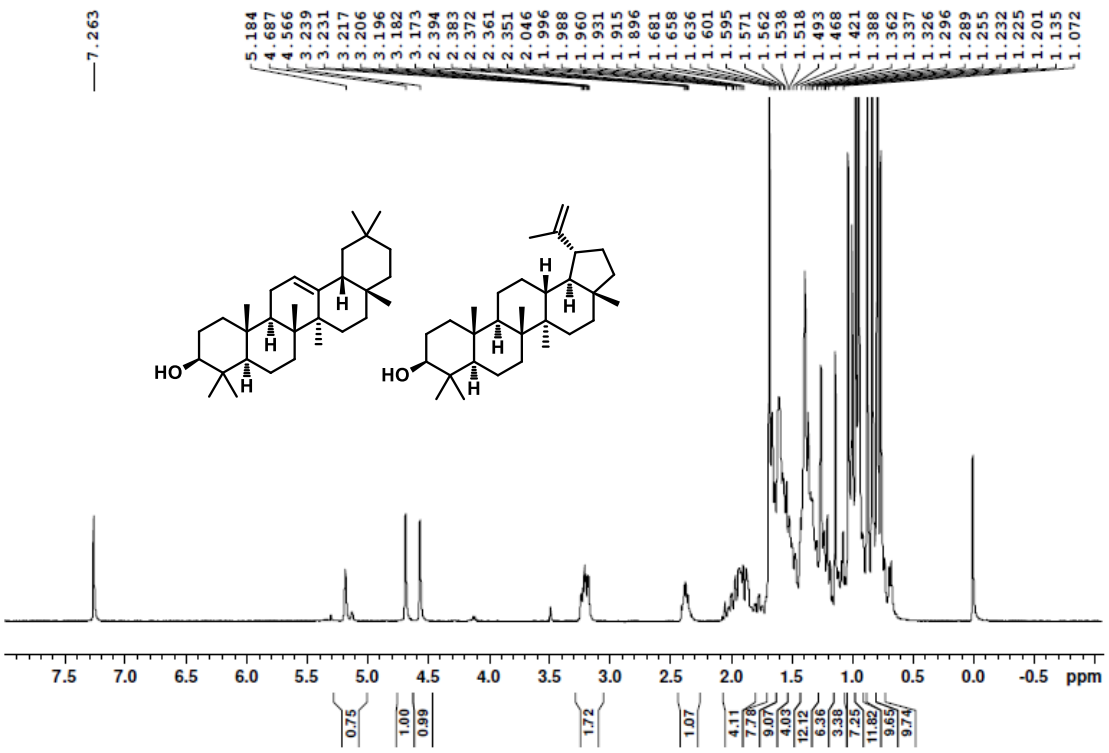


Figure 3A.4. ^{1}H NMR spectrum of compound **15 & 16** in $CDCl_3$

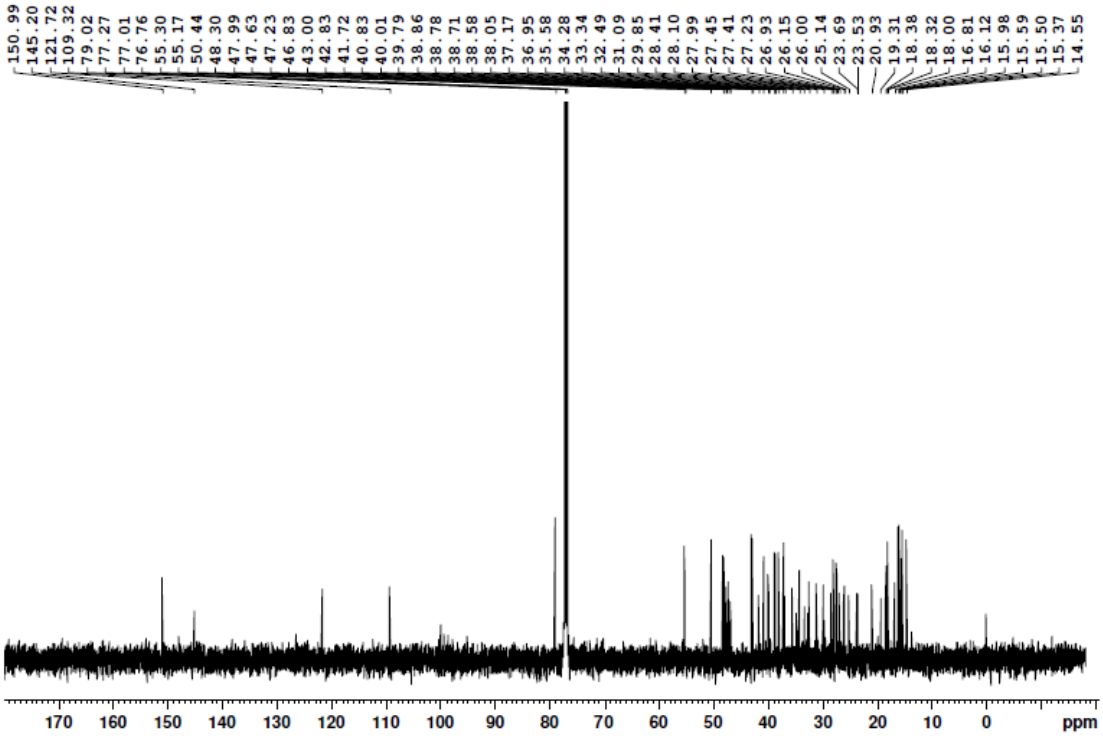
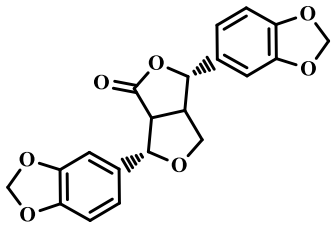


Figure 3A.5. ^{13}CNMR spectrum of compound **15 & 16** in $CDCl_3$

type of lignan. The HRESIMS analysis showed a parent molecular ion peak at *m/z* 391.0793 $(M+Na)^+$. So, the molecular formula of the compound assigned as $C_{20}H_{16}O_7$ which was supported by the spectral data. The ^1H NMR spectrum (**Fig. 3A.6**) represents two methylenedioxy groups [δ 5.98 (s, 2H), 5.96 (s, 2H)], two sets of ABX systems due to the 1,3,4-trisubstituted aromatic ring system [δ 6.87-6.75 (m, 6H)], two benzylic oxymethine groups [δ 5.29 (d, J = 4.0 Hz), 5.31 (d, J = 4.0 Hz)], and one oxymethylene group [δ 4.33 (dd, J = 9.5, 7.0 Hz), 4.01 (dd, J = 10.0, 4.5 Hz)]. The ^{13}C NMR spectrum (**Fig. 3A.7**) gave 20 carbon signals, including one carbonyl carbon (δ 176.6), two methylenedioxy carbons (δ 101.5 & 101.2), four methine (δ 84.4, 83.4, 53.3, & 50.0), one methylene (δ 72.7) and twelve aromatic carbons corresponding to two benzene rings. The spectral details were similar to that of (+)-sesamine previously reported from *Bridelia retusa*; the difference is there was an additional carbonyl group in the C-4 position, supported by the HMBC and HMQC correlations. Hence compound **17** was identified as **4-Oxosesamine** and is reported for the first time from this species.[15]



Compound **17- 4-Oxosesamine**

A dark yellow solid was obtained from fraction pool 5 (Fr. 80-98); it was again washed with acetone and purified. A colourless solid was obtained, labelled as compound **18** (53 mg). The ^1H NMR spectrum (**Fig. 3A.8**) displayed a sharp singlet at δ 7.02 (s, 2H) and a broad singlet at δ 8.53 (s, 2H) ppm, respectively. The ^{13}C spectrum (**Fig. 3A.9**) showed five distinct aromatic carbons in which a carbonyl carbon appeared at δ 172.9 ppm. After a detailed analysis, the compound was characterized as **Gallic acid**, which was well supported with a molecular ion peak at *m/z* 171.0292, the $(M+H)^+$ peak. [16] Gallic acid is a naturally occurring polyphenolic compound found in several plants. It is highly oxidative and plays a protective role in disease caused by oxidative stress, such as cancer, cardiovascular, degenerative and metabolic diseases.[17] Other pharmacological activities include neuroprotective, anti-inflammatory, wound healing, hepatoprotective, antidepressant, antiviral, *etc*.[18]

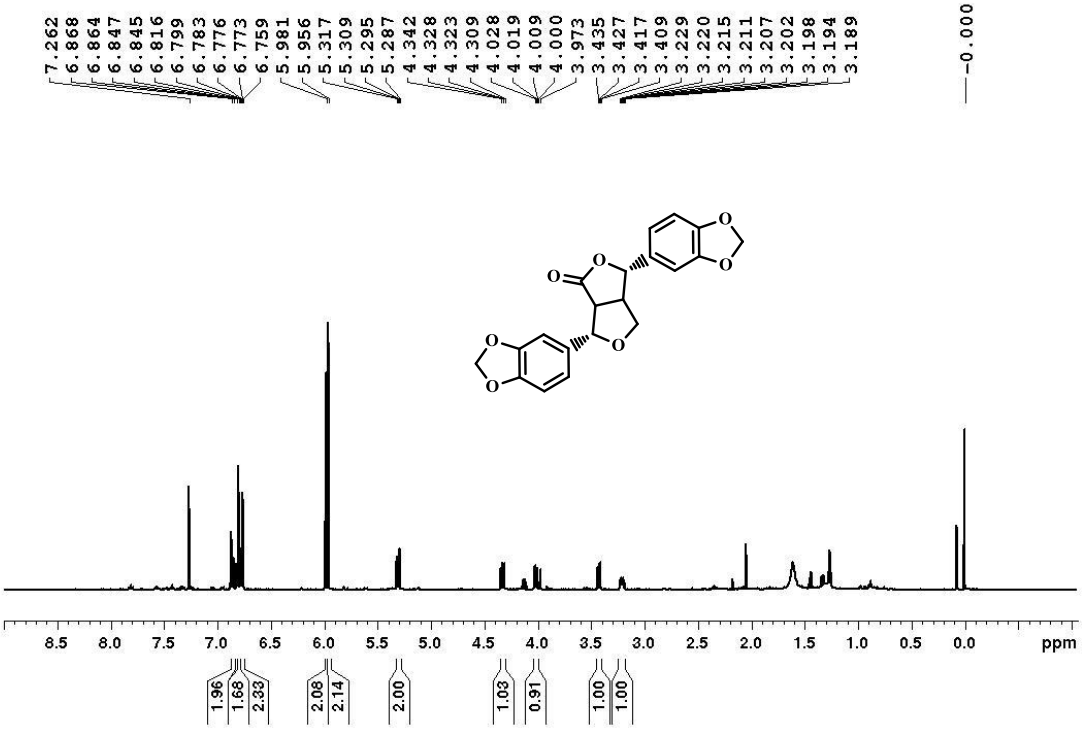


Figure 3A.6. ^{1}H NMR spectrum of compound **17** in $CDCl_3$

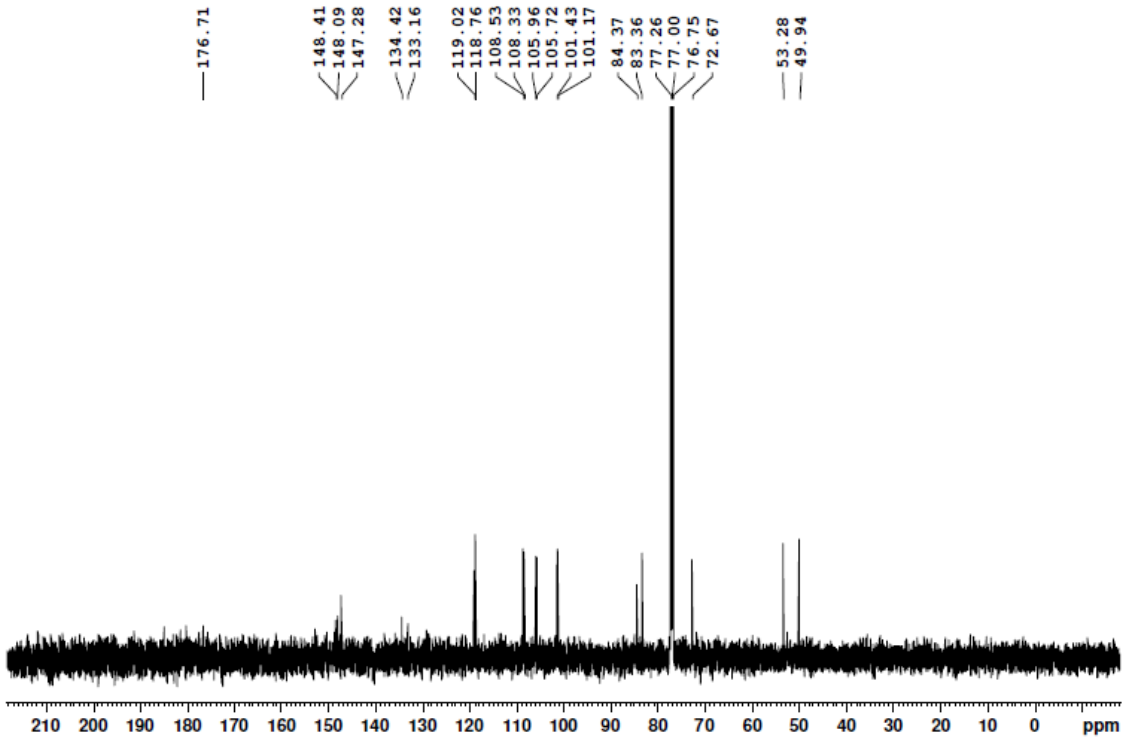


Figure 3A.7. ^{13}C NMR spectrum of compound **17** in $CDCl_3$

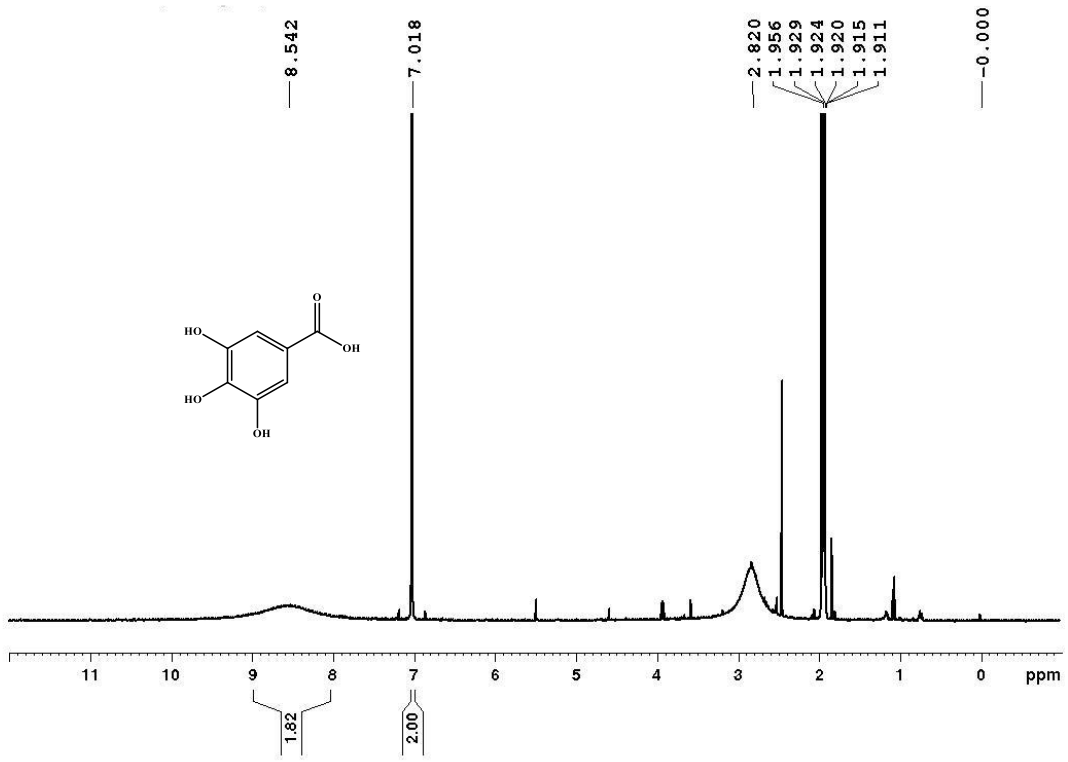


Figure 3A.8.^{1}H NMR spectrum compound **18** in Acetone-d6

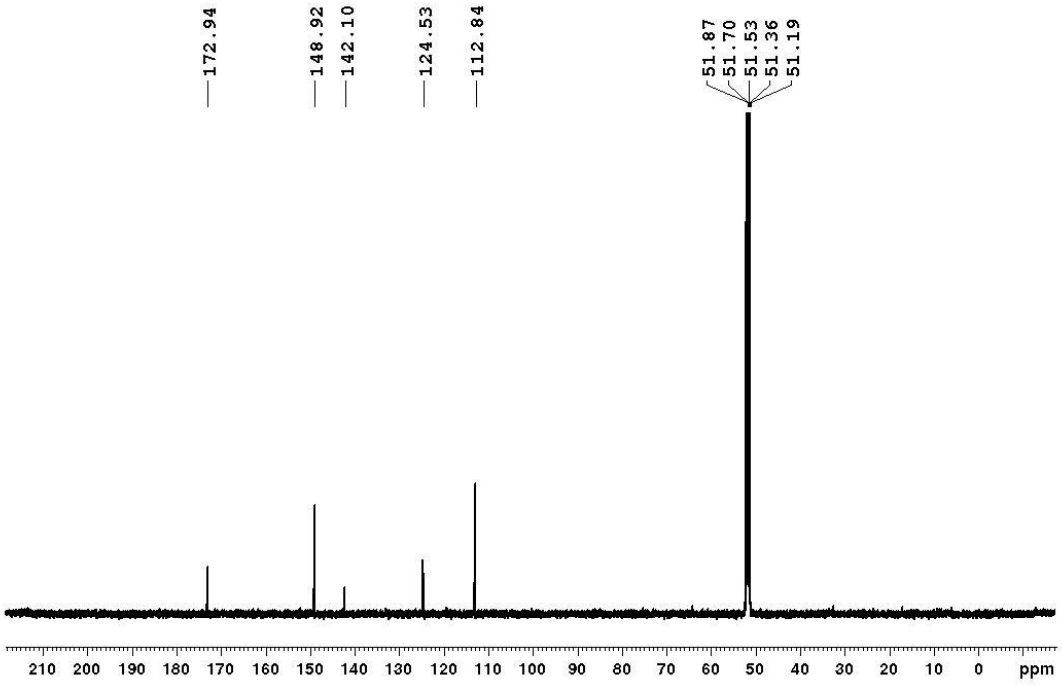
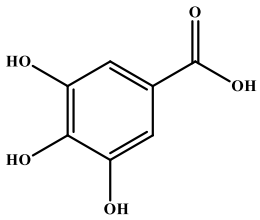
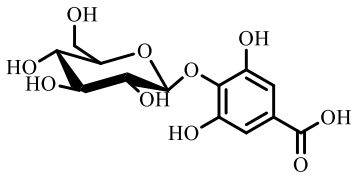


Figure 3A.9.^{13}C NMR spectrum of compound **18** in CD_3OD



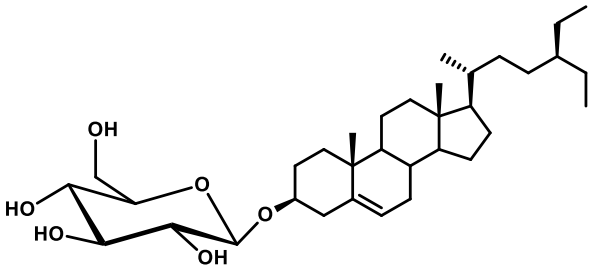
Compound **18- Gallic acid**

Fraction pool 7 (Fr. 104-106) obtained from eluting the column with 90 % ethyl acetate-hexane mixture was dissolved in 25 ml methanol and precipitated with DCM. The precipitated compound was again washed with ethyl acetate, compound **19** obtained as a pure yellow solid (3 g). The ^{1}H NMR spectrum (**Fig. 3A.10**) was similar to that of the previous compound **18**. A singlet peak was observed at δ 7.07 (s, 2H) with an additional glucoside moiety and gave a molecular ion peak at *m/z* 355.0644 $(M+Na)^{+}$. The presence of a doublet at δ 4.69 ppm with a coupling constant of 8.0 Hz confirmed that the system consists of a β-anomeric linkage. Finally, comparing the spectroscopic data of the compound with literature reports, compound **19** was identified as **Gallic-4-*O*-*β*-*D*-glucopyranoside**.[19]



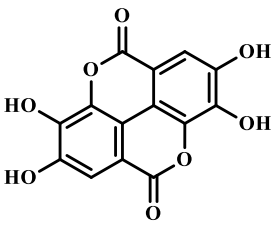
Compound **19- Gallic-4-*O*-*β*-*D*-glucopyranoside**

Fraction pool 8 (Fr. 117-119) afforded a solid, which was purified by washing with acetone and methanol. A colourless solid was isolated and designated as compound **20** (4 mg). This was characterized as the ***β*-sitosterol-*β*-*D*- glucoside**, using various spectroscopic data and on comparison with literature reports. The structure of the compound is as shown below.



Compound **20-*β*- sitosterol- *β*- *D*- glucoside**

Fraction pool 10 (Fr. 133-136) when subjected to column chromatography on Sephadex LH-20 using methanol as the solvent, yielded a solid with some minor impurities. The solid was washed with acetone and purified. Compound **21** was isolated as a yellow solid (333 mg), which melted sharply at 358° C. The HRESIMS analysis showed a parent molecular ion peak at *m/z* 303.0141 $(M+H)^+$. These results, along with ^1H and ^{13}C NMR data suggested a molecular formula of $C_{14}H_6O_8$. The ^1H NMR spectrum (**Fig. 3A.12**) showed a sharp singlet at δ 7.46 (s, 2H) and a broad singlet at δ 10.67 (s, 4H) ppm. The ^{13}C NMR spectrum (**Fig. 3A.13**) displayed fourteen distinct carbons which consisted of two overlapped ester (-COOR) carbons at δ 159.1 due to unsaturated lactones, six oxygenated aromatic carbons (δ 148.1, 139.6 & 136.3), four aromatic quaternary carbons (δ 112.3 & 110.2) and two isolated aromatic methane carbons at δ 107.6 respectively. A detailed analysis using COSY, HMBC and HMQC suggested the compound as **Ellagic acid**, and the results are in good agreement with literature reports.[20] Ellagic acid is a polyphenol found in fruits and vegetables such as raspberries, grapes, strawberries, pomegranate, camu-camu, mango, black currants, guava, almonds, walnuts, longan seeds and green tea. It was reported to possess numerous activities *viz*., antibacterial, antiviral, antimalarial, antiallergic, neuroprotective, anti-inflammatory, antiatherogenic antidiabetic, nephroprotective, antidepressant, antiepileptic, antianxiety, cardioprotective and hepatoprotective activities.[21–23]



Compound **21- Ellagic acid**

The residue obtained after the acetone extraction was subjected to hot extraction with ethanol to yield about 25 g of crude extract. The extract contained some solid precipitate which was removed by filtration. The solid was washed with acetone for further purification and submitted to NMR spectral studies. The spectra were similar to that of the compound **21** and characterized as **Ellagic acid** (2 g).

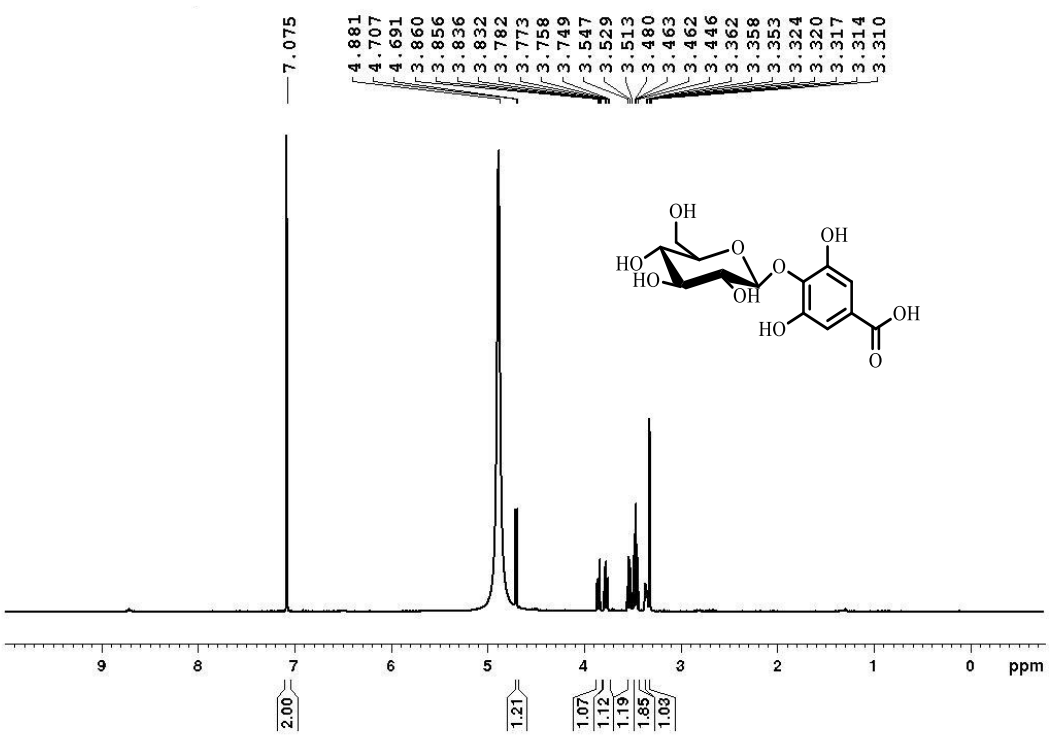


Figure 3A.10. ^{1}H NMR spectrum of compound **19** in CD_3OD

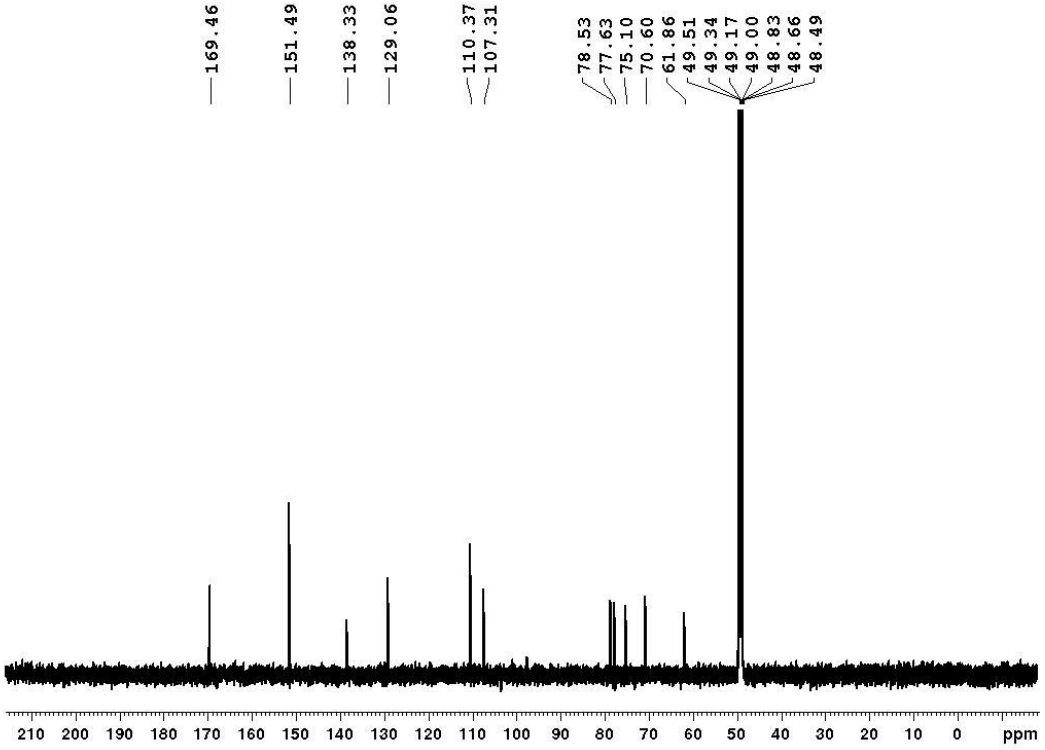


Figure 3A.11. ^{13}C NMR spectrum of compound **19** in CD_3OD

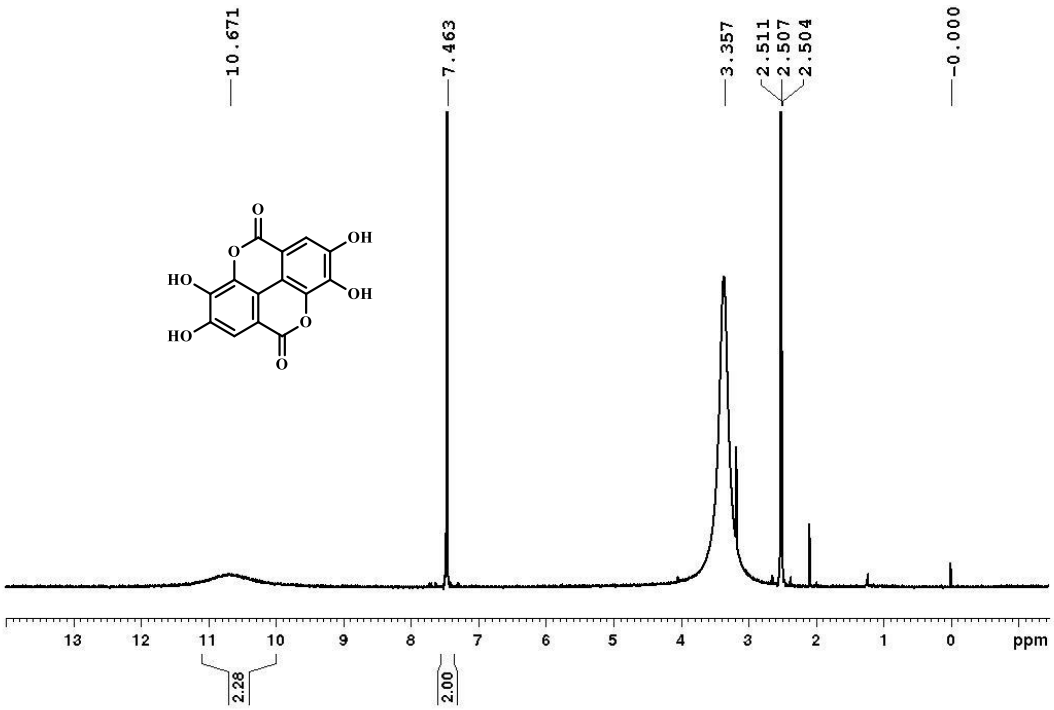


Figure 3A.12.[1]H NMR spectrum of compound **21** in DMSO-d6

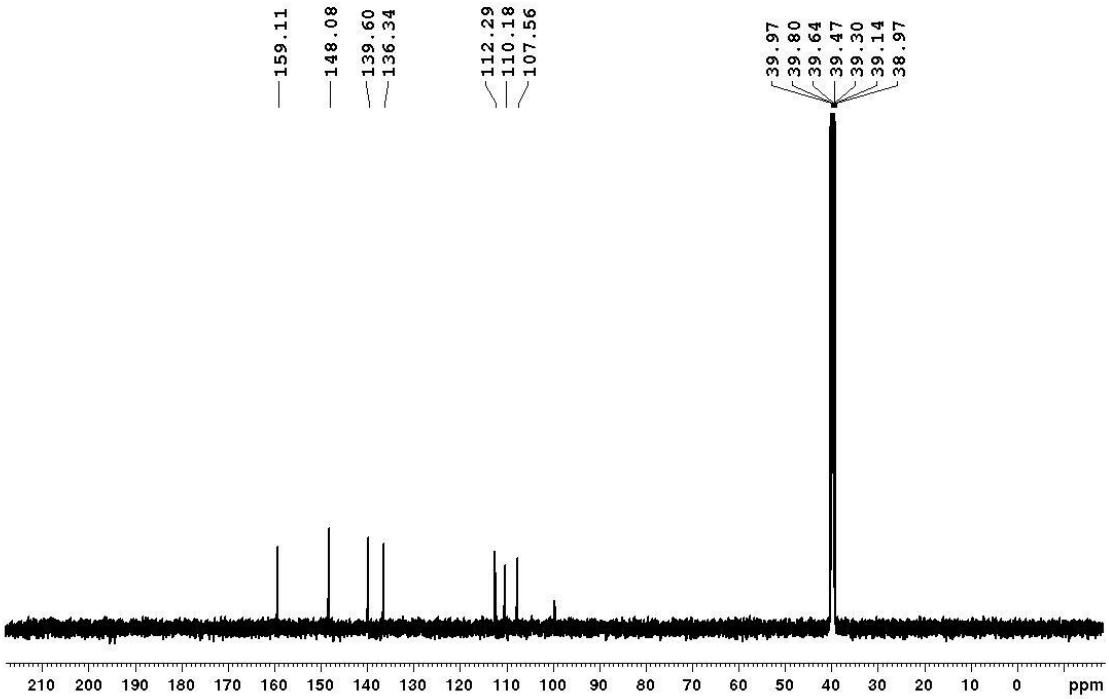


Figure 3A.13.[13]C NMR spectrum of compound **21** in DMSO-d6

The filtrate was subjected to CC on silica gel (100-200 mesh). The elution started with hexane and continued with hexane- ethyl acetate mixture of increasing polarity and the column completed by eluting with 50 % methanol-ethyl acetate mixture. The fractions were collected in 500 ml conical flasks and a total of 41 fractions were collected. According to the similarity in TLC of the fractions collected, they were pooled into seven fraction pools. From fraction pool 3 (fr. 15-20), we could isolate a colourless solid, which was submitted to IR, NMR and mass spectral analysis. The structure of the compound was confirmed as ***β*–sitosterol glucoside** (40 mg).

Fraction pool 4 (Fr. 21-23) was precipitated with ethyl acetate, which yielded a crystalline yellow solid. After spectroscopic analysis, the compound was confirmed as **Gallic acid** (270 mg). Fraction pool 6 (Fr.30-31) precipitated with DCM and methanol, the precipitate filtered. About 60 mg of a yellow solid was obtained and was submitted for spectroscopic analysis. The spectra were similar to that of the compound **19** and confirmed as **glucoside of Gallic acid**. A pictorial representation of the isolation is given in the chart (**Fig. 3A.3**).

3A.6. Extraction, isolation and characterization of compounds from the stem bark of *Bridelia retusa*

The dried stem bark (500 g) of *Bridelia retusa* was crushed, powdered and extracted with acetone (5L* 3 days) followed by ethanol and water. The solvents were evaporated in vacuum at the rotary evaporator to get the crude extracts. The amount of extracts obtained were 35 g, 20 g and 5 g for acetone, ethanol and water, respectively.

3A.6.1. Preliminary antibacterial studies of stembark of *B. retusa*

All the extracts were tested for their antibacterial activity against the ESKAP pathogen panel. Minimum inhibitory concentration (MIC) was determined by performing the antibiotic susceptibility testing on the extracts in accordance with the standard CLSI guidelines. Levofloxacin was used as the reference compound. The MIC of the compounds ranges from 16-64 µg/mL. Acetone and water extract exhibited significant activity against *S. aureus* with MIC value 16 µg/mL The results are given in **Table 3A.2**.

Table 3A.2. MIC values (μg/mL) of the extracts against ESKAP panel of bacteria

Extract	*E. coli* ATCC 25922	*S. aureus* ATCC 29213	*K. pneumoniae* BAA 1705	*A. baumannii* BAA 1605	*P. aeruginosa* ATCC 27853
Acetone	>64	16	>64	>64	>64
Ethanol	>64	64	>64	>64	>64
Water	>64	16	>64	>64	>64
Levofloxacin	0.0156	0.25	64	8	1

3A.6.2. Isolation and characterization of compounds from the stem bark of *Bridelia retusa*

About 35 g of acetone extract was collected. When we checked the TLC of the acetone extract, no clear spots were observed. Even though we have subjected about 10 g of the extract to usual silica gel CC using hexane, ethyl acetate and methanol. We have collected about 114 fractions; each collected about 150 ml in conical flask. But we could isolate only some sterols as pure compounds. The isolated compounds were a mixture of ***β*–sitosterol and stigmasterol** (**22** & **23**-15 mg)**, friedelin (24)**, and the **glucoside of *β*–sitosterol** (7 mg). Other fractions on purification did not afford pure compounds; the spectra obtained were full of impurity peaks.

Compound **24** was isolated from fractions 8-40 as colourless crystalline solid (8 mg). The FTIR spectrum of the compound showed an intense band at 1745 cm^{-1}, which is consistent with a six-membered ring ketone. The comparison of the ^{1}H NMR spectral (**Fig. 3A.14**) features with existing literature reports suggested that the compound was a pentacyclic triterpene. The ^{13}C NMR spectrum (**Fig. 3A.15**) revealed the presence of 30 carbon atoms with a carbonyl carbon at δ 213.3 ppm. Based on COSY, HMBC and HMQC experiments, the structure was assigned as triterpene typical with a friedelane skeleton. The mass spectrum showed a molecular ion peak at *m/z* 427.3934, which could be the $(M+H)^{+}$ peak. Hence the compound was characterized as **Friedelin** and the spectral data were consistent with the literature reports.[24] Friedelin exhibits antimycobacterial, antinociceptive, anti-inflammatory, analgesic and antipyretic activities.[25–27]

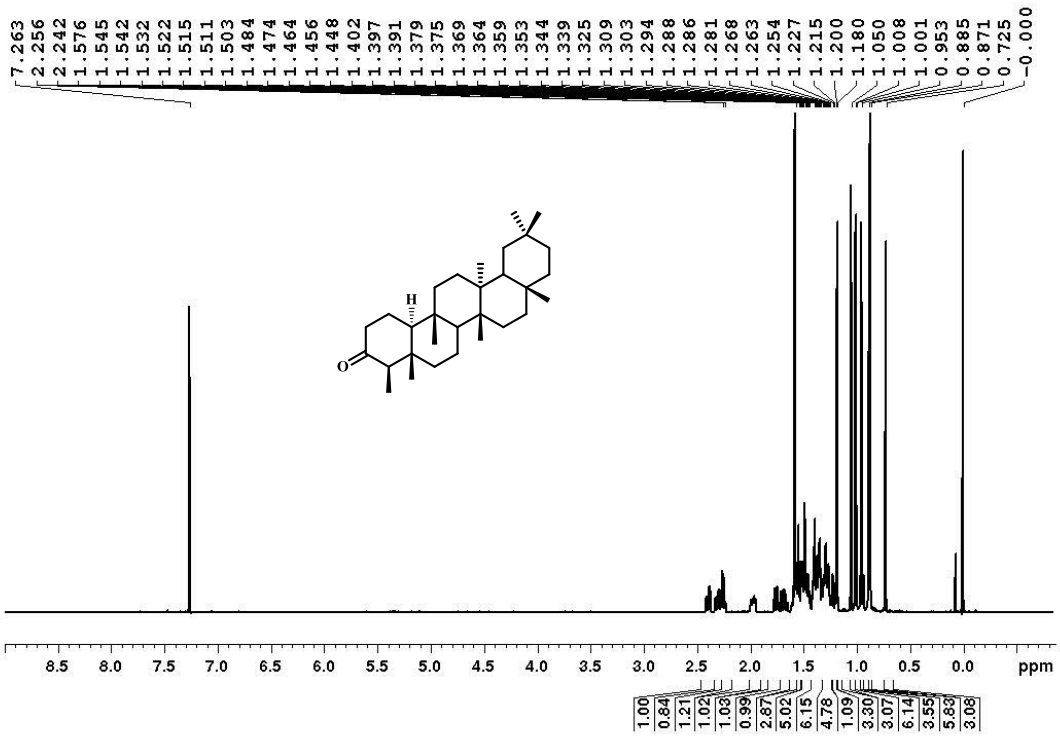


Figure 3A.14. ^{1}H NMR spectrum of compound **24** in $CDCl_3$

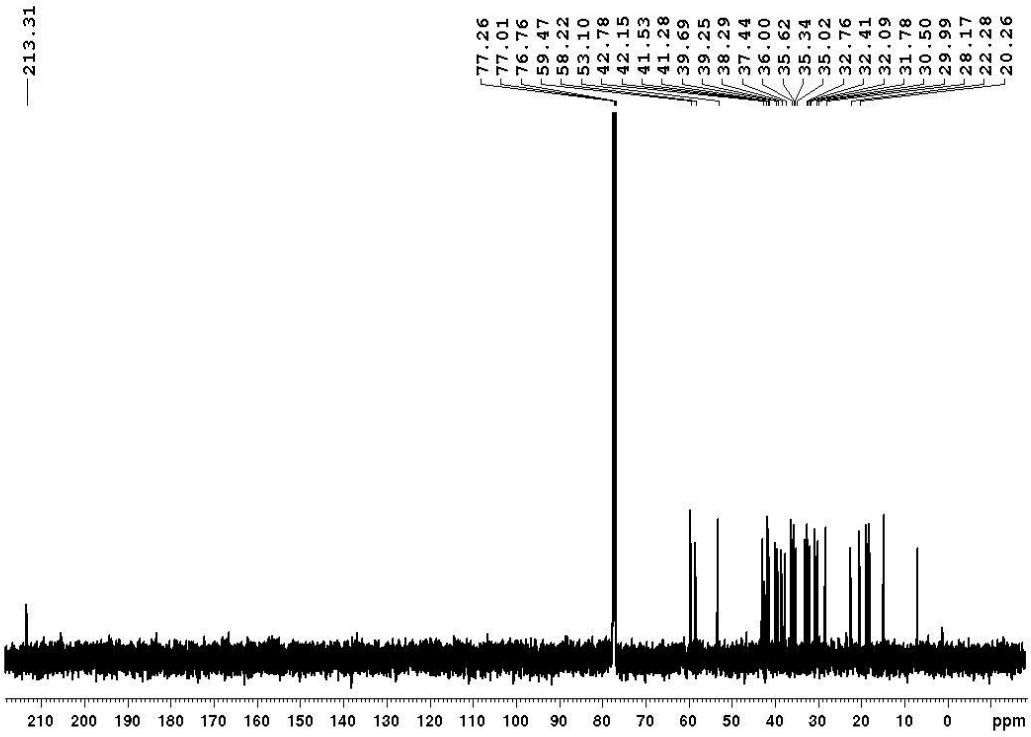


Figure 3A.15. ^{13}C NMR spectrum of compound **24** in $CDCl_3$

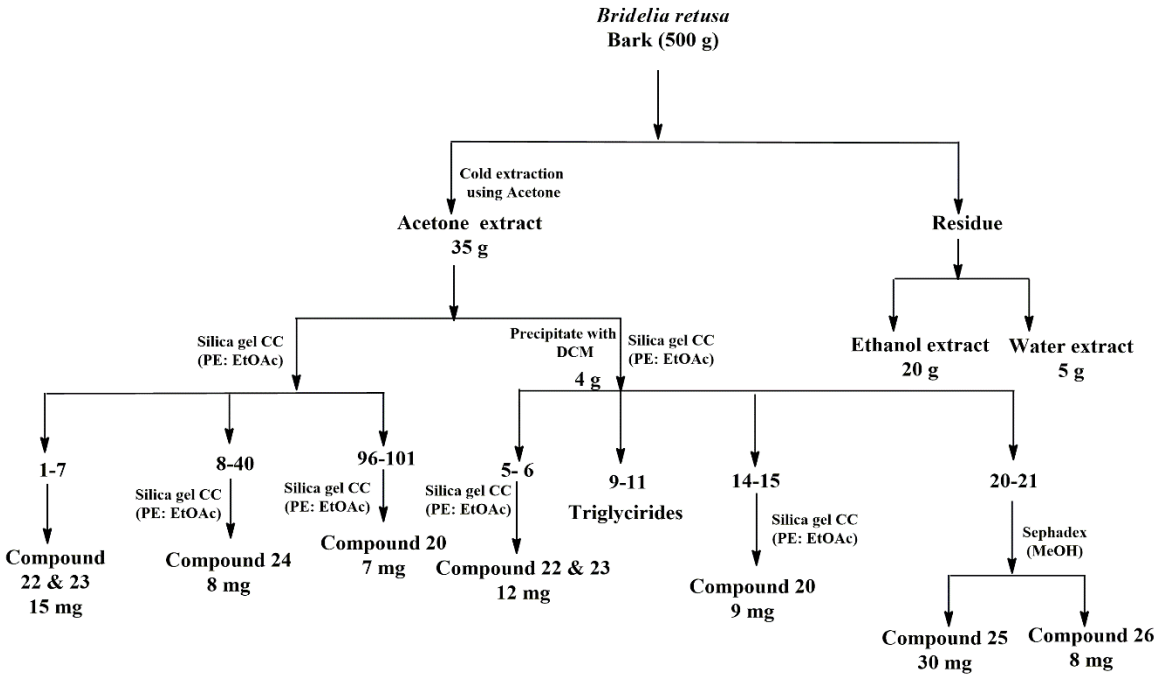
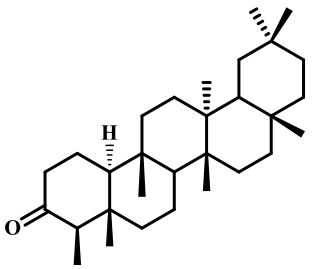


Figure 3A.16. Extraction Process

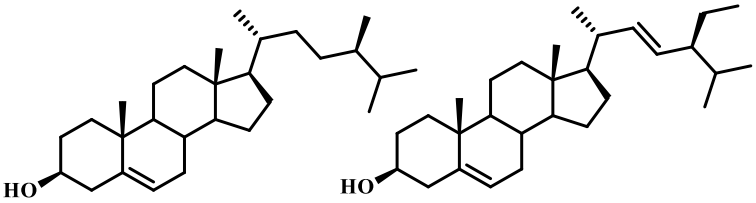


Compound **24- Friedelin**

The plant *Bridelia retusa* contains lots of tannins (polyphenols), making it difficult to separate and identify the compounds. Hence, we have precipitated the remaining acetone extract (25 g) with dichloromethane to remove the polyphenols. The DCM fraction was spotted in TLC and checked with UV. The spots were visible than before. The DCM was evaporated in the rotatory evaporator, affording 4 g of the extract and subjected to further purification by silica gel CC (100-200 mesh), as mentioned before. A total of 25 fractions of 150 ml each were collected. A pictorial representation of the extraction and isolation is given in the chart (**Fig. 3A.16**).

After examining the TLC, it was pooled into nine fraction pools. Fraction pools 2 (fr.5-6) and 6 (Fr. 14-15) afforded the common sterols, a mixture of **stigmasterol (22)** and ***β*–sitosterol (23)** and the **glucoside of *β*–sitosterol (20)**. Compounds

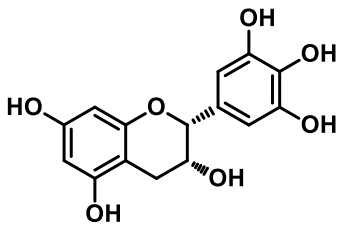
were characterized based on the spectral data obtained and in comparison, with literature reports. The structures of the compounds are shown below.



Compound **22**-***β*–sitosterol**

Compound **23**-**stigmasterol**

Two compounds were isolated from fraction pool 8 (Fr. 20-21) using silica gel CC. The compounds contain some minor impurities, hence purified by Sephadex-LH 20 CC using methanol as the solvent. The compound **25** was obtained as a UV active yellow-coloured solid (30 mg). The molecular formula of the compound was established as $C_{15}H_{14}O_7$ based on the spectral data and HRESIMS analysis in which the parent peak was observed at *m/z* 329.0637 $(M+Na)^+$. The ^1H NMR spectrum (**Fig. 3A.17**) showed the characteristic signals due to the C-ring protons of a flavan-3-ol at δ 4.54 (d, J = 7.0 Hz, H-2), 3.98 (dd, J = 12.5, 7.5 Hz, 1H, H-3), 2.83 (dd, J = 16.0, 5.0 Hz, 1H, H-4a), and 2.52 (dd, J = 16.0, 8.0 Hz, 1H, H-4b) and the meta-coupled aromatic proton signals at δ 5.94 (d, J = 2.5 Hz, 1H, H-6), 5.88 (d, J = 2.5 Hz, 1H, H-8), revealed the presence of a flavan-3-ol skeleton. A two-proton singlet was observed at δ 6.42 ppm which corresponded to the trisubstituted B-ring. The ^{13}C NMR spectrum (**Fig. 3A.18**) exhibited signals for 15 carbons in which the methylene carbon was found at δ 27.9 ppm. The ^1H and ^{13}C NMR data of the compound were consistent with that of (-)- epigallocatechin reported in the literature. Hence compound **25** was identified as **(-)-epigallocatechin**.[28] It is one of the main component in green tea and exhibits anti-inflammatory, antioxidant and cytotoxic activity.[29,30]



Compound **25- (-)-epigallocatechin**

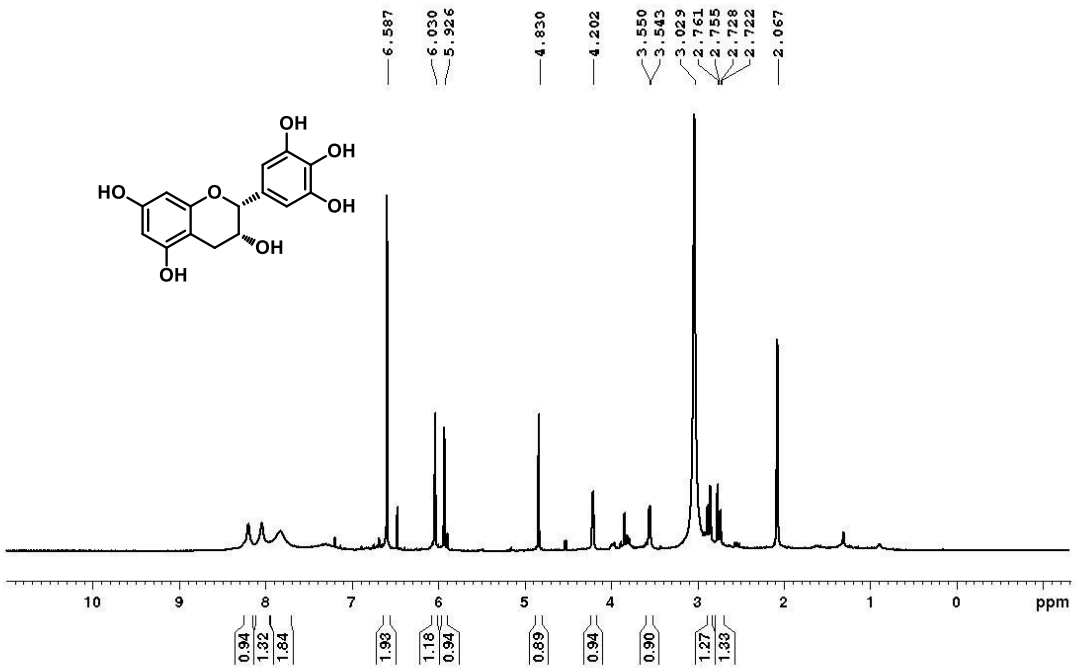


Figure 3A.17. ^{1}H NMR spectrum of compound **25** in Acetone-d6

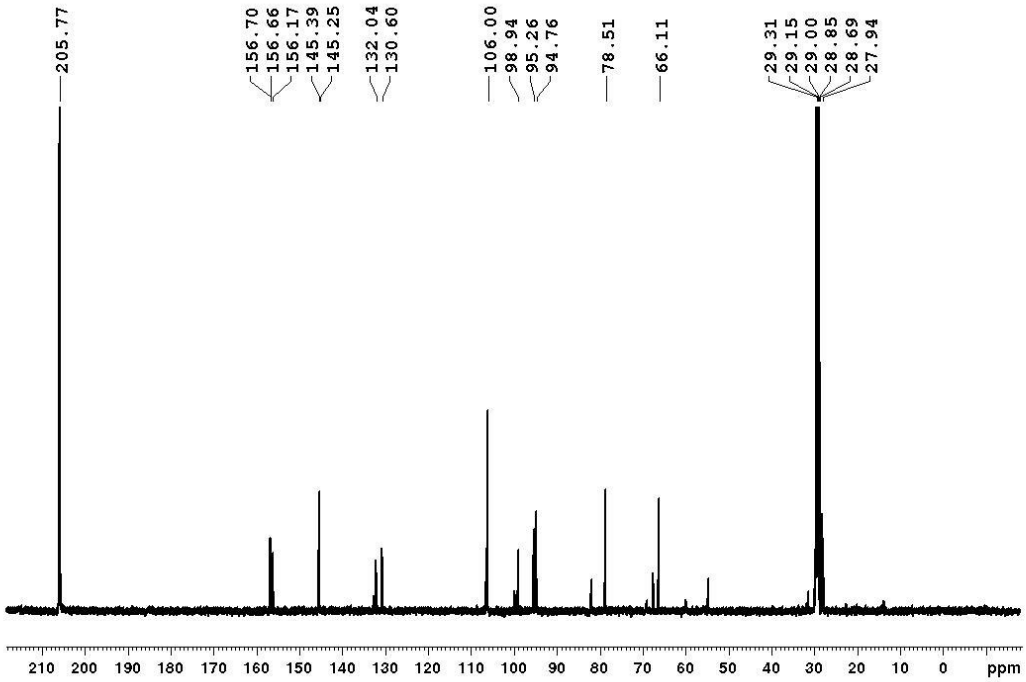
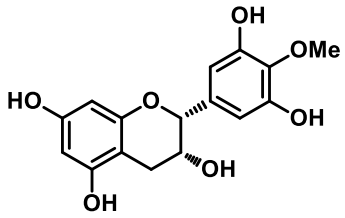


Figure 3A.18. ^{13}C NMR spectrum of compound **25** in Acetone-d6

Compound **26** was obtained as a UV active yellow-coloured solid (8 mg). The mass spectral analysis of the compound showed a molecular ion peak at *m/z* 343.0793 $(M+Na)^+$ which corresponded to a compound with molecular formula $C_{16}H_{16}O_7$. The ^1H and ^{13}C NMR spectra were similar to that of the previous compound. Also, the appearance of a sharp singlet at δ 3.79 indicated the presence of a methoxy group and the corresponding carbon was detected at δ 60.8 ppm in the ^{13}C NMR spectrum (**Fig. 3A.20**). The HMBC spectrum established the correlation between the protons of methoxy and the C-4' confirming the structure as **4'-*O*-methyl-(-)-epigallocatechin**. For the best of our knowledge, the compound is reported for the first time from this species.[31] 4'-*O*-methyl-(-)-epigallocatechin was previously reported from *Cassine transvaalensis* and possessess antioxidant property.[32]



Compound **26- 4'-*O*-methyl-(-)-epigallocatechin**

3A.7. Extraction, isolation and characterization of compounds from *Bridelia retusa* leaves

750 g of the dried leaves of *Bridelia retusa* was extracted with hexane (5L×3) at room temperature. The total extract was then concentrated under reduced pressure to yield approximately 20 g of the crude extract. The process was repeated with acetone and ethanol, which on solvent removal yielded 47 g and 37 g of crude extract, respectively.

3A.7.1. Preliminary antibacterial studies

All the extracts were assessed for their antibacterial activity against ESKAP pathogen panel. Minimum inhibitory concentration (MIC) was determined by performing the antibiotic susceptibility testing on the extracts in accordance with the standard CLSI guidelines. Levofloxacin was used as the reference compound. The MIC of the extracts ranges > 64 µg/mL. The results are given in **Table 3A.3**.

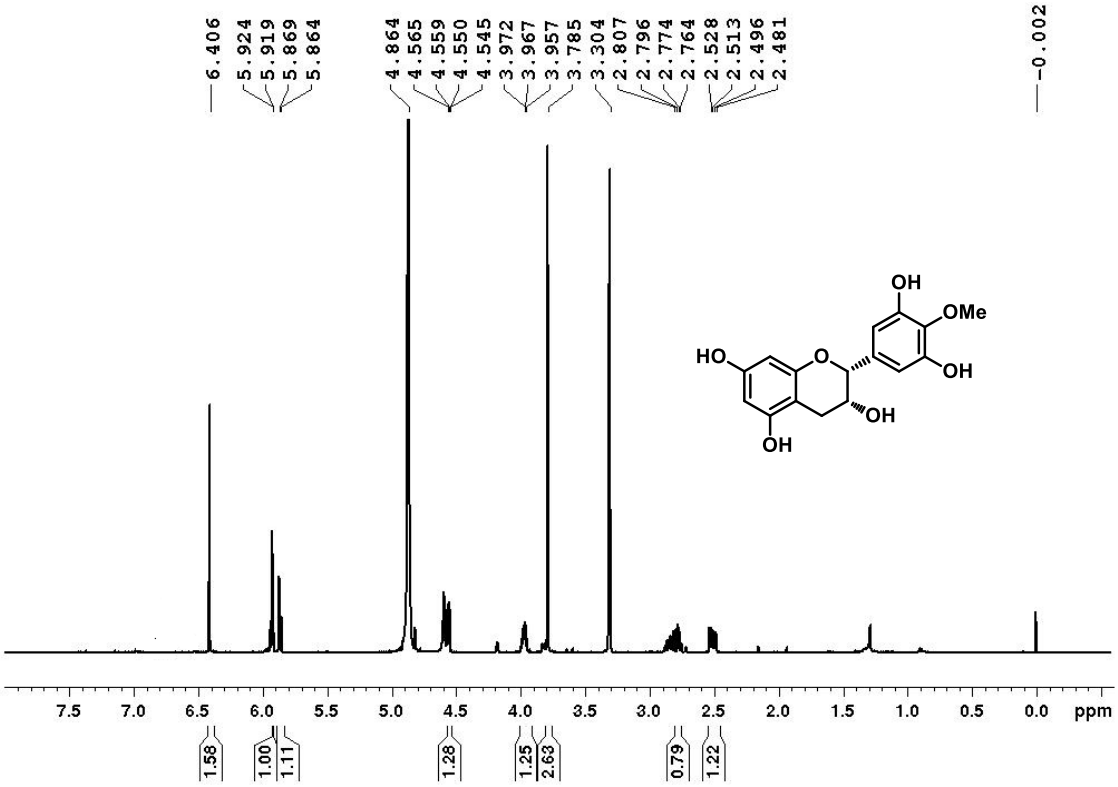


Figure 3A.19. ^{1}H NMR spectrum of compound **26** in CD_3OD

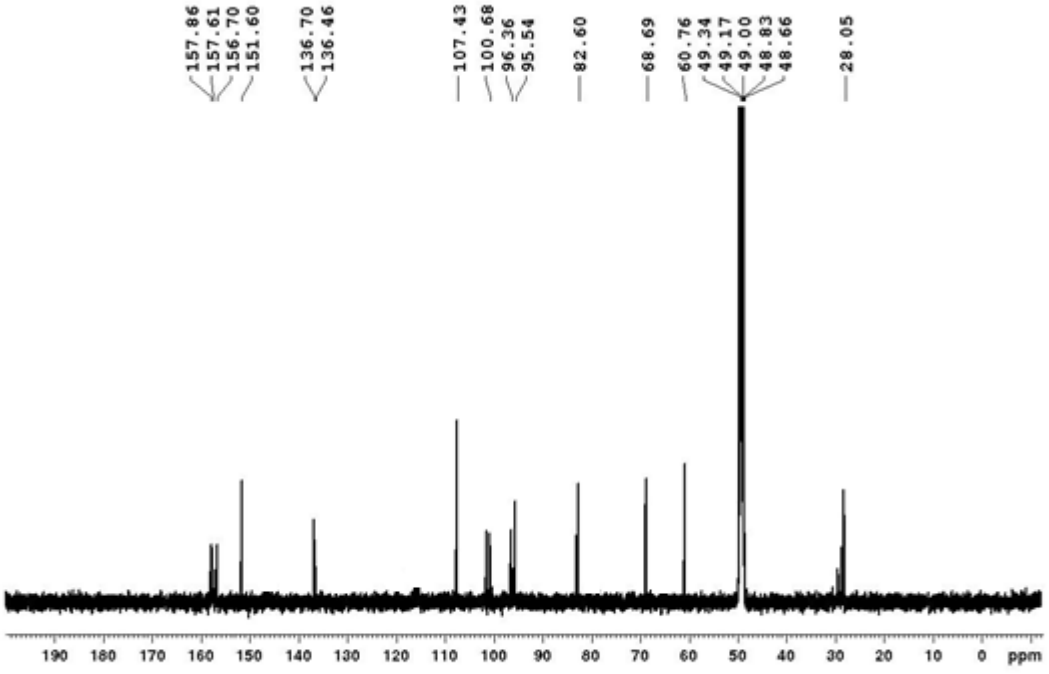


Figure 3A.20. ^{13}C NMR spectrum of compound **26** in CD_3OD

Table 3A.3. MIC values (μg/mL) of the extracts against ESKAP panel of bacteria

Extract	*E. coli* ATCC 25922	*S. aureus* ATCC 29213	*K. pneumoniae* BAA 1705	*A. baumannii* BAA 1605	*P. aeruginosa* ATCC 27853
Hexane	>64	>64	>64	>64	>64
EA	>64	>64	>64	>64	>64
Ethanol	>64	>64	>64	>64	>64
Levofloxacin	0.0156	0.25	64	8	1

3A.7.2. Isolation and characterization of compounds from *Bridelia retusa* leaves

After examining the TLC patterns of the three crude extracts, we selected ethanol extract for further isolation processes. 33 g of the ethanol extract was subjected to column chromatographic purification to isolate compounds using silica gel (100-200 mesh). Column elution was started with hexane and a various mixture of hexane-ethyl acetate and ethyl acetate in increasing order of polarity. Finally, the column was eluted with methanol. A total of 112 fractions of approximately 250 ml each were collected. According to the similarity in TLC, the fractions were pooled into six fraction pools. Compound **20** was isolated from fraction pool 2(fr. 57-65) as a white solid (35 mg). This was characterized as the ***β*-sitosterol-3-*O*-*β*-D-glucoside** using various spectroscopic techniques and on comparison with literature reports.

Fraction pool 4 (Fr. 73-88) obtained by eluting the column with ethyl acetate yielded 15 mg of yellow amorphous solid as compound **27**. The mass spectral analysis gave a molecular ion peak at *m/z* 487.0846 $(M+Na)^+$, which may corresponded to the molecular formula $C_{21}H_{20}O_{12}$. Its UV absorption at 256 and 359 nm was consistent with the presence of 3,5,7,3',4'-pentahydroxyflavone structure. The IR spectra showed an absorption at 1655 $cm^{-1,}$ corresponded to the carbonyl group. In the ^1H NMR spectrum (**Fig. 3A.22**) the aromatic region exhibited ABX system at δ 7.74 (d, J = 2.5 Hz, 1H, H-2'), 7.59 (dd, J = 8.5, 2.5 Hz, 1H, H-6') and 6.86 (d, J = 8.5 Hz, 1H, H-5') due to a 3',4'-disubstitution of ring B and *meta*-coupled

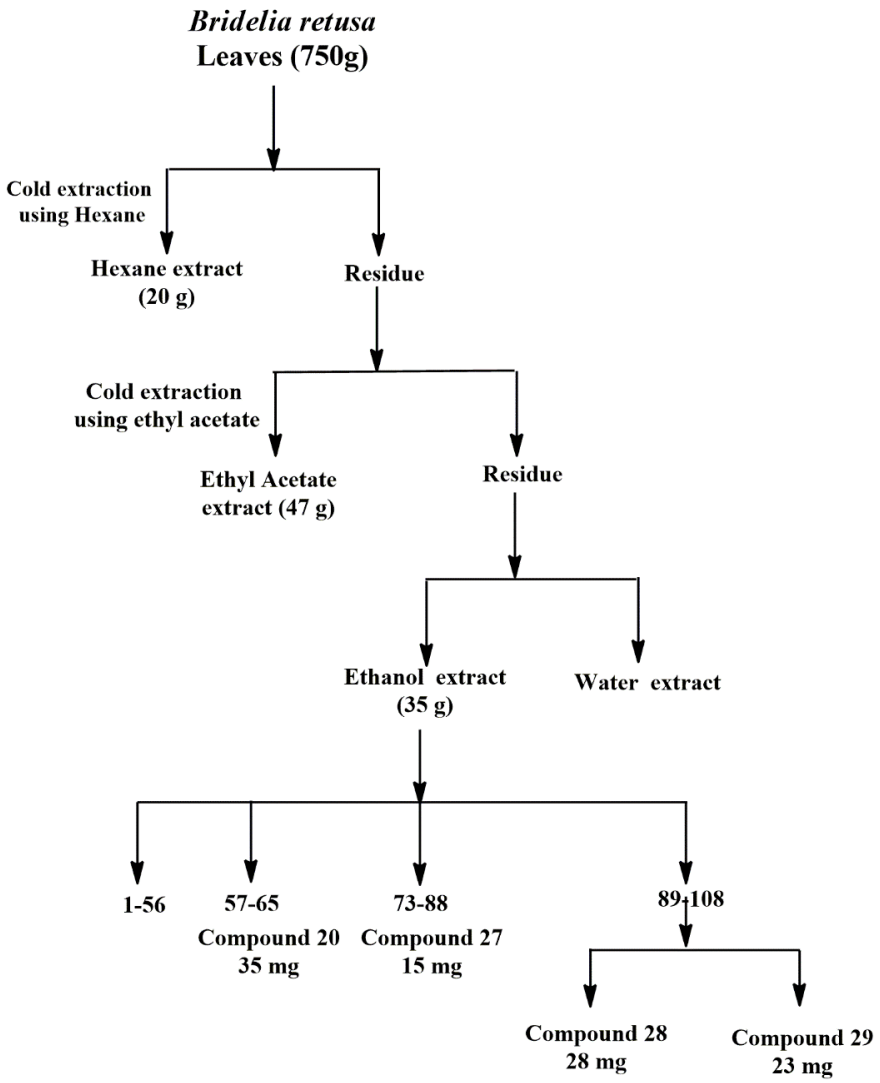
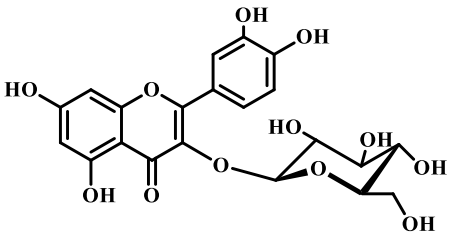


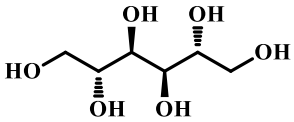
Figure 3A.21. Extraction Process

pattern for H-6 and H-8 protons [δ 6.39 (d, J = 1.5 Hz, 1H, H-8), 6.20 (d, J = 1.5 Hz, 1H, H-6)]. The signals observed between δ 3.71-3.21 indicated the presence of a glucoside moiety in which the anomeric proton appeared at δ 5.26 (d, J = 7.5 Hz). The ^{13}C NMR spectrum (**Fig. 3A.23**) showed 21 carbon peaks; the peak at δ 179.6 corresponded to the carbonyl group in the structure and the anomeric carbon resonated at δ 104.3 ppm. Considering all the spectral data and on comparison with the literature reports, the compound was identified as **Quercetin-3-O-β-glucopyranoside** (Isoquercitrin).[33] Isoquercitrin is widely distributed in fruits, vegetables and cereals and the first isolation was reported from the seed pods of *Cercis canadensis* L.[34] It possesses various pharmacological activities, such as antiviral, anticancer, hepatoprotective, antimicrobial and antioxidant.[35–37]



Compound **27- Quercetin-3-*O*-*β*-glucopyranoside**

A white amorphous solid was isolated from fraction pool 5 (Fr. 89-108), labelled as compound **28** (28 mg). In the ^{1}H NMR spectrum (**Fig. 3A.24**) the peaks were observed in the range δ 3.59-3.81 ppm and in the ^{13}C NMR spectrum (**Fig. 3A.25**) only three carbon signals were present at δ 71.6, 70.0 & 63.7 ppm revealing that the compound was a monosaccharide type symmetric substance. The molecular formula was assigned as $C_6H_{14}O_6$ based on the mass and NMR analysis in which the molecular ion peak was observed at 205.0683, which is the $(M+Na)^+$ peak. Based on an extensive literature survey, the compound was identified as **D-Mannitol** and the spectral data were in good agreement with the reported values.[38]



Compound **28- D-Mannitol**

The residue after isolating compound was subjected to silica gel CC using 40 % methanol- ethyl acetate polarity, afforded compound **29** as yellow solid (23 mg). The ^{1}H NMR spectrum (**Fig. 3A.26**) showed two ortho-coupled aromatic protons [δ 7.63 (dd, J = 8.5, 2.5 Hz, 1H), 6.87 (d, J = 8.5 Hz, 1H)] and three meta-coupled aromatic protons [δ 7.66 (d, J = 2.5 Hz, 1H), 6.40 (d, J = 2.5 Hz, 1H), 6.21 (d, J = 2.5 Hz, 1H)]. It also supported the presence of two sugar moieties with anomeric proton signals at δ 5.11 and 4.52 ppm. The coupling constant of the anomeric protons (J = 7.5 Hz & 1.0 Hz) confirms α- linkage of the rhamnose moiety. The methyl protons of rhamnose were observed as a doublet at δ 1.12 ppm. The remaining protons of the glucose resonated between δ 3.25-3.80 ppm. From the ^{13}C NMR spectrum (**Fig. 3A.27**), it is evident that the compound contains 27 carbon atoms, the methyl group of the rhamnose appeared at δ 17.9 ppm and the signal at δ 179.5 confirms the presence of the carbonyl group.

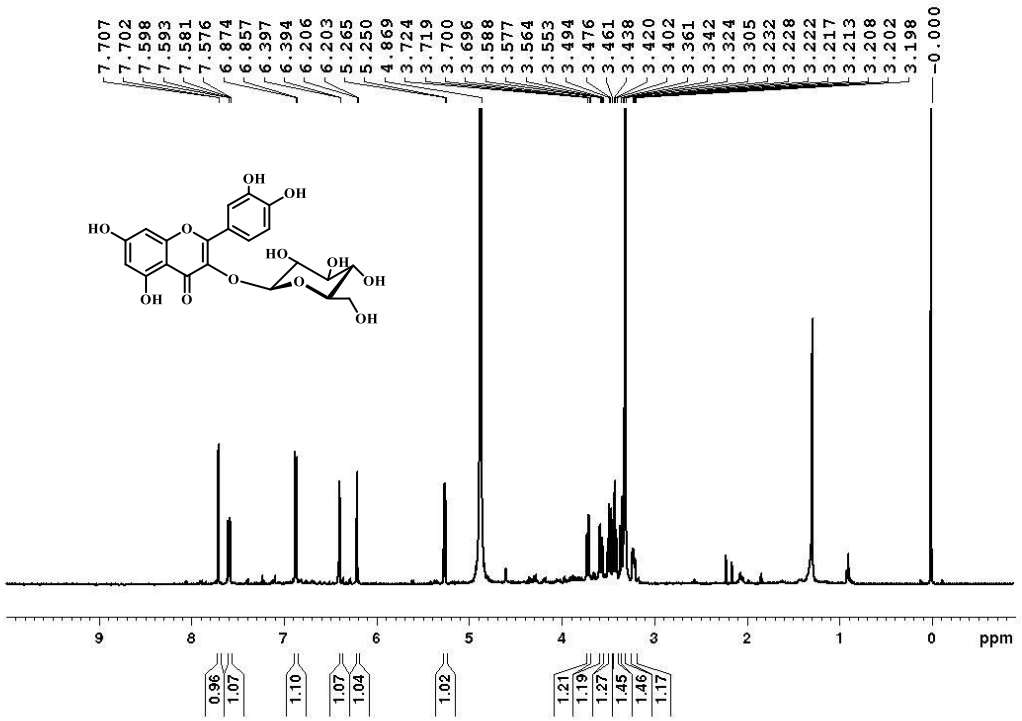


Figure 3A.22.^{1}H NMR spectrum of compound **27** in CD_3OD

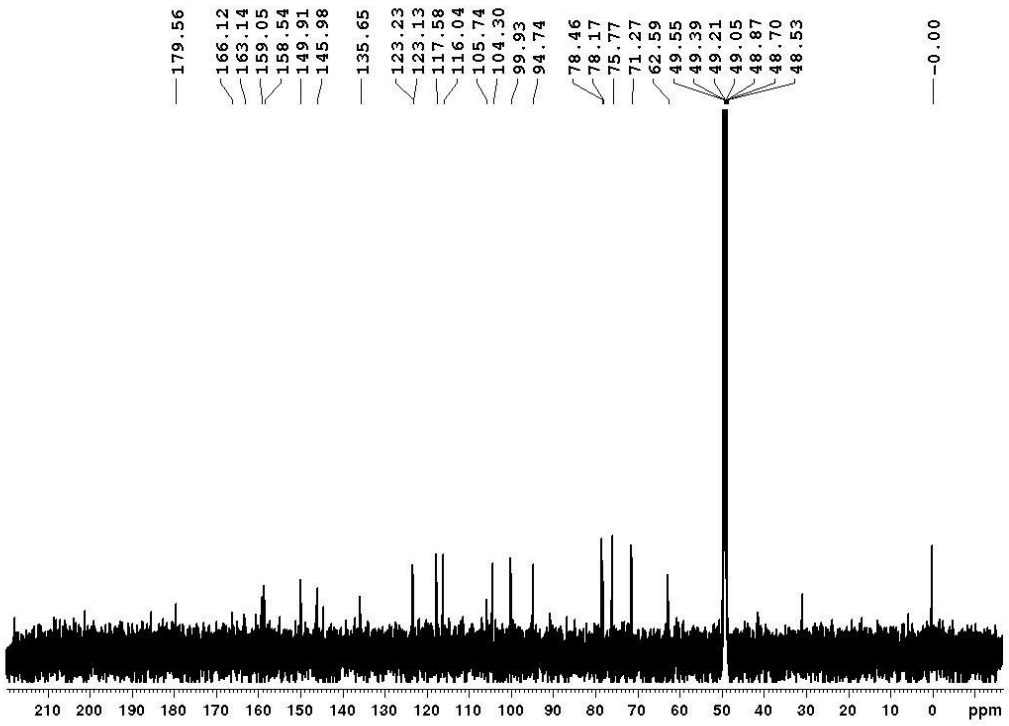


Figure 3A.23.^{13}C NMR spectrum of compound **27** in CD_3OD

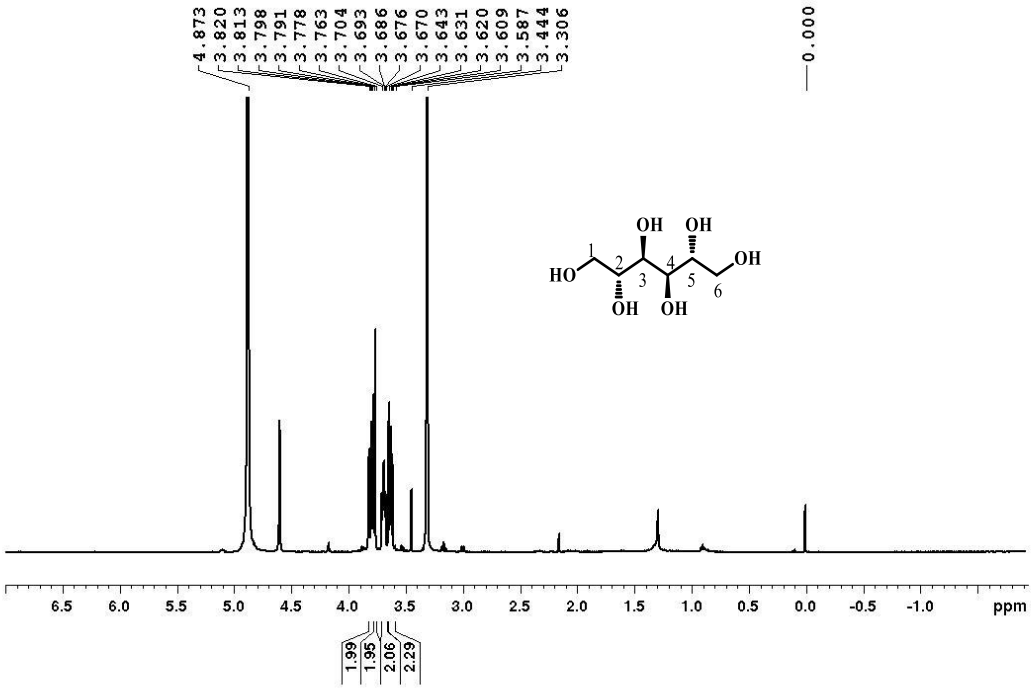


Figure 3A.24. ^{1}H NMR spectrum of compound **28** in CD_3OD

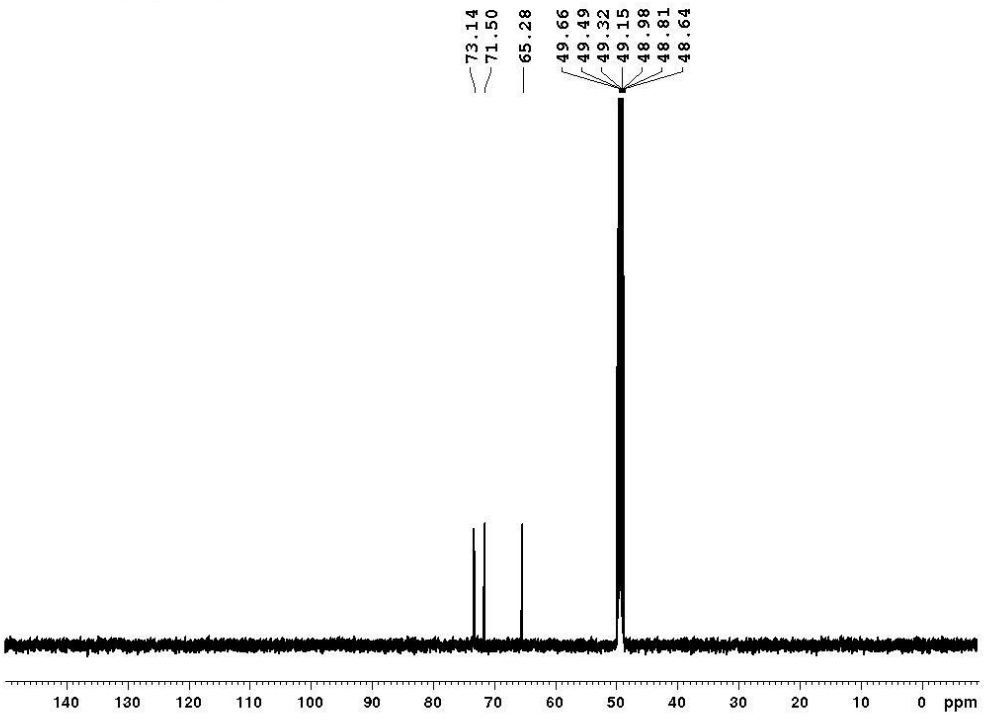
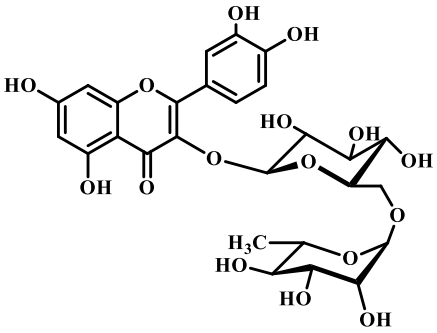


Figure 3A.25. ^{13}C NMR spectrum of compound **28** in CD_3OD

The mass spectrum of the compound showed a molecular ion peak at *m/z* 633.1423 which is the $(M+Na)^+$ peak. From all the above spectral details and on comparing with literature reports the compound was confirmed as **Rutin**.[33] Rutin is abundantly found in plants, such as passion flower, buckwheat, citrus fruits, tea and apple. It possesses a number of pharmacological activities, including antioxidant, antiallergic, cytoprotective, hypolipidemic, vasoprotective, antiprotozoal, anticarcinogenic, antidiabetic, neuroprotective and cardioprotective activities.[39–41]



Compound **29- Rutin**

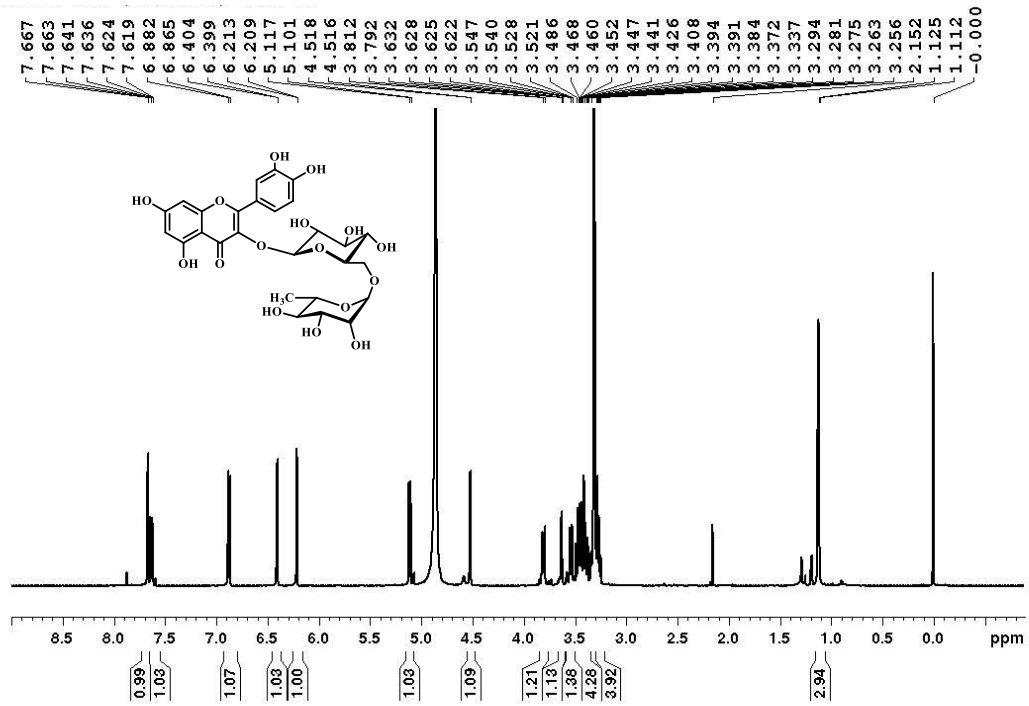


Figure 3A.26. ^{1}H NMR spectrum of compound **29** in CD_3OD

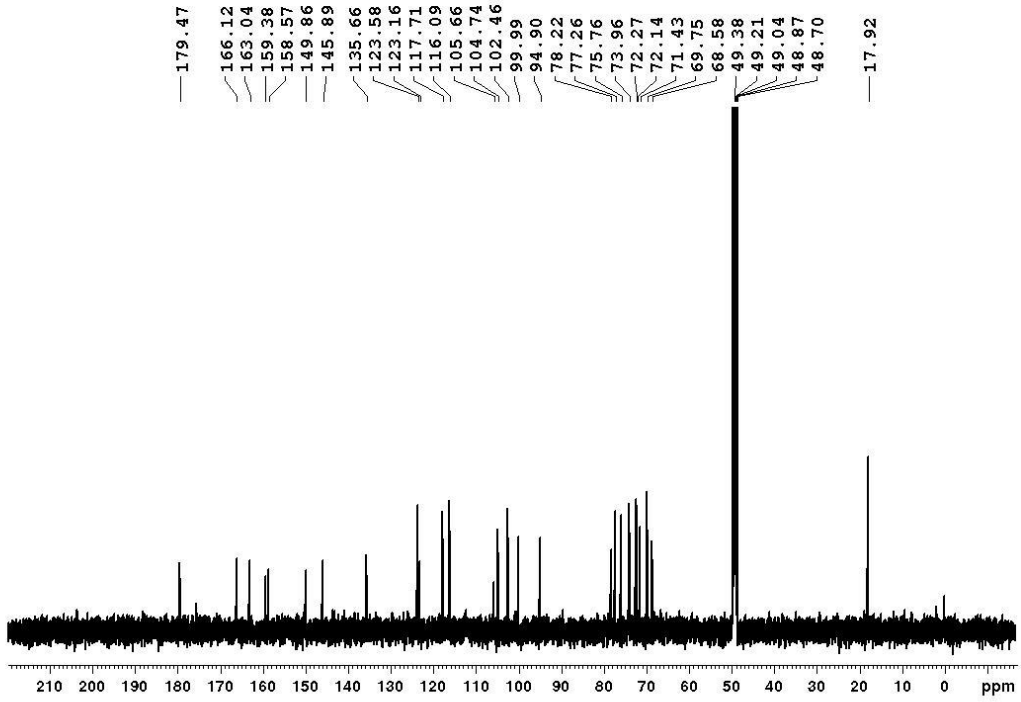


Figure 3A.27. ^{13}CNMR spectrum of compound **29** in CD_3OD

3A.8. Preliminary cytotoxicity analysis of extracts and compounds

Since most of the compounds present in *Bridelia retusa* are polyphenols, we checked some of the extracts and compounds for preliminary cytotoxicity studies against four different cell lines, such as Oral cancer (SAS), Breast cancer cells (MDA-MB-231), Lung cancer cells (A549) and Human colon carcinoma (HCT15). The ethanol extract of seed exhibited 16.48 % of inhibition against SAS cell line and acetone extract exhibited 14.79 % of inhibition against A549 cell lines. It could be due to the presence of gallic acid and ellagic acid present in it. The ethanol extract of bark exhibited 75.13 % of inhibition against SAS cell lines. The values are presented in **Table 3A.4** and **3A.5**.

In the case of compounds, ellagic acid exhibited 66.41 % of inhibition against SAS cell lines at a concentration of 10 µM. Gallic acid inhibited 232.13 and 110.92 % of inhibition against SAS and A549, respectively. The results were in well agreement with the extract level studies.

Table 3A.4. Preliminary cytotoxicity studies of extracts

		Percentage of Inhibition			
	Extracts	**SAS** (10 µg/ml)	**MDA-MB-231** (10 µg/ml)	**A549** (25 µg/ml)	**HCT15** (25 µg/ml)
Seed	Acetone	-32.47	-17.293	**14.792**	-8.623
	Ethanol	**16.48**	-8.656	-13.294	-9.2171
Stembark	Acetone	5.48	7.4726	-1.9794	-15.96239
	Ethanol	**75.13**	12.166	4.88657	-21.53025
	Water	-1.20	0.25743	18.8036	-14.81

Table 3A.5. Preliminary cytotoxicity studies of compounds

	Percentage of Inhibition			
Compounds	**SAS** (10 µM)	**MDA-MB-231** (25 µM)	**A549** (25 µM)	**HCT15 Cells** (25 µM)
18	**232.136**	28.6808	**110.929**	32.5253
19	22.81	-12.7639	-2.525	25.4311
20	56.47	9.20299	34.8125	35.4173
21	**66.41**	42.6384	-0.19607	25.2961

3A.9. Conclusion

We have successfully isolated six compounds from the seeds, six compounds from the stem bark and four compounds from the leaves of *Bridelia retusa*. Lupeol, gallic acid. Gallic acid-*O*-*β*-D-glucoside, ellagic acid and epigallocatechin reported for the first time from *Bridelia retusa*. 4-Oxosesamnine and 4-*O*-methylepigallocatechin are reported for the first time from this genus. All the compounds isolated from leaves are reported for the first time from this plant species and D-mannitol is

reported for the first time from the *Bridelia* genus. Most of the extracts exhibited good antibacterial activity against *Staphylococcus aureus*.

3A.10. Experimental section

General experimental procedure as described in section 2A.6 Chapter 2A.

3A.10.1. Bacterial strains and media

The ESKAP pathogens panel consisted of *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (BAA-1705), *Acinetobacter baumannii* (BAA-1605), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29213). These strains were procured from BEI/NARSA/ATCC (Biodefense and Emerging Infections Research Resources Repository/Network on Antimicrobial Resistance in Staphylococcus aureus/American Type Culture Collection, USA) and routinely cultivated on Mueller-Hinton Agar (MHA). Prior to the experiment, a single colony was picked from MHA plate, inoculated in Mueller-Hinton cation supplemented broth II (CA-MHB) and incubated overnight at 37 °C with shaking for 18–24 h to get the starter culture.

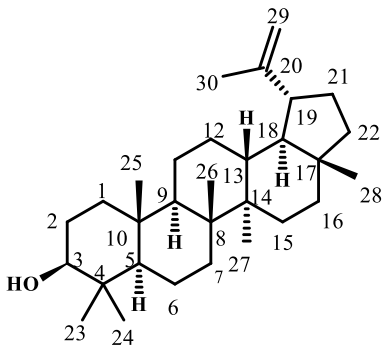
3A.10.2. Cell proliferation assay

Same as Chapter 2A.7.2

3A.11. Spectral data

Compound 15 & 16 (Lupeol & β-Amyrin)

Compound **15 & 16** was isolated from the hexane extract as a colourless solid (38 mg). Based on various spectroscopic techniques the compound was characterized as the mixture of phytosterols, **Lupeol and β-Amyrin**.



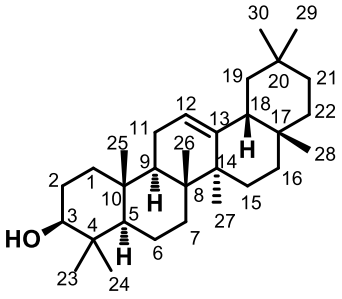
Molecular formula: $C_{30}H_{50}O$

FT-IR (Neat, υ_{max} cm^{-1})**:** 3449, 2931, 2311, 2117, 1587, 1433, 1258, 899 cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 4.69 (s, 1H, H-29a), 4.57 (s, 1H, H-29b), 3.19 (dd, J = 11.5, 4.5 Hz, 1H, H-3), 2.37 (m, 1H, H-19), 1.68 (s, 3H, H-30), 1.65-1.01 (m, 23H), 1.03 (s, 3H, H-26), 0.97 (m, 7H, H-15, H-23, H-27), 0.83 (s, 3H, H-25), 0.79 (s, 3H, H-28), 0.76 (s, 3H, H-24), 0.68 (m, 1H, H-5) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 151.0 (C-20), 109.3 (C-29), 79.0 (C-3), 55.3 (C-5), 50.4 (C-9), 48.3 (C-18), 48.0 (C-19), 43.0 (C-17), 42.8 (C-14), 40.8 (C-8), 40.0 (C-22), 38.8 (C-4), 38.7 (C-1), 38.1 (C-13), 37.2 (C-10), 35.6 (C-16), 34.3 (C-7), 29.8 (C-21), 28.0 (C-23), 27.4 (C-2), 27.2 (C-15), 25.1 (C-12), 20.9 (C-11), 19.3 (C-30), 18.3 (C-6), 18.0 (C-28), 16.1 (C-25), 16.0 (C-26), 15.4 (C-24), 14.6 (C-27) ppm.

HRMS (ESI): *m/z* calcld for $C_{30}H_{50}O$ is 426.3861. Found 427.3943 $(M+H)^+$.



Molecular formula: $C_{30}H_{50}O$

^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 5.18 (s, 1H, H-12), 3.22 (dd, J = 11.0, 4.0 Hz, 1H, H-3), 2.05-1.30 (m, 24 H), 1.13 (s, 3H, H-27), 1.00 (s, 3H, H-24), 0.94 (s, 3H, H-26), 0.91 (s, 3H, H-25), 0.87 (s, 3H, H-30), 0.83 (s, 3H, H-29), 0.79 (s, 3H, H-28), 0.76 (s, 3H, H-23) ppm.

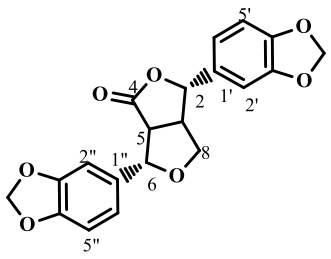
^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 145.1 (C-13) , 121.8 (C-12), 79.0 (C-3), 55.2 (C-5), 47.6 (C-9), 47.2 (C-18), 46.8 (C-19), 41.7 (C-14), 38.9 (C-4), 38.8 (C-8), 38.6 (C-1) 37.2 (C-10), 37.0 (C-21), 34.7 (C-22), 33.3 (C-29) 32.6 (C-7), 32.5 (C-17), 31.2 (C-20), 28.4 (C-28), 28.1 (C-24), 27.2 (C-2), 26.9 (C-16), 26.2 (C-15), 26.0 (C-27), 23.7 (C-30), 23.5 (C-11), 18.4 (C-6), 16.8 (C-26), 15.6 (C-25), 15.5 (C-23) ppm.

HRMS (ESI): *m/z* calcld for $C_{30}H_{50}O$ is 426.3861. Found 427.3943 $(M+H)^+$.

Compound 17)

Fraction pool 3 (Fr. 43-52) obtained by eluting the column with 30 % ethyl acetate-hexane afforded a colourless solid of 15 mg as compound **17**, which was

characterized as the furofuran lignan **4-Oxosesamine** based on the spectral data obtained as shown below.



Molecular formula: $C_{21}H_{18}O_7$

FT-IR (Neat, υ_{max} cm^{-1}): 1761, 1036, 924 cm^{-1}.

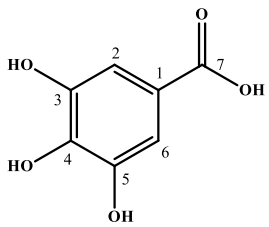
^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 6.87 (s, 1H, H-2”), 6.86 (d, *J* = 8.0 Hz, 1H, H-6”), 6.80 (m, 1H, H-6’), 6.79 (m, 1H, H-2’), 6.77 (d, *J* =7.0 Hz, 1H, H-5”), 6.76 (d, *J* = 7.0 Hz, 1H, H-5’), 5.31 (d, *J* = 3.5 Hz, 1H, H-6), 5.29 (d, *J* = 4.0 Hz, 1H, H-2), 4.32 (dd, J = 9.0, 6.5 Hz, 1H, H-8α), 4.01 (dd, *J* = 9.5, 5.0 Hz, 1H, H-8β), 3.42 (dd, *J* = 9.0, 3.5 Hz, 1H, H-5), 3.21 (m, 1H, H-1) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 176.6 (C-4), 148.4 (C-3”), 148.1 (C-3’), 147.3 (C-4 & 4’), 134.4 (C-1”), 133.0 (C-1’), 119.0 (C-6”), 118.8 (C-6’), 108.6 (C-2”), 108.4 (C-2’), 106.0 (C-5”), 105.7 (C-5’), 101.5 (-OCH_2O-), 101.2 (-OCH_2O-), 84.4 (C-6), 83.4 (C-2), 72.7 (C-8), 53.3 (C-5), 50.0 (C-1) ppm.

HRMS (ESI): *m/z* calcd for $C_{21}H_{18}O_7$ is 368.0896. Found 391.0793 $(M+Na)^+$.

Compound 18 (Gallic acid)

A colourless solid was obtained from fraction pool 5 (Fr. 80-98) as compound **18** (53 mg**).** The compound was characterized as **Gallic acid** based on the spectral data and literature reports.



Molecular formula: $C_7H_6O_5$

Melting point: 252 ± 2° C

FT-IR (Neat, υ_{max} cm^{-1}): 3407, 2960, 2931, 2865, 1639, 1509, 1420, 1153, 1097, 777 cm^{-1}.

^{1}H NMR (500 MHz, Acetone-d6, TMS): δ 8.53 (bs, 2H), 7.02 (s, 2H, H-2 & H-6) ppm.

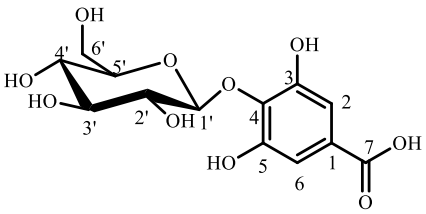
^{13}C NMR (125 MHz, CD_3OD, TMS): δ 172.9 (C-7), 148.9 (C-3 & C-5), 142.1 (C-4), 124.5 (C-1), 112.8

(C-6 & C-2) ppm.

HRMS (ESI): *m/z* calcd for $C_7H_6O_5$ is 170.0215. Found 171.0292 $(M+H)^+$.

Compound 19 (Gallicacid glucoside)

Compound **19** was isolated from fractions pool 7 (Fr. 104-106) by precipitating with methanol-DCM combination yielded 3 g of yellow solid. The ^1H NMR was similar to that of compound **18** with an additional glucose moiety. The compound was characterized as **Gallic-4-*O*-*β*-D-glucopyranoside**.



Molecular formula: $C_{13}H_{16}O_{10}$

Melting point: 152 ± 2° C

^{1}H NMR (500 MHz, CD_3OD, TMS): *δ* 7.07 (s, 2H, H-2 & H-6), 4.69 (d, *J* = 8.0 Hz, 1H), 3.86-3.74 (m, 2H), 3.54-3.49 (m, 1H), 3.47-3.42 (m, 2H), 3.36-3.33 (m, 1H) ppm.

^{13}C NMR (125 MHz, CD_3OD, TMS): *δ* 169.5 (C-7), 151.5 (C-3 & C-5), 138.3 (C-4), 129.1 (C-1), 110.4 (C-6 & C-2), 107.3 (C-1'), 78.5 (C-3'), 77.6 (C-5'), 75.1 (C-2'), 70.6 (C-4'), 61.9 (C-6') ppm.

HRMS (ESI): *m/z* calcd for $C_{13}H_{16}O_{10}$ is 332.0743. Found 355.0644 $(M+Na)^+$.

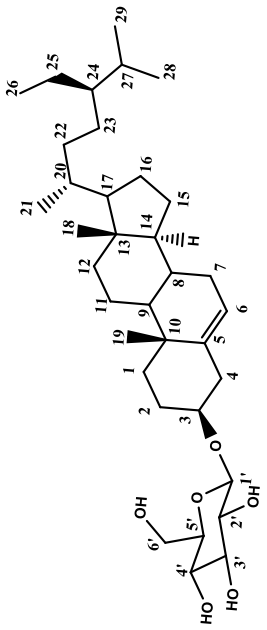
Compound 20

A colourless amorphous solid was isolated from fraction pool 8 (Fr. 117-119) and designated as compound **20** (4 mg). This was characterized as the ***β*- sitosterol-*β*-D- glucoside**, using various spectroscopic data and comparison with literature reports.

Molecular formula: $C_{35}H_{60}O_6$

FT-IR (Neat, υ_{max} cm^{-1}): 3409, 3266, 2995, 2863, 1257, 1190, 1090, 1054, 1029, 968, 844 cm^{-1}.

1H NMR (500 MHz, DMSO-d6, TMS) : *δ* 5.34 (d, *J* = 5 Hz, 1H), 4.88 (m, 3H), 4.44(m, 1H), 4.23 (d, *J* = 8 Hz, 1H), 3.65 (m, 1H), 3.46 (m, *J* = 8 Hz, 1H), 3.13 (m, 1H), 3.08 (m, 1H), 3.02 (m, 1H), 2.89 (m, 1H),



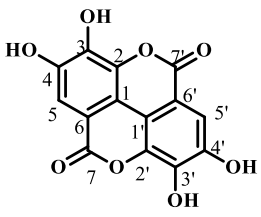
2.36 (m, 1H), 2.13 (m, 1H), 1.94 (m, 2H), 1.80 (m, 3H), 1.64 (m, 1H), 1.51-1.40 (m, 6H), 1.28-1.23 (m, 6H), 1.16 (m, 4H), 0.96 (s, 3H), 0.91 (s, 5H), 0.82 (m, 9H), 0.66 (s, 3H) ppm.

^{13}C NMR (125 MHz, DMSO-d6, TMS): δ 140.4 (C-5), 121.2 (C-6), 100.7 (C-1'), 76.9 (C-5), 76.7 (C-3'), 73.4 (C-2'), 70.1 (C-4'), 61.1 (C-6'), 56.1 (C-14), 55.4 (C-17), 49.6 (C-9), 45.1 (C-24), 41.8 (C-13), 38.3 (C-12), 36.8 (C-1), 36.2 (C-10), 35.4 (C-20), 33.3 (C-22), 31.4 (C-7), 31.3 (C-8), 29.2 (C-2), 28.7 (C-25), 27.8 (C-16), 25.4 (C-23), 23.8 (C-15), 22.6 (C-28), 20.6 (C-11), 19.7 (C-26), 19.1 (C-19), 18.9 (C-27), 18.6 (C-21), 11.8 (C-29), 11.6 (C-18) ppm.

HRMS (ESI): *m/z* Calcd for $C_{35}H_{61}O_6$ is 577.4468. Found 577.4013.

Compound 21 (Ellagic acid)

Fraction pool 10 (Fr. 133-136) was subjected to column chromatographic separation on Sephadex LH-20 using methanol as the solvent yielded a solid with some minor impurities, which was then washed with acetone and purified. Compound **21** was isolated as yellow solid (333 mg) with melting point 358° C. The compound was characterized as **Ellagic acid** based on spectral data and literature reports.



Molecular formula: $C_{14}H_6O_8$

Melting point: 358 ± 2° C

FT-IR (Neat, υ_{max} cm^{-1}): 3600, 1725, 1669, 1588, 1190, 1052 cm^{-1}.

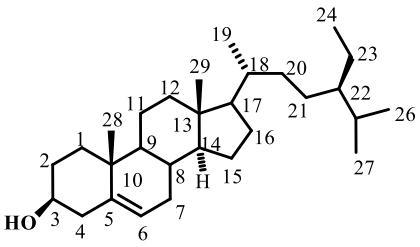
^{1}H NMR (500 MHz, DMSO-d6, TMS): δ 7.46 (s, 2H, H-5 & H-5'), 10.67 (br s, 4H) ppm.

^{13}C NMR (125 MHz, DMSO-d6, TMS): δ 159.1 (C-7 & C-7'), 148.1 (C-4 & C-4'), 139.6 (C-3 & C-3'), 136.3 (C-2 & C-2'), 112.3 (C-1 & C-1'), 110.2 (C-5 & C-5'), 107.6 (C-6 & C-6') ppm.

HRMS (ESI): *m/z* calcd for $C_{14}H_6O_8$ is 302.0062. Found 303.0141$(M+H)^+$.

Compound 22 & 23

Fraction pool 2(Fr. 5-6) afforded a colourless needle-like crystals of compound. The compound was successfully characterised as the common phytosterols; a mixture of ***β*–sitosterol and stigmasterol**, based on the spectral data and comparison with the literature reports.



Molecular formula: $C_{29}H_{50}O$

Melting point: 128-130° C

FT-IR (Neat, υ_{max} cm^{-1}): 3408, 3272, 2935, 2863, 1645, 1459, 1374, 1316, 1257, 1190, 1099, 1054, 1024, 958, 802 cm^{-1}.

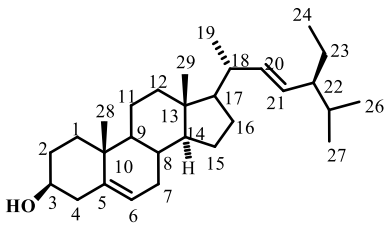
^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 5.37 (d, 1H, *J* = 5.0 Hz, H-6), 3.53-3.54 (m, 1H, H-3), 2.30-2.29 (m, 2H), 2.04-1.87 (m, 2H), 1.87-1.84 (m, 3H), 1.68-1.66 (m, 2H), 1.60-1.45 (m, 7H), 1.32-1.23 (m, 6H), 1.20-1.10 (m, 3H), 1.09-1.96 (m, 3H), 1.02 (s, 5H), 0.94-0.93 (m, 3H), 0.87-0.71 (m, 9H), 0.69 (s, 3H) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 140.8 (C-5), 121.7 (C-6), 71.8 (C-3), 56.8 (C-14), 56.1 (C-17), 50.1 (C-9), 45.8 (C-24), 42.3 (C-4/13), 39.8 (C-12), 37.3 (C-1), 36.5 (C-10), 36.2 (C-20), 33.9 (C-22), 31.9 (C-7/8), 31.7 (C-2), 29.2 (C-25), 28.3 (C-16), 26.1 (C-23), 24.3 (C-15), 23.1 (C-28), 21.1 (C-11), 19.8 (C-26), 19.4 (C-19), 19.0 (C-27), 18.8 (C-21), 12.0 (C-29), 11.9 (C-18) ppm

HRMS (ESI): *m/z* calcd for $C_{29}H_{50}O$ is 414.3861. Found 415.10970 $(M+H)^+$.

Molecular formula: $C_{29}H_{48}O$

FT-IR (Neat, υ_{max} cm^{-1}): 3547, 3232, 1638, 1462



cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 5.35 (m, 1H, H-6), 5.15 (dd, J = 15.5, 9.0 Hz, 1H, H-22), 5.02 (dd, 15.5Hz, 8.5 Hz, 1H, H-23),3.52 (m, 1H, H-3), 2.31, 2.23, 2.06, 1.96, 1.86, 1.84, 1.50, 1.47, 1.29, 1.22, 1.18, 1.15, 1.09, 1.04, 1.01, 0.93, 0.92, 0.83, 0.81, 0.70 ppm.

^{13}C NMR ($CDCl_3$, 125 MHz, TMS): δ 140.8 (C-5), 138.3 (C-22), 129.3 (C-23), 121.7 (C-6), 71.8 (C-3), 56.9 (C-14), 56.0 (C-17), 51.2 (C-24), 50.2 (C-9), 42.3 (C-4), 42.2 (C-13), 40.5 (C-20), 39.7 (C-12), 37.3 (C-1), 36.5 (C-10), 31.9 (C-7/8/25), 31.7 (C-2), 28.9 (C-16), 25.4 (C-28), 24.4 (C-15), 21.2 (C-21), 21.1 (C-11/26), 19.4 (C-19), 19.0 (C-27), 12.3 (C-29), 12.1 (C-18) ppm.

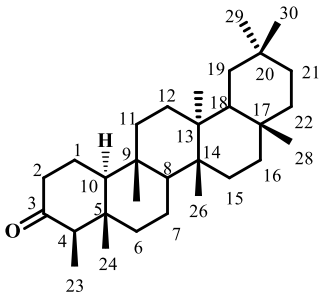
HRMS (ESI): *m/z* calcd for $C_{29}H_{48}O$ is 412.3705. Found 413.6908 $(M+H)^+$.

Compound 24 (Friedelin)

A colourless crystalline solid was isolated from fractions 8-40. The compound was confirmed as **Friedelin** based on spectral data and literature values.

Molecular formula: $C_{30}H_{50}O$

FT-IR (Neat, υ_{max} cm^{-1}): 2954, 2860, 1745, 1460, 1072 cm^{-1}.



^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 2.41-2.37 (m, 1H, H-2b), 2.32-2.28 (m, 1H, H-2), 2.27-2.23 (m, 1H, H-4), 1.98-1.94 (m, 1H, H-1a) 1.77-1.73 (m, 1H, H), 1.69-1.64 (m, 1H, H), 1.54-1.30 (m, 20H), 1.05 (s, 3H, H- 27), 1.01 (s, 3H, H-26), 1.00 (s, 3H, H-26), 0.95 (s, 3H, H), 0.88 (d, 3H, H-23), 0.87 (s, 3H, H-25), 0.73 (s, 3H, H-24) ppm.

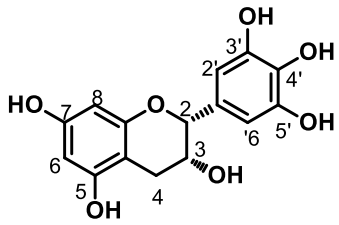
^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 213.3 (C-3) , 59.5 (C-10), 58.2 (C-4), 53.1 (C-8), 42.8 (C-18),

42.2 (C-5), 41.5 (C-2), 41.3 (C-6), 39.7 (C-22), 39.3 (C-14), 38.3 (C-13), 37.4 (C-9), 36.0 (C-16), 35.6 (C-11), 35.3 (C-19), 35.0 (C-29), 32.8 (C-21), 32.4 (C-15), 32.1 (C-28), 31.8 (C-30), 30.5 (C-12), 30.0 (C-17), 28.2 (C-20), 22.3 (C-1), 20.3 (C-26), 18.7 (C-27), 18.2 (C-7), 17.9 (C-25), 14.7 (C-24), 6.8 (C-23) ppm.

HRMS (ESI): *m/z* calcd for $C_{30}H_{50}O$ is 426.3861. Found 427.3934 $(M+H)^+$.

Compound 25 ((-)-Epigallocatechin)

Compound **25** was isolated from fraction pool 8 (Fr. 20-21) as a yellow amorphous solid (30 mg), was characterized as **(-)-Epigallocatechin** based on spectral data and comparison with the literature reports.



Molecular formula: $C_{15}H_{14}O_7$

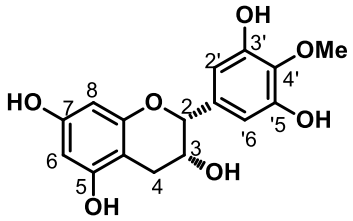
^{1}H NMR (500 MHz, Acetone-d6, TMS): δ 6.42 (s, 2H, H-2' & H-6'), 5.94 (d, *J* = 2.5 Hz, 1H, H-6), 5.88 (d, *J* = 2.5 Hz, 1H, H-8), 4.54 (d, *J* = 7.0 Hz, 1H, H-2), 3.98 (dd, *J* = 12.5, 7.5 Hz, 1H, H-3), 2.83 (dd, *J* = 16.0, 5.0 Hz, 1H, H-4), 2.52 (dd, *J* =16.0, 8.0 Hz, 1H, H-4) ppm.

^{13}C NMR (125 MHz, Acetone-d6, TMS): δ 156.7 (C-5), 156.7 (C-7), 156.2 (C-1), 145.4 (C-3' & 5'), 132.0 (C-4'), 130.6 (C-1'), 106.0 (C-2' & 6'), 98.9 (C-4a), 95.2 (C-6), 94.8 (C-8), 78.5 (C-2), 66.1 (C-3), 27.9 (C-4) ppm.

HRMS (ESI): *m/z* calcd for $C_{15}H_{14}O_7$ is 306.0739. Found 329.0637 $(M+Na)^+$.

Compound 26 (4'-O-methyl- (-) epigallocatechin)

Compound **26** was isolated along with compound 25 as yellow solid of 8 mg, which was characterised as **4'-O-methyl- (-) epigallocatechin**, based on spectral data as given below and on comparison with literature reports. The compound was reported for the first time from *Bridelia* genus.



Molecular formula: $C_{15}H_{16}O_7$

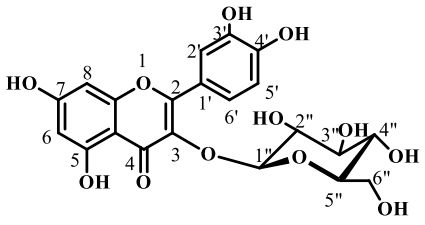
^{1}H NMR (500 MHz, CD_3OD, TMS): δ 6.41 (s, 2H, H-2' & H-6'), 5.92 (d, *J* = 2.5Hz, 1H, H-6), 5.87 (d, *J* =2.5 Hz, 1H, H-8), 4.56 (d, *J* =7.0 Hz, 1H, H-2), 3.97 (m, 1H, H-3), 3.79 (s, 3H), 2.78 (dd, *J* = 16.5, 5.5 Hz, 1H, H-4), 2.50 (dd, *J* =16.0, 3.0 Hz, 1H, H-4) ppm.

^{13}C NMR (125 MHz, CD_3OD, TMS): δ 157.9 (C-5), 157.6 (C-7), 156.7 (C-9), 151.6 (C-3' & C-5'), 136.7 (C-1'), 136.5(C-4'), 107.4 (C-2' & C-6'), 100.7 (C-10), 96.4 (C-6), 95.5 (C-8), 82.6 (C-2), 68.7 (C-3), 60.8 (-OMe), 28.1 (C-4) ppm.

HRMS (ESI): *m/z* calcd for $C_{15}H_{16}O_7$ is 320.0896. Found 343.0793$(M+Na)^+$.

Compound 27 (Quercetin-3-*O*-*β*-D-glucoside)

15 mg of compound **27** was obtained as yellow amorphous solid from fraction pool 4 (Fr. 73-88) was characterised as the derivative of Quercetin; **Quercetin-3-*O*-*β*-D-glucoside**. The NMR values are assigned base on HMBC, HMQC and literature reports.



Molecular formula: $C_{21}H_{20}O_{12}$

Melting point: ± 2° C

FT-IR (Neat, υ_{max} cm^{-1}): 3422, 2958, 1655, 1605, 1460, 1300, 1088 cm^{-1}.

^{1}H NMR (500 MHz, CD_3OD, TMS): δ 7.74 (d, *J* = 2.5 Hz, 1H, H-2'), 7.59 (dd, *J* = 8.5, 2.5 Hz, 1H, H-6'), 6.86 (d, *J* = 8.5 Hz, 1H, H-5'), 6.39 (d, *J*=1.5 Hz, 1H, H-8), 6.20 (d, *J* = 1.5 Hz, 1H, H-6), 5.26 (d, *J* = 7.5 Hz, 1H, H-1), 3.71 (dd, *J* =12.0, 2.5 Hz, 1H, H-6a), 3.57 (dd, *J* = 5.5, 12.0 Hz, 1H, H-6b), 3.48 (t, *J* =9.0 Hz, H-2), 3.42 (t, *J* = 9.0 Hz, H-3), 3.34 (t, *J* = 9.5 Hz, 1H, H-4), 3.21 (m, 1H, H-5) ppm.

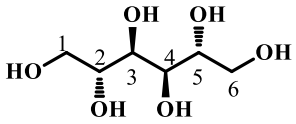
^{13}C NMR (125 MHz, CD_3OD, TMS): δ 179.6 (C-4),

166.1 (C-7), 163.1 (C-5), 159.0 (C-9), 158.5 (C-2), 149.9 (C-3'), 146.0 (C-4'), 135.6 (C-3), 123.2 (C-1'), 123.1 (C-6'), 117.6 (C-5'), 116.0 (C-2'), 105.7 (C-10), 104.3 (C-1), 99.9 (C-6), 94.7 (C-8), 78.5 (C-5"), 78.2 (C-3"), 75.7 (C-2"), 71.3 (C-4"), 62.6 (C-6") ppm.

HRMS (ESI): *m/z* calcd for $C_{21}H_{20}O_{12}$ is 464.0954. Found 487.0846 $(M+Na)^+$.

Compound 28 (D-Mannitol)

A white amorphous solid isolated from fraction pool 5 (Fr. 89-108) was identified as the monosaccharide, **D-mannitol** (28 mg), based on the spectral data obtained as given below.



Molecular formula: $C_6H_{14}O_6$

FT-IR (Neat, υ_{max} cm^{-1}): 3280, 2970, 2902, 1416, 1299, 1017 cm^{-1}.

^{1}H NMR (500 MHz, CD_3OD, TMS): δ 3.81 (dd, *J* = 11.0, 3.5 Hz, 2H, H-1 & H-2), 3.77 (d, *J* = 8.0 Hz, 2H, H-1 & H-2), 3.67-3.70 (m, 2H, H-2 & H-5), 3.62 (dd, *J* = 11.5, 6.0 Hz, 2H, H-3 & H-4) ppm.

^{13}C NMR (125 MHz, CD_3OD, TMS): δ 71.6 (C-2 & C-5), 70.0 (C-3 & C-4), 63.7 (C-1 & C-6) ppm.

HRMS (ESI): *m/z* calcd for $C_6H_{14}O_6$ is 182.0790 Found 205.0683 $(M+Na)^+$.

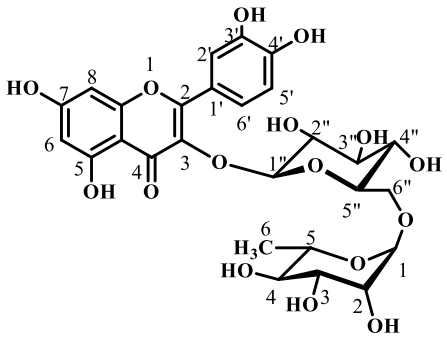
Compound 29 (Rutin)

Fraction pool (Fr. 89-108) 5 obtained by eluting the column at 40 % methanol-ethyl acetate polarity afforded a yellow solid of 23 mg as compound. The compound was characterized as **Rutin** based on the spectral data and is in well agreement with literature reports.

Molecular formula: $C_{27}H_{30}O_{16}$

Melting point: 240 ± 2° C

FT-IR (Neat, υ_{max} cm^{-1}): 3407, 1727, 1654, 1610, 1500, 1450, 1383, 1310, 1210, 1060, 1020, 940,



810 cm^{-1}.

^{1}H NMR (500 MHz, CD_3OD, TMS): δ 7.66 (d, *J* = 2.5 Hz, 1H, H-2'), 7.63 (dd, *J* = 8.5, 2.5 Hz, 1H, H-6'), 6.87 (d, *J* = 8.5 Hz, 1H, H-5'), 6.40 (d, *J* = 2.5 Hz, 1H, H-8), 6.21 (d, *J* = 2.5 Hz, 1H, H-6), 5.11 (d, *J* = 7.5 Hz, 1H, H-1"), 4.52 (d, *J* =1.0 Hz, 1H, Rha-H1), 3.80 (dd, *J* = 11.0, 1.0 Hz, 1H, H-6"), 3.63 (dd, *J* = 3.5, 2.0 Hz, 1H, Rha-H2), 3.53 (dd, *J* = 9.5, 3.0 Hz, 1H, Rha-H3), 3.55-3.25 (m, 10H), 1.12 (d, *J* = 6.5 Hz, 3H, Rha-H6) ppm.

^{13}C NMR (125 MHz, CD_3OD, TMS): δ 179.5 (C-4), 166.1 (C-7), 163.0 (C-5), 159.4 (C-9), 158.6 (C-2), 149.9 (C-4'), 145.9 (C-3'), 135.7 (C-3), 123.6 (C-1'), 123.2 (C-6'), 117.7 (C-2'), 116.1 (C-5'), 105.7 (C-10), 104.7 (C-1'), 102.5 (Rh-C1), 100.0 (C-6), 94.9 (C-8), 78.2 (C-3"), 77.3 (C-5"), 75.8 (C-2"), 74.0 (Rh-C4), 72.3 (Rh-C3), 72.2 (Rh-C2), 71.4 (C-4"), 69.8 (Rh-C5), 68.6 (C-6"), 17.9 (Rh-C6) ppm.

HRMS (ESI): *m/z* calcd for $C_{27}H_{30}O_{16}$ is 610.1533. Found 633.1423 $(M+Na)^+$.

3A.12. References

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Chapter 3B: Phytochemical Investigation of the Aerial Parts of *Bridelia stipularis* (L) Blume

3B.1. Introduction

Bridelia stipularis (L.) Blume (syn. *Bridelia scandens*) is a woody evergreen climber distributed in the tropical and subtropical regions of India, Sri Lanka, Nepal, Bangladesh, Malay Peninsula, Sumatra, Java, N Borneo and Philippines. *B. stipularis* grows as a climber or scrambling shrub, and sometimes as small trees. Traditionally, this plant species is a highly valued medicinal plant and is in use as a remedy for several diseases. The Kurichyas tribe of Kannur district, Kerala uses the fruits for mouth ulcer; the fresh fruits are chewed and taken as such.[1] The leaves are used to treat jaundice by the tribe and village people of Palakkad and Coimbatore districts of Kerala and Tamilnadu.[2] For the treatment, a handful of tender leaves of *B. stipularis.* together with a piece of fresh rhizomes of *Curcuma longa L*. are ground into a paste, mixed with cow's milk, and is given seven times at three-hour interval. The patients are asked not to take fruits of *Cucurbita pepo L* and *Capsicum annum L*. during the treatment. A decoction of wood is administrated orally to treat malaria.[3] The Kanikkar tribals of Agasthiarmalai Western Ghats, Tamil Nadu, India uses the plant for jaundice; a paste made of the stem bark in water is taken orally for a period of time to treat jaundice.[4] The bark decoction has been used to treat intestinal worms, asthma and cough and leaves are used against colic. The leaves are used for oral health; leaves boiled in water with a pinch of wild salt is inhaled into the mouth, relieves tooth pain and decay.[5]

3B.2. Phytochemistry and Pharmacology

Kumar *et al.,* reported the antioxidant activity of various extracts of the whole plant of *B. stipularis* and reported that the plant extract is a better source of natural antioxidants.[6] Sreenivas *et al.,* reported the *in vitro* production of anthocyanins in *B. stipularis.*[7] Biozid *et al*., investigated the thrombolytic activity of the methanolic

extract of leaves and the extract showed promising activity.[8] The leaves also exhibited hypoglycaemic activity in *Streptozotocin*-induced diabetic rats.[9]

Figure 3B.1. *Bridelia stipularis* Bl.

The methanolic extract of *B. scandens* showed significant hepatoprotective activity against CCl_4 induced hepatotoxicity in rats.[10] Sengupta and Ghosh reported friedelin and *β*-sitosterol from the bark. Further investigations on the leaves led to the isolation of fatty alcohol, $C_{22}H_{46}O$, named bridelyl alcohol and a phlobatannin.[11] Taraxerone was isolated from the hexane extract of roots.[12] Anjum *et al.,* reported the isolation of three compounds from the stem bark extract of *B. stipularis* namely glut-5(6)-en-3-one, glut-5(6)-en-3α-ol, and (22E)-7-hydroxy-28-methylcholesta-4,22-dien-3-one. The compounds exhibited cytotoxic activity moderate to strong antimicrobial activity against 13 Gram-positive and Gram-negative bacterial strains and three fungi.[13]

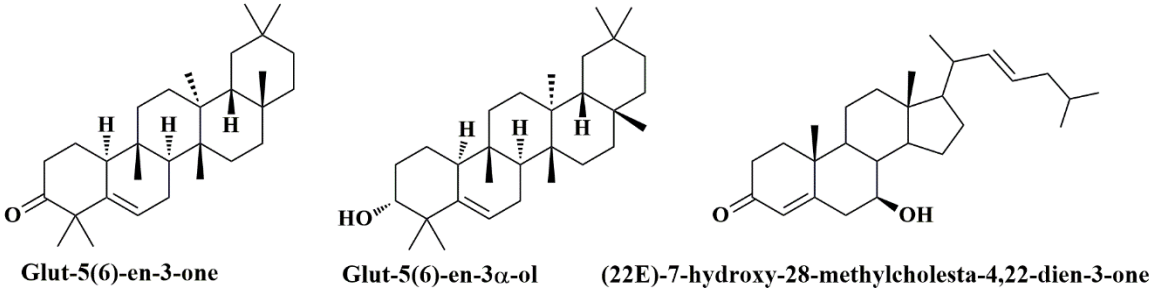


Figure 3B.2. Compounds previously reported from *Bridelia stipularis Bl.*

3B.3. Extraction, isolation and characterization of marker compounds from *Brielia stipularis*

3B.3.1. Collection and Extraction of plant material

The aerial parts (stem and bark) of *Bridelia stipularis* were collected from the Western Ghats region (Wayanad District) of Kerala state, India. The plant material was authenticated by the taxonomist of M. S. Swaminathan Research Foundation (MSSRF), Kerala and a voucher specimen (M.S.S.H. 805) was deposited in the herbarium repository of the institute. The plant material was crushed and dried in an air oven maintained at 50˚C and powdered. About 1 Kg of the powdered material was extracted with hexane (5L × 3 days) at room temperature. The process was repeated thrice. The solvent was removed under reduced pressure in the rotary evaporator, which provided 2.5 g of the hexane extract. The same procedure was repeated with acetone (18 g), ethanol (27 g) and finally, water extract was also collected. A pictorial representation of the extraction procedure is given in **Figure 3B.3.**

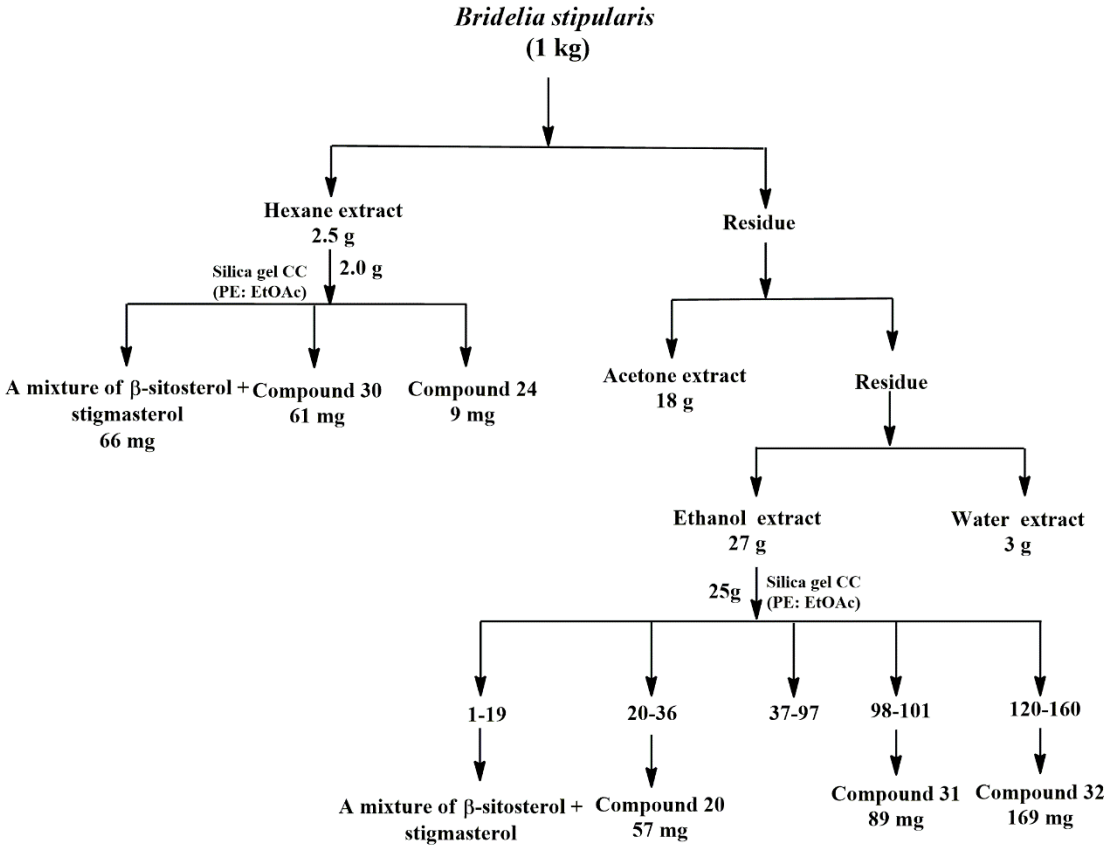


Figure 3B.3. Extraction Process

3B.3.2. Preliminary antibacterial studies of the extracts of *B. stipularis*

All the extracts were tested for their antibacterial activity against ESKAP pathogen panel. Minimum inhibitory concentration (MIC) was determined by

performing the antibiotic susceptibility testing on the extracts in accordance with the standard CLSI guidelines. Levofloxacin was used as the reference compound. The MIC of the compound's ranges > 64 µg/mL. The results are given in **Table 3B.1**.

Table 3B.1. MIC values (µg/mL) of the extracts against ESKAP panel of bacteria

Sample	*E. coli* ATCC 25922	*S. aureus* ATCC 29213	*K. pneumoniae* BAA 1705	*A. baumannii* BAA 1605	*P. aeruginosa* ATCC 27853
Hexane	>64	>64	>64	>64	>64
Acetone	>64	>64	>64	>64	>64
Ethanol	>64	>64	>64	>64	>64
Water	>64	>64	>64	>64	>64
Levofloxacin	0.0156	0.25	64	8	1

3B.3.3. Isolation and characterization of compounds

Around 2 g of the hexane extract was fractionated on a silica gel CC (230-400 mesh). The elution began with hexane and the polarity was increased gradually by adding the desired amount of ethyl acetate and finally eluted with 30 % ethyl acetate in hexane. A total of 30 fractions of approximately 150 ml were collected in conical flasks. After examining the TLC, the fractions were pooled into three fraction pools. Fraction pool 2 (Fr. 7-11) mainly delivered the phytosterols ***β*-sitosterol**, **stigmasterol**, **friedelin** and **taraxerone**. Stigmasterol is reported for the first time from this species and taraxerone was reported from this plant part for the first time. Previously taraxerone was reported from the roots and friedelin and *β*-sitosterol were reported from the bark. The structures of the compounds are given in **Figure 3B.4.**

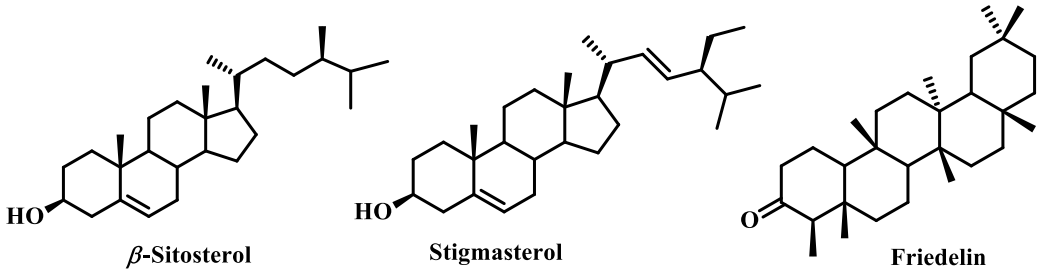
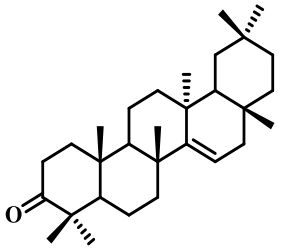


Figure 3B.4. Compounds isolated from Hexane extract

Compound **29** was isolated as a colourless crystalline solid. The presence of a carbonyl was indicated by a strong absorption of C=O stretch at 1709 cm^{-1}. In the ^{1}H NMR spectrum (**Fig. 3B.5**), the appearance of a peak at δ 5.56 integrating to one proton corresponded to an olefinic proton. The ^{13}C NMR spectrum (**Fig. 3B.6**) of the compound showed the presence of 30 carbon peaks. The peak at δ 217.7 ppm confirming the presence of a carbonyl group. The DEPT-135 spectrum along with ^{13}C NMR spectrum suggested that there were eight methyl, ten methylene and four methine carbons. The eight methyl groups detected in the ^{1}H NMR spectrum as singlets indicate that they are attached to the quaternary carbons. This type of structural skeleton is observed in oleanane type triterpenes. Mass spectral analysis showed an *m/z* value of 447.3598, corresponding to $(M+Na)^{+}$ peak. Furthermore, the structure was confirmed with the help of 2D NMR techniques (COSY, HMBC, HMQC) and on comparison with literature reports, as **Taraxerone**.[14] Taraxerone has been reported to exhibit anticancer effects in human chronic myelogenous leukaemia cell line and lung cancer cell line.[15,16]

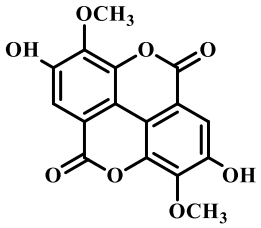


Compound **30- Taraxerone**

The ethanol extract was then subjected to silica gel (100-200 mesh) CC separation. About 25 g of the ethanol extract was loaded on the column; the elution started with 40 % ethyl acetate-hexane mixture and finally with 40 % methanol-ethyl acetate mixture. The fractions collected were pooled into five fraction pools based on similarity in TLC. Fractions 1-19 afforded a mixture of ***β*-sitosterol and stigmasterol**. Another compound was isolated from fraction pool 2 (fr.20-36) as a colourless solid, which was washed with acetone for purification. On comparing the ^{1}H, ^{13}C NMR and HR-ESIMS data with that of the literature reports, the compound was identified as the **glycoside of *β*-sitosterol (20**).

Compound **31** was isolated from the fraction pool 4 (Fr .98-101), as a brown amorphous powder (89 mg); it was purified by washing with acetone. The IR

spectrum showed a strong absorption at 1730 cm^{-1} indicating the presence of a carbonyl group. The ^{1}H NMR spectrum (**Fig. 3B.7**) of the compound suggested the presence of an aromatic peak at δ 7.53 and a methoxy group at δ 4.04 ppm. A peak at δ 10.75 corresponds to a hydroxyl group. The ^{13}C NMR spectrum (**Fig. 3B.8**) displayed eight carbon peaks. DEPT-135 showed one methyl carbon, one methine and six tertiary carbons. The peak at δ 158.5 indicated that the carbonyl group is in the cyclic ester or lactone group. The HRESI-MS measured with positive ion mode showed a parent peak at *m/z* 331.0452 which could be the $(M+H)^{+}$ peak suggesting that the compound has a symmetrical skeleton. Compiling all the data, it was found that the compound contains two aromatic protons, two aromatic hydroxyl and two methoxy group. Hence the molecular formula proposed from the analysis is $C_{16}H_{10}O_8$. The protons and carbons were assigned based on COSY, HMBC and HMQC analysis. In comparison with the literature data compound **31** was characterized as **3, 3'-di-*O*-methylellagicacid**.[17] It was reported to exhibit potent antioxidant and α-glucosidase inhibitory activity.[18,19]



Compound **31- 3, 3'-di-*O*-methylellagicacid**

Fraction pool 5 (Fr. 120-160) was dissolved in methanol and precipitated with DCM, yielded a light brown coloured precipitate. It was washed with ethyl acetate and purified, yielding 169 mg of the solid labelled as compound **32.** The ^{1}H NMR spectrum (**Fig. 3B.9**) of the compound showed the presence of two aromatic protons as singlets at δ 7.82 and δ 7.55 ppm and two methoxy protons at δ 4.09 and δ 4.05 ppm. Five oxygenated methine protons resonated between δ 3.23-3.71 ppm (two protons merged with the water peak of DMSO) and hydroxyl protons [δ 5.48 (s), 5.18 (s), 5.08 (s), 4.60 (s)] were observed along with a doublet at δ 5.15 (d, J = 6.5 Hz), indicating the presence of a glucoside moiety. The ^{13}C NMR spectrum (**Fig. 3B.10**) of the compound exhibited the presence of 22 carbon peaks including two ester carbons (-COOR) at δ 158.4 and 158.3 ppm which are due to α, β-unsaturated lactones.

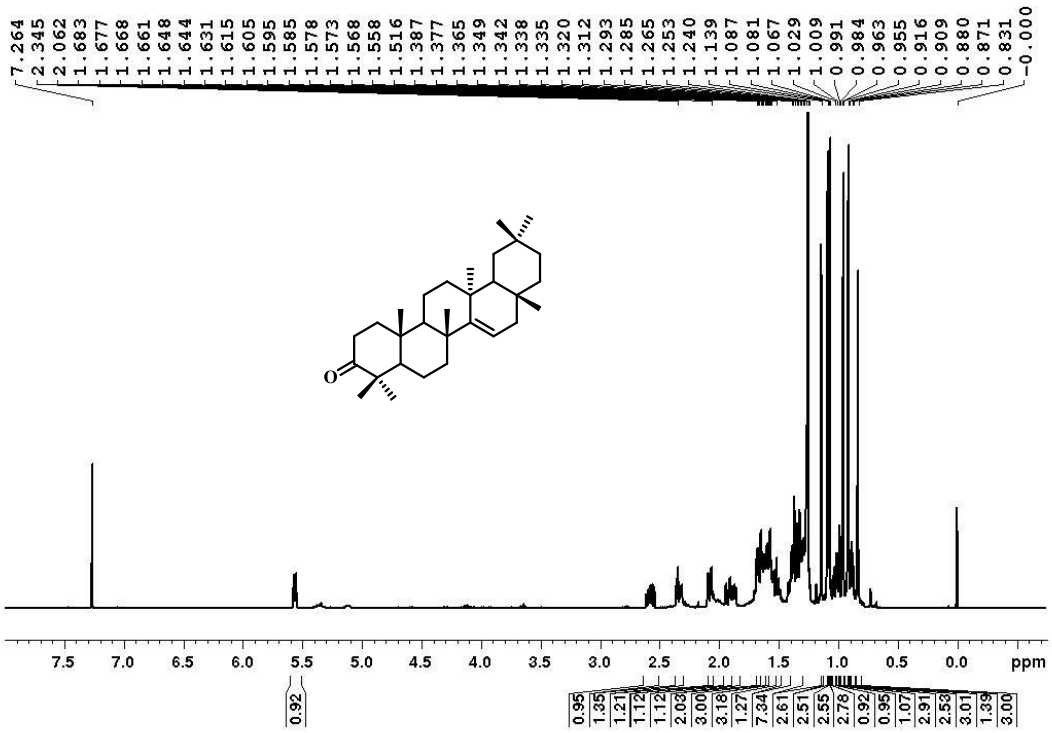


Figure 3B.5. ^{1}H NMR spectrum of compound **30** in $CDCl_3$

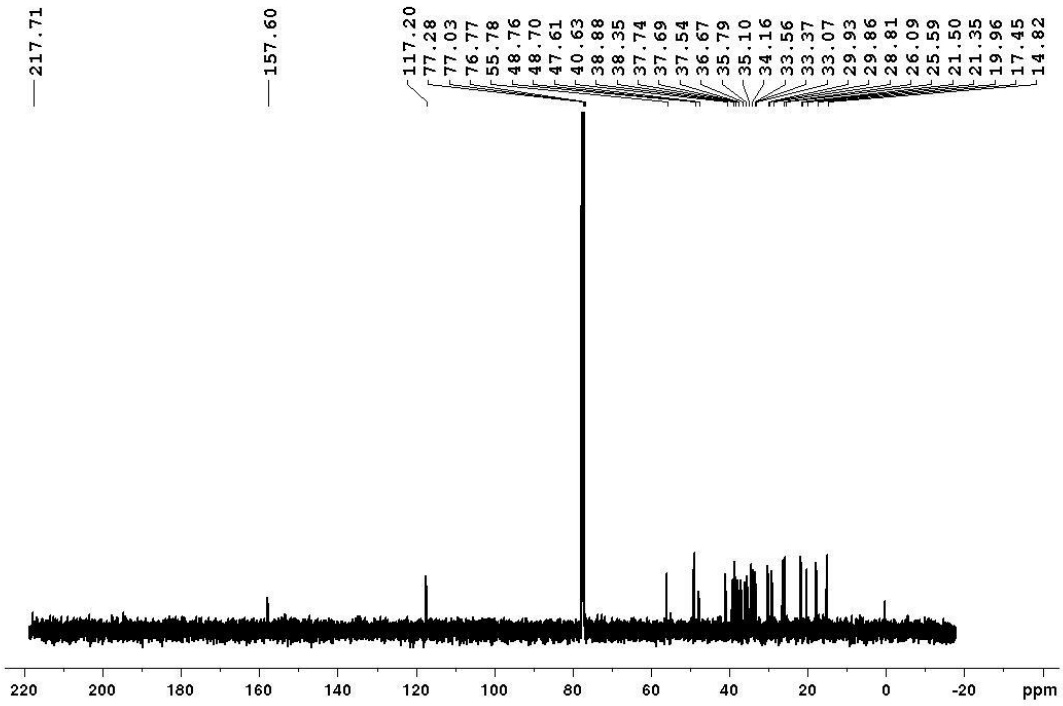


Figure.3B.6. ^{13}C NMR spectrum of compound **30** in $CDCl_3$

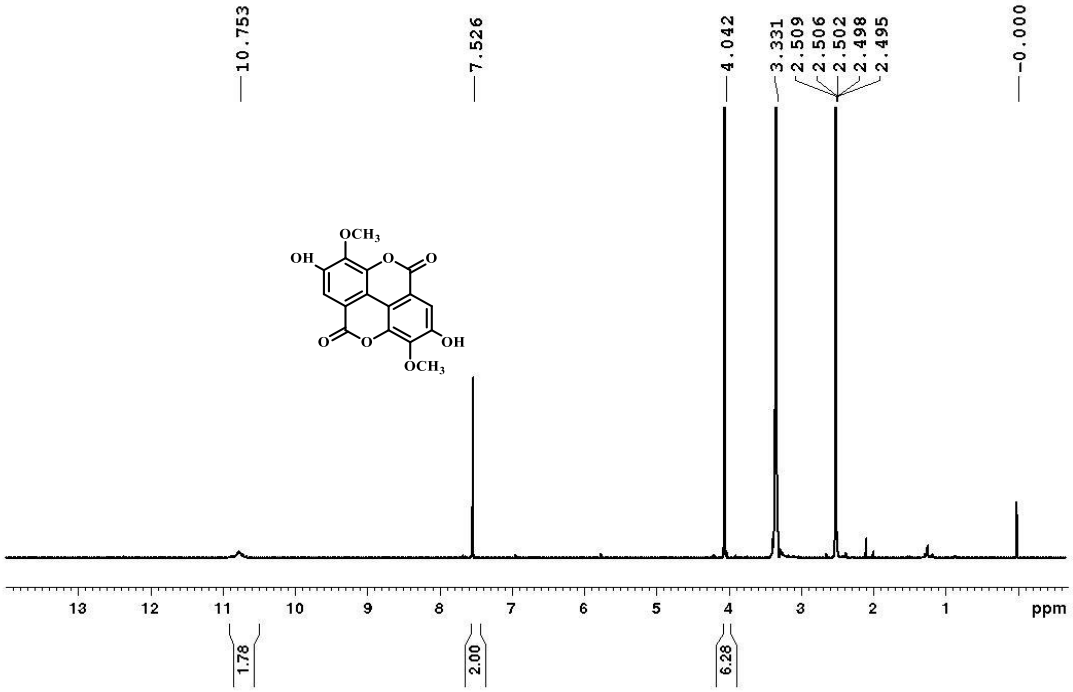


Figure 3B.7. ^{1}H NMR spectrum of compound **31** in DMSO-d6

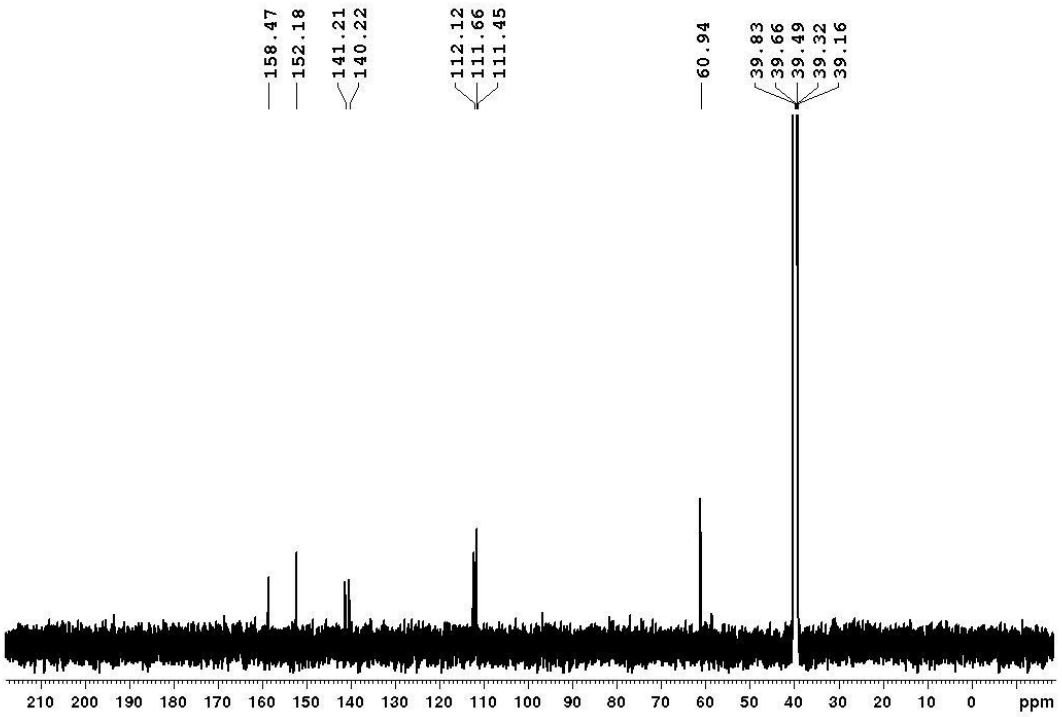


Figure.3B.8. ^{13}C NMR spectrum of compound **31** in DMSO-d6

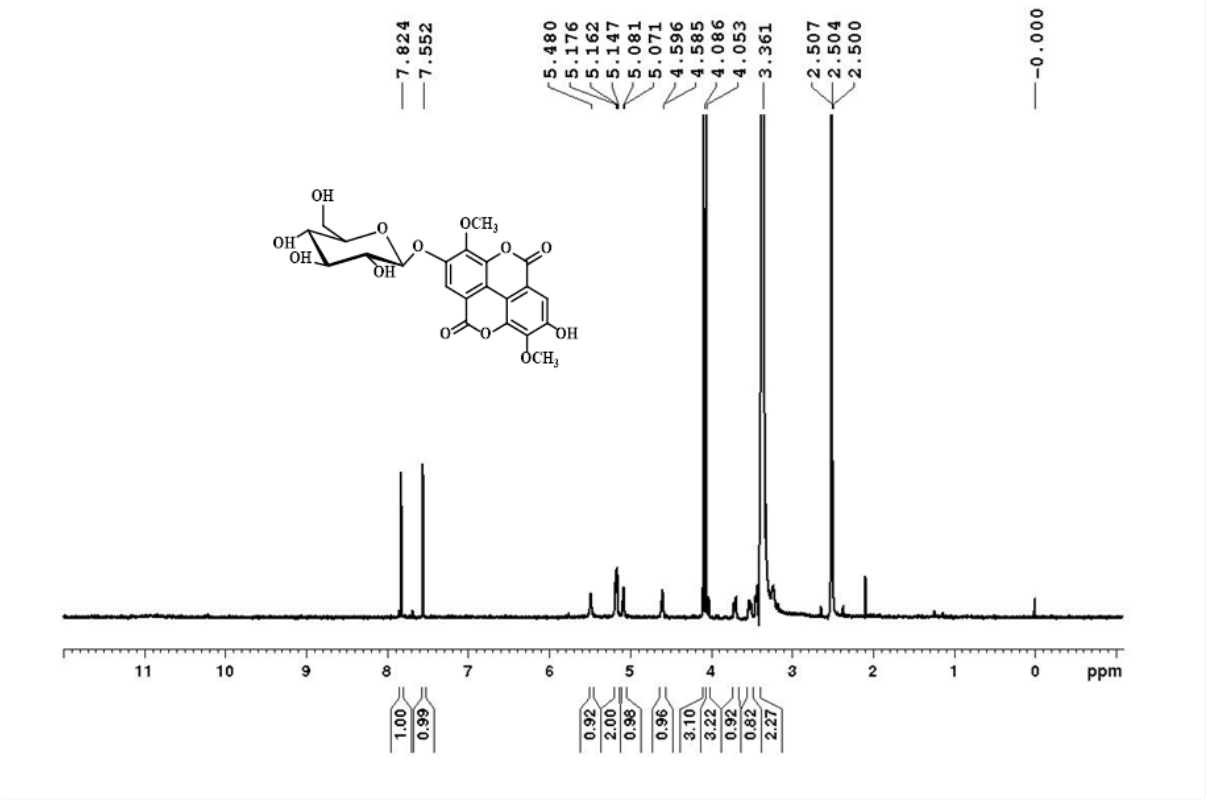


Figure.3B.9. ^{1}H NMR spectrum of compound **32** in DMSO-d6

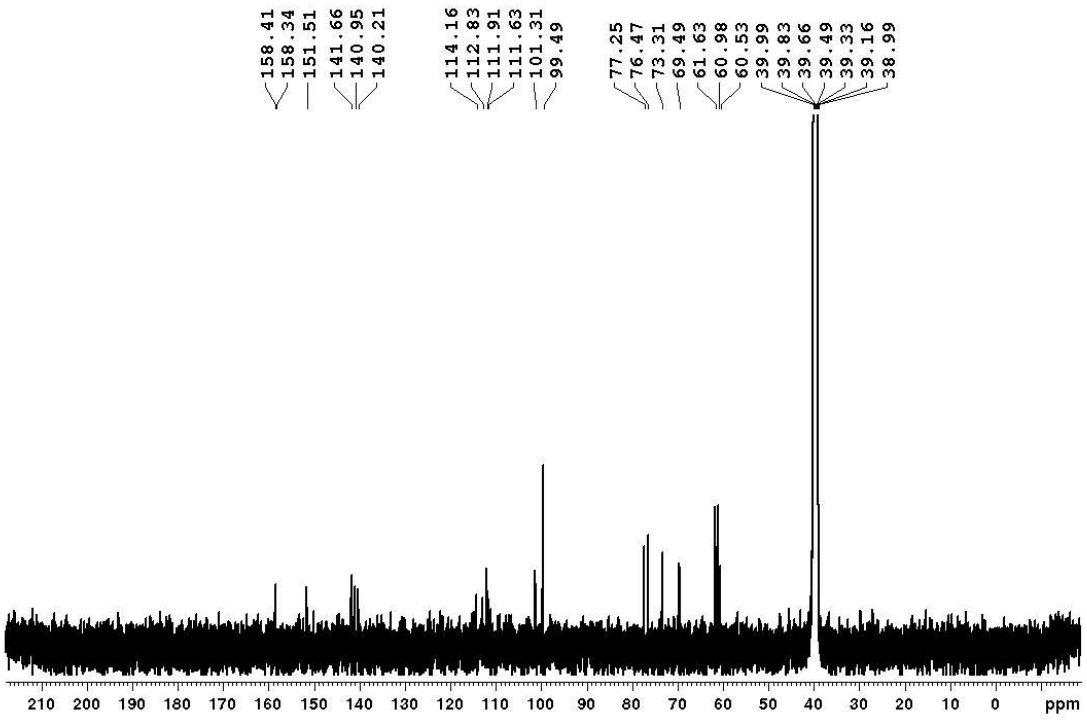
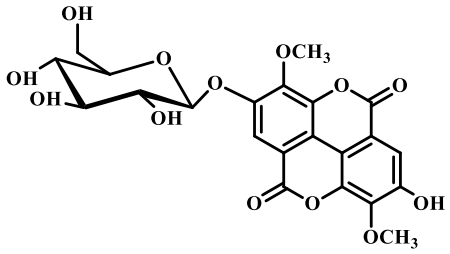


Figure.3B.10. ^{13}C NMR spectrum of compound **32** in DMSO-d6

Six oxygenated aromatic carbons (δ 152.8, 151.5, 141.8, 141.6, 140.9 & 140.2 ppm), four aromatic quaternary carbons (δ 114.2, 112.8, 111.6 & 111.1 ppm), two isolated methine carbon (δ 111.9 ppm) and two methoxy carbons (δ 61.6 & 60.9 ppm) were observed. The anomeric carbon appeared at δ 101.3 ppm. The mass spectrum showed two fragmented peaks at *m/z* 515.0809 and 331.0456 confirms that compound **32** is a glucoside of compound **31.** Based on the literature reports and NMR analysis the compound **32** was identified as **3,3'-di-*O*-methylellagicacid-4-*O*-*β*-D-gluco pyranoside**.[20]



Compound **32- 3,3'-di-*O*-methylellagicacid-4-*O*-*β*-D-glucopyranoside**

3B.4. Preliminary cytotoxicity analysis

We evaluated the cytotoxicity of the ellagic acid derivatives against HeLa cell line using Doxorubicin as standard. The results are shown in **Table 3B.2**. None of the compounds exhibited significant activity up to 100 µM at 24 h. Even though 3,3'-di-*O*-methylellagicacid (**31**) showed better activity than its glucoside (**32**) at 48 h of incubation with an IC_{50} value of 84.26 µM. The variations in different concentrations are shown in **Figure 3B.11**.

Table 3B.2. Preliminary cytotoxicty analysis of compounds

Compound	**IC_{50} of compounds in µM**	
	HeLa	
	24 h	48 h
31	>100	84.26
32	>100	>100
Doxorubicin	>100	9.99

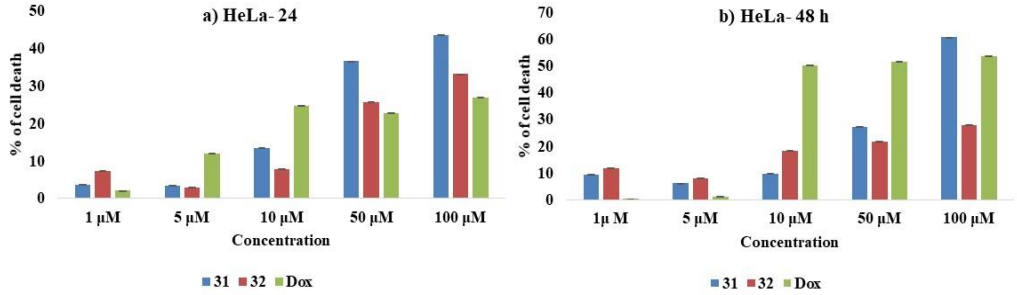


Figure 3B.11. MTT assay of compounds in HeLa cell line at a) 24 and b) 48 h

3B.5. Conclusion

In conclusion, we have isolated seven compounds from the aerial part (stem and bark) of *Bridelia stipularis.* The isolated compounds include the sterols: *β*-sitosterol, stigmasterol, taraxerone and friedelin, ellagic acid derivatives: 3,3'-di-*O*-methylellagicacid and 3,3'-di-*O*-methylellagicacid-4-*O*-*β*-D-glucopyranoside and *β*-sitosterol glucoside. Stigmasterol and the derivatives of ellagicacid are reported for the first time from this plant species and taraxerone reported for the first time from the stem bark.

3B.6. Experimental section

General experimental procedure and cytotoxicity analysis as described in section 2A.6 Chapter 2A and antibacterial studies as in Chapter 3A.8.

3B.7. Spectral data

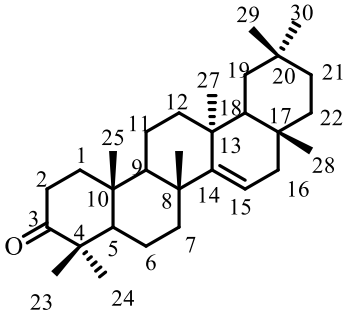
Compound 30 (Taraxerone)

Compound **30** (61 mg) was obtained as a colourless crystalline solid on eluting the column (silica gel 100-200 mesh) with 5 % EtOAc - hexane. The ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound in comparison with literature values, confirmed the molecule as **Taraxerone**.

Molecular Formula: $C_{30}H_{48}O$

FT-IR (Neat, υ_{max} cm^{-1}): 3048, 2939, 2864, 1709, 1450, 1117, 816 cm^{-1}

^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 5.56 (dd, J = 8.0, 3.0 Hz, 1H, H-15), 2.54-2.61 (m, 1H, H-2b), 2.30-2.36 (m, 1H, H-2a), 2.07 (dt, J = 13.0, 3.5 Hz, 1H, H-19b), 1.92 (dd, J = 11.5, 3.0 Hz, 1H, H-16b), 1.85-1.89 (m, 1H, H-1b), 1.48-1.67 (m, 9H), 1.26-1.39 (m,



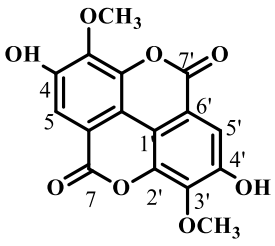
6H), 1.14 (s, 3H, H-27), 1.09 (s, 3H, H-23), 1.08 (s, 3H, H-25), 1.07 (s, 3H, H24), 1.04 (m, 1H), 1.01 (m, 1H), 0.99 (d, *J* = 3.5 Hz, H-18), 0.95 (s, 3H, H-29), 0.92 (s, 3H, H-28), 0.91 (s, 3H, H-30), 0.87 (m, 1H, H-5), 0.83 (s, 3H, H-26) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 217.8 (C-3), 157.6 (C-14), 117.2 (C-15), 55.8 (C-5), 48.8 (C-9), 48.7 (C-18), 47.6 (C-4), 40.6 (C-19), 38.9 (C-8), 38.3 (C-1), 37.7 (C-13), 37.5 (C-10), 36.7 (C-16), 35.1 (C-7), 34.2 (C-2), 33.6 (C-21), 33.4 (C-29), 33.1 (C-22), 29.9 (C-28), 29.9 (C-26), 28.8 (C-20), 26.1 (C-23), 21.5 (C-24), 21.3 (C-30), 20.0 (C-6), 14.8 (C-25) ppm.

HRMS (ESI): *m/z* calcd for $C_{30}H_{48}O$ is 424.3705. Found 447.3598 $(M+Na)^+$.

Compound 31 (3, 3'-di-*O*-methylellagicacid)

Compound **31** (89 mg) was isolated from fraction pool 4 as colourless amorphous solid. The detailed analysis of IR, ^{1}H NMR, ^{13}C NMR, and HRESIMS analysis gave the compound as **3, 3'-di-*O*-Methylellagicacid**.



Molecular Formula: $C_{16}H_{10}O_8$

Melting point: 302 ± 2° C

FT-IR (Neat, υ_{max} cm^{-1}): 3232, 1730, 1612 cm^{-1}.

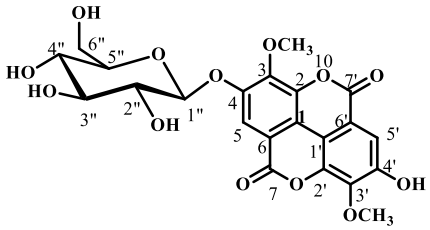
^{1}H NMR (500 MHz, DMSO-d6, TMS): δ 10.75 (s, 2H, -OH), 7.53 (s, 2H, H-5 & H-5'), 4.04 (s, 6H, $-OCH_3$) ppm.

^{13}C NMR (500 MHz, DMSO-d6, TMS): δ 158.5 (C-7 & C-7'), 152.2 (C-4 & C-4'), 141.2 (C-2 & C-2'), 140.2 (C-3 & C-3'), 112.1 (C-6 & C-6'), 111.7 (C-1 & C-1'), 111.4 (C-5 & C-5'), 60.9 ($-OCH_3$) ppm.

HRMS (ESI): *m/z* calcd for $C_{16}H_{10}O_8$ is 330.0375. Found 331.0452 $(M+H)^+$.

Compound 32 (3,3'-di-*O*-methylellagicacid-4-*O*-β-D-glucopyranoside)

Compound **32** was precipitated from fraction pool five as brown coloured amorphous solid. The detailed Spectral and mass analysis gave the compound as 3**,3'-di-*O*-Methylellagicacid-4-*O*-*β*-D-glucopyranoside**.



Molecular Formula: $C_{22}H_{20}O_{13}$

Melting point: 268 ± 2° C

^{1}H NMR (500 MHz, DMSO-d6, TMS): *δ* 7.82 (s, 1H, H-5), 7.55 (s, 1H, H-5'), 5.48 (br s, 1H), 5.18 (br s, 1H), 5.15 (d, *J* = 6.5 Hz, H-5"), 5.08 (s, 1H), 4.60 (s, 1H), 4.07 (s, 3H, -OCH_3), 4.05 (s, 3H, -OCH_3), 3.70 – 3.23 (m, 6H, glycoside) ppm.

^{13}C NMR (500 MHz, DMSO-d6, TMS): *δ* 158.4 (C-7), 158.3 (C-7'), 152.8 (C-4'), 151.5 (C-4), 141.8 (C-3), 141.6 (C-2'), 140.9 (C-2), 140.2 (C-3'), 114.2 (C-1), 112.8 (C-6), 111.9 (C-5), 111.9 (C-5'), 111.6 (C-1'), 111.1 (C-6'), 101.4 (C-1"), 77.2 (C-5"), 76.5 (C-3"), 73.3 (C-2"), 69.5 (C-4"), 61.6 (3-OCH_3), 60.9 (3'-OCH_3), 60.5 (C-6") ppm.

HRMS (ESI): *m/z* calcd for $C_{22}H_{20}O_{13}$ is 492.0903. Found 515.0804 $(M+Na)^+$.

3B.8. References

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Chapter 4A: Isolation of Chemomarkers from the heartwood of *Butea monosperma* (Lam.) Taub

4A.1. Introduction

Butea monosperma (Lam.) Taub is popularly known as the Flame of the forest or Palash, belongs to the family Fabaceae. It is one of the most beautiful trees in our country due to its gorgeous canopy of scarlet of flowers, which looks like a flame. The genus *Butea* named after John Stuart, the third Earl of Bute, a patron of Botany and monosperma meaning 'having one seed.'[1] *Butea monosperma* is a native plant of the Indian subcontinent and south-east Asia: India, Indonesia, Japan, Laos, Myanmar, Nepal, Sri Lanka, Thailand and Vietnam.[2] From ancient times, Palash stick and wood were used in different Indian rituals. It is believed to be a sacred tree; dry stem pieces are used to make sacred fire.[3] The plant is used in Ayurvedic, Unani and Siddha medicine for various ailments and has become a treasure of modern medicine. For example, Pippali Rasayana, an ayurvedic drug prepared from *B. monosperma* and pippali (*Piper longum*), is used in the management of giardiasis.[4] Bark, leaves, flowers, seeds and gum of *B. monosperma* has been traditionally used as anthelmintic, appetizer, aphrodisiac and laxative.[5] In Unani medicine, the temperament of leaves and flowers is cold and dry, while seeds and gum are considered hot and dry. Nadkarni with reference to 'Chakradatta'(a treatise in Hindu medicine), stated the use of its gum as an external astringent application, leaves and flowers are tonic, astringent, aphrodisiac and diuretic, and seeds are laxative and anthelmintic.[6] Almost all the parts of the plant *viz.* root, fruit, leaves, stem bark, flowers, gum, young branches are utilized for medication and different purposes.

4A.1.1. Vernacular names in India

Palasha, Kimshuka, Raktapushpaka (Ayurvedic), Palasa (Sanskrit), Dhak, Palas (Hindi), Flame of forest, Parrot Tree (English), Kesudo (Gujarati), Moduga (Telugu), Palasamu, Palasam, Purasus (Tamil), Muthuga (Kannada), Chamatha, Brahmavriksham, Kimshukam,(Malayalam).[2]

(a) **(b)** **(c)**

Figure 4A.1. a) flower b) fruit and c) stem bark of *Butea monosperma*

4A.1.2. *Butea* species

There are various species of *Butea monosperma* available over the world they are as; *B. acuminate, B. africana, B. affinis, B. apoensis, B. balansae, B. bracteolate, B. braamiana, B. cuneiforms, B. crassfolia, B. dubia, B. ferruginous, B. gyrocarpa, B. harmandii, B. laotica, B. littoralis, B. listeri, B. loureirii, B. maingayi, B. macroptera, B. merguensis, B. minor, B. oblong folia, B. parviflora, B. peltata, B. pellita, B. philippinensis, B. potting, B. pulchara, B. purpurea, B. ridleyi, B. riparia, B. rosea, B. sanguinea, B. spirei, B. sericophylla, B. squirmier, B. superba, B. suberecta, B. varians, B. volubilis, etc.*[7]

4A.2. Ethnopharmacology

Almost all parts of *B. monosperma* are being utilized for decades in traditional or folklore medicine for numerous diseases. Roots are used as a remedy against impotence, night blindness and elephantiasis. The powdered roots are used to apply to injuries caused by snake bites. Stem juice is taken for goiter, paste of the stem bark can be applied for body swellings. Powder of stem bark is used to apply on injuries caused by axe. The ash of young branches in combination with other drugs is prescribed for scorpion sting. The bark is useful in biliousness, liver disorder, dysmenorrhea, gonorrhoea and purifying the blood. Leaf juice is taken to cure a cold, cough, stomach disorders and conjunctivitis. Leaf extract possesses antidiabetic property and is also used for menstrual problems. The decoction of leaves is used for sweating of phthisis, as retention enema in cases of diarrhoea and dysentery, and into the vagina in cases of leucorrhoea. Crushed flowers, along with milk taken for one month, can reduce body heat and fever. Drinking the infusion made by soaking the flowers in water overnight can cure leucorrhoea and strangury. The dried flower

is soaked into water for the whole night to get saffron-coloured water used for bathing of small children to protect from different skin problems and as a coloring matter in Holi festivals. The flower extract with water is used for gastritis (hyperacidity). Flowers are used as medicine for removing urine blockage. Fruits and seeds are useful in piles, eye diseases and inflammation. Seed decoction and flower infusion are used as a diuretic in dysuria and retention of urine. Seeds are crushed in milk and taken orally to treat kidney stones and urinal complaints. Powdered seeds are given to children as a remedy for intestinal worms. *Butea* gum (Bengal kino), the red juice from the bark, have medicinal properties. The plant's gum is also useful in curing dysentery and can be applied for cracks in foot sole. [6,8–12]

4A.3. Phytochemistry

Stem bark: Bandara *et al.,* reported medicarpine, 5-methoxygenistein, prunetin, lupenone, lupeol and *β*-sitosterol from the stem bark of *B. monosperma*.[13,14] Yadav *et al.,* reported a flavonoid glycoside 8-C-prenylquercetin 7,4'-di-O-methyl-3-O-*α*-L-rhamnopyranosyl-(1→4)-*α*-L-rhamnopyranoside.[15] Maurya *et al.,* identified lupeol, buteaspermin A, buteaspermin B, buteaspermanol, lupeonone, flemmichapparin C, medicarpin, prunetin, 3-O-acetyl-8,9-methylenedioxycoumestan, 3-methoxy-8,9-methylenedioxypterocarp-6-ene, nonacosanoic-2',3-dihydroxypropyl ester, cajanin, pentacosanoicacid 2,3-dihydroxypropyl ester, docosanoic acid, 2-methyl-7-hydroxy-4-methoxyisoflavone, formonentin, isoformonentin, cladrin, daidzein, genistein, 2',4',5,7-tetrahydroxy- isoflavone and ononin from the stem bark.[16] Mishra *et al.,* reported stigmasterol, stigmasterol-*β*-D-glucopyranoside, nonacosanoic acid, 3α-hydroxyeuph-25-ene and 2,14-dihydroxy-11,12-dimethyl-8-oxo-octadec-11-enyl cyclohexane.[17] Shukla *et al*. reported stigmasterol-3-*α*-L-arabinopyranoside, 3-methoxy-8,9-methylenedioxypterocarp-6-ene, 4-pentacosanylphenol, 21-methylene -22-hydroxy-24-oxooctacosanoicacid methyl ester and pentacosanyl-*β*-D-gluco pyranoside.[18] Jafri *et al.,* reported tetratriacont-15-ene, heptacos-11-ene, 15-hydroxylethyl heptadec-12-enoate and 10-hydroxy dodecyltridec-5-enoate.[19] Kaur *et al.,* reported gallic acid, quercetin, ellagic acid, rutin, coumaric acid, umbelliferone, caffeic acid, epicatechin, chlorogenic acid, catechin and kaempferol.[20] Flavone quercetin and cholesterol were also reported from the bark. [21,22]

Leaves: 3,9-dimethoxypterocarpon, a triterpenoid ester 3*α*-hydroxyeuph-25-enyl hepta- cosanoate, gallic acid, *β*-carotene and stigmasterol isolated from the leaves.[23–25] GC-MS analysis of the chloroform extract revealed the presence of major steroids: *β*-sitosterol, γ-sitosterol, stigmasterol acetate, *β*-sitosterol acetate, stigmastan-3,5-diene, cholesterol, cholesta-3,5-diene, dihydrotachysterol, 26-hydroxycholesterol and retinol.[26]

Flowers: In 1955, Puri and Seshadri reported a flavonoid glycoside palasitrin.[27] Later, Gupta *et al.,* reported another group of flavonoid glycosides isocoreopsin, sulphurein, coreopsin, butrin, isobutrin, butin, butein, monospermoside and isomonospermoside.[28] Chokchaisiri *et al.,* reported dihydromonospermoside, isoliquiritigenin, (-)-liquiritigenin and afrormosin.[29] Yadava and Tiwari isolated a flavone molecule 5,7-dihydroxy-3,6,4'-trimethoxyflavone-7-O-*α*-L-xylopyranosyl-(1→3)-O-*α*-L-arabinopyranosyl - (1→4)-O-*β*-D-galactopyranoside from the methanol soluble fraction of flower.[30] Shah *et al*., reported glucose, fructose, histidine, aspartic acid, alanine and phenylalanine.[31] Ahmed *et al.* reported sulphuretin 3'-O-*β*-glucopyranoside, sulfurein, butein, monospermoside, isobutrin, coreopsin, butin, isomonospermoside, formononetin, aformosin, kaempferide, umbelliferone, 7,8-dihydroxychromone, methyl-3,4,5-trihydroxybenzoate and 2,4-dihydroxybenzoic acid.[32] Oberoi *et al.,* reported a plant pigment lanceoletin from the petals. [33] Namratha *et al.,* reported a flavanone di-glucoside, butaspermin from the alcoholic extract of flowers.[34] Subramaniyan *et al.,* reported a novel compound sodium salt of butrin.[35] Rutin, quercetin and gallic acid are also reported from the flower extracts.[36,37]

Seeds: Mehta *et al.,* reported the alkaloid monospermin and Bochis *et al.,* reported palasonin from the seeds.[38,39] Singh *et al.,* reported acid esters, jalaric ester-I, jalaric ester-II, laccijalaric ester-I and laccijalaric ester-II from the soft resin fraction of seedlac.[40] Porwal *et al.,* reported a triazine 4-carbomethoxy-3,6-dioxo-5-hydro-1,2, 4-triazine, allophanic acid and a derivative of allophanic acid 2-hydroxy-ω-methylallophanic acid from the seed coat. [41,42] Yadava and Tiwari isolated a flavone glycoside 5,2'-dihydroxy-3,6,7-trimethoxyflavone-5-*O*-*β*-D-xylopyranosyl-(1→4)-*O*-*β*-D-glucopyranoside from the seeds.[43] n-Docosanoic, n-octacosanoic, n-

dotriacontanoic acids, *β*-sitosterol xyloside and buteagibrenol glycoside were reported from methanolic extract of seeds.[44]

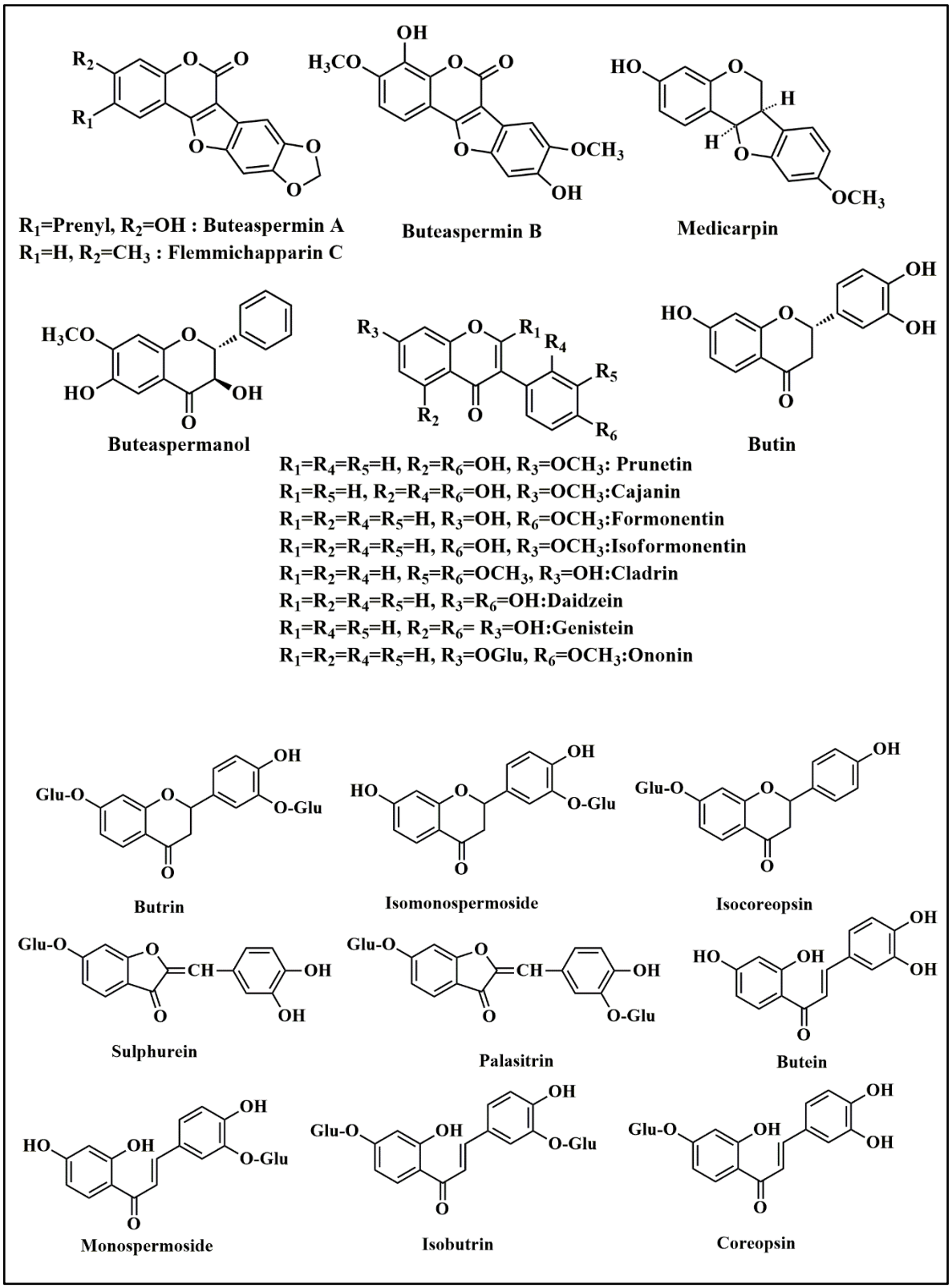


Figure 4A.2. Chemical structure of some compounds reported from *B. monosperma*

Sharma *et al.,* isolated 15-hydroxypentacosanoic acid and 1-carbomethoxy-2-carbomylhydrazine from the ethanolic extract.[45] Saxena *et al.,* reported an isoflavone glycoside 5,6,7,4'-tetrahydroxy-8-methoxyisoflavone-6-O-rhamnopyranoside.[46] Bishnoi and Gupta reported a *δ*-lactone of *n*-heneicosanoic acid.[47] Chandra *et al.,* reported *α*-amyrin, *β*-sitosterol, *β*-sitosterol-*β*-D-glucoside and sucrose.[48] Butin, 16-dihydroxyhexadecanoic acid, oleic, linolic, palmitic and lignoceric acids, an acid imide and a lectin reported from the seeds.[49–53] Phospholipids identified from the seeds were phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, lysophosphatidyl choline and cardiolipin.[54]

Pods: Guha *et al.,* reported an imide named palasonin-*N*-phenylimide (palasimide) from the pods of *B. monosperma*.[55]

Gum: Tannins, (-)-leucocyanidin (5,7,3',4'-tetrahydroxyflavan-3,4-diol) and tetramers of leucocyanidin were reported from the gum of *B. monosperma* .[56–58]

4A.4. Pharmacology

Different parts of *Butea monosperma* possess various biological activities such as antimicrobial, antihelmintic, anticonvulsive, antifertility, antidiarrhoeal, antigiardiasis, wound healing, hepatoprotective, antitumor, antihypertensive, antidiabetic, antiinflammatory and antioxidant activity. Sharma *et al.,* evaluated the antihyperlipidemic, antihyperglycemic and antioxidative properties of hydroethanolic extract of *B. monosperma* bark in alloxan-induced diabetic mice. Diabetic animals were daily treated with crude ethanolic extract of *B. monosperma* bark (300 mg kg^{-1}), significantly lowered blood glucose level and elevated hepatic glycogen content, bringing the values close to those observed in normal control and glibenclamide-treated diabetic mice.[59] Deore *et al.,* reported the hypoglycemic activity of the bark extracts (aqueous and methanol) in normal and alloxan-induced diabetic albino rats. The aqueous extract significantly reduced the blood glucose level in both normal and diabetic mice.[60] Yadav *et al.,* studied the antidiabetic potential of aqueous extract of bark in insulin-dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM). The aqueous extract of bark substantially declined the plasma glucose level in both IDDM and NIDDM animal subjects by 7.2 % and 26.6 %, respectively and also lowered the serum lipid

profile.[61] The ethanolic extract of the bark modulates dyslipidemia in streptozotocin-induced diabetic rats.[62]

Sharma and Garg reported the antidiabetic and antioxidant potential of ethanolic extract of leaves in alloxan-induced diabetic mice. Fasting blood glucose level was decreased significantly following 45 days of treatment of extract from 172 to 117.143 mg/dl, as compared to normal control (79.286 mg/dl).[63] The antidiabetic effect of ethanolic extract of leaves on adrenaline-induced and glucose fed diabetic rabbits displayed the most productive activity in reducing blood sugar level at 8 h reading 182.5 ± 3.83 mg/dl for 400 mg dose.[64] Harish *et al.,* evaluated the antidiabetic potential of aqueous extract of leaves and stem bark using various *in vitro* techniques. Both leaves and bark promoted the glucose uptake in yeast cells. Leaves inhibited α-amylase, α-glucosidase and sucrase enzymes in succession to varying degrees. Still, bark inhibited only α-amylase and slightly activated other two.[65] Samad *et al.,* reported the antihyperglycemic activity of ethanolic extract of leaves in type 2 diabetic model rats and isolated rat Islets. Significant insulin secretagogue activity of *B. monosperma* was found in serum insulin assay of extract-treated type 2 diabetic rats.[66] Aqueous extracts of *B. monosperma* leaves and bark does not produce significant antihyperglycemic activity on blood glucose level in severely diabetic rats.[67]

Das *et al.,* reported the antiepileptic activity of the crude methanol extract of stem and lupeol using Pentylenetetrazole (PTZ) induced convulsion (chemically induced convulsion) and Maximal electroshock (MES) induced convulsion (electrically induced convulsion) models in Swiss albino mice. Lupeol exhibited anticonvulsant effect in PTZ induced convulsions at 20 mg/ kg p.o. *via* the GABA mediated action. [68] The methanolic extract of leaves exhibited anticonvulsant activity. The extract significantly delayed the onset of convulsions induced by pentylenetetrazole and maximal electroshock seizure in a dose-dependent manner.[69] Kasture *et al*., reported anticonvulsant activity of the acetone-soluble part of the petroleum ether extract of *Butea monosperma* flowers.[70] Further studies revealed that the triterpene isolated from the n-hexane: ethyl acetate fraction of the petroleum ether extract of flowers showed anticonvulsant activity in pentylenetetrazol-induced seizures.[71]

Kaur *et al.,* reported the antiproliferative activity of different extracts of bark against the MCF-7 breast cancer cells *via* the apoptotic pathway. Apoptosis induction in MCF-7 cells caused by both chloroform and ethyl acetate fractions observed to be associated with DNA damage, ROS generation, and the loss of MMP. [20] Tablets formulated by using the methanolic extract of stem bark using microcrystalline cellulose as filler and PVP-k30 as a binder by direct compression method analyzed for anticancer activity. The results indicated a moderate activity of the stem bark compared to the standard drug.[72] Banurekha and Jaykar reported the anticancer activity of the ethanolic extracts of leaves against Ehrlich Ascites Carcinoma in Swiss albino mice. The extract showed significant anticancer properties at a dose of 200 and 400 mg/kg body weight. [73] Hiremath *et al*., reported the anticancer activity of seeds attributed to the presence of a lectin.[74] Choedon *et al*., reported antioxidative, anti-inflammatory, hepatoprotective and anticancer activities of the aqueous extract of *B. monosperma* flowers. The aqueous extract of the flower was tested for its antiproliferative, proapoptotic and anticarcinogenic effects in hepatoma cell lines. Extract exhibited a strong anticancer activity (growth inhibition, cell cycle arrest, pro-apoptotic activity and interference with mitogenic signaling) in hepatoma cells and showed a minimal cytotoxic effect on non-transformed AML12 hepatocytes.[75] The flavonoids isocoreopsin, butrin and isobutrin isolated from the flowers examined for their anticancer activity and isocoreopsin showed significantly higher efficacy in cell death on human colon and liver cancer cell lines (50 µg/mL in HT-29 and 100 µg/mL in HepG2) than butrin (100 µg/mL in HT-29 and 500 µg/mL in HepG2) and isobutrin (80 µg/mL in HT-29 and 150 µg/mL in HepG2).[76]

Mehta *et al.,* reported the in *vitro* antimicrobial activity of seed oil in several human pathogenic bacteria and fungi, showed a significant effect.[77] Similarly, the methanolic extract of seed displayed antimicrobial activity against *E. coli*, *P. aeruginosa* and *S. aureus*.[78] Dhale *et al.,* reported antimicrobial activity of various extracts of leaves (ethanol, chloroform and petroleum ether). The ethanol extract was found to be more effective against *B. subtilis* and *S. aureus* (13 mm at 100 mg/ml).[79] A combination of leaf extracts of *Cardiospermum halicacabum* and *Butea monosperma* extracts exhibited good antimicrobial activity.[80] Sahu and Padhy reported the antibacterial activity of petroleum ether, acetone, ethanol, methanol

and water extracts of leaves against clinically isolated 12 Gram-positive and -negative multidrug-resistant (MDR) pathogenic bacteria. The water and ethanol extract of leaves shown significant antibacterial activity against all bacteria.[81] Rajput *et al*., reported the antibacterial activity of petroleum ether, chloroform and methanol extracts of leaves against *E. coli*, *B. subailis*, *P. aeruginosa, P. vulgaris*, *S. aureus*, and *K. pneumonia*. The methanol and petroleum ether extract exhibited potent activity as compared to chloroform extract.[82] Similarly, Zafar *et al.,* also reported the antibacterial activity of chloroform, methanol and aqueous extracts.[83] Tarannum *et al*., screened the various extracts of bark (ethanol, methanol and water) for antivenom and antifertility activity. The ethanol extract successfully inhibited the ovine, mouse testicular as well as *Vipera russelli* snake venom hyaluronidase enzyme activities, with IC_{50} values of 12.00 ± 0.45, 49.40 ± 1.58 and 125.42 ± 2.82 μgmL^{-1}. The activities are ascribed to the antioxidant potential and the phytochemical constituents of the plant, mainly polyphenols.[84] Reddy *et al.,* reported the antifertility activity of methanolic leaf extract in male albino rats. Administration of extract (200-400 mg/kg body weight) for 21 days significantly reduced sperm count, sperm motility and also reduced reproductive organ weights.[85]

The methanolic extract of the bark of *B. monosperma* is effective against free radical-mediated gastrointestinal ulcerative diseases and showed both anti-inflammatory and analgesic activity in a dose-dependent manner.[86,87] The flavonoid fraction isolated from the stem bark exhibited anti-inflammatory activity against carrageenan-induced rat paw oedema and cotton-pellet induced granuloma by modulating cyclooxygenase, lipoxygenase enzymes and augmenting antioxidant defense system in the inflammation bearing rat.[88] The methanolic extract of the leaves exhibited significant activity (400 mg/kg body weight), by the inhibition of mediators of inflammation such as histamine, serotonin and prostaglandin, in albino rats.[89] Leaves arka gel displayed ocular anti-inflammatory activity in rabbits.[90] The petroleum ether extract of *B. monosperma* leaves possesses antinociceptive and anti-inflammatory activities. Extract at a dose of 200 and 400 mg/kg significantly reduced writhings and stretchings induced by acetic acid (nociceptive stimuli) and the formation of edema.[91] Butrin, isobutrin and butein, isolated from *B. monosperma* flower, inhibited the PMACI-induced activation in human mast cells. The

polyphenols down regulated the TNF-α, IL-6, and IL-8 production *via* repressing the NF-κB activation in HMC-1 mast cells.[92] Methanolic extract of flowers exhibited anti-inflammatory activity against carrageenan-induced paw edema and cotton pellet granuloma in albino rats at oral doses of 600 mg/kg and 800 mg/kg. The serum levels of lysosomal enzymes and lipid peroxidation were significantly reduced by the administration of methanolic extract of B*. monosperma* flowers.[93] The protective effect hydroethanolic extract flowers against skin inflammation was studied in normal human keratinocyte cells (cells which involved in the skin inflammatory response). The extract was able to decrease the secretion of IL-1β, IL-6 and IL-8 pro-inflammatory cytokines of 32, 33 and 18 %, respectively.[94] A flavone glycoside with potential antiviral activity was reported from the seeds.[43] Ansari *et al.,* reported the chondroprotective effect of hydromethanolic extract of flowers standardized to the concentration of butein on human osteoarthritis (OA) chondrocytes stimulated with IL-1β. The extract has strong potential to activate autophagy and suppress IL-1β induced expression of IL-6 and MMP-3, -9 and -13 in human OA chondrocytes.[95] The methanol and aqueous extracts of bark exhibited aphrodisiac activity. The methanolic extract inhibited Rho-kinase 2 (ROCK-II) enzyme activity and relaxed phenylephrine precontracted isolated CCSM of rats significantly.[96,97]

4A.5. Aim and Scope of the present study

Butea monosperma, an important medicinal tree in India, is extensively used in Ayurveda for its innumerable medicinal properties. The plant is highly used by the tribal and rural people in curing various diseases. In Ayurveda, during Kayakalpa treatment, the patient is allowed to eat a specially prepared Rasayana called Amalaki Rasayana (described in chapter 4B). The Rasayana is made by heating it in a vessel made by the heartwood of *B. monosperma*. Since the majority of the population is dependent on traditional and ayurvedic medicines for primary health needs, therefore it is necessary to validate the quality of ayurvedic products. So, we undertook a study to analyse the role of Palash in the therapeutic property of amla, when it is processed in the wooden vessel. For that, first we carried out the phytochemical reinvestigation of the heartwood of *Butea monosperma* (Chapter 4A) followed by the biological evaluation of Amalaki Rasayana (Chapter 4B).

4A.6. Extraction, isolation and characterization of compounds from the heartwood of *Butea monosperma*

4A.6.1. Collection and extraction of plant material

The plant material of *Butea monosperma* was collected from Paravur region of Kollam district of Kerala, India. Voucher samples of the plant was deposited at the Department of R and B, Government Ayurveda College, Kannur (voucher number 2018-06-05). Approximately 1.5 kg of the plant material was coarsely powdered and subjected to repeated extraction with acetone (2.5 L × 48 h) using mechanical stirrer for about four times at room temperature. Thin-layer chromatography indicated that the extraction was completed after nine days. The total extract was then concentrated under reduced pressure using a Heidolph rotatory evaporator, yielded 33 g of the crude extract. In the same way, we obtained ethanol extract of about 20 g. A pictorial representation of the entire isolation procedure is given in **Figure 4A.3.**

4B.6.2. Preliminary cytotoxicity analysis

The acetone and ethanol extract were tested for cytotoxicity analysis against various cell lines such as SAS (oral cancer), MDAMB (breast cancer), A549 (lung cancer) and HCT15 (human colon carcinoma). The results are shown in **Table 4A.1**. The results indicated that the acetone extract was more cytotoxic towards cancer cell lines than the ethanol extract. Hence further isolation was focused on the acetone extract.

Table 4A.1. Percentage of inhibition of acetone and ethanol extract of *Butea monosperma*

Extract	Percentage of Inhibition			
	SAS (10 µg/ml)	**MDAMB 231** (10 µg/ml)	**A549** (25 µg/ml)	**HCT15** (25 µg/ml)
Acetone	42.97	15.8073	24.3039	31.6089
Ethanol	21.29	9.37585	-2.2654	-0.4194

4A.6.3. Isolation and characterization of compounds

After studying the TLC extensively, 33 g of the crude acetone extract was subjected to silica gel column chromatography (100-200 mesh) and elution started with 100 % hexane and increased the polarity by adding an adequate amount of ethyl acetate. A total of 101 fractions were collected; approximately 500 ml were collected. Fractions were pooled into ten fraction pools based on TLC profile. The fraction pools on further purification resulted in the isolation of compounds **32-39.** The extraction process is shown in **Figure 4A.3.**

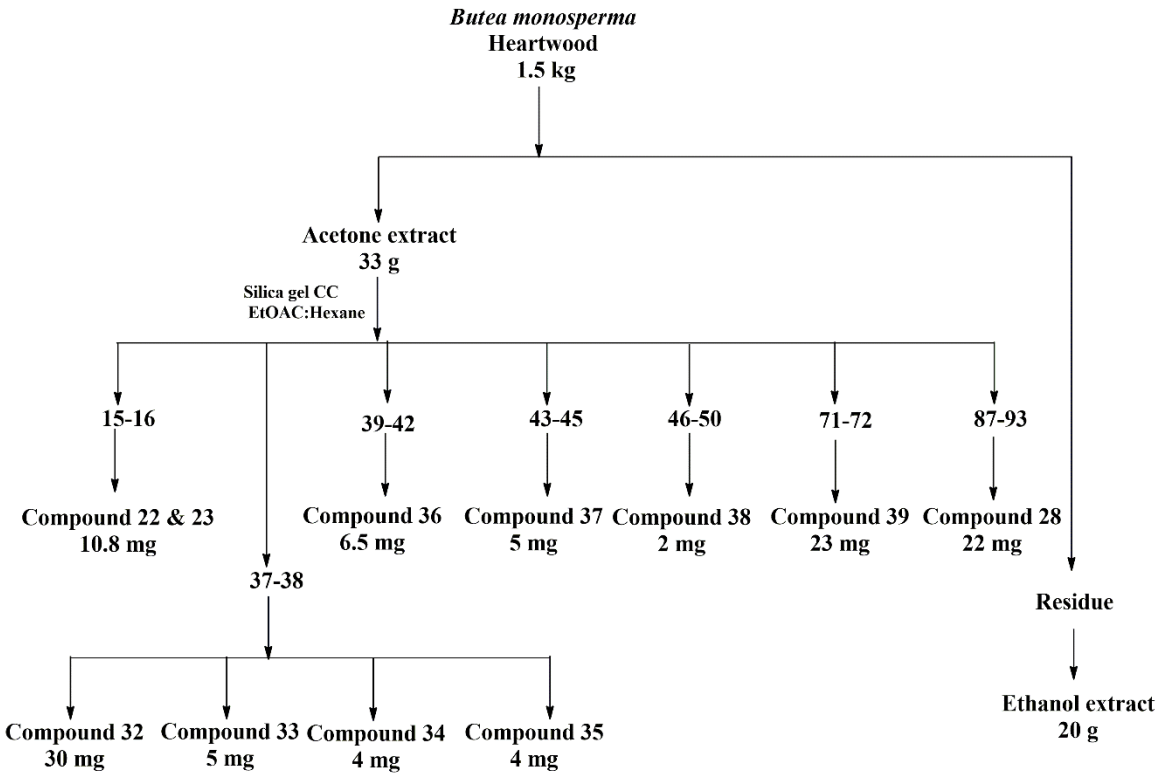
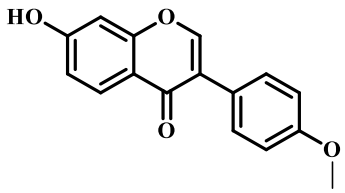


Figure 4A.3. Extraction Process

The fraction pool 2 (fr.15-16) obtained by column chromatographic separation of acetone extract were found to contain colourless needle-like crystals of 10.5 mg. It was characterized as a mixture of common phytosterols, ***β*-sitosterol** and **stigmasterol** based on various spectral data and literature reports.

Compound **32** was obtained from fraction pool 4 (Fr. 37-38) as a white amorphous powder of about 30 mg from the main column. The melting point of the compound was found to be 252-256° C. The HRESIMS analysis established the

molecular formula as $C_{16}H_{12}O_4$ with a parent ion peak at *m/z* 269.0818, which is the $(M+H)^+$ peak. The ^1H NMR spectrum (**Fig. 4A.4**) showed the presence of eight aromatic protons. One singlet observed at *δ* 8.16 was the characteristic peak of an isoflavone skeleton and corresponding olefinic oxymethine signal observed at *δ* 153.7 ppm attributed to the H-2 and C-2 of A ring of an isoflavone. A singlet at *δ* 9.61 corresponds to the –OH proton and three proton singlet at *δ* 3.83 indicated the presence of $–OCH_3$ group. The spectrum showed the presence of two pairs of *ortho*-coupled doublets, integrating for two protons each, centered at *δ* 7.46 (d, *J* = 8.5 Hz, 2H) and *δ* 6.98 (d, J = 8.5 Hz, 2H), which could be attributed to the B-ring aromatic protons of an isoflavone. The ^{13}C NMR spectrum (**Fig. 4A.5**) displayed 16 carbon peaks which were assigned to eight methine, seven quaternary carbons, including the carbonyl group (C-4) at *δ* 175.6 and one methoxy group at *δ* 55.6 ppm was in agreement with the molecular formula. The carbon attached to –OH group appeared at *δ* 163.1 (C-7). The HMBC spectrum showed the correlation between the methoxy carbon and C-4' carbon confirms that the methoxy group is attached to the C-4' carbon. Hence from all the spectral data and literature report compound **32** was identified as **Formononetin**.[98]



Compound **32**- **Formononetin**

Analyzing the TLC of the remining portion of fraction pool 4 after the separation of compound **32** showed two UV active spots. It was further subjected to silica gel CC separation which resulted in the isolation of two compounds. An orange solid (5 mg) obtained was labelled as compound **33**. The HRESIMS analysis showed the molecular ion peak at *m/z* 255.0661, which is the $(M+H)^+$ peak and gave a molecular formula $C_{15}H_{10}O_4$. In ^1H NMR spectrum (**Fig. 4A.6**) the signals displayed at *δ* 7.47 (d, *J* = 8.5 Hz, 2H) & 6.89 (d, *J* = 8.5 Hz, 2H), respectively corresponded to two o*rtho*-coupled aromatic protons (2', 6' and 3', 5') which was the characteristics peak pattern for a disubstituted isoflavone skeleton, while *meta*-coupled aromatic protons resonated at *δ* 6.90 (d, *J* = 2.5 Hz, 1H) ppm.

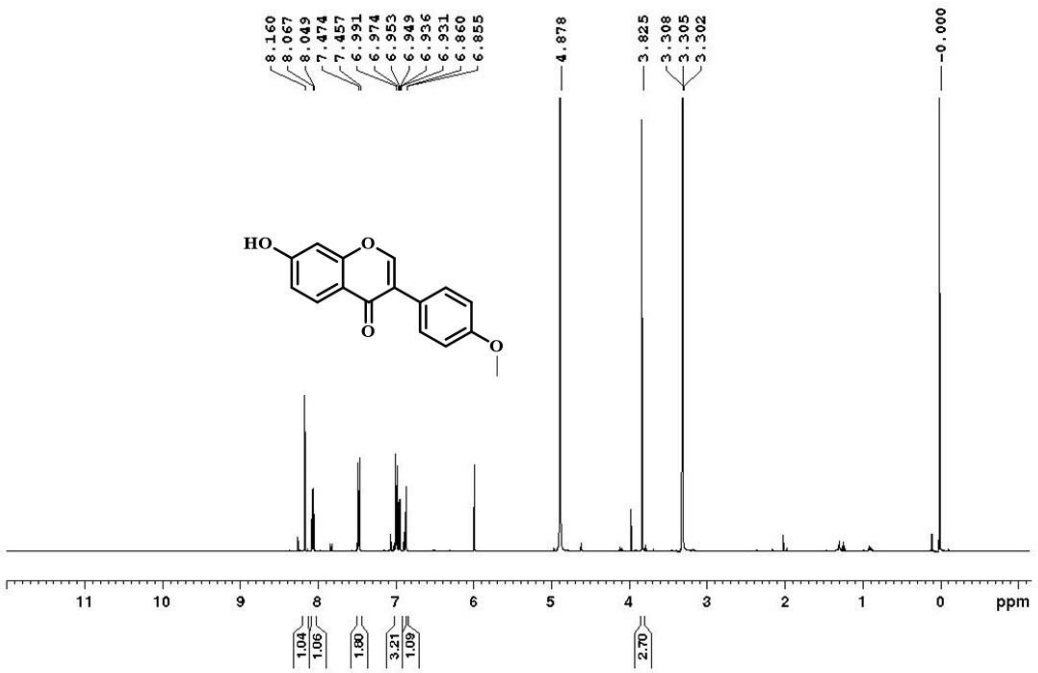


Figure 4A.4. ^{1}H NMR spectrum of compound **32** in MeOD-d4

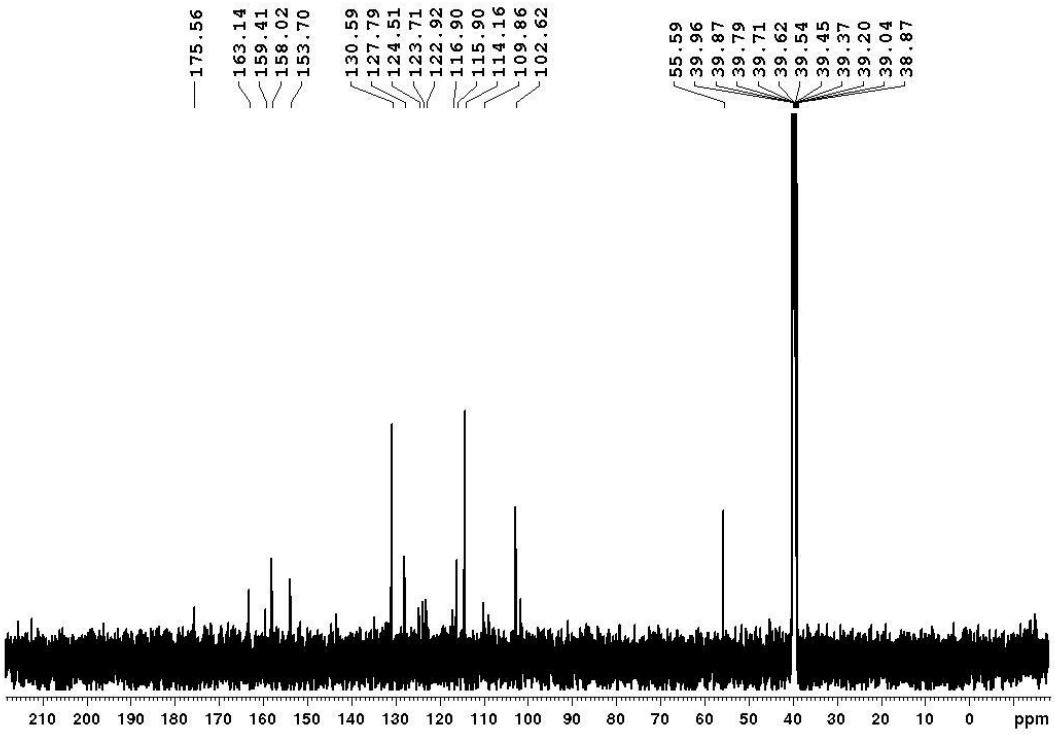


Figure 4A.5. ^{13}C NMR spectrum of compound **32** in DMSO-d6

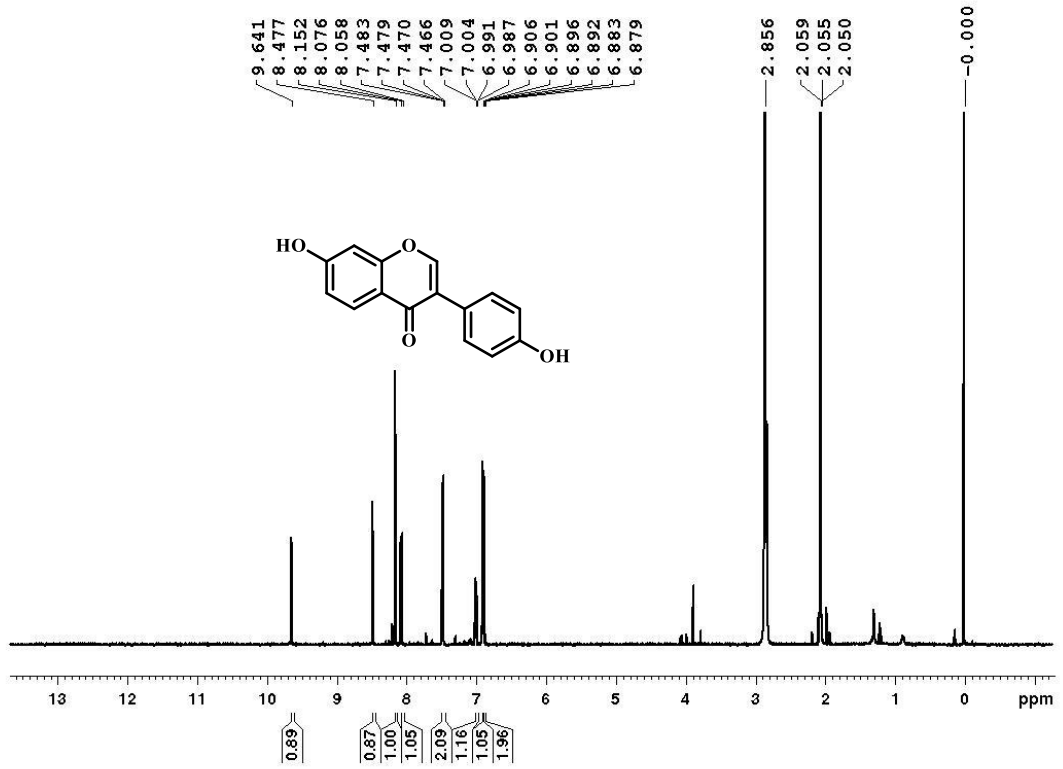


Figure 4A.6. ^{1}H NMR spectrum of compound **33** in Acetone-d6

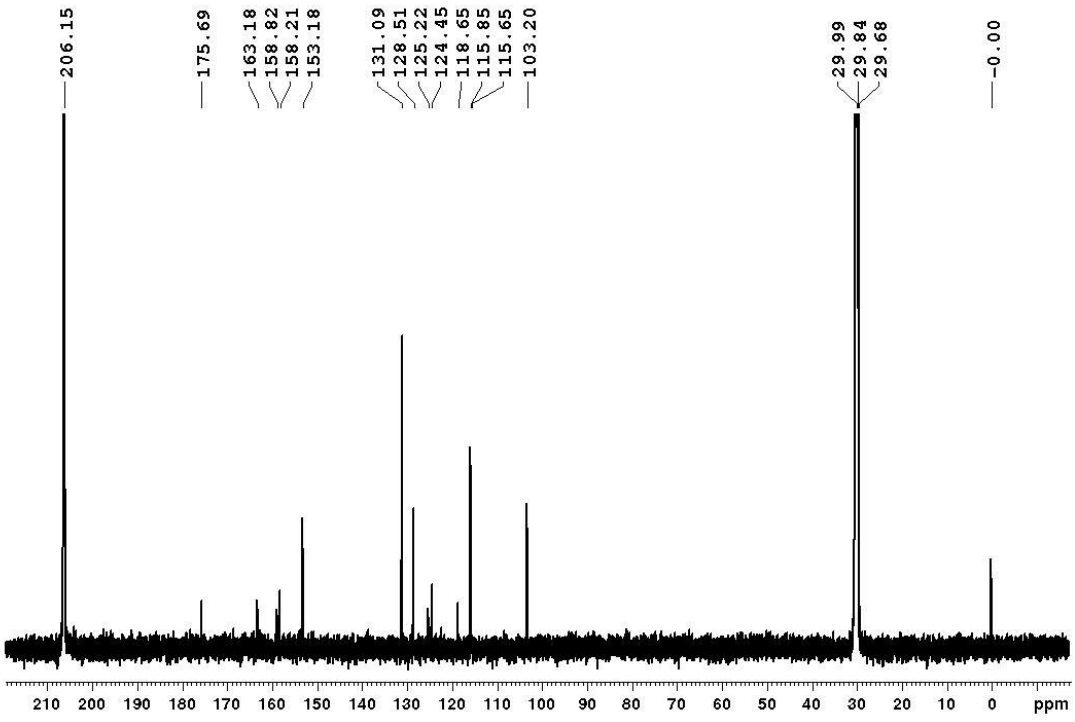
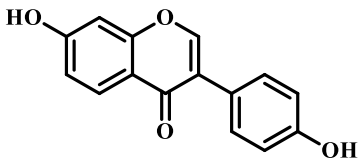


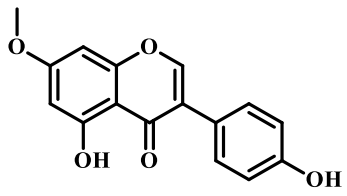
Figure 4A.7. ^{13}C NMR spectrum of compound **33** in Acetone-d6

The ^{13}C NMR spectrum (**Fig. 4A.7**) revealed the presence of 15 carbons which consisted of the carbonyl carbon at δ 175.6 and C-4', C-7 (-OH attached carbon) at δ 163.1 and δ 158.8 ppm, respectively. The placement of the phenolic hydroxyl group was identified based on COSY and HMBC correlations. Finally, the structure was assigned as **Daidzein** (4',7-dihydroxyisoflavone), consistent with the literature reported values. [99]



Compound **33- Daidzein**

On repeated chromatographic separation technique, one more solid was obtained from 37-38 fractions labelled as compound **34**. As in the previous compounds, this compound also showed the pattern of an isoflavonoid moiety in the ^{1}H NMR spectrum (**Fig. 4A.8**). The singlet peak at δ 8.23 (s, 1H, H-2), and two pairs of *ortho*-coupled doublets at δ 7.46 (dd, J = 8.5, 2.0 Hz, 2H, H-2'/H-6') and δ 6.91 (dd, J = 8.5, 2.0 Hz, 2H, H-3'/H-5'), confirmed the skeleton of isoflavonoid. The ^{1}H NMR spectrum showed the presence of a methoxy group at δ 3.93 ppm, which correlated with C-7 carbon atom in the HMBC spectrum. ^{13}C NMR spectrum (**Fig. 4A.9**) revealed the presence of 16 carbon atoms in which the carbonyl carbon appeared at δ 181.7 ppm and methoxy carbon at δ 56.4 ppm, respectively. Hence from the spectral data the structure of compound **34** is as shown below which was supported by HRMS analysis with a parent peak at *m/z* 285.0764 which is the $(M+H)^{+}$ peak and is in well agreement with the literature reports. Hence compound **34** was characterised as **Prunetin**.[100]



Compound **34- Prunetin**

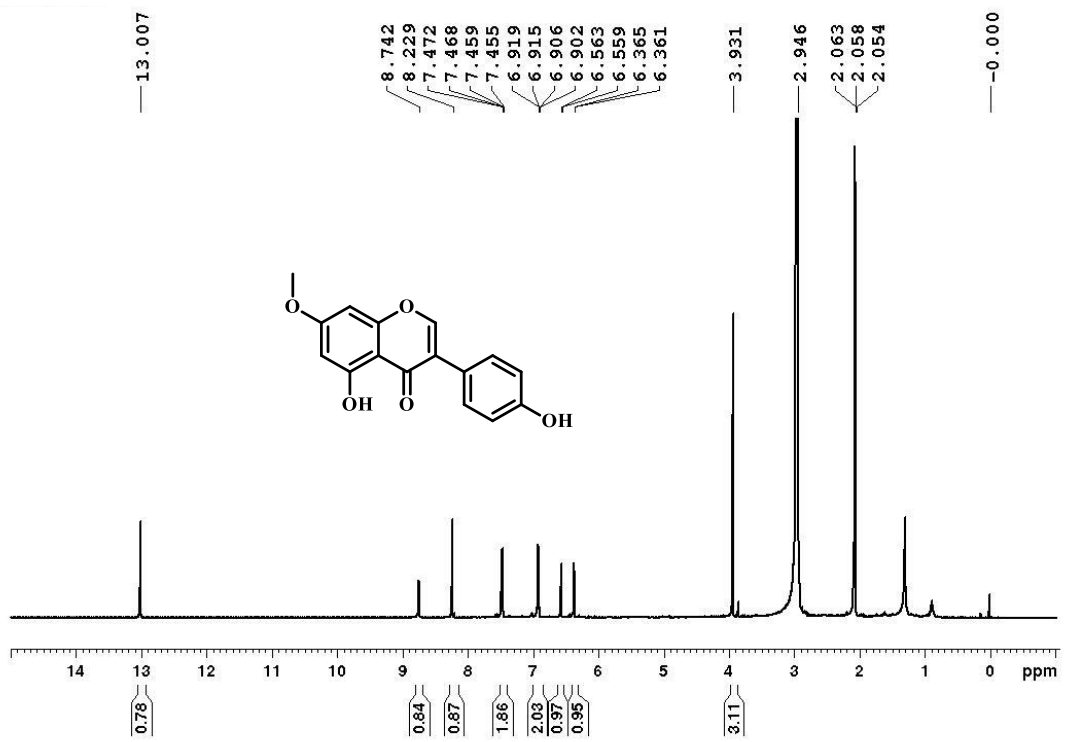


Figure 4A.8. ^{1}H NMR spectrum of compound **34** in Acetone-d6

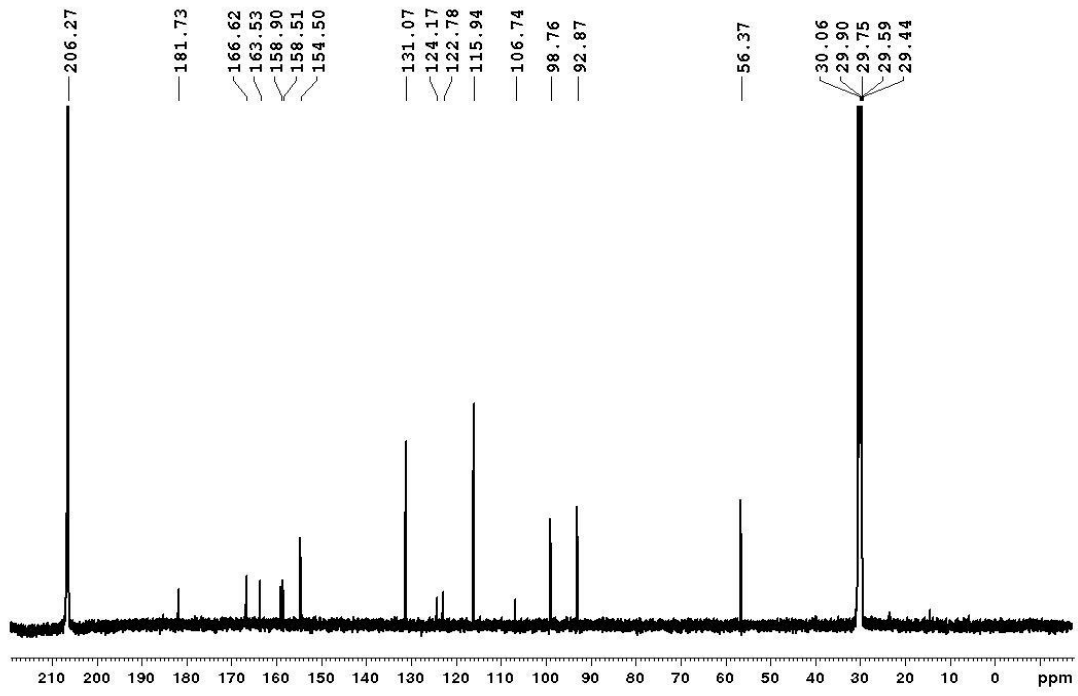
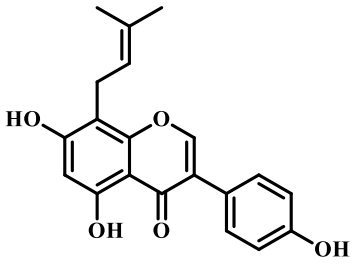


Figure 4A.9. ^{13}C NMR spectrum of compound **34** in Acetone-d6

Compound **35** (4 mg) was obtained as a white solid from fraction pool 4 (Fr. 37-38). The compound has almost the typical spectral pattern as that of an isoflavanoid with a singlet at δ 8.27 and ^{13}C NMR signals at δ 180.0 (C-4), 154.4 (C-2), and 123.2 (C-3) ppm. The ^{1}H NMR spectrum (**Fig. 4A.10**) showed two CH_3 group at δ 1.89 and 1.66 ppm, a multiplet at δ 5.25 and two proton doublets at δ 3.45 ppm confirmed the presence of a prenyl group. Further, the ^{13}C NMR spectrum (**Fig. 4A.11**) indicated the presence of carbonyl group at δ 182.0 and the methyl groups resonated at δ 25.8 & 17.9 ppm. DEPT-135 showed a $-CH_2-$ group at 22.0 ppm. The HMBC correlation between the proton at δ 3.45 (C-1") and carbon at δ 107.2 (C-8) ppm confirmed the attachment of C-prenyl group at C-8 carbon. The structure was well supported by HRMS analysis with a molecular ion peak at 339.1185 $(M+H)^+$. Thus compound **35** was identified as **Lupiwighteone** based on the spectral data obtained and literature reports. For the best of our knowledge this compound is reported for the first time from *Butea* genus.[101]



Compound **35- Lupiwighteone**

Fraction pool 5 (Fr. 39-42) on repeated silica gel CC afforded a white solid as compound **36**. The ^{1}H NMR spectrum (**Fig. 4A.12**) contain two sets of protons in the aromatic region which consisted of a two proton doublet and a singlet. The doublets correspond to the para-substituted rings [δ 7.58 (d, J = 9.0 Hz, 2H), 6.99 (d, J = 9.0 Hz, 2H)] and a sharp singlet at δ 8.19 confirms the compound is having an isoflavone skeleton. The peaks at δ 3.98 and 3.84 ppm indicated the presence of two methoxy group in the structure. The carbonyl carbon appeared at δ 175.5 and the methoxy carbons at δ 56.6 & 55.6 ppm, respectively in the ^{13}C NMR spectrum (**Fig. 4A.13**). The protons were assigned based on HMBC and HMQC correlations. The structure was characterized as **Afrormosin** and it is well supported by HRMS analysis with a molecular ion peak at *m/z* 299.0874 $(M+H)^+$. This compound is being reported for the first time from the heartwood, previously reported from the flowers of *B. monosperma*.[102]

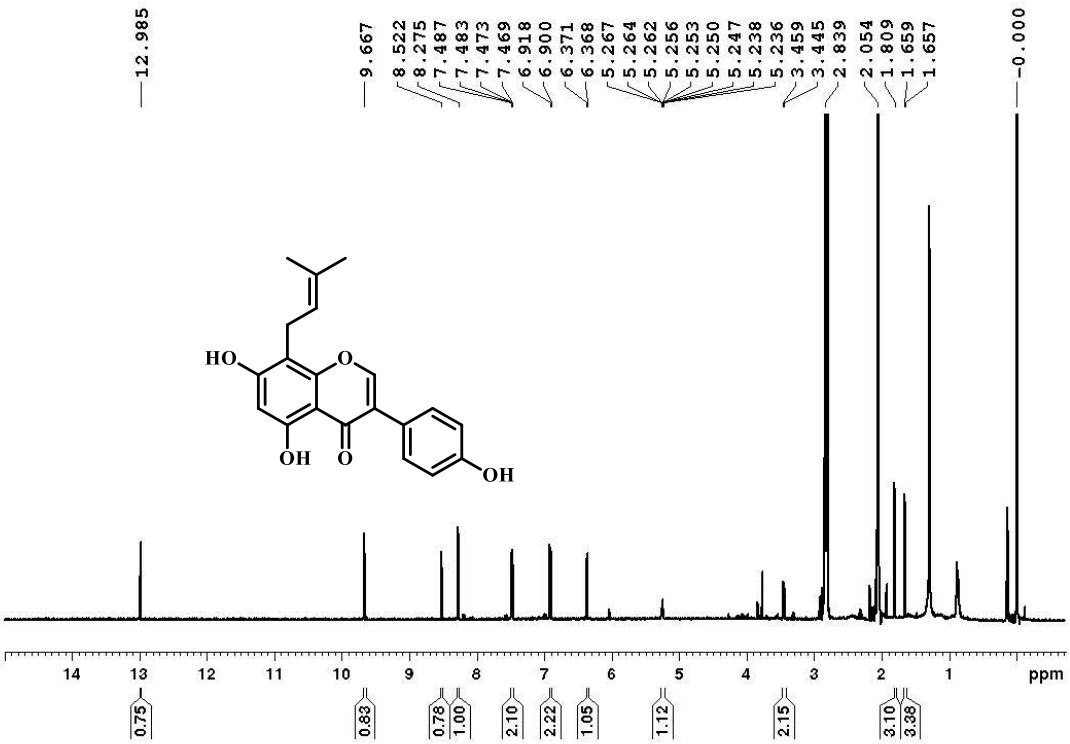


Figure 4A.10. ^{1}H NMR spectrum of compound **35** in Acetone-d6

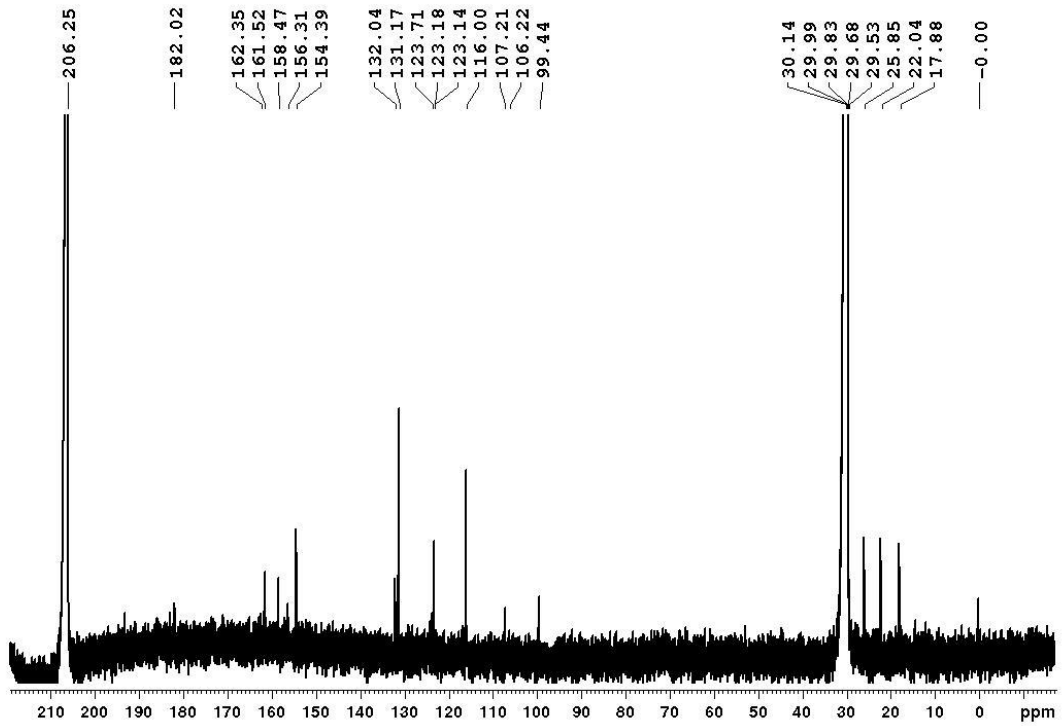


Figure 4A.11. ^{13}C NMR spectrum of compound **35** in Acetone-d6

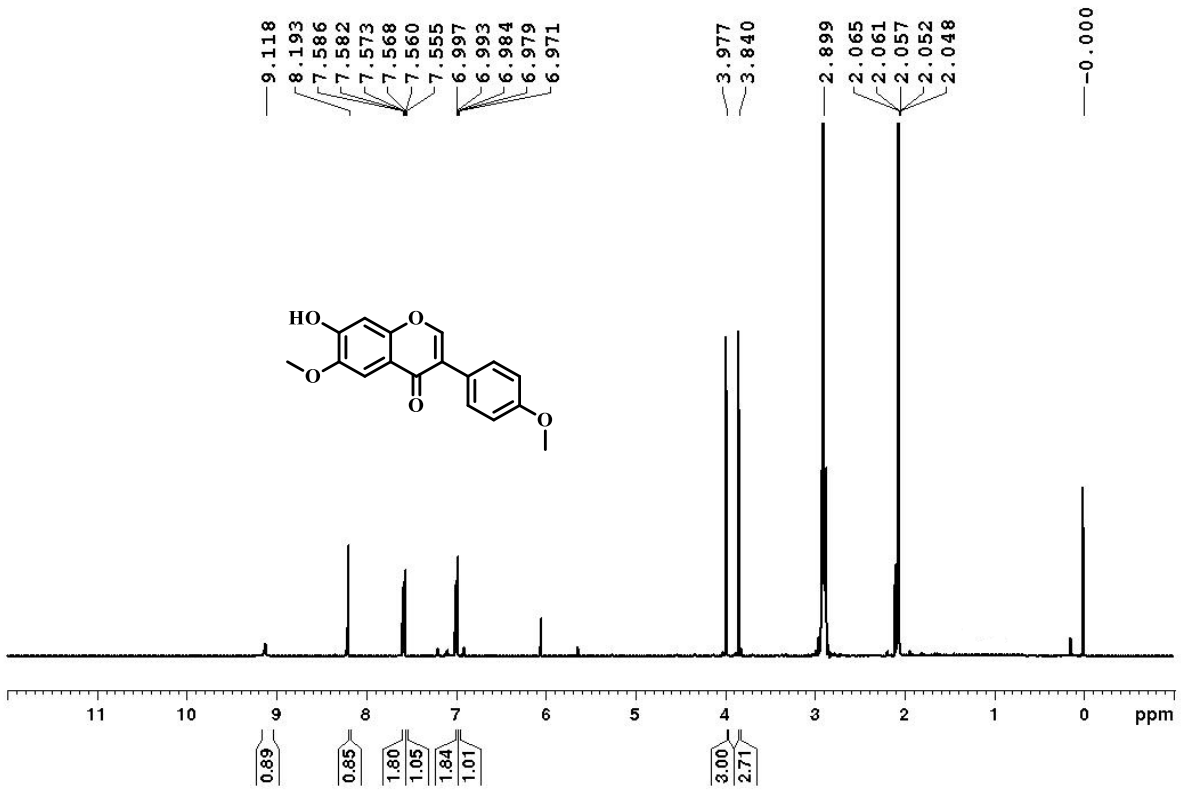


Figure 4A.12. ^{1}H NMR spectrum of compound **36** in Acetone-d6

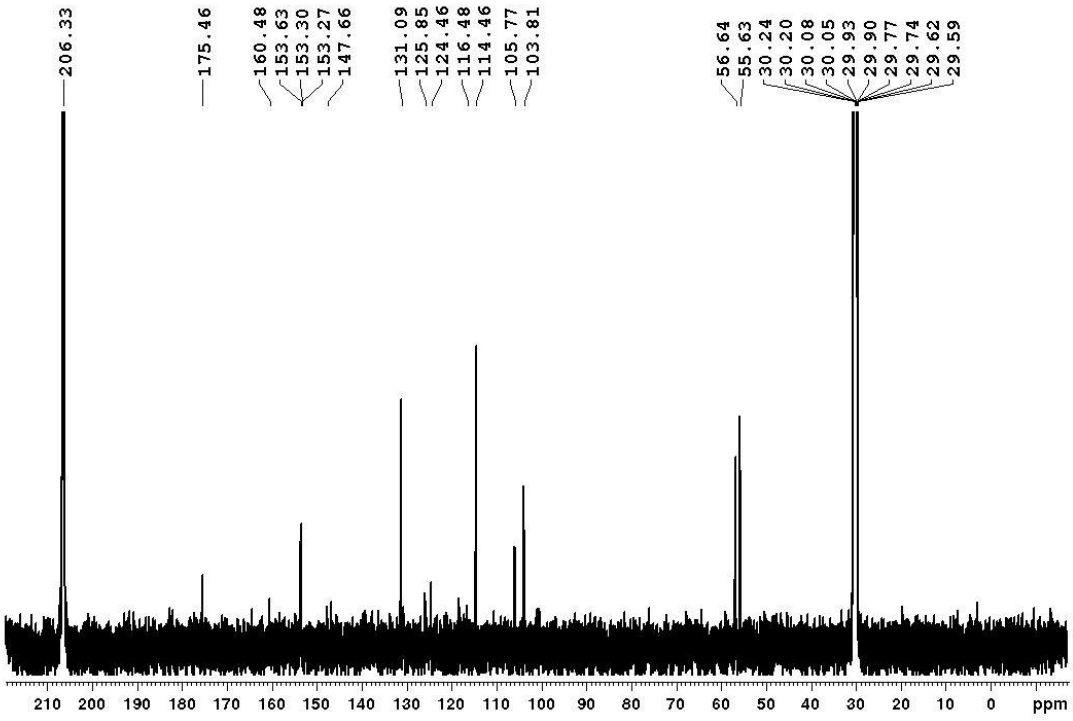
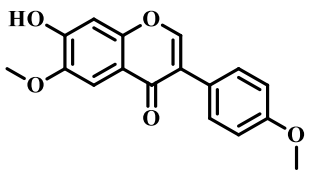
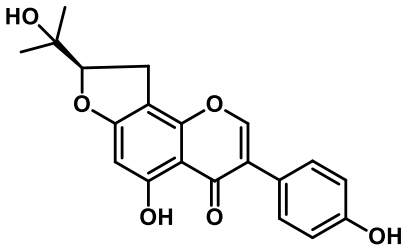


Figure 4A.13. ^{13}C NMR spectrum compound **36** in Acetone-d6



Compound **36- Afrormosin**

Fraction pool 7 (Fr. 43-45) showed a fluorescent nature. On CC separation, we could isolate a UV active yellow crystalline solid (5 mg) as compound **37**. The compound has assigned the molecular formula $C_{20}H_{18}O_5$ based on HRESIMS analysis with a parent peak at *m/z* 355.1127, which is the $(M+H)^+$ peak. As in the previous compounds, the ^1H NMR spectrum (**Fig. 4A.14**) showed a singlet at *δ* 8.19 (s, 1H, H-2), two pairs of *ortho*- doublets at *δ* 7.45 (dd, *J* = 8.5, 2.0 Hz, 2H, H-2'& H-6',) and δ 6.91 (dd, *J* = 8.5, 2Hz, 2H, H-3'& H-5'), attributing to the presence of an isoflavonoid system. The singlet at *δ* 6.21 ppm corresponded to the proton at the C-6th position. The three proton singlets at *δ* 1.25 & 1.30 ppm indicated the presence of two methyl groups and a doublet of doublet at δ 3.30 and δ 3.28 ppm, the presence of a $-CH_2$ - proton, respectively. In the ^{13}C NMR spectrum (**Fig. 4A.15**), the carbonyl carbon appeared at δ 181.7 ppm and the carbon attached to –OH protons at *δ* 167.7, 164.4 & 158.5 ppm. The $-CH_3$, $-CH_2$- & -CH carbons resonated at *δ* 25.5, 26.0, 27.1 & 71.4 ppm, respectively. The HMBC spectrum showed a correlation between H-3" and C-8 indicating a dihydrofuran ring fused to the C-7 and C-8th position. Hence by analyzing the spectral data and comparing with literature reports compound **37** was confirmed as **Erypoegin K**. For the best of our knowledge, this compound is being reported for the first time from *Butea* genus.[103]



Compound **37- Erypoegin K**

Fraction pool 8 (Fr. 46-50) on silica gel CC separation yielded a yellow solid with some minor impurities, which was further purified by passing through a Sephadex LH-20 column. We could obtain 2 mg of a yellow amorphous solid as compound **38**. The molecular formula of the compound was established as $C_{15}H_{10}O_5$ based on the HRESIMS analysis in which the parent molecular ion peak observed at

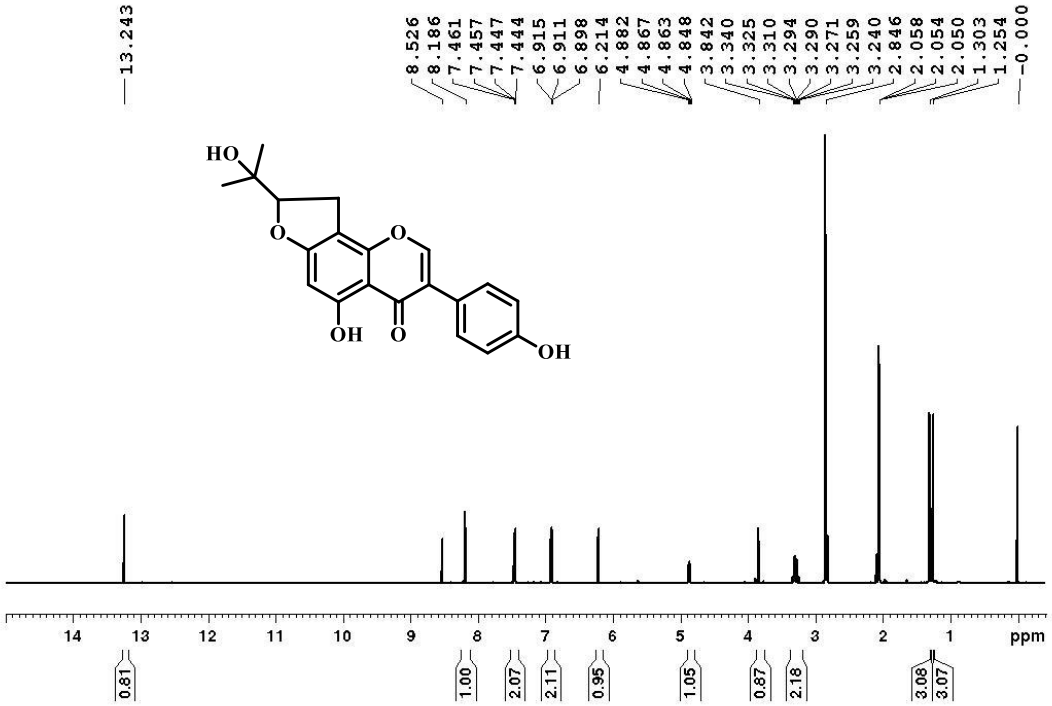


Figure 4A.14. ^{1}H NMR of compound **37** in Acetone-d6

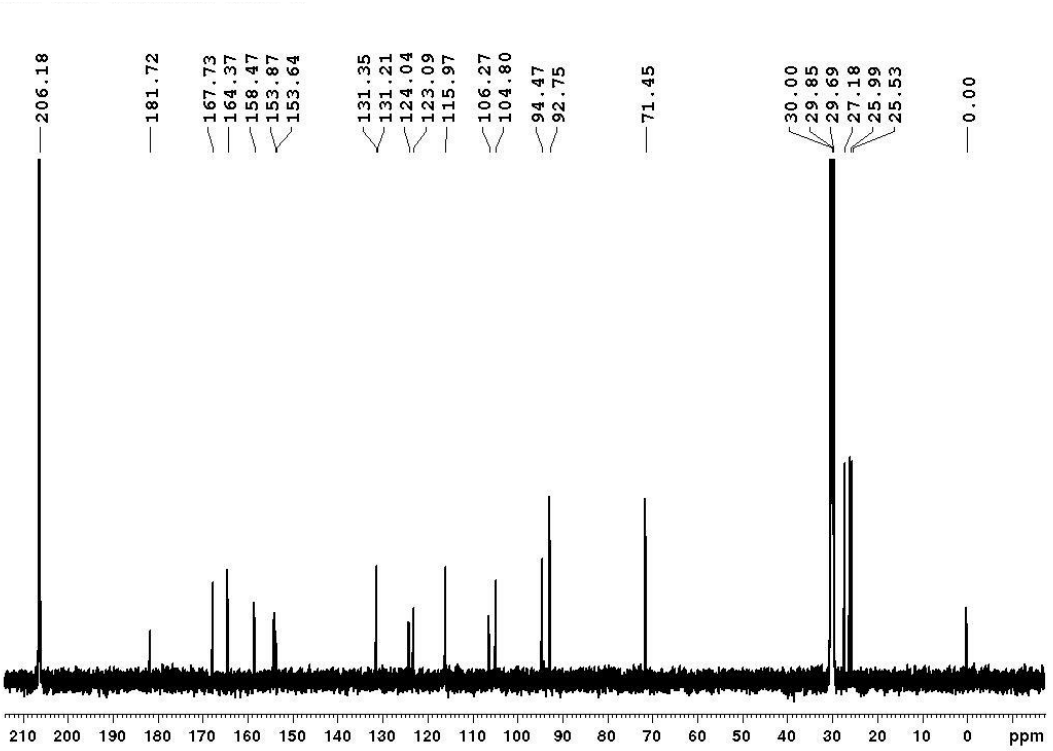


Figure 4A.15. ^{13}C NMR of compound **37** in Acetone-d6

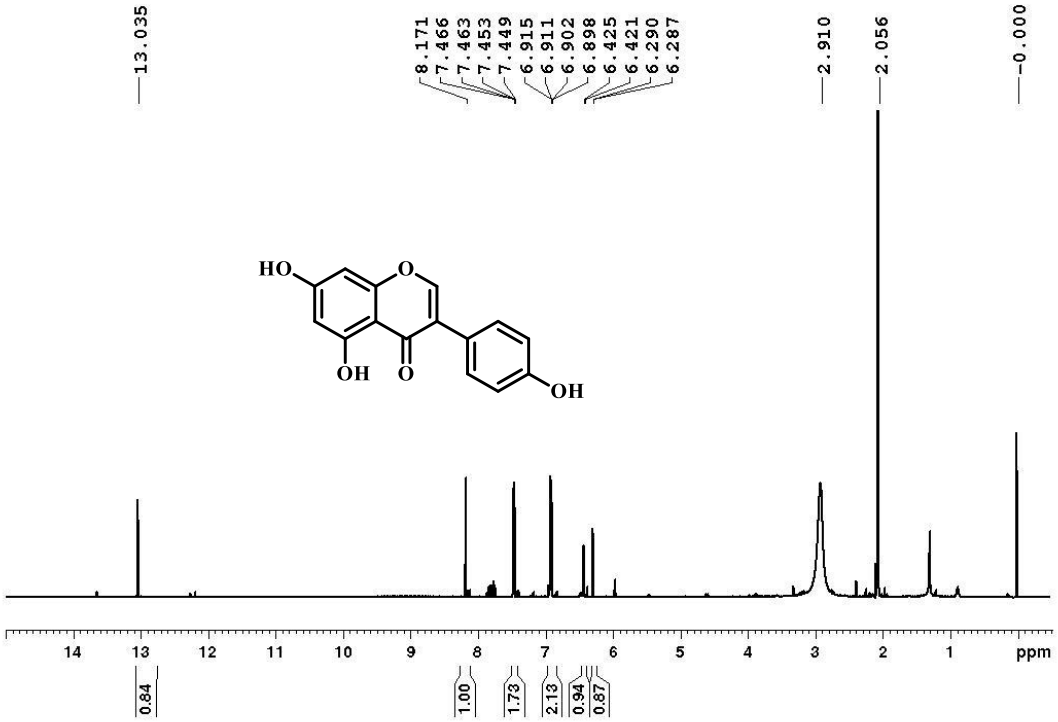


Figure 4A.16. ^{1}H NMR spectrum of compound **38** in Acetone-d6

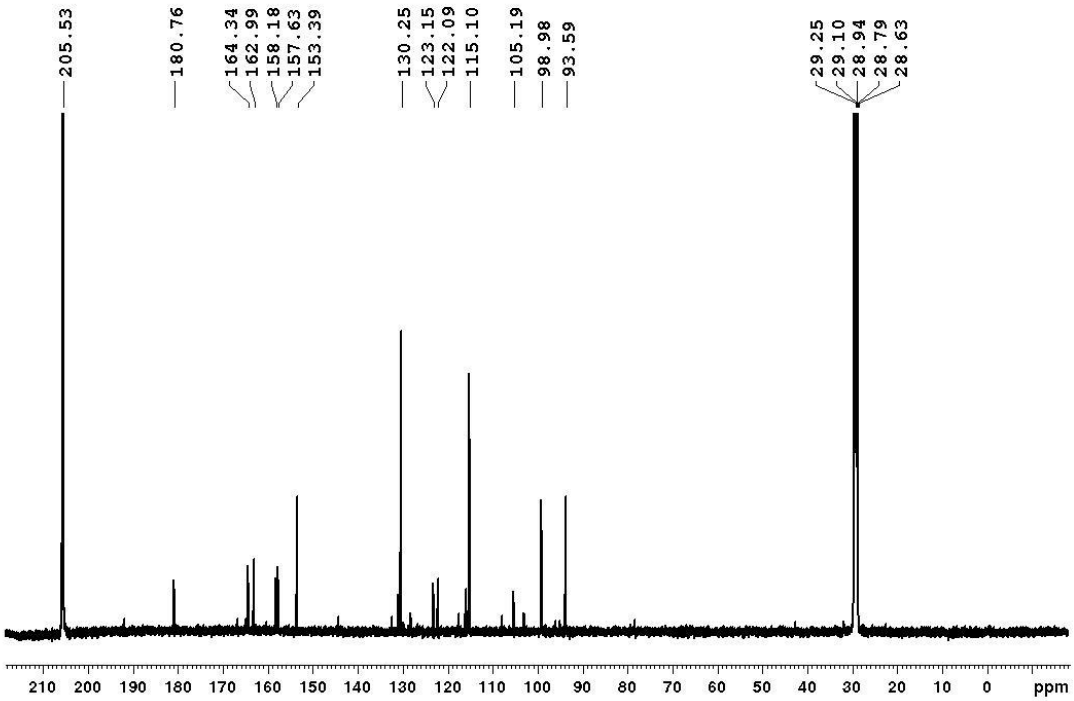
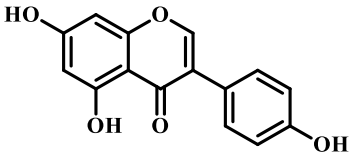


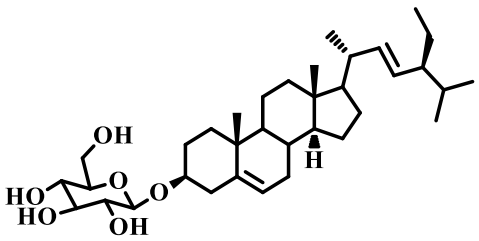
Figure 4A.17. ^{13}C NMR spectrum of compound **38** in Acetone-d6

m/z 269.0818 which could be the $(M-H)^+$ peak. The ^1H NMR spectrum (**Fig. 4A.16**) showed the presence of two *meta*-coupled protons at *δ* 6.42 (d, *J* = 2.0 Hz, 1H, H-8) and *δ* 6.29 (d, *J* = 2.0 Hz, 1H, H-6) and two pairs of ortho-coupled protons at *δ* 7.46 (d, *J* = 8.5 Hz, 2H, H-2'& H-6'), *δ* 6.91 (d, *J* = 8.5 Hz, 2H, H-3'& H-5') are characteristics for the 4',5,7-trisubstituted isoflavone. The ^{13}C NMR (**Fig. 4A.17**) spectrum displayed the carbonyl carbon at *δ* 180.8 (C-4) and C-7, C-5 & C-4' (-OH attached carbons) at *δ* 164.4, 163.0 and 158.2 ppm, respectively. The ^1H and ^{13}C values were assigned on the basis of COSY, HMBC, HMQC and the structure was in good agreement with literature reports. Finally, the structure was assigned as **Genistein** (4',5,7-trihydroxyisoflavone).[99]



Compound **38- Genistein**

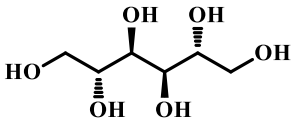
A white solid was obtained from fraction pool 9 (Fr. 71-72) in silica gel CC separation. The compound was labelled as compound **39**. The olefinic protons at H-20 & H-21 showed the peaks at *δ* 5.16 & 5.06 ppm. The protons corresponding to sugar moiety resonated in the range *δ* 3.2 - 3.8 ppm. The ^{13}C NMR spectrum showed peaks at *δ* 24.3, 19.9, 19.3 (C-33), 18.9 (C-38), 12.1 (C-40), 19.1 (C-41) due to methyl groups. The olefinic carbon atoms showed the peaks at *δ* 141.4 (C-20), 121.3 (C-21), 138.0 (C-34), 129.0 (C-35) ppm respectively. The mass spectrum showed molecular ion peak at *m/z* 397 due to [(M+l) - Glucose]. Thus, from all the above spectral data and literature reports, the structure of compound **39** was assigned as **Stigmasterol glucoside**.



Compound **39- Stigmasterol glucoside**

Fraction pool 11 (Fr. 87-93) on silica gel CC separation on 90 % ethyl acetate-hexane polarity afforded an amorphous colourless solid. The ^1H and ^{13}C NMR data were similar to that of compound **28** from chapter 3A. Hence, the compound was

characterized as **D-Mannitol**, based on spectral data and comparing with literature reports. The compound is reported for the first time from *Butea* genus. [104]



Compound **28- D-Mannitol**

4B.7. Conclusion

Phytochemical investigation of the medicinally important plant *Butea monosperma* was carried out. We have isolated eleven compounds from the acetone extract of the heartwood of *B. monosperma* and successfully characterized all molecules using various spectroscopic techniques. The compounds are a mixture of *β*–sitosterol and stigmasterol and stigmasterol glucoside and seven isoflavones-formononetin, daidzein, prunetin, 8-prenylgenistein, afromosin erypoegin K, and genistein. Erypoegin K and Lupiwighteone are being reported for the first time from the genus *Butea*. Afromorsin reported for the first time from the heartwood of *Butea monosperma*. It is found that this species contains mostly isoflavones and are reported to have anticancer activities.

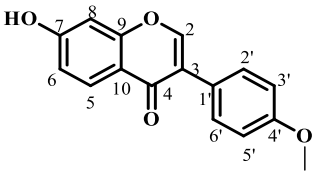
4B.8. Experimental section

General experimental procedure as described in section 2A.6 Chapter 2A.

4B.9. Spectral data

Compound 32 (Formononetin)

Fraction pool 4 obtained by eluting the column with 40 % ethyl acetate-hexane yielded 30 mg of compound **32** as a colourless solid. The compound was successfully characterized as an isoflavone, **Formononetin** based on the spectral data obtained.



Molecular formula: $C_{16}H_{12}O_4$

Melting point: 252-256˚C

FT-IR (Neat, υ_{max} cm^{-1}): 3132, 1637, 1620, 1600, 1513 cm^{-1}.

^{1}H NMR (500 MHz, MeOD-d4, TMS): δ 8.16 (s, 1H, H-

2), 8.06 (d, *J* = 9.0 Hz, 1H, H-5), 7.46 (d, *J* = 8.5 Hz, 2H, H-2'& H-6'), 6.98 (d, *J* = 8.5 Hz, 2H, H-3' & H-5'), 6.95 (d, *J* = 2.5 Hz, 1H, H-6), 6.93 (d, *J* = 2.5 Hz, 1H, H-8), 3.82 (-OCH_3) ppm.

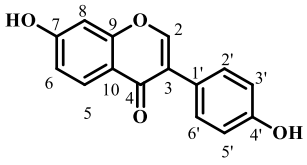
^{13}C NMR (125 MHz, DMSO-d6, TMS): *δ* 175.6 (C-4), 163.1 (C-7), 159.5 (C-4'), 158.0 (C-9), 153.7 (C-2), 130.6 (C-2' & C-6'), 127.8 (C-5), 124.5 (C-3), 122.9 (C-1'), 116.9 (C-10), 115.9 (C-6), 114.2 (C-3'& C-5'), 102.6 (C-8), 55.6 (O-CH_3) ppm.

HRMS (ESI): *m/z* calcld for $C_{16}H_{12}O_4$ is 268.0735.

Found 269.0818 $(M+H)^+$.

Compound 33 (Daidzein)

After the isolation of compound **32** from fraction pool 4, the residue subjected silica gel CC eluting with 40 % ethyl acetate-hexane polarity, we could isolate an orange coloured solid as compound **33** (5 mg). The compound was successfully characterized as an isoflavone, **Daidzein** based on the spectral data obtained as given below.



Molecular Formula: $C_{15}H_{10}O_4$

FT-IR (Neat, υ_{max} cm^{-1}): 3355, 2927, 1650, 1610, 843 cm^{-1}.

^{1}H NMR (500 MHz, Aetone-d6, TMS): *δ* 8.15 (s, 1H, H-2), 8.07 (d, *J* = 9.0 Hz, 1H, H-5), 7.47 (d, *J* = 8.5 Hz, 2H, H-2'& H-6'), 7.00 (dd, *J* = 6.5, 2.5 Hz, 1H, H-6), 6.90 (d, *J* = 2.5 Hz, 1H, H-8), 6.89 (d, *J* = 8.5 Hz, 2H, H-3'& H-5'), 8.47 (s, 1H, C-4'-OH), 9.64 (1H, s, C-7-OH) ppm.

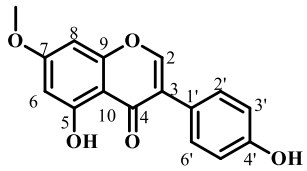
^{13}C NMR (125 MHz, Acetone-d6, TMS): *δ* 175.7 (C-4), 163.2 (C-7), 158.8 (C-4'), 158.2 (C-9), 153.2 (C-2), 131.1 (C-2' & C-6'), 128.5 (C-5), 125.2 (C-3), 124.4 (C-1'), 118.6 (C-10), 115.8 (C-3'& C-5'), 115.6 (C-6), 103.2 (C-8) ppm.

HRMS (ESI): *m/z* calcld for $C_{15}H_{10}O_4$ is 254.0579.

Found 255.0661 $(M+H)^+$.

Compound 34 (Prunetin)

A white solid (4 mg) isolated along with compound **33** from fraction pool 4 as compound **34**. It was identified as **Prunetin** based on IR, NMR and HRESIMS analysis. The NMR data were assigned by the 2D NMR and literature reports.



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

FT-IR (Neat, υ_{max} cm^{-1}): 3400, 2950, 1645 cm^{-1}.

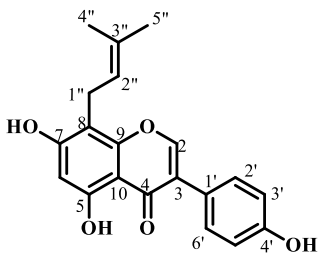
^{1}H NMR (500 MHz, Acetone-d6, TMS): δ 8.23 (s, 1H, H-2), 7.46 (dd, *J* = 8.5, 2.0 Hz, 2H, H-2' & H-6'), 6.91 (dd, *J* = 8.5, 2.0 Hz, 2H, H-3' & H-5'), 6.56 (d, *J* = 2.0 Hz, 1H, H-8), 6.36 (d, *J* = 2.0 Hz, 1H, H-6), 3.93 (s, 3H, -OCH_3), 8.74 (s, 1H, -OH at C-4'), 13.00 (s, 1H, -OH at C-5) ppm.

^{13}C NMR (125 MHz, Acetone-d6, TMS): δ 181.7 (C-4), 166.6 (C-7), 163.5 (C-5), 158.9 (C-4'), 158.5 (C-9), 154.5 (C-2), 131.1 (C-2' & C-6'), 124.2 (C-3), 122.8 (C-1'), 115.9 (C-3' & C-5'), 106.7 (C-10), 98.8 (C-6), 92.9 (C-8), 56.4 (-OCH_3) ppm.

HRMS (ESI): *m/z* calcld for $C_{16}H_{12}O_5$ is 284.0684. Found 285.0764 $(M+H)^+$.

Compound 35 (Lupiwighteone)

Fraction pool 4 yielded 4 mg of a white solid labelled as compound **35**. From detailed analysis of spectroscopic data and on comparison with literature report, the compound was confirmed as **Lupiwighteone**. The compound is reported for the first time from this plant species.



Molecular formula: $C_{16}H_{12}O_4$

FT-IR (Neat, υ_{max} cm^{-1}): 3522, 3396, 3165, 1651, 1512, 1431, 1367, 1203, 1064, 827 cm^{-1}.

^{1}H NMR (500 MHz, Acetone-d6, TMS): δ 8.27 (1H, s, H-2), 7.48 (d, *J* = 9.0 Hz, 2H, H-2' & H-6'), 6.91 (d, *J* = 9.0 Hz, 2H, H-3' & H-5'), 6.37 (d, *J* = 1.5 Hz, 1H, H-8), 5.25 (m, 1H, H-2"), 3.45 (d, *J* =7.0 Hz, 2H, H-1"), 1.89

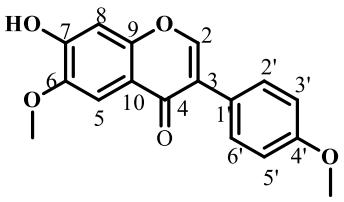
(s, 3H, H-5”), 1.66 (s, 3H, H-4”), 8.52 (C-4’-OH), 9.67 (C-7-OH), 12.99 (C-5- OH) ppm.

^{13}C NMR (125 MHz, Acetone-d6, TMS): *δ* 182.0 (C-4), 162.4 (C-7), 161.5 (C-5), 158.5 (C-4’), 156.3 (C-9), 154.4 (C-2), 132.0 (C-3”), 131.2 (C-2’ & C-6’), 123.7 (C-1’), 123.2 (C-3), 123.1 (C-2”), 116.0 (C-3’ & C-5’), 107.2 (C-8), 106.2 (C-10), 99.4 (C-6), 25.8 (C-5”), 22.0 (C-1”), 17.9 (C-4”) ppm.

HRMS (ESI): *m/z* calcld for $C_{16}H_{12}O_4$ is 338.1154. Found 339.1185$(M+H)^+$.

Compound 36 (Afrormosin)

Fraction pool 5 (Fr. 39-42) on repeated silica gel CC using 40 % ethyl acetate-hexane polarity afforded a yellow coloured solid as compound **36**. Based on ^{1}H, ^{13}C and 2D NMR analysis and mass spectrum, the structure was assigned as the isoflavone **Afrormosin.**



Molecular formula: $C_{17}H_{14}O_5$

Melting point: 220 ± 2° C

FT-IR (Neat, υ_{max} cm^{-1}): 3232, 1606, 1511, 1449, 1381 cm^{-1}.

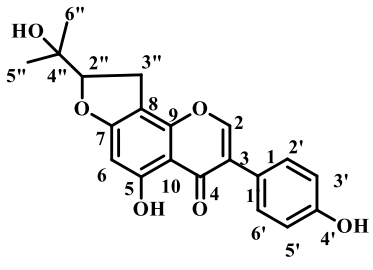
^{1}H NMR (500 MHz, Acetone-d6, TMS): *δ* 8.19 (s, 1H, H-2), 7.58 (d, *J* = 9.0 Hz, 2H, H-2’ & H-6’), 7.56 (s, 1H, H-5), 6.99 (d, *J* = 9.0 Hz, 2H, H-3’ & H-5’), 6.97 (s, 1H, H-8), 3.98 (s, 3H, C-6), 3.84 (s, 3H, C-4’), 9.12 (s, 1H, C-7) ppm.

^{13}C NMR (125 MHz, Acetone-d6, TMS): *δ* 175.5 (C-4), 160.5 (C-4’), 153.6 (C-7), 153.3 (C-2), 153.3 (C-8), 147.6 (C-6), 131.1 (C-2’ & C-6’), 125.8 (C-3), 124.5 (C-1’), 116.5 (C-10), 114.5 (C-3’& C-5’), 105.8 (C-5), 103.8 (C-8), 56.6 (-OCH_3), 55.6 (-OCH_3) ppm.

HRMS (ESI): *m/z* calcld for $C_{17}H_{14}O_5$ is 298.0841. Found 299.0874 $(M+H)^+$.

Compound 37 (Erypoegin K)

Fraction pool 6 on silica gel CC yielded 5 mg of yellow crystalline solid as compound **37**. Based on NMR and mass spectral data the compound was identified as **Erypoegin K**, and the result was well supported with literature reports. This compound is reported for the first time from this species.



Molecular Formula: $C_{20}H_{18}O_5$

FT-IR (Neat, υ_{max} cm^{-1}): 3691, 3019,1654 cm^{-1}.

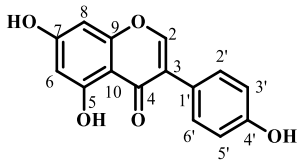
^{1}H NMR (500 MHz, Acetone-d6, TMS): δ 8.19 (s, 1H, H-2), 7.45 (dd, J = 8.5, 2.0 Hz, 2H, H-2'& H-6'), 6.91 (dd, J =8.5, 2.0 Hz, 2H, H-3'& H-5'), 6.20 (d, J = 1.5 Hz, 1H, H-6), 4.87 (dd, J = 9.5, 7.5 Hz, 1H, H-2"), 3.30 (d, J = 8.0 Hz, 1H, H-3"), 3.28 (d, J = 9.5 Hz, 1H, H-3"), 1.30 (s, 3H, H-5"), 1.25 (s, 3H, H-6"), 3.84 (C4"-OH), 8.53 (s, 1H, C4'-OH), 13.24 (s, 1H, C5-OH) ppm.

^{13}C NMR (125 MHz, Acetone-d6, TMS): δ 181.7 (C-4), 167.7 (C-7), 164.4(C-5), 158.5 (C-4'), 153.9 (C-2), 153.6 (C-9), 131.2 (C2' & C-6'), 124.0 (C-3), 123.1(C-1'), 116.0 (C-5'/C-3'), 106.3 (C-10), 104.8 (C-8), 94.5 (C-6), 92.8 (C-2"), 71.4 (C-4"), 27.2 (C-3"), 26.0 (C-6"), 25.5 (C-5") ppm.

HRMS (ESI): *m/z* calcld for $C_{20}H_{18}O_5$ is 354.1103. Found 355.1127 $(M+H)^+$.

Compound 38 (Genistein)

Fraction pool 7 on silica gel CC followed by purification with Sephadex LH-20 column in methanol yielded 2 mg of yellow solid as compound **38**. The compound was successfully characterized as the isoflavone **Genistein** based on the spectral data.



Molecular formula: $C_{15}H_{10}O_5$

FT-IR (Neat, υ_{max} cm^{-1}): 3352, 2935, 1654, 1613, 848 cm^{-1}.

^{1}H NMR (500 MHz, Acetone-d6, TMS): δ 8.17 (s, 1H, H-2), 7.46 (d, J = 8.5 Hz, 2H, H-2'& H-6'), 6.91 (d, J = 8.5

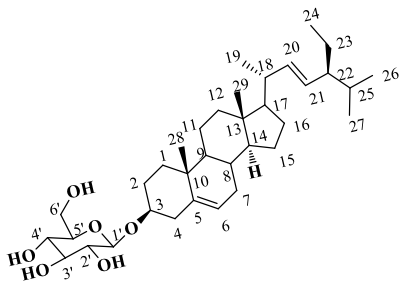
Hz, 2H, H-3'& H-5') 6.42 (d, *J* = 2.0 Hz, 1H, H-8), 6.29 (d, *J* = 2.0 Hz, 1H, H-6), 13.03 (s, 1H, C5- OH) ppm.

^{13}C NMR (125 MHz, Acetone-d6, TMS): δ 180.8 (C-4), 164.3 (C-7), 163.0 (C5), 158.2(C-4'), 157.6(C-9), 153.4 (C-2), 130.2 (C-2' & C-6'), 123.2 (C-3), 122.1(C-1'), 115.1 (C-3'& C-5'), 105.2 (C-10), 99.0 (C-6), 93.6 (C-8) ppm.

HRMS (ESI): *m/z* calcld for $C_{15}H_{10}O_5$ is 270.0528. Found 269.0818 $(M-H)^+$.

Compound 39 (Stigmasterol glucoside)

A white amorphous solid (23 mg) isolated from fraction pool 9 was characterized as **Stigmasterol glucoside** based on spectral data and literature analysis.



Molecular Formula: $C_{35}H_{58}O_6$

FT-IR (Neat, υ_{max} cm^{-1}): 3450, 2955, 1610, 1155 cm^{-1}.

^{1}H NMR (MeOD-d4, 500 MHz, TMS): δ 5.36 (d, 1H, *J* = 7.0 Hz), 5.18 (m,1H), 5.06 (m, 1H), 4.38 (d, *J* = 7.6 Hz, 1H), 3.84 (d, *J* = 7.0 Hz, 1H), 3.25-3.26 (m, 4H), 3.14 (t, 1H), 2.40-2.43 (m, 1H), 2.22-2.27 (s, 1H), 1.84-2.05 (m, 4H), 1.50-1.62 (m, 7H), 1.04-1.21(m, 5H), 0.86-0.88 (m, 8H), 0.73 (d, *J* = 6.7 Hz, 2H) ppm.

^{13}C NMR (DMSO-d4, 125 MHz, TMS): δ 140.4 (C-20), 138.5 (C-21), 129.3, 121.6 (C-6), 101.3, 77.4, 77.2, 73.9, 70.6, 61.6, 56.7, 51.0, 40.6, 40.5, 40.4, 40.3, 40.2, 40.1 40.0, 39.9, 38.8, 37.3, 36.7, 31.9, 31.8, 29.7, 29.6, 29.5, 29.1, 25.3, 24.3, 22.6, 21.6, 18.7, 18.3, 11.5, 11.4 ppm.

HRMS (ESI): *m/z* calcld for $C_{35}H_{58}O_6$ is 574.4233. Found 597.4138 $(M+Na)^+$.

4A.10. References

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Chapter 4B: Antiproliferative Potential of Amalaki Rasayana on Cervical Carcinoma and the Effect of *Butea monosperma* (Lam.) Taub on the Cytotoxicity

4B.1. Introduction

Ayurveda, the traditional Indian system of medicine, signifies the science of long life. It revolves around three basic senses of humor of the body, *i.e.*, *Vata* (responsible for body movement), *Pitta* (responsible for bodily chemical reactions such as metabolism and temperature) and *Kapha* (responsible for growth, protection, lubrication and sustenance) called the *tridoshas.* These three factors in equilibrium are the state of health and an imbalance in this stability causes diseases.[1] The treatment of Ayurveda targets to bring the vitiated *dosha* back to the state of equilibrium. Sushruta defines a healthy man as one who has an equilibrium of *doshas*, normal functioning of Agni, normal condition of seven Dhatus, beside his soul, the sense organs and mind should be happy and cheerful.[2] Rasayana therapy is an active step towards achieving this, and it constitutes one of the eight major divisions of Ayurveda. Rasayana includes a set of specific therapies that can reverse the physical degeneration of the body caused by aging.[3] Rasayana chikitsa ('Rasa': plasma; Ayana: path, which means the path that 'Rasa' takes) or therapy improves longevity, intelligence, memory, health, youth, voice, complexion, motor, and sensory strength.[4] According to sages, rejuvenation therapy is of two types (i) *Kutipraveshika* and (ii) *Vata tapika.* The first one is the most beneficial treatment. "*Kuti*" is a specially build hut in which no light enters and the patient is sequestered there for a long time period by taking the various Rasayana herbs.[5] Amalaki Rasayana is one of such rejuvenation medicines prepared from Amla (*Emblica officinalis* or Indian Gooseberry). The Rasayana formulation mentioned in Charaka Samhita was the raw fruits of Amla was heated in a covered tube made of fresh Palash wood, pasted with mud and get cooked with wild cow-dung fire. After self-

cooling, the fruits were taken out, remove seeds and crushed. It is then mixed with pippali powder, vidanga powder, sugar, oil, honey and ghee. The preparation is stored for 21 days thereafter, it is used.[6]

Amalaki Rasayana (AR) is grouped under Vayasthapana Rasayana, which is reported to promote longevity and healthy aging. AR was used since time immemorial by sages or ascetics to extend their lives to achieve liberation in their lifetime. Previous studies on AR indicates that it possesses significant immunostimulant activity and moderate cytoprotective activity.[7] The combination of AR with milk is found to be very effective in treating aging elements.[8] AR alleviates suffering from neurodegenerative disorder like Alzheimer's diseases and promote healthy aging with enhanced physical and mental strength.[9] Dietary supplement of AR and Rasa-Sindoor in *Drosophila* models where shown to substantially suppress neurodegeneration in fly models of polyQ and Alzheimer's disorders without any side effects.[10] One of the significant features of neurodegeneration is the apoptotic death of the affected neurons. Both these formulations were very effective in suppressing the apoptosis induced and also elevated the levels of anti-apoptotic proteins.[11] AR feeding in flies *Drosophila melanogaster* significantly improved the oxidative stress tolerance and reduced ROS accumulation with age, reflecting improved cellular redox homeostasis.[12] AR triggers a protective effect on UV-induced DNA damage in aged individuals without altering the NER (Nucleotide Excision Repair) and BER (Constitutive Base Excision Repair).[13] It is also efficient in the management of anaemia (*Pandu*).[14]

As mentioned in the literature, Amalaki Rasayana is prepared in the wooden vessel made of Palash (*Butea monosperma)*. From ancient time, Palash stick and wood were used in different Indian rituals. The plant is used in Ayurvedic, Unani and Siddha medicine for various ailments and has become a treasure of modern medicine. Palash (*Butea monosperma)* is popularly known as the Flame of the forest, belongs to the family Fabaceae. *Butea monosperma* is a native plant of the Indian subcontinent and south-east Asia. It is said that the tree is a form of Agnidev 'The god of fire' and is believed to be a sacred tree. *Butea monosperma* has been traditionally reported to possess antibacterial, antimicrobial, antifungal, astringent,

anticonceptive, anticonvulsant, anti-tumor, anti-fertility, hepatoprotective, radical scavenging and wound healing activity [15].

Chemoprevention with food phytochemicals is considered as one of the most important strategies to control cancer. *Emblica officinalis* possess potent free radical scavenging activity, thereby prevents carcinogenesis.[16] Since the preparation of Amalaki Rasayana makes use of the plants amla (*E. officinalis*) and Palash (*B. monosperma*), our study mainly aims at the comparative evaluation of the anticancer property of amla and Amalaki Rasayana along with the phytochemical reinvestigation of *B. monosperma* (Chapter 4A). An attempt has been made to validate the method of drug processing (heating amla inside Palash wood) and its influence on the therapeutic property of amla after the processing.

4B.2. Materials and Methods

4B.2.1. Plant Materials

The materials used for the present study were collected from the Paravur region of Kollam district of Kerala, India. The plants, *Butea monosperma* and *Emblica officinalis* were identified and authenticated by using Gamble's Flora of Presidency of Madras. Sufficient quantities of fresh berries of *E. officinalis* (EO) were collected from the trees, and stem pieces from the matured branch of *B. monosperma* (BM) tree were used for making the wooden vessels for processing the amla fruits. A voucher sample of the plants are kept at the Department of R and B, Government Ayurveda College, Kannur with voucher number 2018-06-03 and 2018-06-05 for EO and BM respectively.

4B.2.2. Amalaki Rasayana Preparation

Three cylindrical stem pieces, each having 25 cm length with bark was removed from the straight medium-sized BM branch. The central pith portions of the stem pieces were scrapped out to form a central hollow cavity of about 5 cm circumference and 23 cm length. The whole structure thus resembled as a vessel. Three circular lids were made using the same stem piece of palash (BM) to enable the tight closure of the mouths of the vessels. Accurately weighed 240 g of freshly collected amla fruits (EO) were filled into each of these BM wooden cylinders and

the prepared wooden lids closed the mouth of the vessels (**Fig. 4B.1**). Then the surface of the vessels was smeared with mud in a specific way to attain a considerable thickness and heated slowly by increasing the temperature from 30 to 400˚C. The entire procedure adopted was as detailed in Ashtangahridaya. After completion of the thermal curing, processed amla was taken out from the wooden vessels, and the seeds from each processed amla fruits were carefully removed using a knife and flesh of the fruits were dried in the shade to achieve uniform weights. [17]

Figure 4B.1. Amalaki Rasayana preparation: filling raw fruits of amla into wooden vessels of Palash

4B.2.3. Extraction Method

Fresh fruits of amla after removing seeds, processed amla and the stem bark of Palash weighing about 10 g was taken and extracted with 70 % ethanol-water mixture at room temperature. Ethanol was removed under vacuum in a rotatory evaporator and the remaining mixture was lyophilized to get the crude extract. The extraction yielded 484.6 mg of Amalaki, 485 mg of amla and 70.2 mg of *Butea monosperma* extract, respectively.

4B.3. Cytotoxicity analysis

The present study evaluates the potential of Amalaki Rasayana, *E. officinalis and B. monosperma* for cytotoxicity against cancer cells and normal cells in comparison with the standard drug, Doxorubicin (DOX). MTT assay was performed to assess the cytotoxicity of all the extracts. The extracts of AR, EO and BM were evaluated against human cervix adenocarcinoma (HeLa) and normal lung fibroblast (WI-38). The results of the cytotoxic studies are shown in **Table 4B.1**. The effect of various

concentrations of BMA, BME, EO, AR & BM on HeLa and WI-38 cell lines at 24 and 48 h are shown in **Figure 4B.2.**

Table 4B.1. Cytotoxicity data for different extracts in HeLa and WI-38 cells over a time of 24 and 48 h

	IC_{50} in µg/mL			
	HeLa		WI-38	
Extracts	24 h	48 h	24 h	48 h
Doxorubicin[a]	13.8 ± 0.05	1.21 ± 0.005	>100	4.23 ± 0.002
EO	65.23 ± 0.00 [b]	50.77 ± 0.02[b]	>100[c]	33.43 ± 0.02[b]
AR	44.77 ± 0.001[b]	2.94 ± 0.005[b]	>100[c]	>100[c]
BM	1.56 ± 0.002[b]	0.545 ± 0.001[b]	96.29 ± 0.002[b]	22.72 ± 0.005[b]

[a]Standard drug; [b]Statistical significance: DOX vs Extracts p<0.001; [c]Not tested; HeLa - human cervix adenocarcinoma; WI-38 - normal lung fibroblast

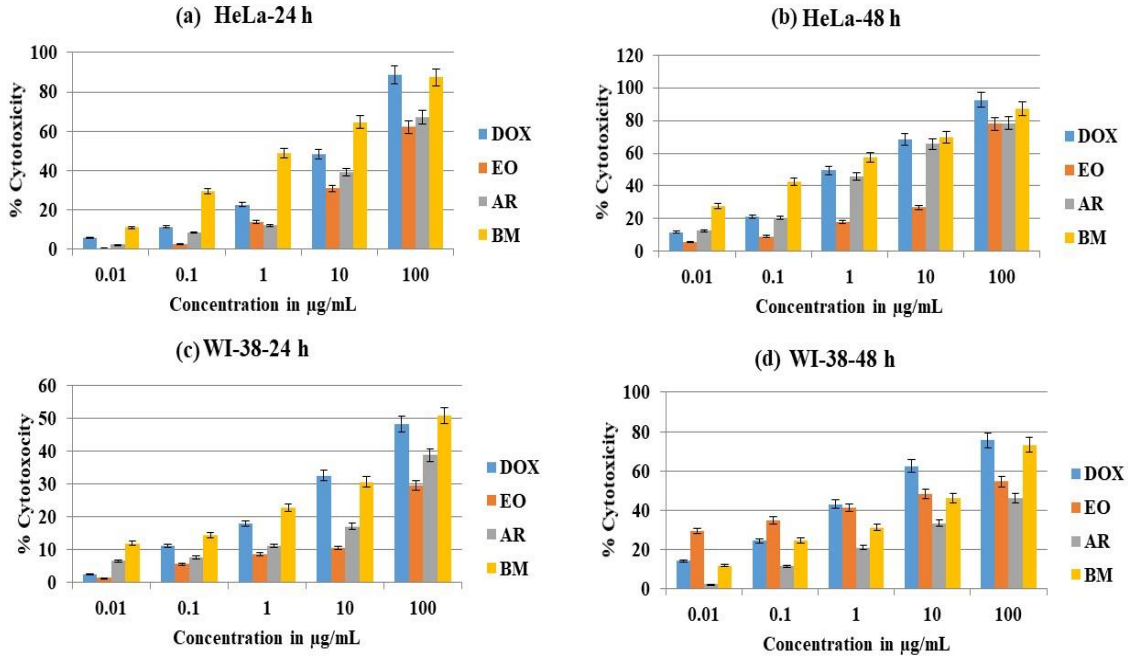

Figure 4B.2. Evaluation of cytotoxicity of extracts by MTT assay on (a) HeLa cells at 24 h (b) HeLa cells at 48 h (c) WI-38 cells at 24 h and (d) WI-38 cells at 48 h

The results showed that AR and BM possessed potent cytotoxic activity against HeLa cells with an IC_{50} value falling 2.94 µg/mL & 0.545 µg/mL at 48 h. With 48 h of incubation, 10 µg/mL of extracts of EO, AR, and BM showed 26.85, 65.7 and 69.75 % of inhibition on cell proliferation. The IC_{50} values for 24 h treatment were 13.8, 65.23, 44.77 and 1.56 µg/mL, respectively, for DOX and extracts. In the case of

WI-38 cells, all the extracts showed the least cytotoxic effect compared to DOX. The IC_{50} values of the extracts were >100 µg/mL at 24 h and that of AR remains the same at 48 h of incubation. But EO and BM exhibited cytotoxicity with an IC_{50} value of 33.43 and 22.72 µg/mL.

4B.4. Discussion

Amlaki or amla (*Emblica officinalis*) is the Indian gooseberry that belongs to the family Euphorbiaceae, has traditionally been used for various medicinal purposes. According to the two primary classic texts on Ayurveda, Charaka Samhita and Sushruta Samhita, amla is regarded as ``the best among rejuvenating herbs,'' "useful in relieving cough and skin diseases,'' and "the best among the sour fruits".[18] This plant has an inevitable role in Ayurveda, having antioxidant, antitumor, hepatoprotective, antidiabetic, cardioprotective, anti-ulcer, oxidative stress and memory-enhancing ability.[19] The phytochemicals reported from fruits includes phyllemblin, hydroxymethylfurfural, phyllemblic acid, gallic acid, emblicol, quercetin, ellagic acid, pectin, putranjivan A, *etc*.[20]

Amalaki referred to as the Maharasayana since the fruits serves as the major ingredient or one of the ingredient in most of the Rasayana preaparation.[21] The classical Ayurvedic drugs such as Amritaprasham, Ashwaganda rasayana, Brahma Rasayana, Chyavanaprasha, Narasimha Rasayana and Triphala are reported to exhibit radioprotective effects.[22] All the Rasayana's have a unique formulation with a number of plants and herbs and the Rasayana treatment has a multidimensional benefits other than memory enhancing, anti-aging *etc*. Vyas et *al*. reported the efficacy of the Rasayana Avaleha, as an adjuvant medication to modern radiotherapy and chemotherapy. The preparation constitutes the plant species Amalaki (*Emblica officinalis*), Ashwagandha (*Withania somnifera*), Guduchi (*Tinospora cordifolia)*, Yashtimadhu (Glycirrhiza glabra), Jivanti (*Leptadenia reticulata*), Tulasi (*Ocimum sanctum*), and Pippali (*Piper longum*).[23] Brahmarasayana, is reported to possess anti stress, adaptogenic and memory enhancing effects. It contains more than 35 ingredients in which Amalaki and T*erminalia chebula* (Indian Gall nut) serves as the major one. Triphala consists of the dried fruits of Haritaki, Bibhitaki (*Terminalia*

bellerica) and Amalaki in equal proportions. Triphala reported to inhibit cancer cell proliferation in colon cancer, breast cancer, pancreatic cancer *etc.* [24,25]

The present study evaluates the potential of Amalaki Rasayana, *E. officinalis and B. monosperma* for cytotoxicity against cancer cells and normal cells in comparison with the standard drug, Doxorubicin (DOX). The extracts were assessed for their *in vitro* cytotoxicity against human cervix adenocarcinoma (HeLa) cell line and showed significant cytotoxicity as reflected in IC_{50} value, *i.e.*, 46.98 µg/mL, 26.12 µg/mL, 50.77 µg/mL, 2.94 µg/mL and 0.545 µg/mL compared to the control doxorubicin (IC_{50} 1.21 µg/mL). Moreover, all the compounds extracts were least toxic towards the normal lung fibroblast (WI-38) cells. The studies revealed the cell-specific cytotoxic effect of the extracts. From the results, it is found that, AR and BM were exhibiting an appreciable cytotoxic effect towards HeLa cells even at a concentration of 2.94 & 0.545 µg/mL at 48 h, while showing a negligible cytotoxic effect in WI-38 cells. AR showed much better cytotoxicity (2.94 µg/mL) than EO (50.77 µg/mL) at 48 h towards HeLa cell line. In the case of WI-38 cells, AR was nontoxic upto a concentration > 100 µg/mL, whereas EO exhibited slight toxicity (33.43 µg/mL) at 48 h. The variation in cytotoxicity indicated that the processed amla (AR) was exhibiting more anticancer potential than raw amla.

From the results, BM was exhibiting excellent cytotoxicity than AR and EO towards cancer cells. To check whether the plant BM has any influence on the activity change of amla, we needed further studies. For that we have carried out the phytochemical investigation of the heartwood of BM (Chapter 4A), resulted in the isolation of eleven compounds. The compounds include seven isoflavones: formononetin, daidzein, prunetin, lupiwighteone, afrormosin, erypoegin K, genistein, a monosaccharide: D-mannitol, sterols: *β*–sitosterol, stigmasterol and stigmasterol-*β*-D- glucoside. Majority of the compounds were isoflavones (**Fig. 4B.3.**) and are reported for potent anticancer activity.

Formononetin exhibits excellent cytotoxicity towards various cancer cells such as breast cancer, prostate cancer, bladder cancer, lung cancer, ovarian cancer, cervical cancer *etc*. [26,27] Afrormosin was previously reported from the flowers of *B. monosperma* [28], heartwood of *Gliricidia sepium* [29], stem bark of *Dipteryx alata* [30],

stems of *Millettia dorwardi* [31] of the Leguminosae family. Afrormosin has antioxidant, anti-inflammatory and antiproliferative activities.[32,33] Erypoegin K was first reported from the stem bark of *Erythrina poeppigiana* (Fabaceae) and possesses significant apoptosis-inducing effect against human leukemia HL-60.[34] Lupiwighteone previously isolated from *Vatairea guianensis* Aubl. (Fabaceae) and *Glycyrrhiza glabra* [35] has been reported to induce apoptosis in breast cancer cells MCF-7 and MDA-MB-231.[36] Lupiwighteone is known to inhibit cell growth and induce apoptosis in human neuroblastoma and prostate cancer cells.[37,38] Genistein and daidzein, known as soy phytoestrogens has been explored as growth inhibitor of several cancer cell lines.[39,40] Both induce antiproliferative effects in prostate and breast cancer cells *via* the inhibition of different signalling pathways.[41,42]

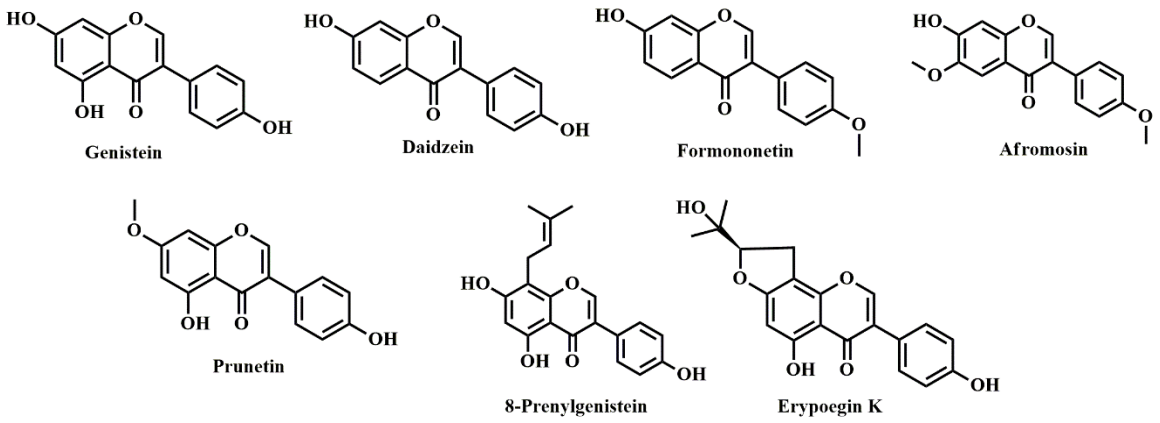

Figure 4B.3. Isoflavones isolated from *B. monosperma*

From the literature review, we could summarize that isoflavones play a significant role in contributing to the anticancer property of the species *B. monosperma*. BM showed high cytotoxicity than AR towards the cancer cell lines but exhibited some toxicity in normal cells. There is significant variation in cytotoxicity of amla and Amalaki Rasayana. The exact mechanism is not known; however, our results suggest that BM significantly contributes to the drastic change in cytotoxicity of Amalaki Rasayana. Moreover, AR has proven to be nontoxic towards the normal cells, whereas amla was showing toxicity. Thus, the method of such drug processing helps to reduce the toxicity of the raw fruits of amla. These findings suggest that the cytotoxicity of BM synergetically enhances the antiproliferative activity of AR or in other words BM helps to boost the therapeutic potential of amla. Also, the nontoxic nature of Amalaki Rasayana gives an immense advantage that AR could be an

effective drug lead in chemoprevention along with other Rasayanas. Hence, the rejuvenation therapy with Amalaki Rasayana will help to improve the quality of life of cancer patients.

4B.4. Conclusions

The present work was undertaken to assess the potential of Amalaki Rasayana as a chemopreventive agent. AR and BM exhibited significant cytotoxicity towards the human cervix adenocarcinoma (HeLa) cell lines. Even though AR is not as effective as doxorubicin against HeLa cell lines but exhibited low toxicity against normal lung fibroblast (WI-38) up to a concentration of 100 μM. Similarly, extracts of BM and EO showed lower toxicity in WI- 38 cells. The results indicate that the cytotoxicity of amla towards normal cells is reduced when it is thermally processed inside the wooden vessels of BM stem. So *Butea monosperma* plays the role of a perfect synergistic therapeutic partner for amla to improve its therapeutic efficacy and reduce toxicity. It is evident from our study that the methods used by our ancestors for drug processing helps to increase the therapeutic potential of the final drug form. Moreover, further studies are needed to uncover the exact mechanism of action of Amalaki Rasayana.

4B.5. Experimental Section

Same as in Chapter 2A.

4B.6. References

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CHAPTER 5

Phytochemical Investigation of Some Selected Plants from the Zingiberaceae Family

5.1. Introduction

Zingiberaceae, the ginger family of flowering plants, is the largest monocotyledonous family in India. It constitutes of 53 genera and more than a thousand species, among which 22 genera and 165 species are confined to the eastern and peninsular regions of India. Zingiberaceous plants have been a part of our culture and tradition both as medicine and spices. The important genera of Zingiberaceae which possess medicinal and aromatic properties are *Curcuma, Hedychium, Kaempferia, Amomum, Alpinia, Zingiber, Elettaria* and *Costus*. The members of this family are small to large perennial plants with creeping horizontal rhizome. An important distinguishing characteristic is the presence of volatile oils and oleoresins of export value.

Within this family, *Curcuma* is a genus comprising over 120 of rhizomatous herbs gaining worldwide importance as a potential source of new drugs to combat a variety of ailments and as an essential natural resource that provides many useful products such as spice, medicine, dyes, food, perfume and aesthetics to people. The generic epithet was derived from the Arabic word Kurkum, meaning yellow, which refers to the colour of the rhizome or flowers and *Curcuma* is the Latinised version. In 1753, Carl Linnaeus established the genus in his Species Plantarum. He described ten species under five genera of the present-day Zingiberaceae, including two of the *Curcuma* species, namely *C. rotunda* and *C. longa*. The first classification of the genus was done by Baker (1890 - 1892), and he reported twenty-nine species from India. Later Roxburgh (1820-1824) in his Flora Indica, described sixty-five species of Zingiberaceae under the class Monandria monogynia distributed over eight genera.[1]

The rhizomes of the *Curcuma* species are usually aromatic, carminative and are used to treat indigestion, jaundice, hepatitis, atherosclerosis, diabetes and bacterial infections. They contain molecules credited with anti-inflammatory, antimicrobial, anti-rheumatic, insect repellent, antiviral, hypocholesterolemic,

antidiabetic, antifibrotic, choleretic, antihepatotoxic, anti-venomous and anticancer properties.[2–6]

5.1.1. Curcuma species

Various Curcuma species occurring in India are *C. aeruginosa*, *C. albifora*, *C. amada, C. amarissima*, *C. angustifolia*, *C. aromatica*, *C. bhatti*, *C. brog*, *C. caesia*, *C. cannonorensis*, *C. caulina*, *C. comosa*, *C. coriacea*, *C. decipiens*, *C. relinata*, *C. oligantha*, *C. ferruginea*, *C. karnatakensis*, *C. kudagensis*, *C. longa*, *C. montana*, *C. mutabilis*, *C. nilamburensis*, *C. raktakanta*, *C. vamana*, *C. zedoaria*, *etc*.

5.2. Literature survey on the Phytochemistry and Pharmacological uses of some important *Curcuma* species

Curcuma species are widely used in traditional medicine for the treatment of a variety of ailments – as a stomachic, carminative, bitter tonic for indigestion and liver disorder. Marked variations have been observed in *Curcuma* species regarding chemical constituents and essential oils. The monoterpenoids present in the genus include limonene, 1,8-cineole, camphor, linalool, camphene, methyl cinnamate, myrcene, fenchone, α-fenchyl acetate, geranyl acetate, *β*-fenchyl alcohol, terpinene-4-ol, *p*-cymene, *α*-terpinene, *γ*-terpinene, bornyl acetate menthe-1,4,8-triene, borneol and isoborneol. Sesquiterpenoids found in this genus were *β*-caryophyllene, germacrene-D, ar-curcumene, trans-*β*-farnesene, zingiberene, caryophyllene oxide, xanthorrhizol, ar-turmerone, *α*-atlantone, turmerone, curdione, and isocurcumenol. Fatty acid like palmitic acid, acyclic hydrocarbons like heptadecane and pentadecane are also present.

5.2.1. *Curcuma longa*

Turmeric (*C. longa*) is commonly used as a spice, food preservative and colouring material in India, China and southeast Asia. It has been used in traditional medicine as a household remedy for various diseases, including biliary disorders, cough, anorexia, rheumatism, diabetic wounds, hepatic disorders and sinusitis.[7] In the Indian Ayurvedic system of herbal medicine, turmeric is known as strengthening and warming to the whole body. Ethnomedicinal uses in India include to improve digestion, to improve intestinal flora, to eliminate worms, for relief of arthritis and swelling, to cleanse and strengthen the liver and gallbladder, to relieve gas, to normalize menstruation, as a blood purifier, to warm and promote proper

metabolism correcting both excesses and deficiencies, for local application on sprains, burns, cuts, bruises, insect bites and itches, for soothing action in cough and asthma, as antibacterial and anti-fungal, and in any condition of weakness or debility.[8] *C. longa* demonstrated a number of pharmacological activities including antioxidant, hepatoprotective, anti-inflammatory, anticarcinogenic, antimicrobial, antidiabetic, antiviral, neuroprotective, cardiovascular protective and gastrointestinal effects.[9]

Ohshiro *et al*., reported germacrone-13-al, 4-methoxy-5-hydroxybisabola-2,10-diene-9-one, 4-hydroxybisabola-2,10-diene-9-one, 2,5-dihydroxy-bisabola-3,10-diene, procurcumadiol, dehydrocurdione, curcumenone, (4S,SS)-germacrone-4,5-epoxide, bisabola-3,10-diene-2-one, epiprocurcumenol, isoprocurcumenol, ar-turmerone, bisacumol, bisacurone, curcumenol, zedoaronediol and procurcumenol from *C. longa.*[10] Khan *et al*., reported turmeronol-A, turmeronol-B, 4-hydroxy bisabola-2,10-diene-9-one, 3,4-dimethoxycinnamic acid, 4-hydroxy-3-methoxy cinnamic acid, 4-hydroxybenzaldehyde and 2,3,5,6-tetrahydroxy-arturmerone.[11] Curcumin was first isolated in 1815, but its chemical structure had not been elucidated until 1910. Its chemical structure was determined by Roughley and Whiting in 1973. Natural curcumin isolated from *C*. *longa* contains curcumin I (Diferuloylmtehane, 94 %), curcumin II (demethoxy curcumin, 6 %) and curcumin III (bisdemethoxycurcumin, 0.3 %). Curcumin, the major component in *Curcuma* species shows a broad range of biological activities. Pharmacological properties of curcumin include anti-inflammatory, reduction of blood cholesterol level, prevention of low density lipoprotein (LDL) oxidation, suppression of symptoms associated with type II diabetes, suppression of thrombosis and myocardial infarction, multiple sclerosis, Alzheimer's disease, rheumatoid arthritis, inhibition of human immunodeficiency virus (HIV) replication, enhancement of wound healing, bile secretion, increase of protection from liver injury, exhibition of anti-leishmaniasis cataract formation and pulmonary toxicity and fibrosis, and antiatherosclerotic properties, as well as prevention and treatment of cancer.[12]

5.2.2. *Curcuma mangga (*syn *C. amada)*

C. *mangga* is traditionally used as carminative and stomachic agent. The rhizomes are used for the treatment of inflammatory conditions as a household remedy on an empirical basis.[13] The rhizomes reported to possess antioxidant, anti-inflammatory, cytotoxic, antiallergic, analgesic and antitubercular activities.[14]

Molecules reported from *C. mangga* are (*E*)-labda-8(17),12-dien-15,16-dial, (*E*)-15,16-bisnor-labda-8(17),11-dien-13-on, β-sitosterol, zerumin A, curcumin, bis-demethoxycurcumin, demethoxycurcumin, labda-8(17),12-diene-15,16-dial, calcaratarin A , scopoletin, zerumin B, 1,7-bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one and *p*-hydroxycinnamic acid.[15,16]

5.2.3. *Curcuma aromatica*

C. *aromatica*, one of the aromatic and pretty ginger is a wild plant found in the forest of Western Ghats and Bengal in India. Traditionally used for the treatment of cancer and lung disease and commonly used as a flavoring agent, condiment and a source of yellow dye. Medicinally it possesses strong antimicrobial properties. It is a well-listed drug in Ayurveda and another indigenous system of medicine. Historically rhizomes are used as a tonic, carminative and externally in combinations with astringents, bitters, and aromatics to bruises, sprains, and snake bite. They are also used for the treatment of skin eruptions, infections and to improve skin complexion.[17] The rhizomes of *C. aromatica* has antiangiogenic, antimicrobial, antitumor, cholerectic cholagogues, anthelminthic, wound healing, cytoprotective, anti-inflammatory, antioxidant and hypoglycemic activity.[18,19] Isozedoarondiol, methylzedoarondiol, neocurdione, germacrone, curdione, (4S,5S)-germacrone 4,5-epoxide, procurcumenol, zedoarondiol, dehydrocurdione and curcumenone were reported from the rhizomes of *C. aromatica.*[20,21]

5.2.4. *Curcuma zedoaria*

C. zedoaria is a well-known ethnomedicinal plant, different plant parts are used in Ayurveda and Indian traditional folk medicine is well recognized. Its rhizome oil is used for stomachic, emmenagogic, vomiting, *etc*. The rhizome of *C. zedoaria* is used as appetizer and tonic. It is an odoriferous ingredient of the cosmetics used for the cure of chronic skin diseases. The powdered rhizome is used as an anti-allergant. Its leaf juice is used in the treatment of dropsy and leprosy. Fresh roots of *C. zedoaria* are used in the treatment of leucorrhoea discharge.[22] Several studies were carried out on the biological activities of C. *zedoaria*. Some of them are antimicrobial, antifungal, larvicidal, antinociceptive, antiallergic, analgesic, antiulcer, hepatoprotective, anti-inflammatory, antimutagenic, antivenom, antioxidant and anticancer activities.[23] Shiobara *et al.,* reported the isolation of cucuminolides: curcuminolide A and curcuminolide B, (+)-germacrone-4,5-epoxide, germacrone, furanodienone,

curzerenone, zederone, dehydrocurdione, curcumenol, isocurcumenol, zedoarol, 13-hydroxygermacrone and curzeone from the rhizomes of *C. zedoaria.*[24,25] Matsuda *et al.,* isolated carabrane-type sesquiterpenes: curcarabranols A and B, a new eudesmane-type sesquiterpene: zedoarofuran, six new guaiane- or seco-guaiane-type sesquiterpenes: 4-epicurcumenol, neocurcumenol, gajutsulactones A and B, zedoarolides A and B, curcumenone, 4S-dihydrocurcumenone, isolated from aqueous acetone extract of rhizomes together with 36 known sesquiterpenes and two diarylheptanoids.[26,27] Makabe *et al.,* reported eleven sesquiterpenes including furanodienone and zedoaronediol.[28] Hong *et al*., reported ar-turmerone and β-turmerone from the rhozomes.[29] Kyoung *et al.* isolated curcumenol-9,10-epoxide and curcuzedoalide B.[30]

5.2.5. *Curcuma haritha*

C. *haritha* is a lesser-known, widely distributed medicinal Zingiber from Kerala. It is common throughout the coastal regions and grows along with *C*. *caesia* and *C*. *raktakanta*. The rhizome is an expectorant, astringent and useful for treating diarrhoea.[31] The essential oil contains curdione, furanogermenone, germacrone, neocurdione, (4S,5S)-germacrone-4,5-epoxide, curcumol and curzerene.[32]

5.2.6. *Curcuma xanthorrhiza*

In traditional medicine, *C*. *xanthorrhiza* is used for the treatment of hepatitis, liver complaints, diabetes, cancer, hypertension, rheumatism and heart disorders.[33] *C. xanthorrhiza* has shown antidiuretic, antioxidant, anti-inflammatory, anti-hypertensive, antirheumatic, anti-hepatotoxic, anti-dysmenorrheal, anti-spasmodic, anti-leucorrhoea, antibacterial and antifungal activities. Curcumin, xanthorrhizol, 10-methylalismoxide, (+)-alismoxide, isozedoarondiol, zedoarondiol, (1R,4S,5S,10R)-10-methyl guaianediol, zedoalactone B, 13-hydroxyxanthorrhizol, 12,13-epoxyxanthorrhizol, *β*-curcumene, *β*-bisabolol, (7R,10R)-10,11-dihydro-10,11-dihydroxyxanthorrhizol 3-O-*β*-D- glucopyranoside and (-)-curcuhydroquinone 2,5-di-O-*β*-D-glucopyranoside are present in this species.[34–36] Xanthorrhizol exhibits antibacterial, antifungal, hepatoprotective, nephroprotective, neuroprotective, antioxidant and anti-inflammatory activities.[37]

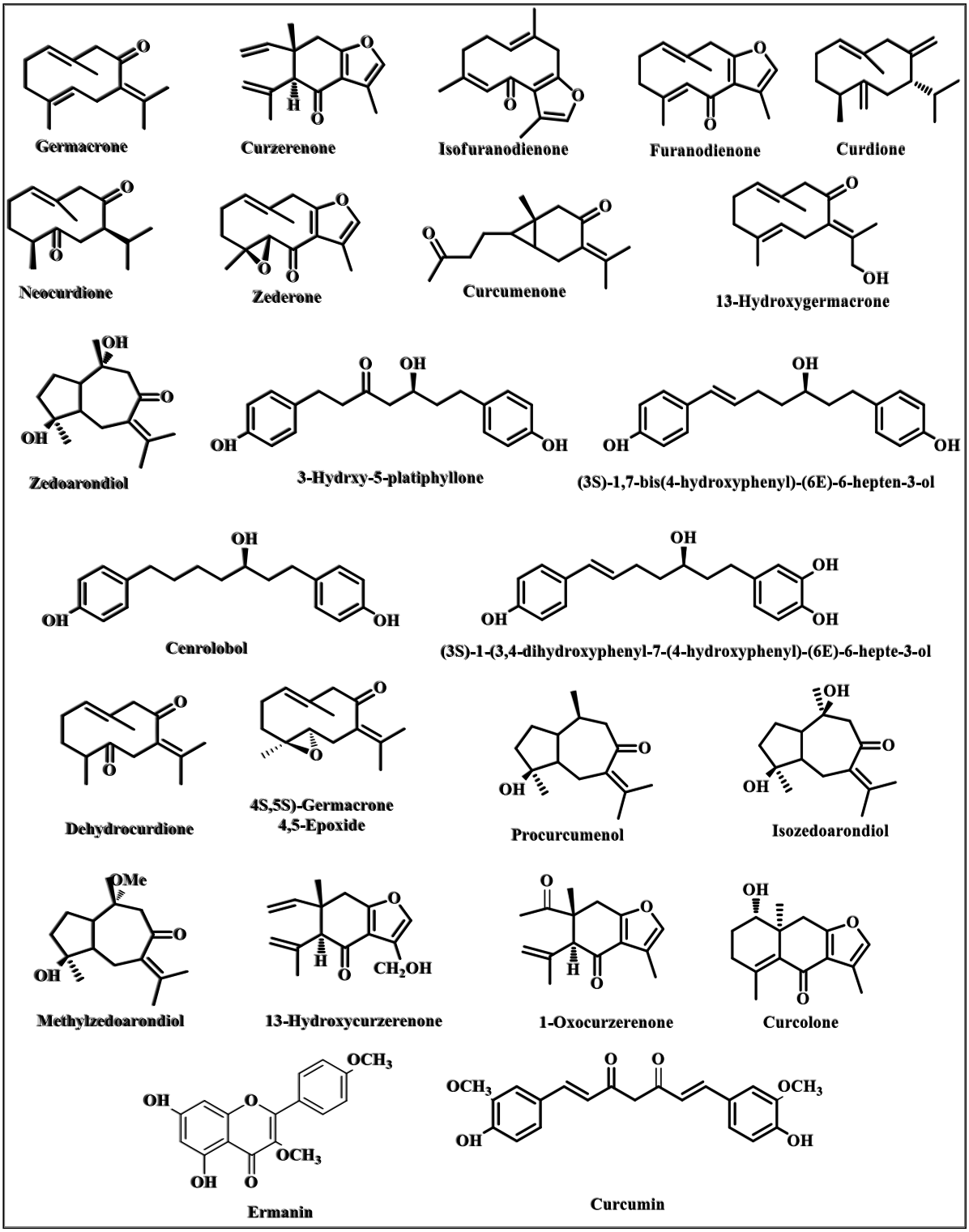

Figure 5.1. Some compounds reported from *Curcuma* genus

5.2.7. *Curcuma caesia* Rox.

Curcuma caesia Roxb. (Black Tumeric), is a perennial herb of distinguishable bluish-black rhizome with a bitter and pungent smell. It is widely cultivated as a

medicinal plant in South East Asian countries. The rhizomes of this plant are used as stimulants, anti-diarrheal, diuretic, anti- emetic, wound cleaner and skin disorder. Traditionally, the rhizomes of *Curcuma caesia* are used in treating leucoderma, asthma, tumours, piles, bronchitis, headaches and rheumatic pains.[38] The essential oil of this plant possessed antifungal, antioxidant and antimutagenic activity.[39] The major constituents were identified from the methanolic extract were α-santalol (46.90 %), retinal (10.72 %) and ar-tumerone (10.38 %).[40]

5.3. Aim and scope of the present study

From the literature survey, it is evident that the plants belong to the Zingiberaceae family are a rich source of bioactive compounds. Although 80 species are reported in *Curcuma*, a perusal through the literature revealed extensive work in *C. longa* followed by *C. amada*, *C. aromatica* and a few reports in *C. zedoaria*. We hardly came across any work on other species of *Curcuma*. Hence we have selected two species from Curcuma *viz, Curcuma raktakanta* and *Curcuma aeruginosa.* For the best of our knowledge, the chemoprofiling of *C. raktakanta* is hitherto investigated. Hence a detailed study was undertaken on the essential oil of the rhizome of *C. raktakanta (Chapter 5B)* and phytochemical reinvestigation of *C. aeruginosa* (Chapter 5A). Synthetic modification of a natural product germacrone is also described in chapter 5B. Another unexplored species *Hedychium flavescens* from *Hedychium* genus is the subject of matter in chapter 5C.

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CHAPTER 5

Chapter 5A: Phytochemical Investigation of the Rhizomes of *Curcuma aeruginosa* Roxb

5A.1. Introduction

Curcuma aeruginosa Roxb is a medicinal plant belongs to Zingiberaceae family known as pink and blue ginger in English and as Neelakoova in Malayalam. The plant is native to Myanmar and also found in West Bengal, Coromandel Coast, Bihar, South Karnataka and Kerala. The species is indigenous from Indo-China to West Malaysia. Rhizomes of *C. aeruginosa* are used in traditional medicine to relieve stomach pain, enteritis, asthma, rheumatic problems, increase appetite, prevent obesity and serve as an anthelmintic. Many biological activities have been reported for *C. aeruginosa,* such as an anti-inflammatory agent, a drug to increase numbers of thrombocytes in dengue fever treatment, an anti-androgenic, anti-microbial agent, a platelet-activating factor and antagonists for treatments of immunological and inflammatory disorders, an anti-nociceptive, an anti-oxidant and as an anti-cancer agent.[1]

Figure. 5A.1. Inflorescence and rhizomes of *C. aeruginosa*

5A.2. Ethnopharmacology

Curcuma aeruginosa is an indigenous medicinal plant found in the south-east Asia region. The rhizomes are considered to be anthelmintic and depurative. They are used externally to treat skin conditions such as scurvy and itchy skins. Rhizomes of *C. aeurginosa* are used medicinally as antidiarrhoeal, antifungal and externally as

astringent for wounds. Rhizomes of *C. aeruginosa* have functional food quality as well as medicinal and health benefits. Tubers/rhizomes used as food by tribals from Pechiparai Social Forest in Kanyakumari district, India. In Indonesia, the rhizome of *C. aeruginosa* is commonly utilized as an ingredient of traditional medicine to treat various diseases.[2] The rhizome of *C. aeruginosa* was traditionally used to treat gastrointestinal problems such as diarrhoea and fungal infections.[3] It also used as an analgesic, antipyretic, anti-inflammatory, to treat cold, cough, asthma and uterine maladies.[4] The plant has been used as an active ingredient in many Thai herbal medicinal recipes used for the treatment of inflammations, postpartum uterine and peri-menopausal bleeding and also as carminative, analgesic and anti-inflammatory agent for uterine inflammation.[5] According to Burkill, it is one of the numerous ingredients in Singapore's universal tonic or 'ubat jamu'. It is prescribed for cough and asthma and used externally pounded in coconut oil for scurf and to treat mental disorders.[2]

5A.3. Phytochemistry

Itokawa *et al.,* reported guaiane type sesquiterpenes, Zedoalactone A, Zedoalactone B and zederoandiol from the rhizomes of *C. aeruginosa*.[6] Zhang *et al.,* reported germacrone, curzerenone, curcumenol and isocurcumenol.[7] Boutsada *et al.,* reported pyrocurzerenone, dehydrochromolaenin, curzeone, linderazulene, curzerenone and 8,12-epoxy-1(10),4(15),7,11-germacratetraen-6-one from the methanolic extract of rhizomes.[4] Sirat *et al*., reported isofuranodiene, furanodienone, dehydrocurdione, curcumenone, 13-hydroxygermacrone and zedoarol.[8] Auregenone, aerugidiol, curcumenone, zederone zedoarondiol and difurocumenone also reported from the rhizomes.[9–12] More than 20 components were identified from the rhizome essential oil, in which methenolone, cycloisolongifolene, propiolic acid, *etc*., were the major compounds.[13] More than fifty components are identified from the leaf essential oil, curzerene (10.5 %), furanogermenone (7.8 %), 1,8-cineole (17.7 %) and camphor (7.5 %), were the major components.[14]

5A.4. Pharmacological activity

Paramita *et al*., reported the anti-inflammatory activity *C. aeruginosa* extracts in carrageenan-induced paw oedema, the extracts exhibited significant activity.[15]

Reanmongkol *et al*., reported the analgesic, antipyretic and anti-inflammatory activities of chloroform, methanol and water extracts. Chloroform extract exhibited good analgesic effect.[16] Hossain *et al.,* evaluated the antinociceptive activity of the methanolic extract of *C. aeruginosa* rhizomes and germacrone. Germacrone showed potent activities in both writhing and licking methods indicating the compound is the central and peripheral antinociceptive principle of *C. aeruginosa* rhizome.[17] Different plant parts of *C. aeruginosa* reported for excellent antioxidant activity.[1819] Nurcholis *et al*., reported the antioxidant activity of ethanolic extract of 20 accessions of *C. aeruginosa*.[20] Sulfianti *et al.,* evaluated the chemo preventive potential of the rhizomes of *C. aeurginosa* in Wistar rat induced by 7,12-Dimethylbenz[a]anthracene (DMBA). Administration of the extracts at different doses promoted an increase in TNF-α, IL-2 and IL-12 levels and the dose of 80 mg/ 200 g BW appeared to be best potential dose as chemo-preventive agent.[21] The chloroform and *n*-hexane fraction of rhizomes exhibited cytotoxicity against MCF-7 and Ca-ski cell lines.[9]

The ethanolic extract of rhizomes exhibited antibacterial activity against *S. aureus* and *E. coli.*[22] Various extracts of rhizomes such as hexane, chloroform, ethyl acetate, acetone, methanol and water were tested against Gram positive and Gram negative bacteria. Among these, hexane extract exhibited maximum inhibition against *B. cereus* and methanol extract against *S. typhi*.[23] *C. aeruginosa* rhizome oil showed strong activity against the growth of *S. aureus*, *B. cereus*, *P. aeruginosa* and *C. albicans*.[24] The essential oil of rhizomes showed moderate activity against the fungi *C. albicans* and weak activity towards *C. neoformans*.[25] Methanolic extract of *C. aeruginosa* and curcumenone exhibited moderate activity against *S. typhi* and *E. coli*.[26] Methanol extract was also effective in inhibiting parasites such as *Fasciola gigantica*.[3] *C. aeruginosa* extracts showed effective insecticidal activity against red spider mites (*Tetranychus urticae*) on *L. rotundifolia* leaves.[27]

C. aeruginosa has high anti-androgenic activity both *in vitro* and *in vivo.* Srivilai *et al.*, reported the anti-androgenic activity of furanodiene isolated from the hexane extract of *C. aeruginosa*.[28] Other sesquiterpenes germacrone, zederone, dehydrocurdione, curcumenol, zedoarondiol and isocurcumenol were also exhibited good activity in which germacrone was the most active.[29,30] (E,E)-8-

hydroxygermacrene B synthesised from germacrone and analogues of germacrone were also showed remarkable *in vitro* activity.[31,32] Thaina *et al*., reported the uterine relaxant effects of chloroform and methanol extract of *C. aeruginosa* rhizomes using isolated uterus strips from estrogen primed rats. The extracts exerted concentration-dependent inhibition of the contractions induced by oxytocin, prostaglandin, Ach and KCI.[5] 5 % hexane extract of *C. aeruginosa* and 5 % minoxidil is efficient for the treatment of androgenetic alopecia.[33] Ethanolic extract of the rhizomes exhibited decrease in spasmolytic activity on isolated guinea pig tracheas indicated good antiasthma effect.[34] The water extract of the rhizomes significantly inhibited human immunodeficiency virus type-1 (HIV-1) by interfering with the adsorption of the virus to the cell.[35] Six members of Zingiberaceae family along with *C. aeruginosa* effectively inhibited platelet activating factor (PAF) with IC_{50} values ranging from 1.2 to 18.4 $mgml^{-1}$.[36]

5A.5. Extraction, isolation and characterization of compounds from the rhizomes of *C. aeruginosa*

5A.5.1. Collection and extraction of plant material

The plant material of *Curcuma aeruginosa* was collected from a medicinal garden in Thiruvananthapuram. The plant material was authenticated by the taxonomist of M. S. Swaminathan Research Foundation (MSSRF), Kerala and a voucher specimen (M.S.S.H. 806) was deposited in the herbarium repository of the institute. Approximately 1 kg of the plant material was coarsely powdered and subjected to repeated extraction with hexane (2.5 L * 3 days) using mechanical stirrer for about three times at room temperature. Thin-layer chromatography indicated that the extraction was completed after nine days. The total extract was then concentrated under reduced pressure using a Heidolph rotatory evaporator, which yielded 25 g of crude extract. In the same way, using the residue after the removal of the hexane, acetone (40 g) and ethanol (28 g) extract were collected.

5A.5.2. Preliminary cytotoxic studies of the extracts

The crude extracts were screened for cytotoxic analysis against cervical carcinoma (HeLa) cell lines using MTT assay. Doxorubicin (DOX) was used as the standard drug. From the results, acetone extract exhibited potent cytotoxicity with

an IC_{50} value of 41.6 µg/mL at 24 h. Hexane and acetone extract showed an IC_{50} value of 14.8 and 8.74 µg/mL at 48 h in HeLa cell line (**Table 5A.1**).

Table 5A.1. IC_{50} values of extracts in HeLa cell line

Extracts	Cytotoxicity (IC_{50}) in µg/mL	
	HeLa	
	24 h	48 h
Hexane	78.64 ± 0.05	14.8 ± 0.02
Acetone	41.6 ± 0.06	20.5 ± 0.04
Ethanol	54.41 ± 0.04	8.74 ± 0.01
Doxorubicin	43.21	<5

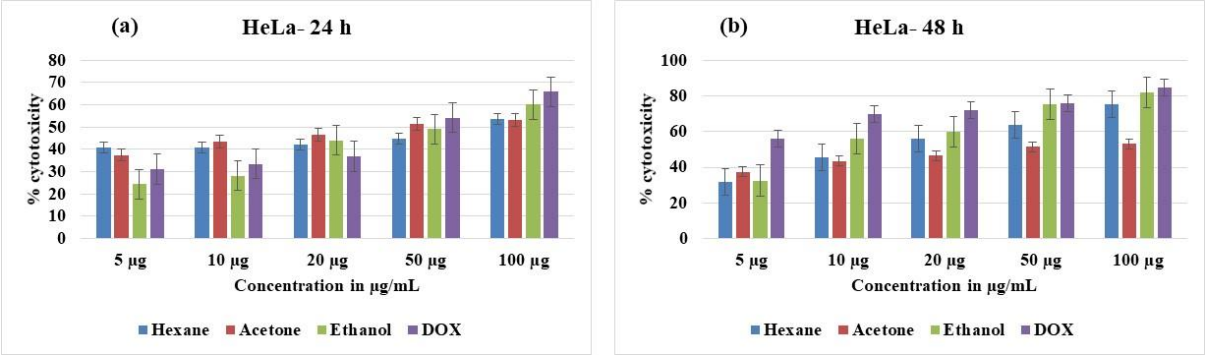


Figure 5A.2. MTT assay of extracts on HeLa cells (a) at 24 h (b) MTT assay at 48 h

5A.5.3. Isolation and characterization of compounds

After examining the TLC profile of hexane and acetone extract, both showed almost similar spots. So, we combined both the extracts for further isolation process. Around 35 g of the extract was subjected to column chromatography on 100-200 mesh sized silica gel and elution was started with 100 % hexane and ethyl acetate-hexane mixture by adding an adequate amount of ethyl acetate to give a total of 45 fractions. Each fraction was collected in a 500 ml conical and pooled into nine fraction pools. Fractions pools on further purification resulted in the isolation of nine marker compounds**.** A pictorial representation of the entire isolation procedure is given in **Figure 5A.3**.

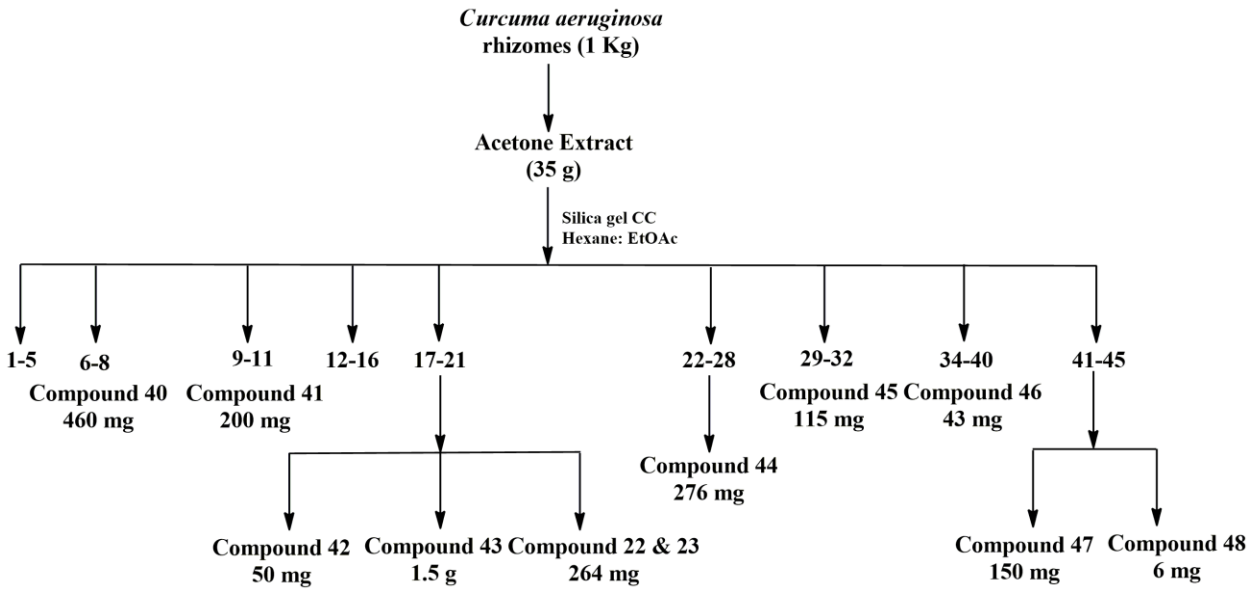
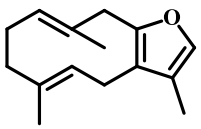


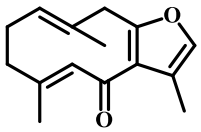
Figure 5A.3. Extraction process

The fraction pool 2 (Fr. 6-8) showed a highly UV active spot. On further purification, we could isolate a colourless oily component as compound **40**. The HRMS analysis showed a molecular ion peak at *m/z* 217.1559 $(M+H)^+$, along with ^1H and ^{13}C NMR data suggested a molecular formula $C_{15}H_{18}O_2$. The characteristic peak at *δ* 7.06 ppm (**Fig. 5A.4**) is due to a methine proton for a trisubstituted furan ring. The multiplet at *δ* 4.94 (m, 1H) and triplet at *δ* 4.74 (t, *J* = 7.5 Hz, 1H) ppm attributed to two olefinic protons. Three methyl protons resonated as a singlet at *δ* 1.92, 1.60 and 1.27 ppm respectively. In the ^{13}C NMR spectrum (**Fig. 5A.5**), the methyl protons appeared at δ 8.9, 16.5 and 16.2 ppm and the aromatic carbon at *δ* 136.0 ppm. In the HMBC spectrum, the proton at *δ* 7.06 (H-12) showed a correlation with carbons at *δ* 149.7 (C-8), 118.9 (C-7) and 8.9 (C-13), supporting the attachment of methyl furan ring at C-7, C-8 of the main skeleton The structure of the compound was identified as **Furanodiene** which was well supported by the literature report.[37] Furanodiene is reported to possess anticancer activity against various types of cancers such as breast cancer, lung cancer and leukaemia cancer cells.[38]



Compound **40 – Furanodiene**

Fraction pool 3 (Fr. 9-11) contains a UV active spot, which on purification by silica gel CC yielded a light yellowish oily compound of 200 mg. The ^{1}H NMR spectrum (**Fig. 5A.6**) showed a sharp singlet at δ 7.08 ppm corresponded to the aromatic proton as in the previous molecule. The singlet at δ 5.81 ppm could be attributed to an olefinic proton. The multiplet at δ 5.18 ppm indicated the presence of another olefinic proton. The three proton singlets at δ 2.13, 1.99 and 1.29 ppm showed the presence of three CH_3-groups. The ^{13}C NMR spectrum (**Fig. 5A.7**) displayed 15 carbon signals, the peak at δ 189.9 ppm confirmed the presence of a carbonyl carbon. The olefinic carbons appeared at δ 130.6 and 132.4 ppm and the methyl groups were resonated at δ 9.6, 15.8 and 19.0 ppm, respectively. In the HMBC spectrum, the carbonyl carbon correlates with the olefinic proton at δ 5.18 (H-5) and the methyl group at δ 1.99 (H-14) confirmed the attachment of C=O in the C-6th position. The mass spectrum of the compound showed a molecular ion peak at *m/z* 231.1410, which is the $(M+H)^+$ peak. Finally, the structure of compound **41** was identified as **Furanodienone,** which was well supported by the literature reports. [37]



Compound **41 - Furanodienone**

Fraction pool 5 (Fr. 17-21) on further CC separation using silica gel gave three different compounds. A colourless crystalline solid was obtained, labelled as compound **42**. The IR spectrum showed absorption corresponding to a hydroxyl group (3400 cm^{-1}) and C=C stretching (1660 cm^{-1}). The ^{1}H NMR spectrum (**Fig. 5A.8**) showed two triplet peaks at δ 4.78 and 4.76 ppm attributed to exocyclic olefinic protons. The singlet at δ 2.84 ppm could be attributed to an –OH proton. The three methyl protons resonated at δ 1.80, 1.62 & 1.00 ppm corresponded to δ 22.5, 19.0, 12.0 ppm peaks in the ^{13}C NMR. ^{13}C NMR spectrum (**Fig. 5A.9**) exhibited 15 carbon signals suggested that the compound has a sesquiterpene skeleton. The ^{1}H and ^{13}C signals were assigned based on HMBC and HMQC analysis. On comparison with reported values, the structure was confirmed as **Isocurcumenol** which was well supported by HRMS analysis with a molecular ion peak at *m/z* 257.1524 $(M+Na)^+$.[39]

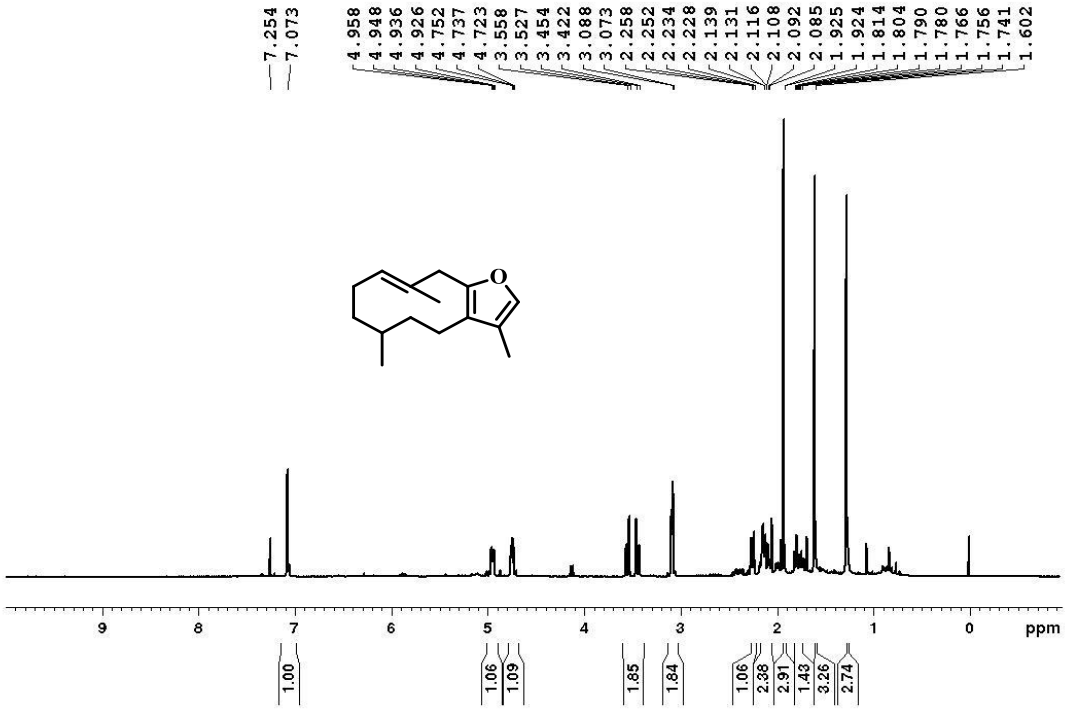


Figure 5A.4. ^{1}H NMR spectrum of compound **40** in $CDCl_3$

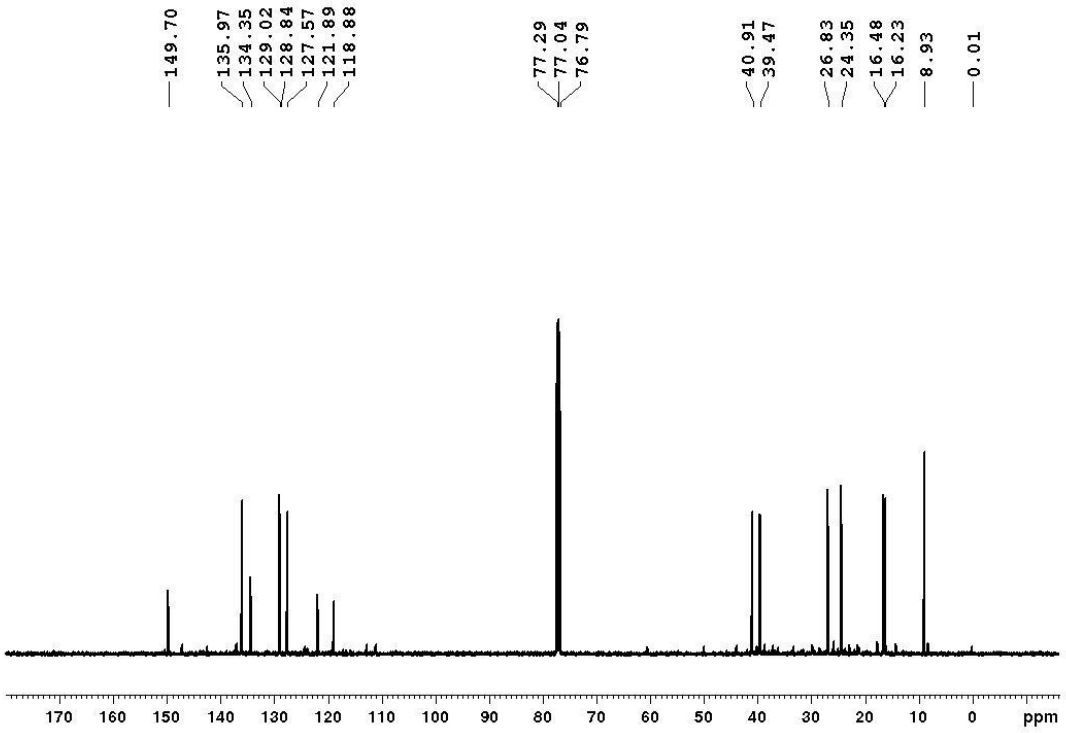


Figure 5A.5. ^{13}C NMR spectrum of compound **40** in $CDCl_3$

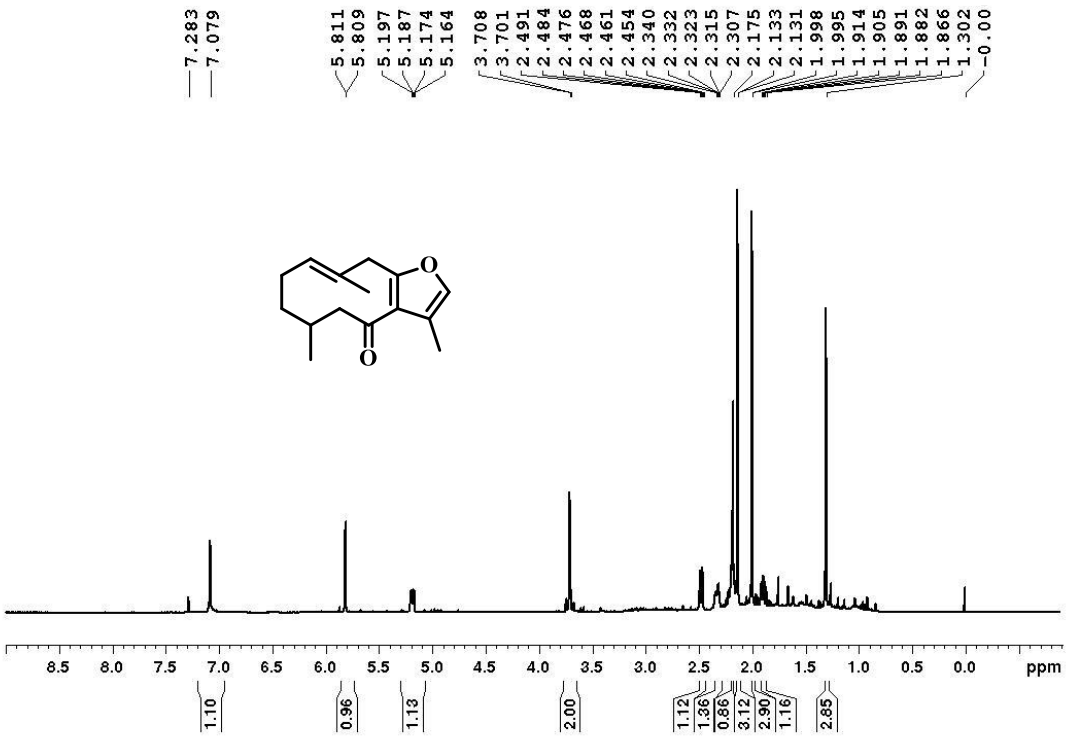


Figure 5A.6. ^{1}H NMR spectrum of compound **41** in $CDCl_3$

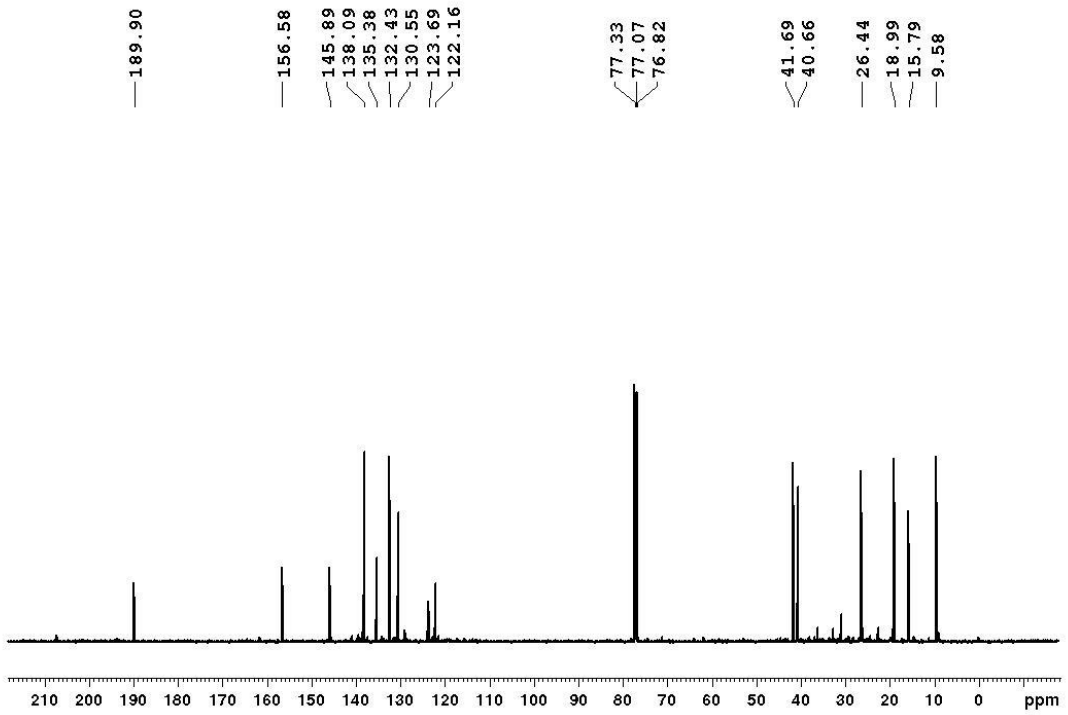


Figure 5A.7. ^{13}C NMR spectrum of compound **41** in $CDCl_3$

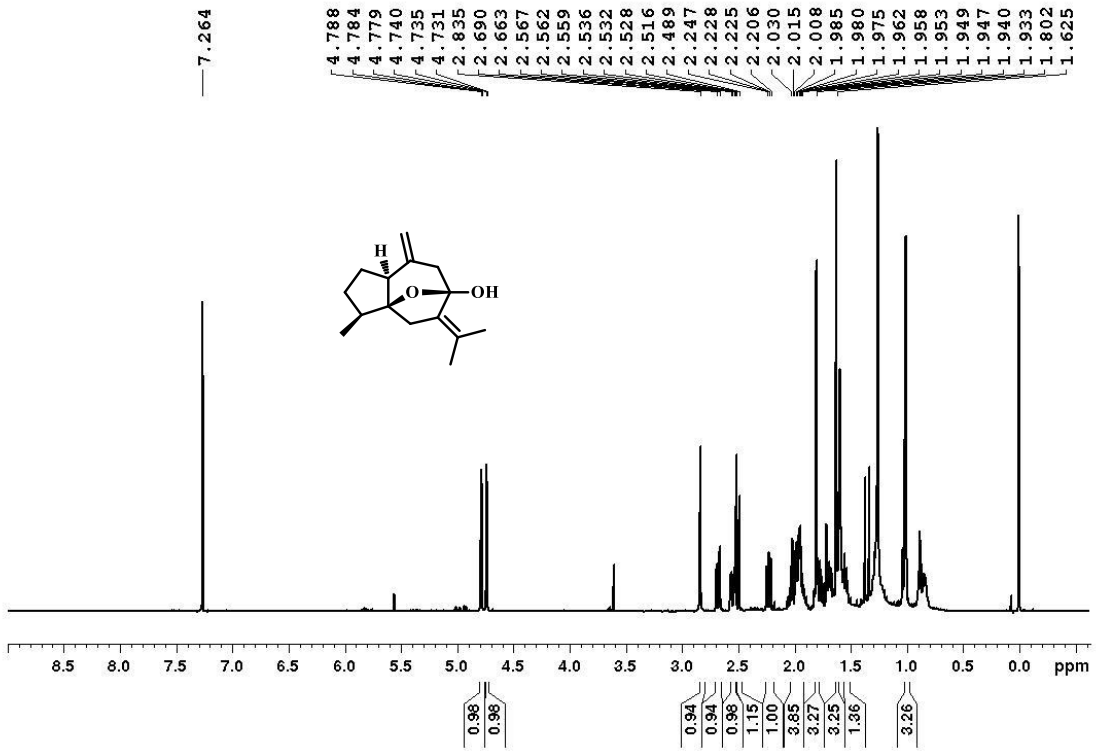


Figure 5A.8. ^{1}H NMR spectrum of compound **42** in $CDCl_3$

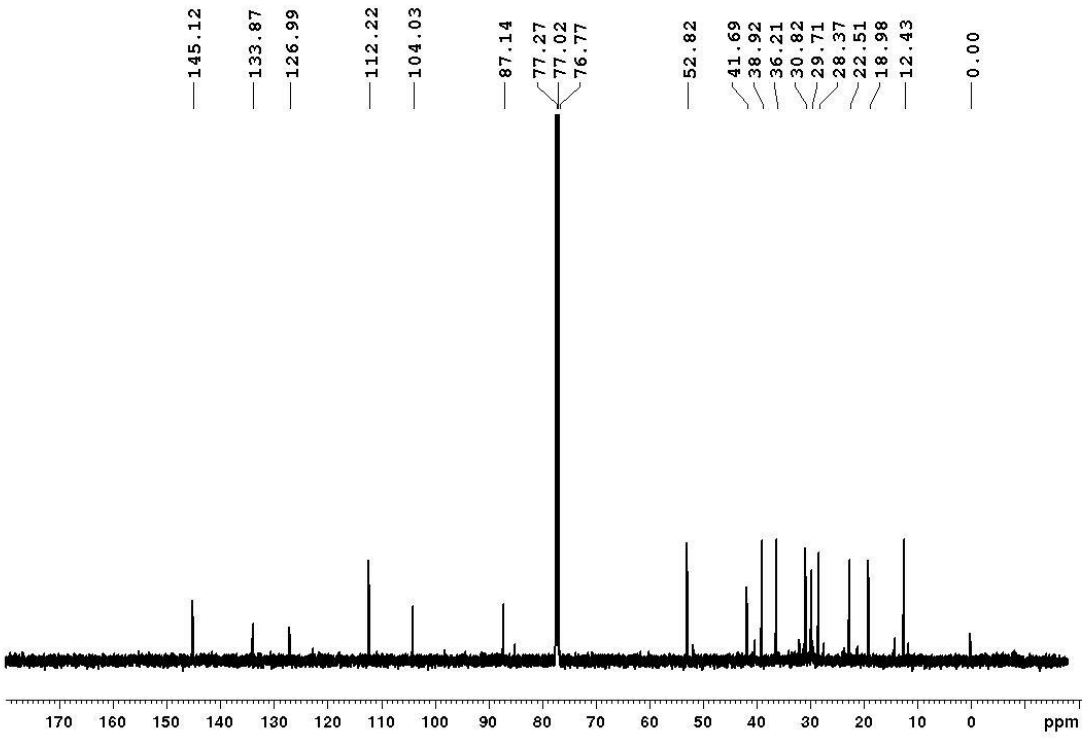
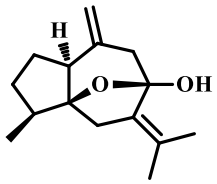


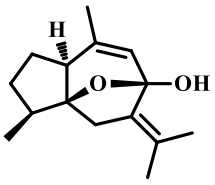
Figure 5A.9. ^{13}C NMR spectrum of compound **42** in $CDCl_3$

Isocurcumenol has been reported to possess anticancer activity against human lung, leukaemia, nasopharyngeal carcinoma and murine lymphoma cells.[40]



Compound **42 – Isocurcumenol**

Fraction pool 5 (Fr. 17-21) obtained by eluting with 10 % ethyl acetate in hexane, upon further purification yielded a colourless crystalline compound of 1.5 g. The HRMS analysis showed a molecular ion peak at *m/z* 301.1410 $(M+H)^+$ suggested a molecular formula $C_{15}H_{22}O_2$. The IR spectrum showed absorption corresponding to a hydroxyl group (3400 cm^{-1}) and C=C stretching (1670 cm^{-1}). The ^1H NMR spectrum (**Fig. 5A.10**) displayed a singlet at δ 5.75 ppm attributed to an olefinic proton and the broad peak at δ 3.37 ppm corresponded to the –OH proton. The four methyl groups resonated at δ 1.81, 1.66, 1.59 & 1.02 ppm and in ^{13}C NMR (**Fig. 5A.11**) at δ 11.9, 18.9, 20.9 & 22.3 ppm, respectively. The structure of compound **43** was assigned as **Curcumenol** based on spectral data as well as literature reports.[41] This was the marker compound obtained from *C. aeruginosa*. Curcumenol reported to exhibit antibacterial, neuroprotective properties and inhibits human liver cytochrome P450 enzymes.[26,42,43]

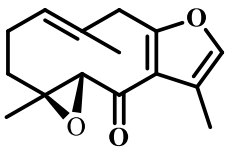


Compound **43 – Curcumenol**

The UV inactive spot obtained from fraction pool 5 (Fr. 17-21) was isolated as a crystalline solid of 264 mg. The crystalline compound was identified as a mixture of β–sitosterol and stigmasterol.

Fraction pool 6 (Fr. 22-28) obtained by eluting the column at 10 % ethyl acetate-hexane polarity, afforded a colourless crystalline solid (276 mg). Mass spectral data provide a molecular ion peak at *m/z* 269.115, which is the $(M+Na)^+$ peak suggested a molecular formula $C_{15}H_{18}O_3$. The IR spectrum indicated the

presence of a conjugated carbonyl group absorption at 1662 cm^{-1}. The ^{1}H NMR spectrum (**Fig. 5A.12**) exhibited characteristic signals at δ 7.09 ppm due to a methane proton for a trisubstituted furan ring and at δ 5.50 (d, J = 11.5 Hz) due to a vinylic proton. The presence of an oxymethine proton at δ 3.81 (H-5) and an oxyquarternary carbon at δ 64.0 confirmed the presence of an epoxide in the molecule. The spectrum showed signals due to three methyl protons at δ 1.35, 1.60 & 2.11 ppm. The ^{13}C NMR (**Fig. 5A.13**) and DEPT-135 spectra of the compound showed the presence of 15 carbon atoms, which includes a carbonyl group (δ 192.2 ppm), three methine (δ 138.1, 131.2 & 66.6 ppm), three methylene (δ 41.9, 38.0 & 24.6 ppm) and three methyl groups (δ 15.7, 15.2 & 10.3 ppm). In the HMBC spectrum the proton at δ 7.09 (H-12) showed a correlation with carbon at δ 157.1 (C-8), 123.2 (C-8) and 10.3 (C-13), supporting the attachment of methyl furan ring at C-7, C-8 of the main skeleton. Thus, based on spectral data and comparison with previously reported values, compound **44** was assigned as **Zederone**.[44] Zederone is one of the major component present in *C. aeruginosa* and it is reported for cytotoxicity against KG1a cell line and produce CYP-inducing and CYP-inhibiting effect.[45,46]



Compound **44- Zederone**

A coloureless oily (115 mg) compound was isolated from fraction pool 7 (Fr. 29-32). IR spectrum showed absorption at 1713 cm^{-1} corresponded to the carbonyl stretching. The HRMS analysis showed a molecular ion peak at m/z 257.1522 $(M+Na)^{+}$, suggested a molecular formula $C_{15}H_{22}O_2$. The ^{1}H NMR spectrum (**Fig. 5A.14**) displayed the presence of a cyclopropane ring in the structure with a peak at δ 0.45 (dt, J =7.1, 4.7 Hz, 1H) and 0.68 ppm (q, J = 4.1 Hz, 1H), two olefinic methyl groups at δ 2.09 and 1.80 ppm and an acetyl group at δ 2.14 ppm, respectively. The ^{13}C NMR spectrum (**Fig. 5A.15**) consists 15 signals with two carbonyl groups (δ 208.9 & 201.7 ppm), four methyl groups (δ 30.0, 23.5, 23.4, & 19.0 ppm), two methine carbons (δ 24.1 & 24.0 ppm), four methylene carbons (δ 48.9, 43.9, 28.0 & 23.4 ppm) and three quarternary carbons (δ 147.4, 128.0, & 20.1 ppm).

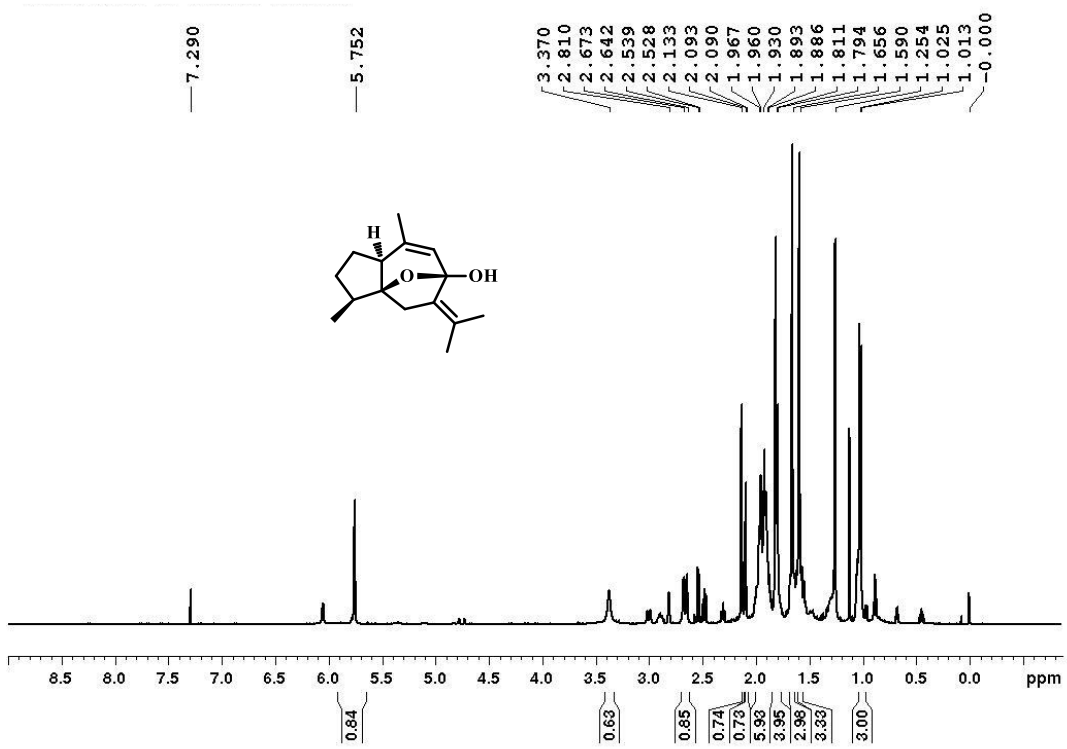


Figure 5A.10. ^{1}H NMR spectrum of compound **43** in $CDCl_3$

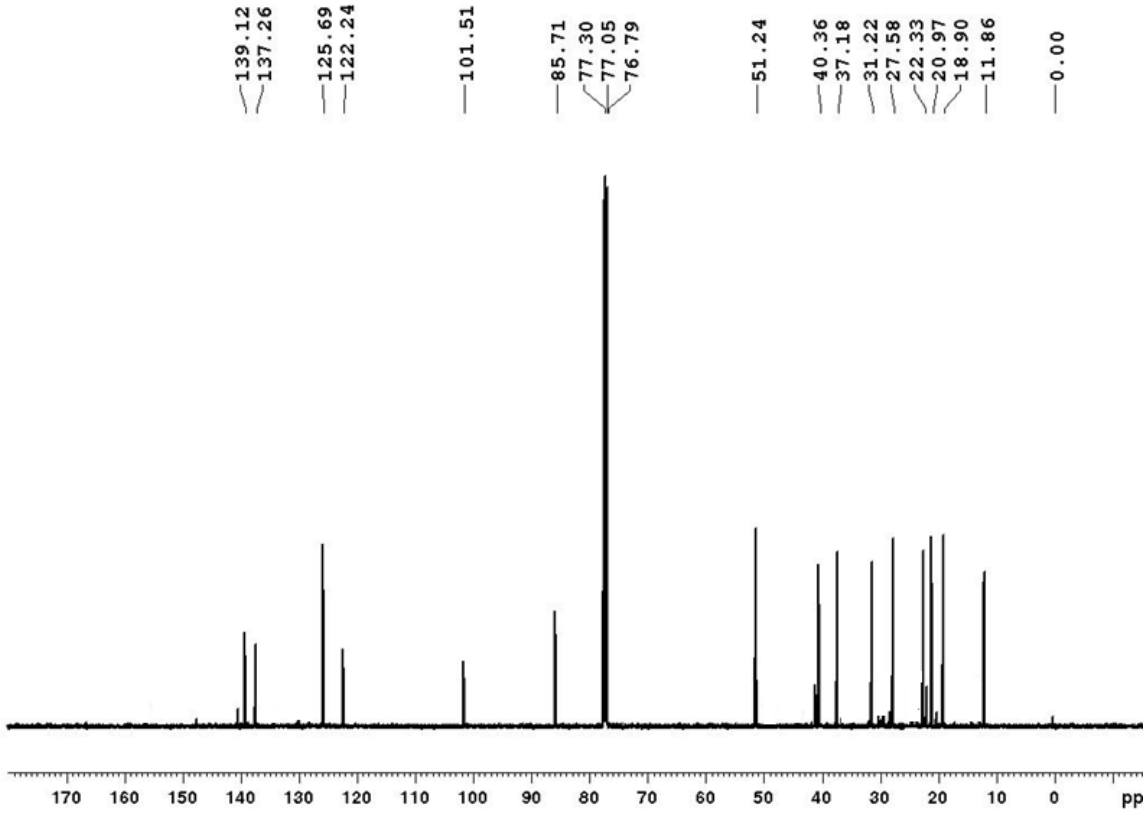


Figure 5A.11. ^{13}C NMR spectrum of compound **43** in $CDCl_3$

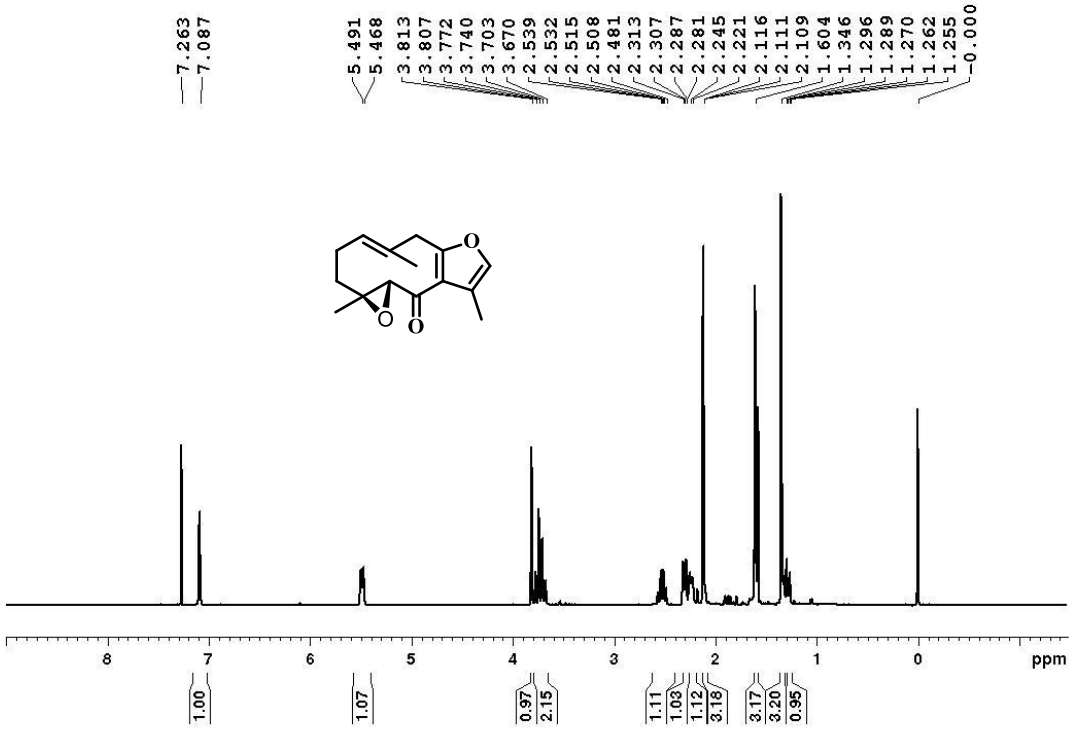


Figure 5A.12. ^{1}H NMR spectrum of compound **44** in $CDCl_3$

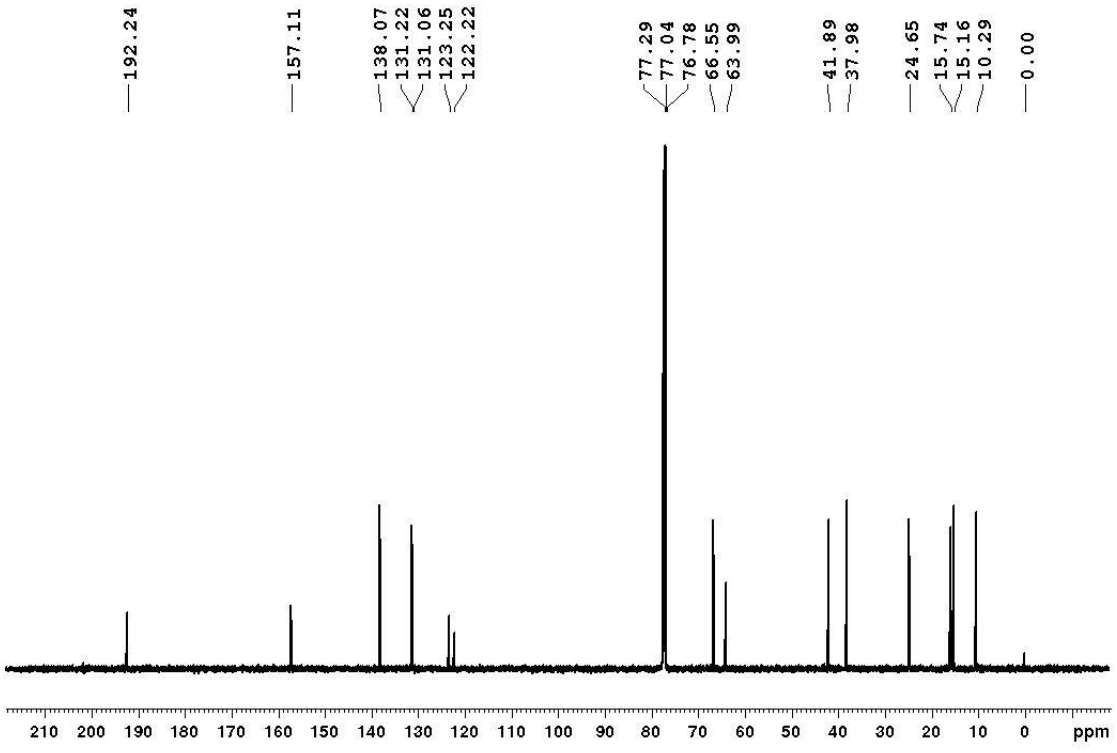
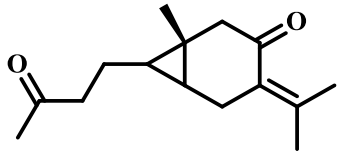


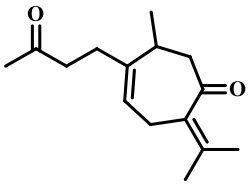
Figure 5A.13. ^{13}C NMR spectrum of compound **44** in $CDCl_3$

The spectral data obtained were characteristic for sesquiterpene having two carbonyl groups. The protons and carbons were assigned by a detailed analysis of the COSY, HMQC and HMBC spectra. From the spectral data and literature reports the structure of compound **45** was confirmed as carabrane type sesquiterpene **Curcumenone**. [47]



Compound **45 – Curcumenone**

From the fraction pool 8 (Fr. 34-40) on CC separation, we could isolate a colourless oily compound, labelled as compound **46** (43 mg). The ^{1}H NMR spectrum (**Fig. 5A.16**) showed an olefinic proton at δ 5.52 ppm, also the presence of two olefinic methyl groups at δ 1.80 and 1.99 ppm and an acetyl group at δ 2.14 ppm and a secondary methyl group at δ 1.07 ppm, respectively**.** The ^{13}C NMR spectrum (**Fig. 5A.17**) exhibited the peaks for two carbonyl groups at δ 208.4 and 205.3 ppm and four olefin carbons at δ 121.1, 134.7, 140.8 and 143.7 ppm. From the HMBC and HMQC data, we assumed that the compound is a seco-guaiane type sesquiterpenes analogs to curcumenone. From the detailed spectral analysis, the structure of the compound was identified as **Curcumadione**. It was also supported by the HRMS analysis with a molecular ion peak at *m/z* 257.1522 $(M+Na)^{+}$. The compound was previously reported from the rhizomes of *Curcuma aromatica*.[48] To the best of our knowledge, **Curcumadione** is reported for the first time from this species.



Compound **46 - Curcumadione**

Compound **47** was (150 mg) obtained from fraction pool 9 (Fr. 41-45) as colourless oil on repeated silica gel CC and further purified by Sephadex LH-20 in methanol. The IR spectrum showed the presence of a hydroxyl group (3450 cm^{-1}) and a conjugated carbonyl group (1680 cm^{-1}). The ^{1}H NMR spectrum (**Fig. 5A.18**) was similar to that of germacrone, but the difference is the signal characteristics of a

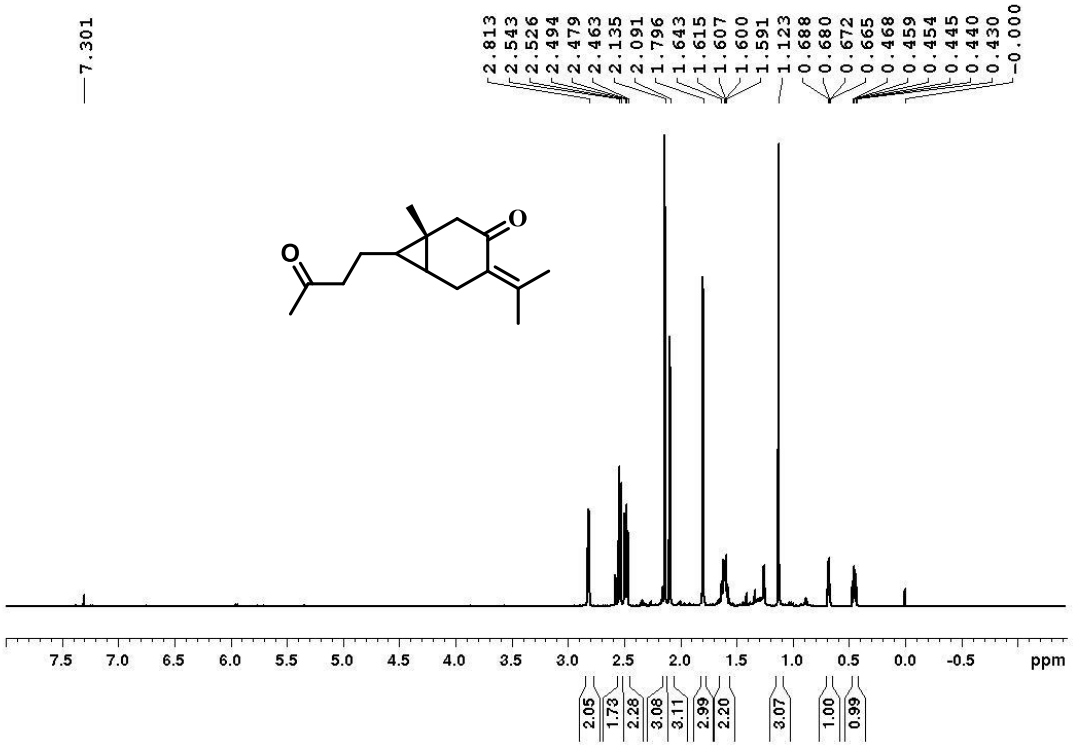


Figure 5A.14. ^{1}H NMR spectrum of compound **45** in $CDCl_3$

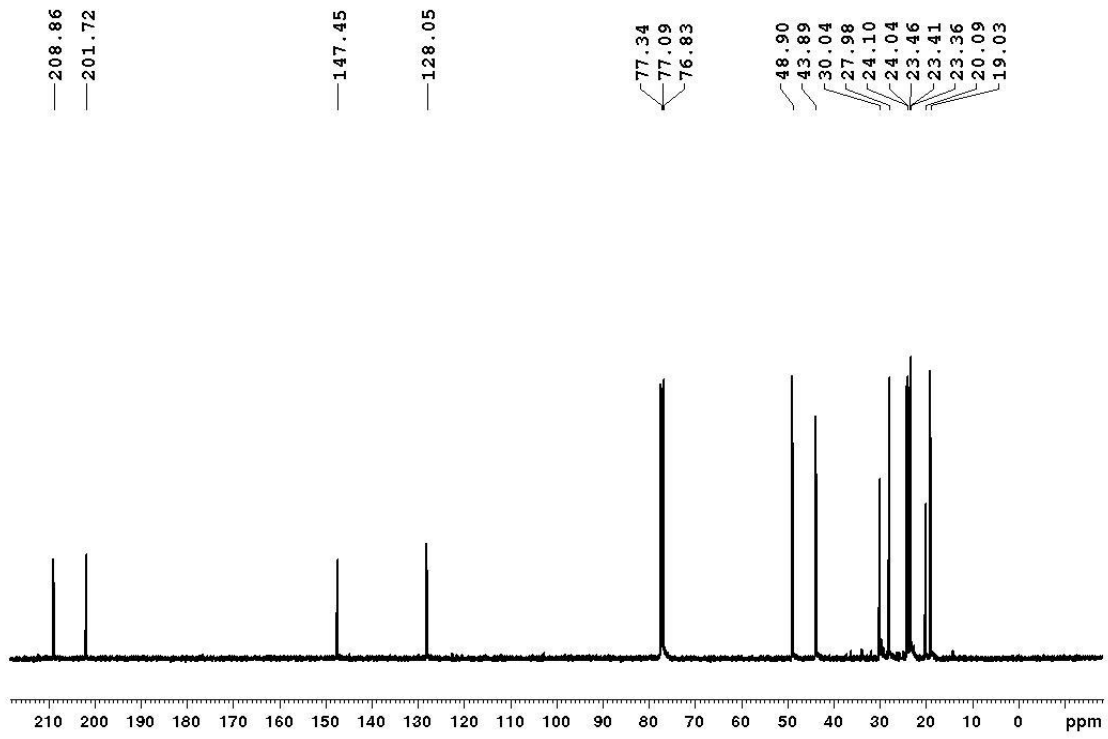


Figure 5A.15. ^{13}C NMR spectrum of compound **45** in $CDCl_3$

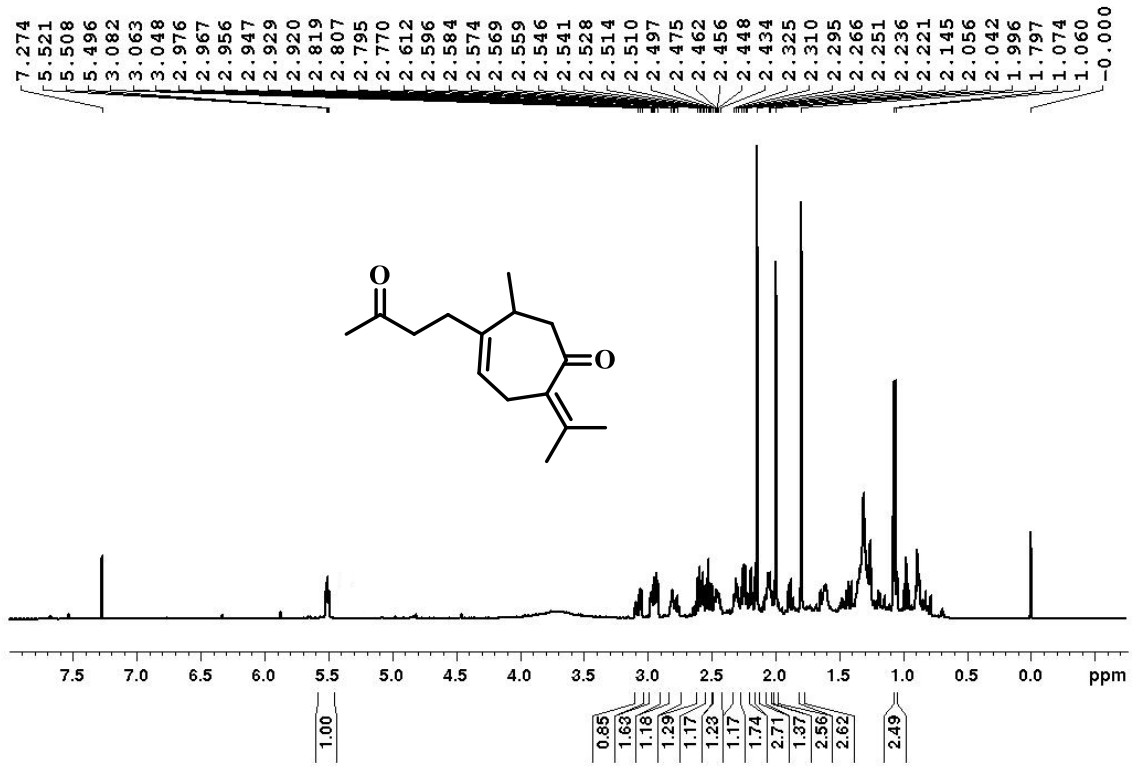


Figure 5A.16. ^{1}H NMR spectrum of compound **46** in $CDCl_3$

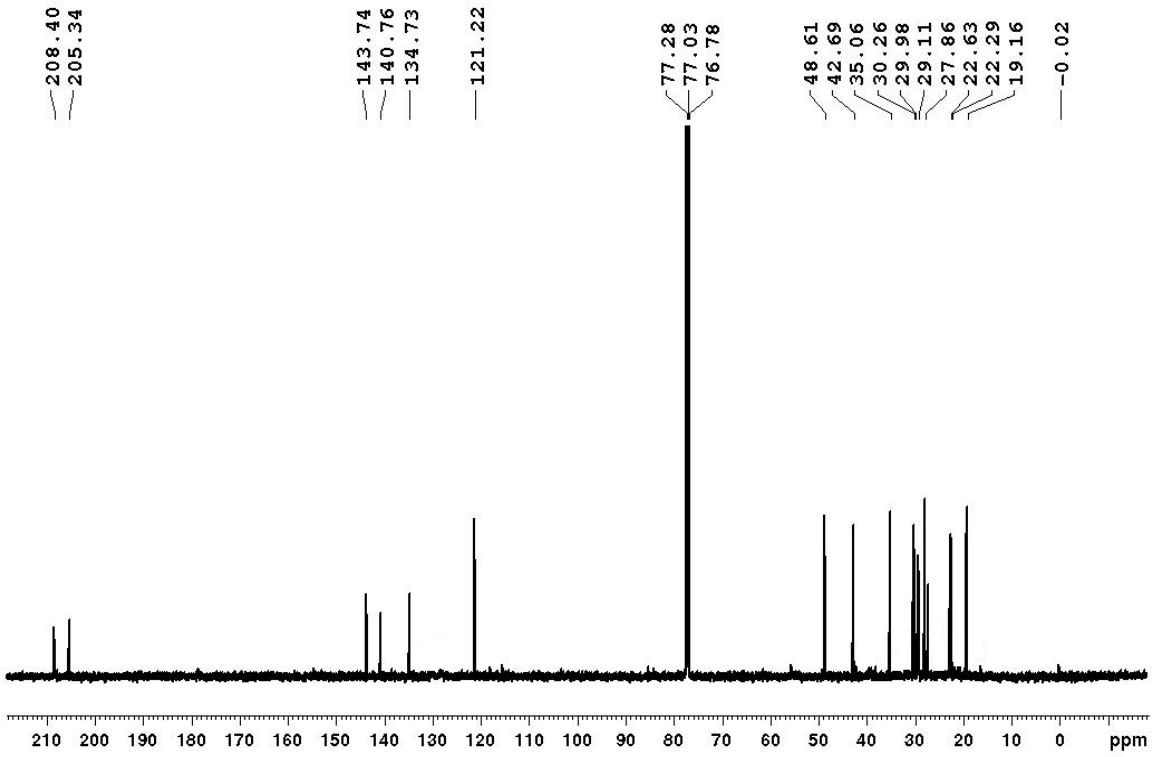
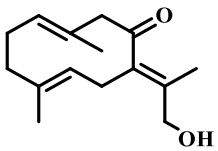


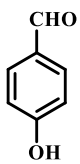
Figure 5A.17. ^{13}C NMR spectrum of compound **46** in $CDCl_3$

hydroxymethyl group replaced the vinyl methyl group resonance. The spectrum showed three methyl groups resonated at δ 1.81, 1.63 and 1.44 ppm, respectively. The methylene protons in the 13th carbon atom appeared as broad doublets at δ 4.30 (br d, J = 12.0 Hz, 1H) & 4.18 (br d, J = 12.5 Hz, 1H) ppm. The ^{13}C NMR spectrum (**Fig. 5A.19**) of the compound exhibited the carbonyl peak at δ 207.3 ppm and the hydroxyl methyl carbon resonated at δ 62.8 ppm. From the spectral data and literature reports, it was confirmed that the –OH group is attached to the 13th carbon atom. Hence the structure of the compound was identified as **13-Hydroxygermacrone**, which was well supported by HRMS analysis with a molecular ion peak at *m/z* 235.1610 $(M+H)^+$.[49]



Compound **47 - 13-Hydroxygermacrone**

Compound **48** (6 mg) was obtained as a brown solid on CC separation of fraction 41-45. First, by silica gel column followed by Sephadex LH-20, we could obtain the pure compound. The IR spectrum showed the presence of a hydroxyl group (3156 cm^{-1}) and carbonyl group (1667 cm^{-1}). The ^{1}H NMR spectrum (**Fig. 5A.20**) displayed characteristic signals of 1,4-disubstituted benzene at δ 7.81 (d, J = 8.5 Hz, 2H) and 6.96 (d, J = 8.5 Hz, 2H) ppm. A sharp singlet observed at δ 9.87 ppm corresponded to an aldehydic proton, which was confirmed by the peak at δ 190.7 ppm in the ^{13}C NMR spectrum (**Fig. 5A.21**). HRMS analysis showed a parent molecular ion peak at *m/z* 123.0367, which is the $(M+H)^+$ peak. Finally, the structure of compound **48** was confirmed as ***p*-Hydroxybenzaldehyde** and was found to be in good agreement with the literature reports. *p*-Hydroxybenzaldehyde was previously reported from the plant species *Curcuma longa*, *Gastrodia elata* Blume (Orchidaceae), and *Gunnera perpensa* (Gunneraceae). [50–52] It is being reported for the first time from this species.



Compound **48 - *p*-Hydroxybenzaldehyde**

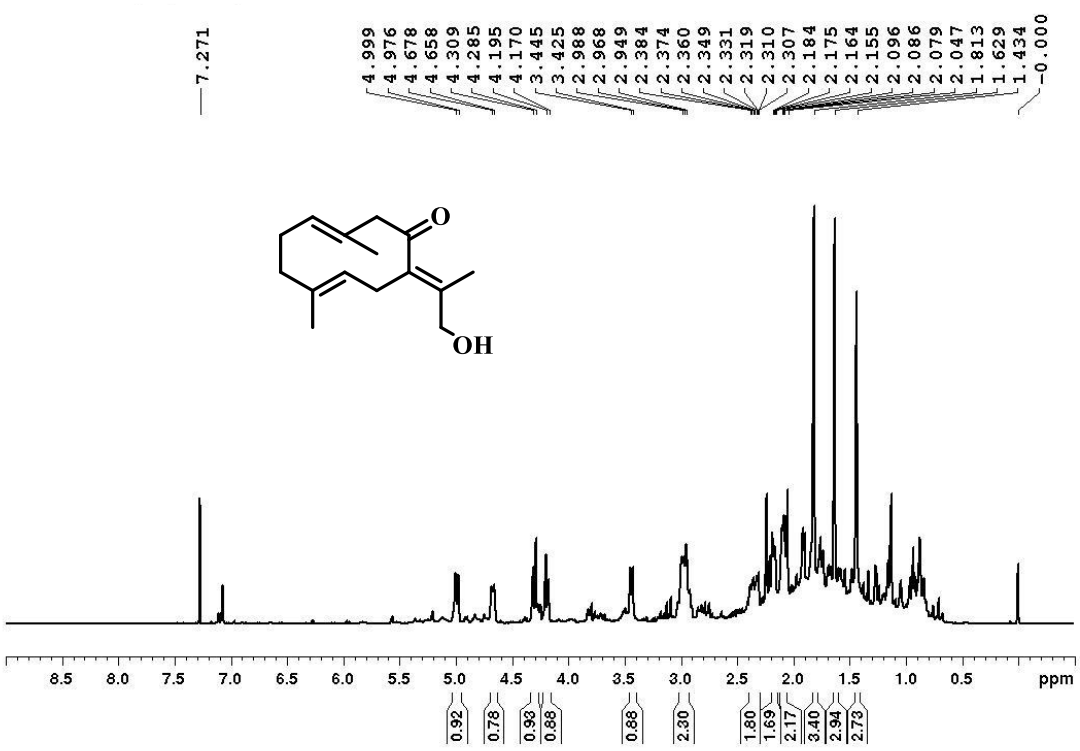


Figure 5A.18. ^{1}H NMR spectrum of compound **47** in $CDCl_3$

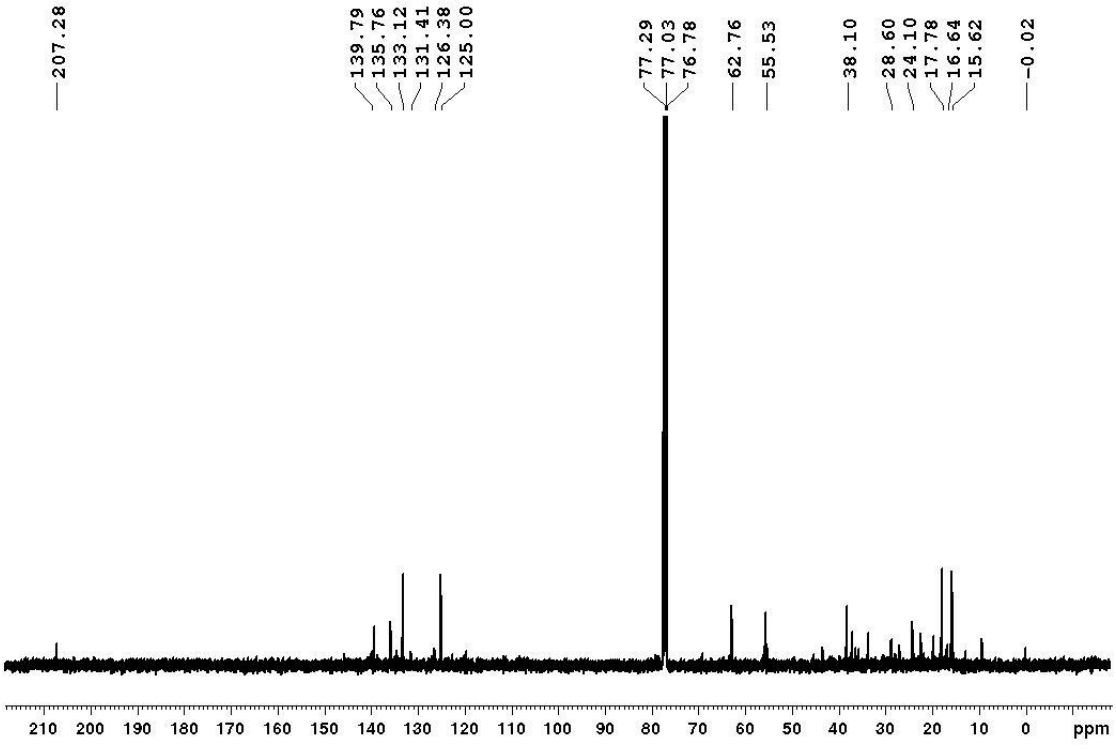


Figure 5A.19. ^{13}C NMR spectrum of compound **47** in $CDCl_3$

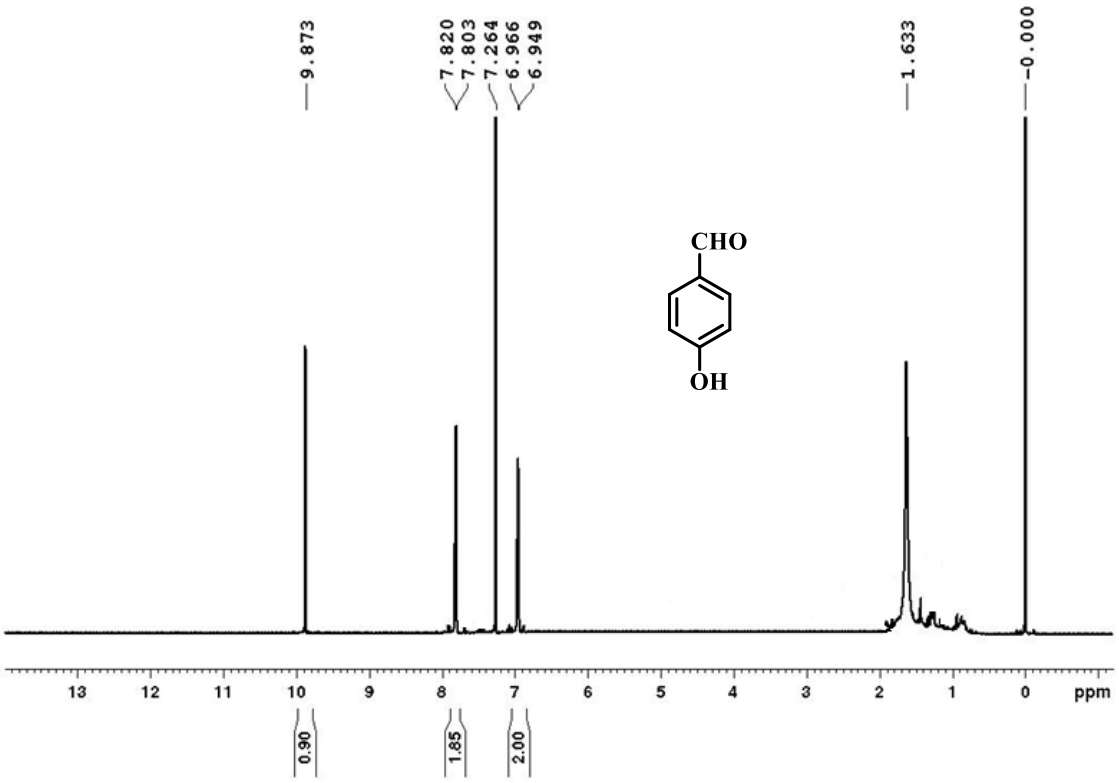


Figure 5A.20. ^{1}H NMR spectrum of compound **48** in $CDCl_3$

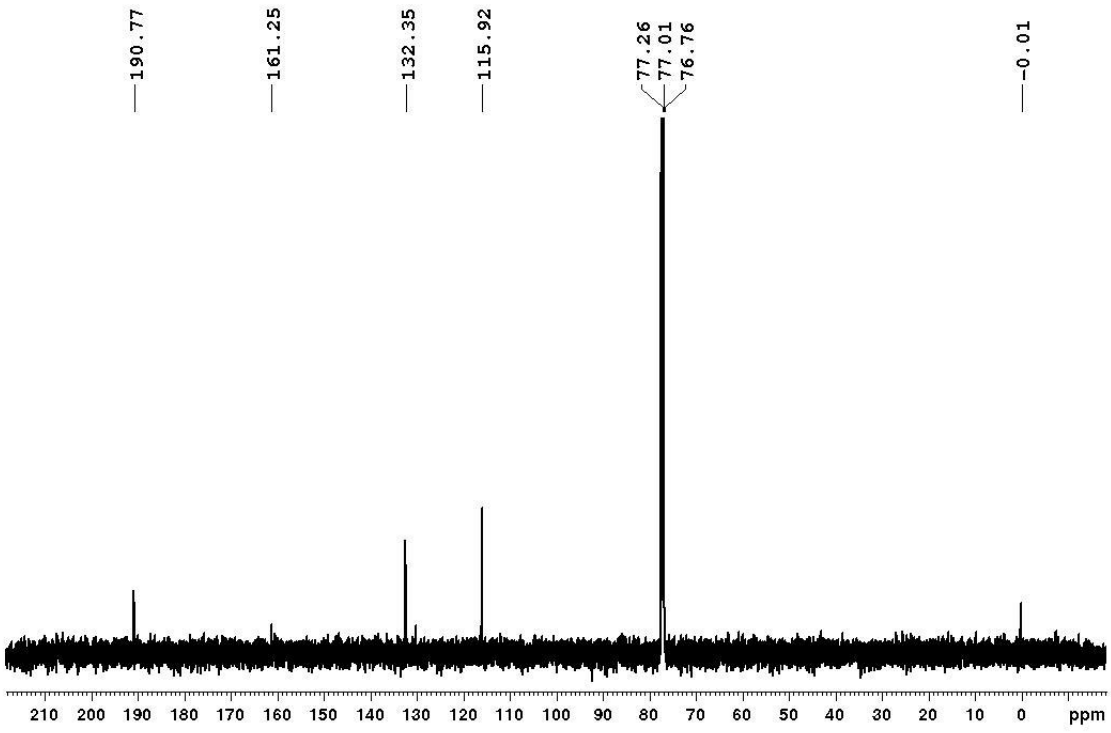


Figure 5A.21. ^{13}C NMR spectrum of compound **48** in $CDCl_3$

5A.6. Conclusion

The phytochemical investigation of the medicinally important plant *C. aeruginosa* was carried out. We have successfully isolated and characterized eleven compounds from the acetone extract of *Curcuma aeruginosa* which includes furanodiene, furanodienone, curcumenol, curcumenone, curcumadione, zederone, isocurcumenol, 13-hydroxygermacrone, curcumenol, *p*-hydroxybenzaldehyde and a mixture of *β*-sitosterol and stigmasterol. Curcumadione and *p*-hydroxybenzaldehyde were reported for the first time from this species. The biological evaluation of the isolated compounds are ongoing in our lab.

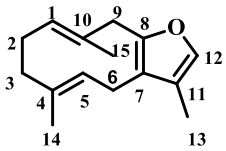
5A.7. Experimental section

Same as in Chapter 2A.

5A.8. Spectra

Compound 40 (Furanodiene)

Compound **40** was isolated from fraction pool 2 directly from the main column by eluting the column in hexane, as colourless oily component of about 460 mg. The compound was confirmed as **Furanodiene** based on spectral data, and comparison with literature reports.



Molecular formula: $C_{15}H_{18}O_2$

FT-IR (Neat, υ_{max} cm^{-1}): 2966, 2925, 2861, 1552, 1440, 1081 cm^{-1}.

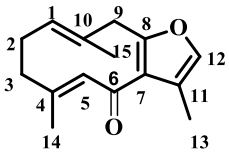
^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 7.07 (s, 1H, H-12), 4.94 (m, 1H, H-1), 4.74 (t, J = 7.5 Hz, 1H, H-5), 3.54 (d, J = 15.5 Hz, 1H, H-9), 3.44 (d, J = 16.0 Hz, 1H, H-9), 3.07 (d, J = 7.5 Hz, 2H, H-6), 1.77-2.26 (m, 4H, H-2 & H-3), 1.92 (s, 3H, H-13), 1.60 (s, 3H, H-14), 1.27 (s, 3H, H-15) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 149.7 (C-8), 136.0 (C-12), 134.4 (C-10), 129.0 (C-1), 128.8 (C-4), 127.6 (C-5), 121.9 (C-11), 118.9 (C-7), 40.9 (C-9), 39.5 (C-3), 26.8 (C-2), 24.4 (C-6), 16.5 (C-14), 16.2 (C-15), 8.9 (C-13) ppm.

HRMS (ESI): *m/z* calcld for $C_{15}H_{18}O_2$ is 216.1550. Found 217.1589 $(M+H)^+$.

Compound 41 (Furanodienone)

Fraction pool 3 (Fr. 9-11) obtained by eluting the column with 5 % ethyl acetate in hexane on further purification yielded 200 mg of yellowish oily compound. The compound was confirmed as **Furanodienone** based on spectral data obtained as shown below. The NMR values were assigned on the basis of 2D NMR and comparison with literature reports.



Molecular formula: $C_{15}H_{18}O_2$

FT-IR (Neat, υ_{max} cm^{-1}): 2861, 1676, 1542, 1089 cm^{-1}.

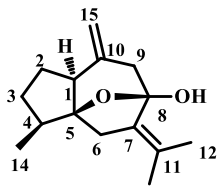
^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 7.08 (s, 1H, H-12), 5.81 (s, 1H, H-5), 5.18 (m, 1H, H-1), 3.70 (d, *J* = 3.5 Hz, 2H, H-9), 2.47 (dt, *J* = 11.5, 4.0 Hz, 1H, H-3), 2.32 (m, 1H, H-2), 2.18 (m, 1H, H-2), 2.13 (s, 3H, H-13), 1.99 (s, 3H, H-14), 1.89 (td, *J* = 11.5, 4.5 Hz, 1H, 1H, H-3), 1.30 (s, 3H, H-15) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 189.9 (C-6), 156.6 (C-8), 145.9 (C-4), 138.1 (C-12), 135.4 (C-10), 132.4 (C-5), 130.6 (C-1), 123.7 (C-11), 122.2 (C-7), 41.7 (C-9), 40.7 (C-3), 26.4 (C-2), 19.0 (C-14), 15.8 (C-15), 9.6 (C-13) ppm.

HRMS (ESI): *m/z* calcld for $C_{15}H_{18}O_2$ is 230.1306. Found 231.1410 $(M+H)^+$.

Compound 42 (Isocurcumenol)

Fraction pool 5 (Fr. 17-21) on silica gel CC afforded three compounds. Compound **42** was isolated as colourless crystalline solid of about 50 mg. The compound was identified as **Isocurumenol** based on spectral data and comparison with literature reports.



Molecular formula: $C_{15}H_{22}O_2$

FT-IR (Neat, υ_{max} cm^{-1}): 3400, 2920, 1660, 1310, 1100, 980, 880 cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 4.78 (t, *J* = 2.0 Hz, 1H, H-15), 4.76 (t, *J* = 2.0 Hz, 1H, H-15), 2.84 (s, 1H, -OH), 2.67 (d, *J* = 13.5 Hz, 1H, H-4), 2.50 (d, *J* = 13.5 Hz, 1H, H-9), 2.23 (t, *J* = 9.5 Hz, 1H, H-1), 1.97 (m, 4H, H9, H-6, H-3), 1.80 (s, 3H, H-15), 1.55 (m, 1H, H-3) 1.62 (s, 3H, H-12),

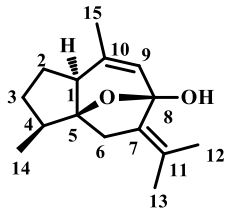
1.00 (d, *J* = 6.0 Hz, 3H, H-14) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 145.1(C-10), 133.8 (C-7), 127.0 (C-11), 112.2 (C-15), 104.0 (C-8), 87.1 (C-5), 52.8 (C-1), 41.7 (C-4), 38.9 (C-6), 36.2 (C-9), 30.8 (C-3), 28.4 (C-2), 22.5 (C-12), 19.0 (C-13), 12.4 (C-14) ppm.

HRMS (ESI): *m/z* calcld for $C_{15}H_{22}O_2$ is 234.1619. Found 257.1524 $(M+Na)^+$.

Compound 43 (Curcumenol)

Compound 43 was the marker compound isolated from *C. aeruginosa,* about 1.5 g crystalline solid was obtained from fraction pool 5. The compound was characterized as **Curcumenol** based on the spectral data and comparison with literature reports.



Molecular formula: $C_{15}H_{22}O_2$

FT-IR (Neat, υ_{max} cm^{-1}): 1670, 2920, 2980, 3400 cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 5.75 (bs, 1H, H-9), 2.66 (d, *J* = 15.5 Hz, 1H, H-6), 2.13 (d, *J* = 15.5 Hz, 1H, H-6), 2.09 (m, 1H, H-1), 1.81 (s, 3H, H-15), 1.66 (s, 3H, H-13), 1.59 (s, 3H, H-12), 1.02 (d, *J* = 6.5 Hz, 3H, H-14) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 11.9 (C-14), 18.9 (C-13), 20.9 (C-15), 22.3 (C-12), 27.0 (C-2), 31.2 (C-3), 37.2 (C-6), 40.4 (C-4), 51.2 (C-1), 85.8 (C-5), 101.5 (C-8), 122.2 (C-11), 125.9 (C-9), 137.1 (C-10), 138.9 (C-7) ppm.

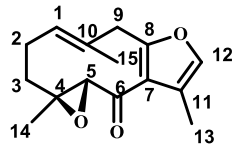
HRMS (ESI): *m/z* calcld for $C_{15}H_{22}O_2$ is 234.1619. Found 235.1619 $(M+H)^+$.

Compound 44 (Zederone)

Fraction pool 6 (Fr. 22-28) obtained by eluting the column with 10 % ethyl acetate in hexane was further subjected to purification using silica gel afforded a colourless crystalline solid as compound **44** (276 mg). The compound was characterized a **Zederone**, based on spectral data obtained and comparison with literature reports.

Molecular formula: $C_{15}H_{18}O_3$

FT-IR (Neat, υ_{max} cm^{-1}): 2929, 1662, 1527, 1404 cm^{-1}.



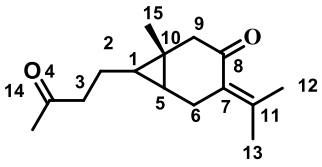
^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 7.09 (1H, s, H-12), 5.48 (d, *J* = 11.5 Hz, 1H, H-1), 3.81 (s, 1H, H-5), 3.76 (d, *J* = 16.0 Hz, 1H, H-9), 3.69 (d, *J* = 16.0 Hz, 1H, H-9), 2.52 (br d, 1H, H-2), 2.30 (1H, dt, , H-3), 2.23 (br d, 1H, H-2), 2.11 (s, 3H, H-13), 1.60 (s, 3H, H-15), 1.35 (s, 3H, H-14), 1.27 (m, 1H, H-3) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 192.2 (C-6), 157.1 (C-8), 138.1 (C-12), 131.2 (C-1), 131.1 (C-10), 123.2 (C-7), 122.2 (C-11), 66.6 (C-5), 64.0 (C-4), 41.9 (C-9), 38.0 (C-3), 24.6 (C-2), 15.7 (C-15), 15.2 (C-14), 10.3 (C-13) ppm.

HRMS (ESI): *m/z* calcld for $C_{15}H_{18}O_3$ is 246.1252. Found 269.1155 $(M+Na)^+$.

Compound 45 (Curcumenone)

A colourless oily compound of 115 mg was isolated from fraction pool 7 (Fr. 29-32) as compound **45**. It was characterized as the sesquiterpene **Curcumenone** based on the spectral data and literature reports.



Molecular formula: $C_{15}H_{22}O_2$

FT-IR (Neat, υ_{max} cm^{-1}): 2922, 1713, 1678 cm^{-1}.

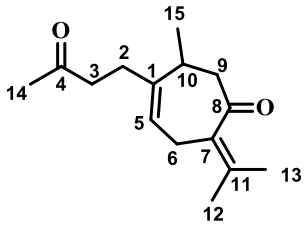
^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 2.81 (s, 2H, H-6), 2.54 (d, *J* = 8.5 Hz, 2H, H-9), 2.48 (t, *J* =7.5 Hz, 2H, H-3), 2.14 (s, 3H, H-14), 2.09 (s, 3H, H-12), 1.80 (s, 3H, H-13), 1.60 (m, 2H, H-2), 1.12 (s, 3H, H-15), 0.68 (q, *J* = 4.1 Hz, 1H, H-5), 0.45 (dt, *J* = 4.5 Hz, 7.0 Hz, 1H, H-1) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 208.9 (C-4), 201.7 (C-8), 147.4 (C-11), 128.0 (C-7), 48.9 (C-9), 43.9 (C-3), 30.0 (C-14), 28.0 (C-6), 24.1 (C-5), 24.0 (C-1), 23.5 (C-13), 23.4 (C-12), 23.4 (C-2), 20.1 (C-10), 19.0 (C-15) ppm.

HRMS (ESI): *m/z* calcld for $C_{15}H_{22}O_2$ is 234.1619. Found 257.1520 $(M+Na)^+$.

Compound 46 (Curcumadione)

Fraction pool 8 (Fr. 34-40) obtained by eluting the column at 20 % ethyl acetate in hexane polarity, on further purification with silica gel CC yielded a colourless oily solid of 43 mg as compound **46**. The compound was confirmed as the sesquiterpene **Curcumadione** based on the spectral data and literature review.



Molecular formula: $C_{15}H_{22}O_2$

FT-IR (Neat, υ_{max} cm^{-1}): 2925, 2858, 1736, 1451, 1376 cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 5.52 (t, *J* = 6.5 Hz, 1H, H-5), 3.05-3.10 (dd, *J* = 7.5 Hz, 1H, H-2), 2.97 (d, *J* = 4.5 Hz, 1H, H-2), 2.92-2.97 (m, 1H, H-9), 2.31 (t, *J* = 7.5 Hz, 1H, H-10), 2.53 (dt, 2H, H-3), 2.43-2.51 (m, 1H, H-9), 2.26 (d, *J* = 7.5 Hz, 1H, H-6), 2.23 (d, *J* = 7.5 Hz, 1H, H-6), 2.14 (s, 3H, H-14), 1.99 (s, 3H, H-13), 1.80 (s, 3H, H-12), 1.07 (d, *J* = 7.0 Hz, 3H, H-15) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 208.4 (C-4), 205.3 (C-8), 143.7 (C-11), 140.8 (C-1), 134.7 (C-7), 121.2 (C-5), 48.6 (C-9), 42.7 (C-3), 35.1 (C-10), 30.2 (C-6), 30.0 (C-14), 27.9 (C-2), 22.6 (C-15), 22.3 (C-14), 19.1 (C-12) ppm.

HRMS (ESI): *m/z* calcld for $C_{15}H_{22}O_2$ is 234.1619. Found 257.1525 $(M+Na)^+$.

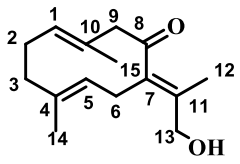
Compound 47 (13-Hydroxygermacrone)

Fraction pool 9 (Fr. 41-45) on silica gel CC afforded a UV active compound with minor impurities, which was further purified by Sephadex LH-20 column using methanol as solvent. Compound **47** isolated as colourless oil (43 mg), was confirmed as **13-Hydroxygermacrone** on detailed analysis of spectral data as well as comparison with literature reports.

Molecular formula: $C_{15}H_{22}O_2$

FT-IR (Neat, υ_{max} cm^{-1}): 3450, 1680 cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 4.99 (br d, *J* = 12.0 Hz, 1H, H-1), 4.67 (br d, *J* = 10.0 Hz, 1H, H-5), 4.30 (br d, *J* = 12.0 Hz, 1H, H-13), 4.18 (br d, *J* = 12.5 Hz, 1H, H-13),



3.44 (br d, *J* = 10.0 Hz, 1H, H-9), 2.97 (m, 2H, H-6 & H-9), 2.36 (m, 1H, H-2) 2.17 (m, 1H, H-3), 2.08 (m, 2H, H-2 & H-3), 1.81 (s, 3H, H-12), 1.63 (s, 3H, H-15), 1.44 (s, 1H, H-14) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 207.2 (C-8), 139.8 (C-11), 135.8 (C-10), 133.1(C-1), 131.4 (C-7), 126.4 (C-4), 125.0 (C-5), 62.8 (C-13), 55.5 (C-9), 38.1 (C-3), 28.6 (C-6), 24.1 (C-2), 17.8 (C-12), 16.7 (C-15), 15.6 (C-14) ppm.

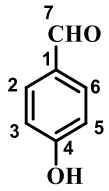
HRMS (ESI): *m/z* calcld for $C_{15}H_{22}O_2$ is 234.1619. Found 235.1610 $(M+H)^+$.

Compound 48 (*p*-Hydroxybenzaldehyde)

Purification of compound **48** in Sephadex LH-20 column yielded a phenolic compound of 6 mg. The compound was identified as ***p*-Hydroxybenzaldehyde** based on spectral data and literature reports.

Molecular formula: $C_7H_6O_2$

FT-IR (Neat, υ_{max} cm^{-1}): 3156, 1779, 1667, 853 cm^{-1}.



^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 9.87 (s, 1H, H-7), 7.81 (d, *J* = 8.5 Hz, 2H, H-2 & H-6), 6.96 (d, *J* = 8.5 Hz, 2H, H-3 & H-5) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 190.7 (C-7), 161.2 (C-4), 132.3 (C-2 & C-6), 129.9 (C-1), 115.9 (C-3 & C-5) ppm.

HRMS (ESI): *m/z* calcld for $C_7H_6O_2$ is 122.0367. Found 123.0367 $(M+ H)^+$.

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Chapter 5B: Phytochemical Investigation of the rhizomes of *Curcuma raktakanta* Mangaly & M. Sabu & Synthetic Modification of Germacrone

5B.1. Introduction

Curcuma raktakanta Mangaly & M. Sabu is a small to large perennial plant, mainly distributed in Kerala, India. It has rhizomes with long, horizontally running branches. The rhizomes are medium-sized, appear as creamy to white colour internally (the younger is white) and whitish towards the periphery. The tubers are sessile and finger-shaped, possessing a pleasant aroma with traces of camphor and menthol.[1] Rajamma *et al*., previously reported the antioxidant and antibacterial activities of *C. raktakanta* oleoresins.[2] Inthirakanthi *et al.,* investigated the antidiabetic potential of ethanolic extract of the rhizomes using *in vitro* inhibitory assays and streptozotocin-induced diabetic animal models. The results suggested that the ethanolic extract possesses potent antidiabetic and antihyperlipidemic activity.[3] Different extracts of the rhizomes have also been reported to exhibit anticancer activities.[4] Besides these, the other pharmacological properties and phytochemical investigation of *C. raktakanta* have not yet been explored, which prompted us to undertake the present study.

Figure. 5B.1. Inflorescence and rhizomes of *C.raktakanta*

5B.2. Extraction, isolation and characterization of compounds from the rhizomes of *C. raktakanta*

5B.2.1. Collection and extraction of plant material

The plant material of *Curcuma raktakanta* was collected from Tropical Botanical Garden and Research Institute Herbarium (JNTBGRI)- Palode, Thiruvananthapuram, Kerala, India. The taxonomist of JNTBGRI authenticated the plant material and a voucher specimen (voucher number TBGRI 60671) was deposited in the herbarium repository of the institute. Approximately 1 kg of the dried plant material was coarsely powdered and subjected to extraction with acetone (5L * 48 h) using a mechanical stirrer for agitation about three times at room temperature. Thin-layer chromatography indicated that the extraction was completed after nine days. The total acetone extract was then concentrated under reduced pressure using a Heidolph rotatory evaporator, which yielded 70 g of crude extract. The residue obtained was further extracted with ethanol yielded 18 g of the crude extract.

5B.2.2. Preliminary cytotoxicity analysis

The acetone and ethanol extract were tested for cytotoxicity analysis against various cell lines such as SAS (oral cancer), MDAMB (breast cancer), A549 (lung cancer) and HCT15 (human colon carcinoma). The results are shown in **Table 5B.1**. The results indicated that the acetone extract exhibited better cytotoxicity towards cancer cell lines than the ethanol. Hence further isolation was focused on the acetone extract.

Table 5B.1. Percentage of inhibition of acetone and ethanol extract of *C. raktakanta*

Extract	Percentage of inhibition			
	SAS (10 µg/ml)	**MDA-MB-231** (10 µg/ml)	**A549** (25 µg/ml)	**HCT15 Cells** (25 µg/ml)
Acetone	-113.868	**13.3853**	-6.22825	-71.531
Ethanol	-21.81	-19.404	-10.82	-12.202

5B.2.3. Isolation and characterization of compounds

Around 50 g of the acetone extract was subjected to column chromatography on 100-200 mesh sized silica gel and eluted with ethyl acetate-hexane mixtures of increasing polarities which gave six fraction pools. Fractions on further CC separation resulted in the isolation of seven compounds**.** A pictorial representation of the entire isolation procedure is shown in **Figure.5B.2**.

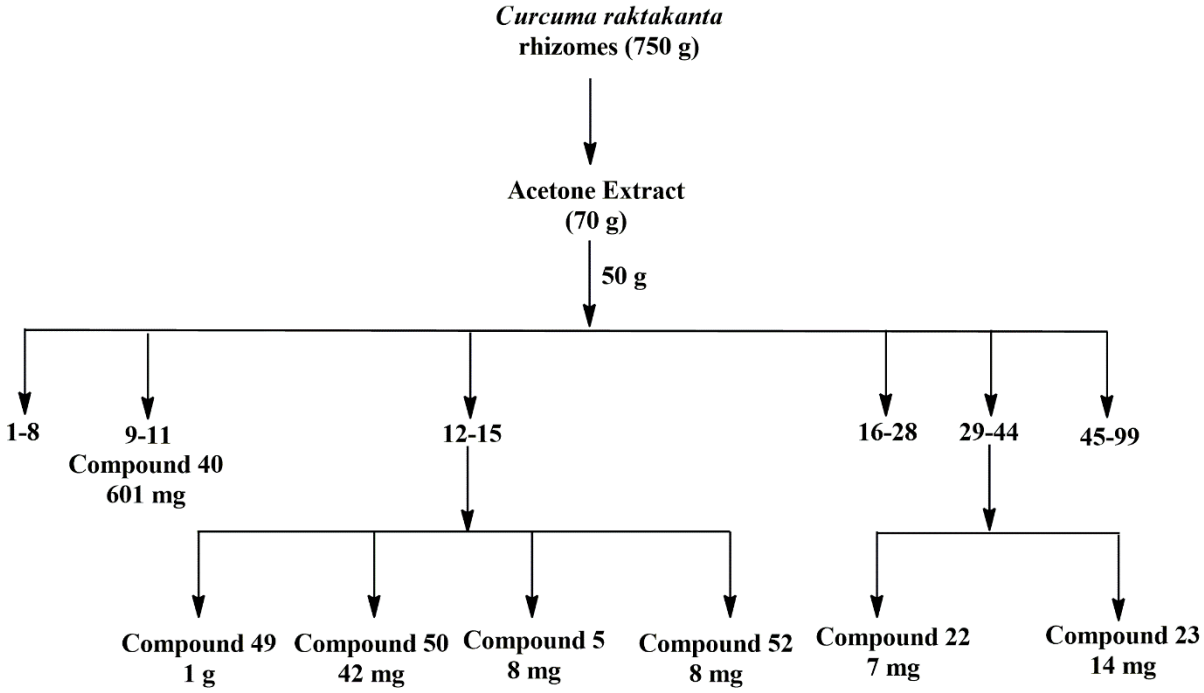
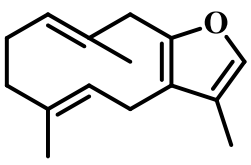


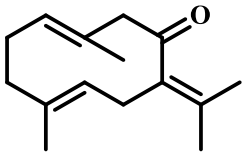
Figure 5B.2. Extraction process

The fraction pool 2 (fr.9-11) showed a highly UV active spot with some minor impurities, and on purification, yielded a colourless oily compound (601 mg). The ^{1}H and ^{13}C spectra were similar to that of compound **40** (chapter 5A). Therefore, the structure of the compound was assigned as **Furanodiene,** which was well supported by the literature report and finally confirmed by HRMS analysis with a molecular ion peak at *m/z* 217.1559 $(M+H)^{+}$.[5]



Compound **40 – Furanodiene**

Fraction pool 3 (Fr. 12-15) obtained by eluting the column using 5 % ethyl acetate-hexane mixture was subjected to further purification on silica gel CC (230-400 mesh), yielded four compounds (compound **49-52**). Compound **49** was isolated as a colourless crystalline solid of about 1.0 g. The IR spectrum showed absorbance at 1671 cm^{-1} indicated the presence of a conjugated carbonyl group. Mass spectral analysis showed a molecular ion peak at *m/z* 219.1722, which is the $(M+H)^+$ peak. The mass spectral data together with ^1H and ^{13}C NMR spectra suggested a molecular formula of $C_{15}H_{22}O$. The presence of 15 carbons indicated that the compound is a sesquiterpenoid type. The ^1H NMR spectrum (**Fig. 5B.3**) displayed a broad doublet at δ 4.98 (br d, J = 11.0 Hz, 1H) and 4.71 (br d, J = 13.7 Hz, 1H), corresponded to the presence of two olefinic methine protons. The three proton singlets at δ 1.77, 1.72, 1.44 and 1.63 ppm were attributed to the presence of four tertiary methyl groups. The ^{13}C NMR spectrum (**Fig. 5B.4**), displayed 15 signals including, a carbonyl carbon (δ 208.0 ppm), four methyl carbons (δ 19.9, 22.4, 15.6 & 16.7 ppm), four methylene carbons (δ 24.1, 29.2, 38.1 & 56.0 ppm) and four quaternary carbons (δ 126.7, 129.6, 132.7, 135.0 & 137.2 ppm). From a detailed spectral analysis and on comparison with the literature reports compound **49** was identified as **Germarone**.[6] Germacrone is reported to exhibit a wide range of biological activities, including depressant, antiulcer, anti-inflammatory, antifeedant, antifungal, antibacterial, antitussive, antitumor, choleretic, vasodilator and hepatoprotective effects.[7]



Compound **49 - Germarone**

Fraction pool 3 gave an orange crystalline solid (42 mg), labelled as compound **50**. The IR spectrum of the compound showed the absorption of a carbonyl group at 1725 cm^{-1}. ^1H NMR spectrum (**Fig. 5B.5**) showed the presence of two aromatic protons at δ 6.90 and 6.75 ppm and three methyl protons resonated at δ 2.25, 2.00, and 1.26 ppm. The ^{13}C NMR spectrum (**Fig. 5B.6**) displayed fifteen carbon atoms which includes a carbonyl carbon (δ 170.6 ppm), three methyl carbons (δ 24.7, 20.0 & 7.6 ppm), two methylene (δ 33.6 & 31.9 ppm) and three methine carbons (δ 117.9, 116.3 & 43.8 ppm).

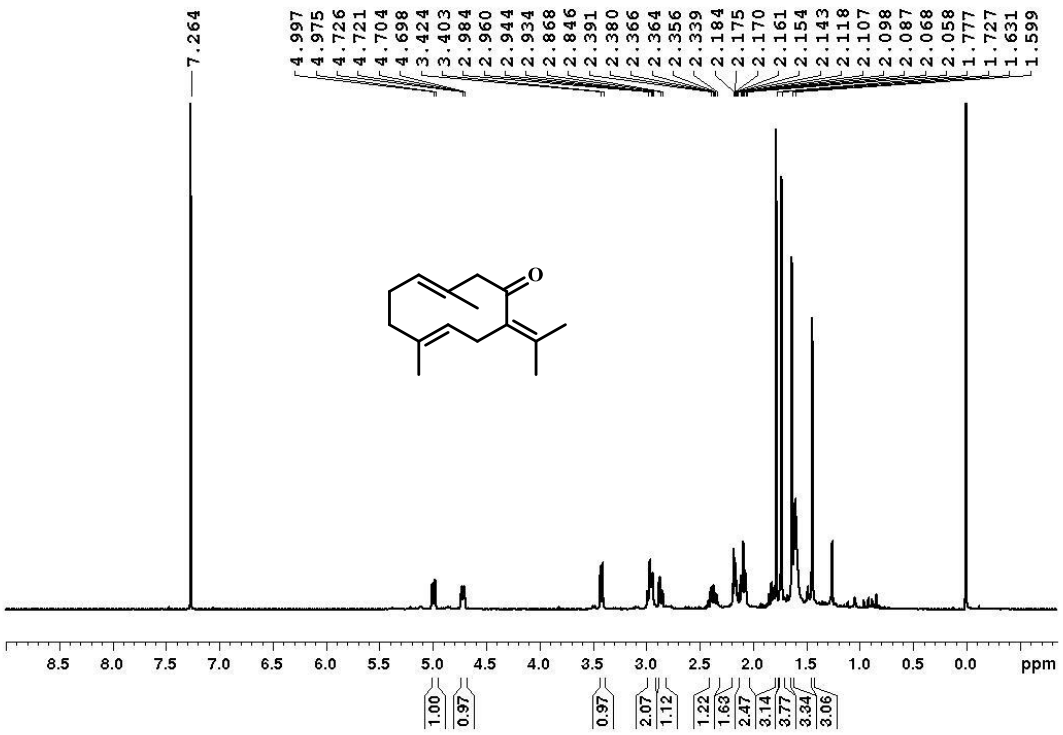


Figure 5B.3. ^{1}H NMR spectrum of compound **49** in $CDCl_3$

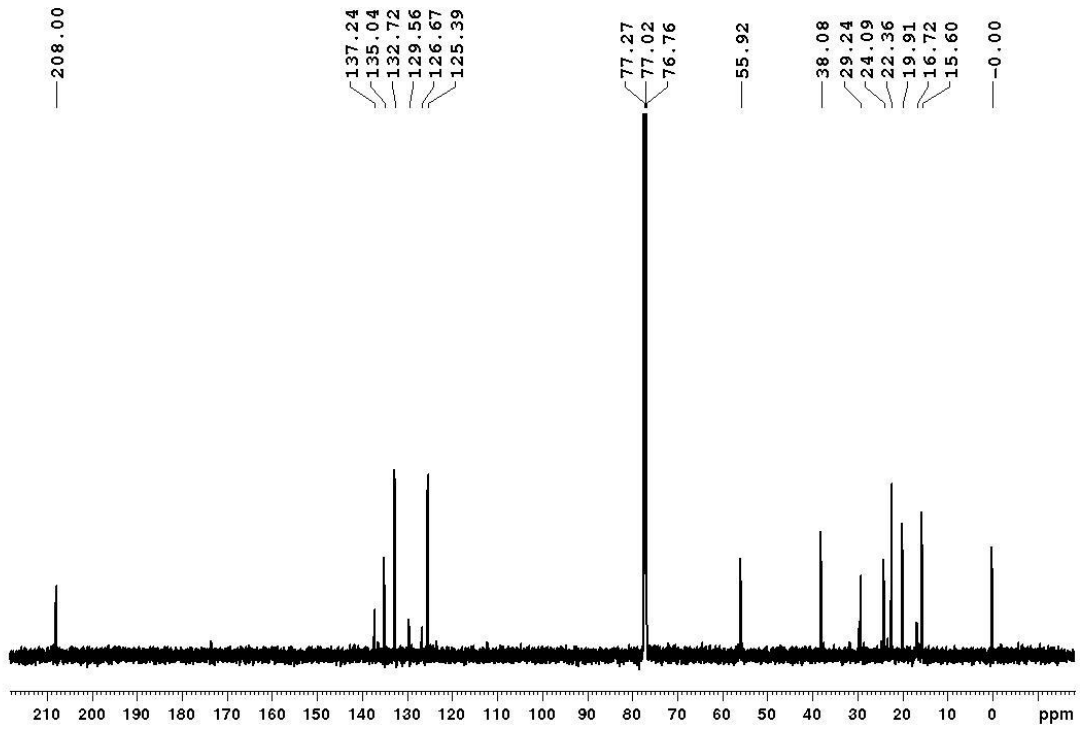
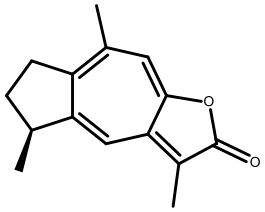


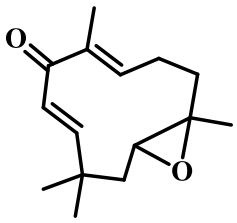
Figure 5B.4. ^{13}C NMR spectrum of compound **49** in $CDCl_3$

NMR spectral data suggested a molecular formula $C_{15}H_{16}O_2$. The spectral values were assigned based on HMBC and HMQC. So, in comparison with literature reports, the compound was identified as guaianoilide type sesquiterpene **Gweicurculactone**.[8]



Compound **50 - Gweicurculactone**

Compound **51** (8 mg) was isolated along with compound **49** & **50** as a colourless crystalline solid. The mass spectrum of the compound showed a molecular ion peak at *m/z* 257.1521, which is the $(M+Na)^+$, suggested a molecular formula $C_{15}H_{22}O_2$. The IR spectrum showed the absorption for a carbonyl group (1630 cm^{-1}) and one epoxy group (1250, 880, 761 cm^{-1}) with a proton signal at δ 2.74 (d, J = 11.5 Hz, 1H) ppm. The ^{1}H NMR spectrum (**Fig. 5B.7**) exhibited a multiplet at δ 6.12 attributed to three olefinic protons at H-10, H-9, and H-6, also four methyl groups resonated at δ 1.86, 1.30, 1.22 and 1.09, respectively. The ^{13}C NMR spectrum (**Fig. 5B.8**) displayed the presence of 15 carbon atoms in which a carbonyl carbon peak was observed at δ 203.0 ppm. DEPT-135 showed three methylene carbons at δ 42.6, 38.2 & 24.7 ppm and three methine carbons at δ 159.5, 147.8 & 128.3 ppm. The protons and carbons were assigned based on HMQC and HMBC spectral data. From a detailed spectral analysis and on comparison with the literature reports compound **51** was confirmed as **Zerumbone epoxide**.[9] Zerumbone epoxide is reported to exhibit anticancer activity, especially against breast cancer cell lines.[10]



Compound **51 - Zerumbone epoxide**

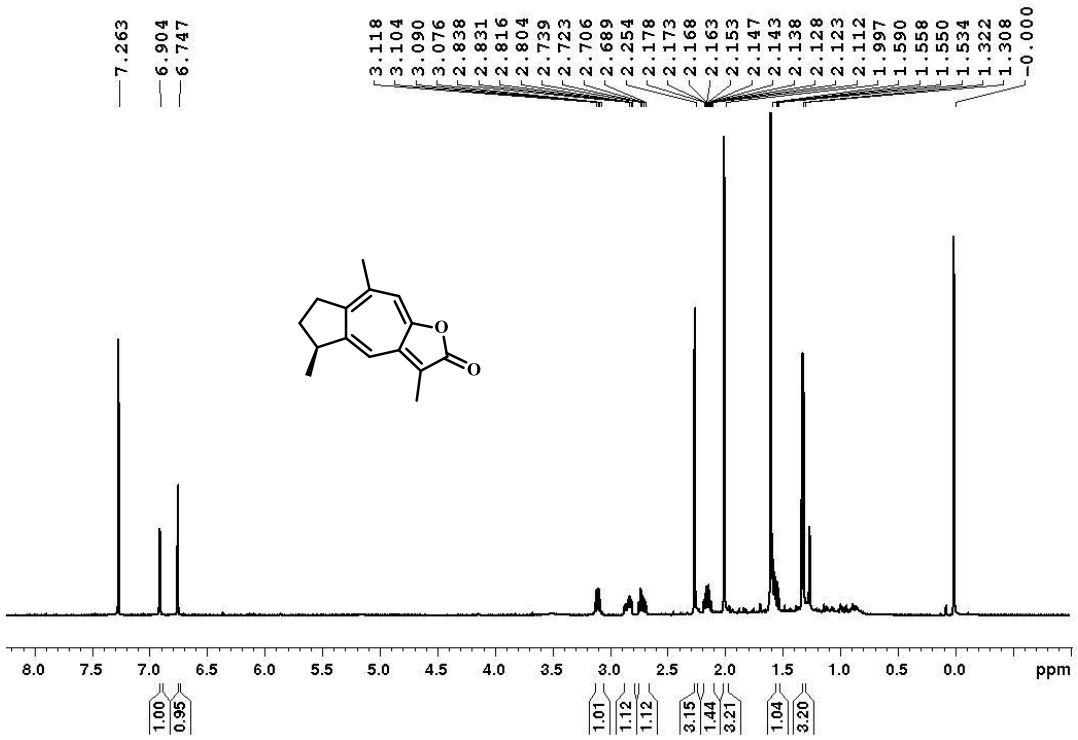


Figure 5B.5. ^{1}H NMR spectrum of compound **50** in $CDCl_3$

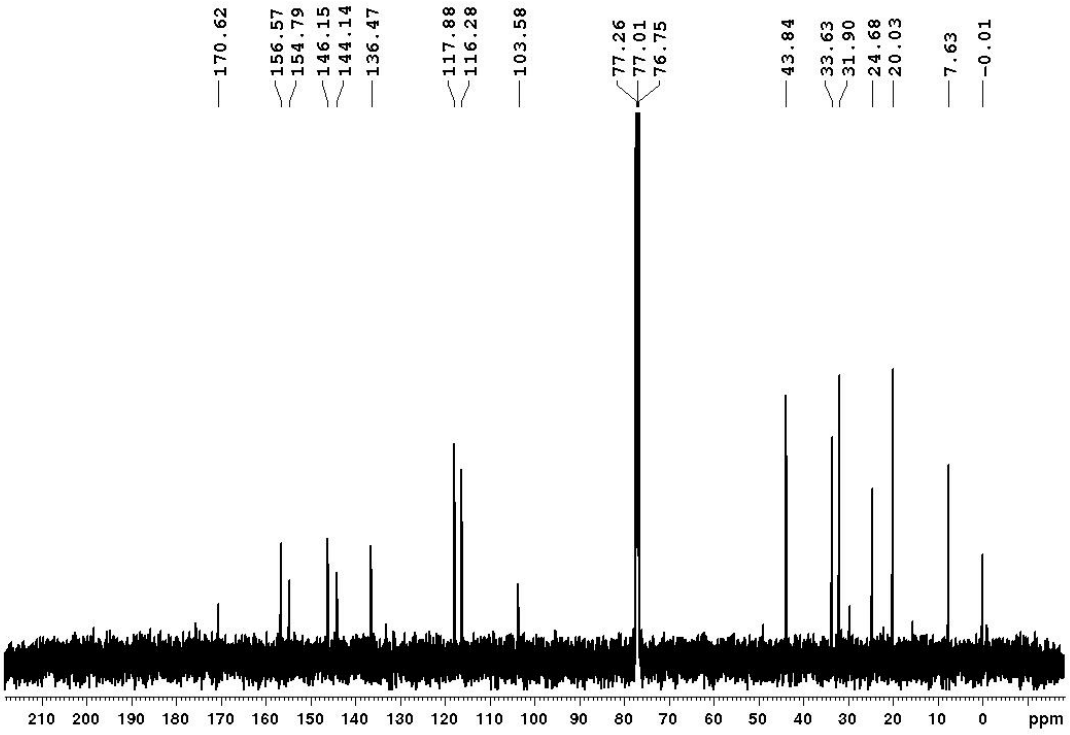


Figure 5B.6. ^{13}C NMR spectrum of compound **50** in $CDCl_3$

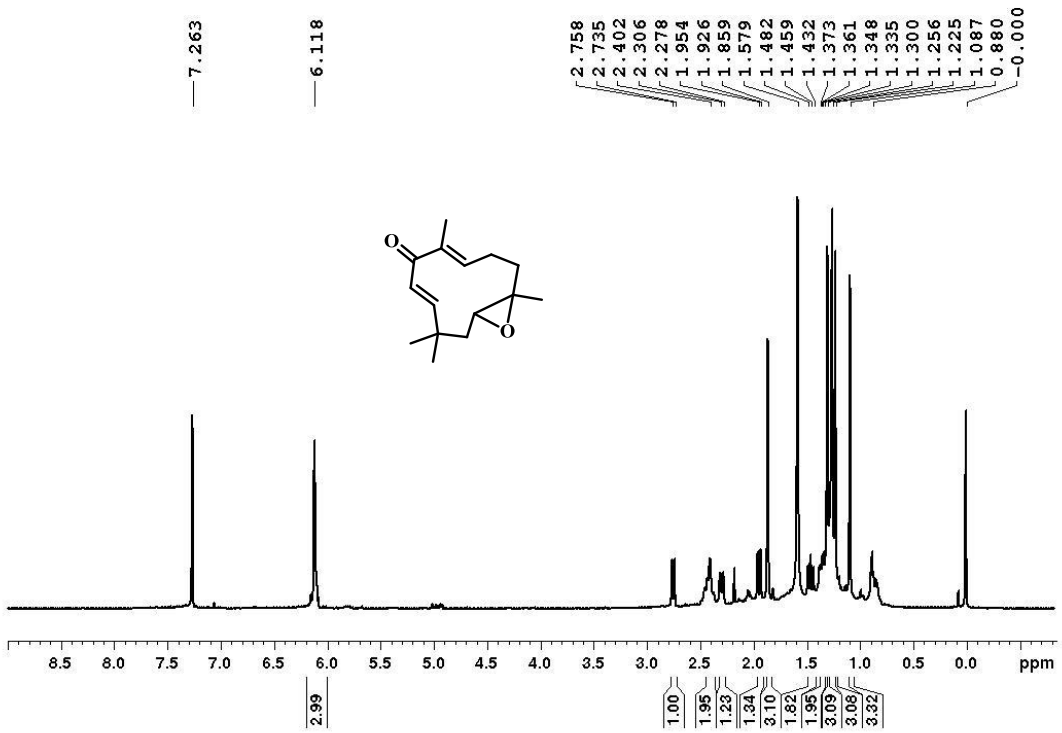


Figure 5B.7. ^{1}H NMR spectrum of compound **51** in $CDCl_3$

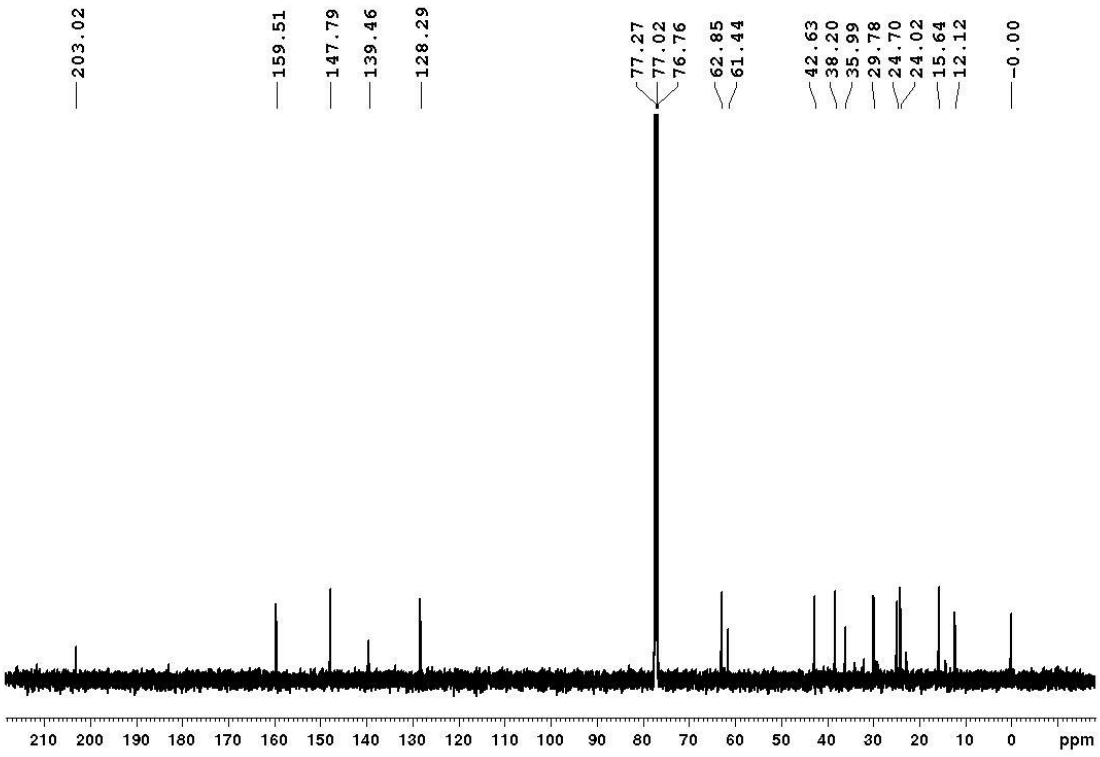
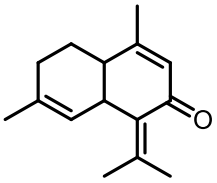


Figure 5B.8. ^{13}C NMR spectrum of compound **51** in $CDCl_3$

Compound **52** was isolated from the same fraction pool 3 as colourless oily substance (8 mg). The molecular formula was established as $C_{15}H_{20}O$ on the basis of mass spectral analysis, which exhibited a parent molecular ion peak at *m/z* 217.1598 $(M+H)^+$. The IR spectrum indicated the presence of a conjugated carbonyl at 1665 cm^{-1}. The ^1H NMR spectrum (**Fig. 5B.9**) showed four vinyl methyl (δ 2.06, 1.93, 1.87 & 1.57 ppm), two methylene (δ 2.20, 1.82 & 1.78 ppm), two methine (δ 3.75 & 2.74 ppm) and two trisubstituted olefin groups (δ 5.90 & 4.92 ppm). The ^{13}C NMR spectrum (**Fig. 5B.10**) displayed the presence of 15 signals, which includes four methyl (δ 21.0, 21.9, 23.0 & 23.5), two methylene (δ 25.3 & 26.0), four methines (δ 38.3, 39.8, 122.0 & 130.8), and five quaternary carbons including a carbonyl group (δ 191.9, 158.6, 141.8, 135.1 & 133.4). Further, the protons and carbons were assigned on the basis of 2D NMR analysis and on comparison with previous literature reports. Thus, compound **52** was identified as **Comosone II**.[11]



Compound **52 - Comosone II**

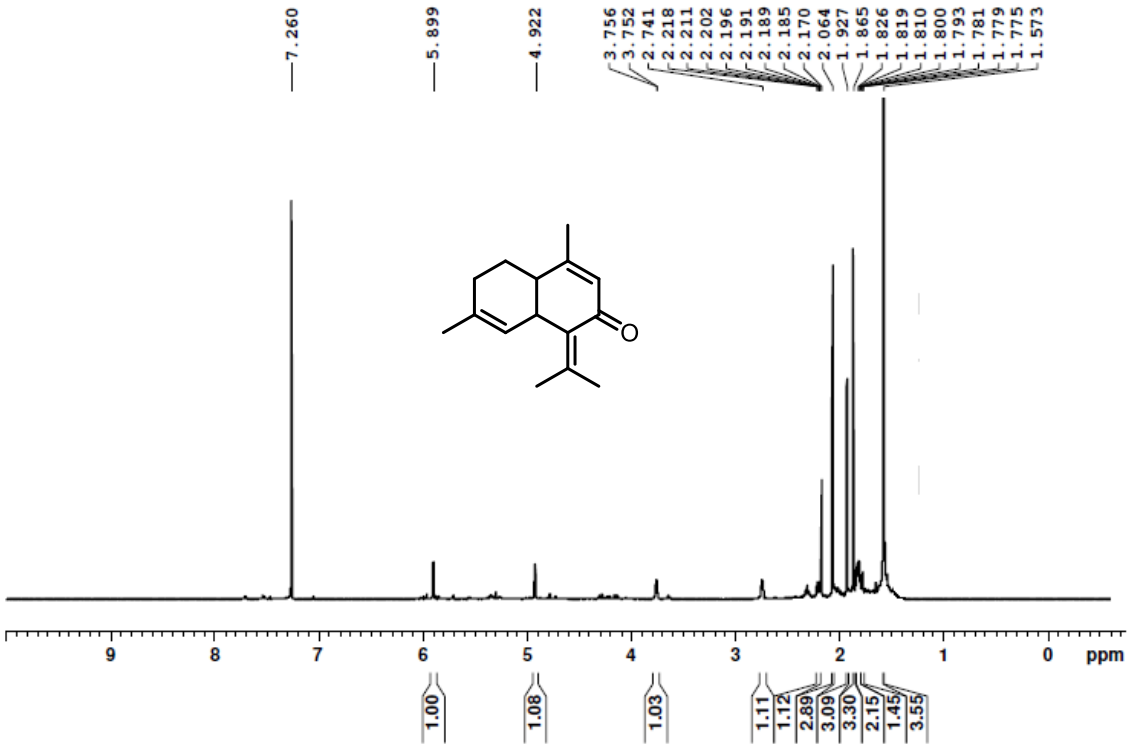


Figure 5B.9. ^1H NMR spectrum of compound **52** in $CDCl_3$

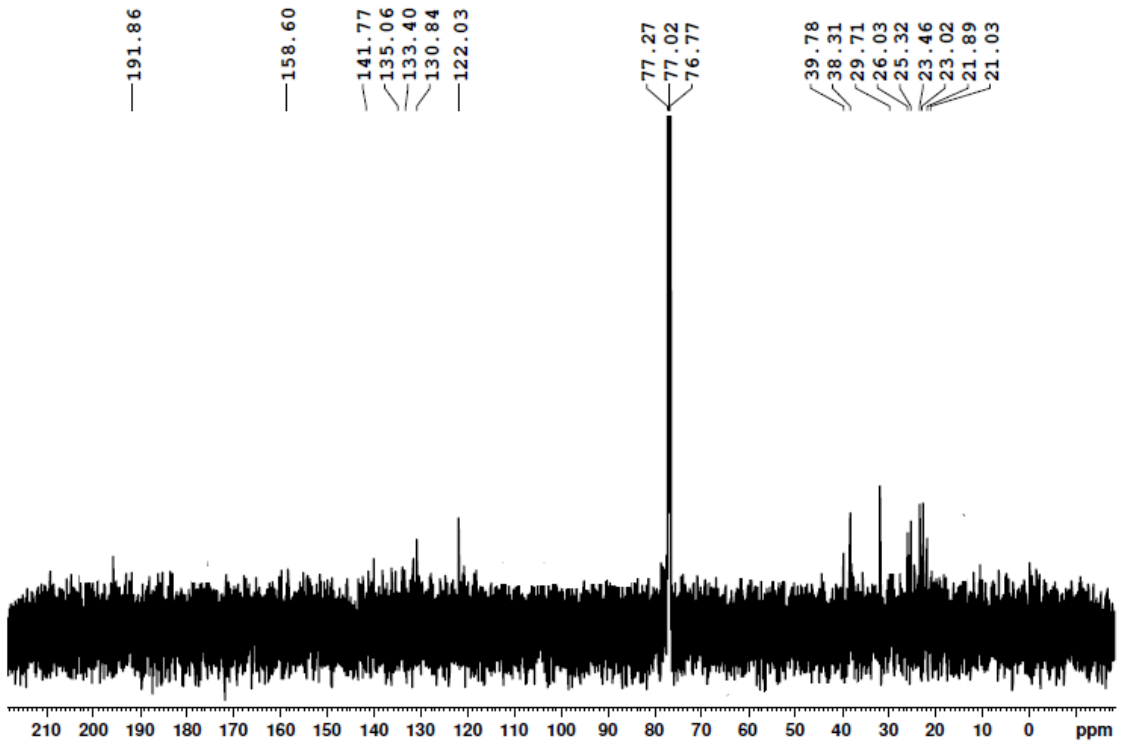


Figure 5B.10. ^{13}C NMR spectrum of compound **52** in $CDCl_3$

The fraction pool 5 (Fr. 29-44) afforded a mixture of ***β*- sitosterol** and **stigmasterol**. On purification, 14 mg of stigmasterol was isolated as a colourless crystalline solid (14 mg) and 7 mg of *β*- sitosterol was obtained.

5B.3. Lewis acid-catalyzed nucleophilic substitution of Germacrone

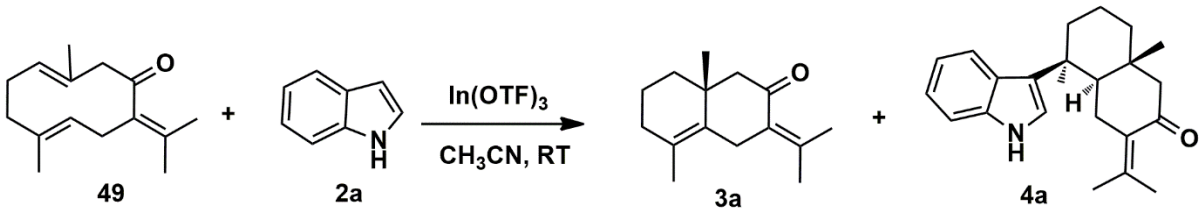
5B.3.1. Definition of Problem

Germacrone is the primary active component found in *Curcuma rakthakanta.* In the field of the synthesis of potent bioactive natural products, it should be remarked that the efficiency of synthetic approaches including the use of renewable starting material, is generally far ahead of the current capabilities of chemical total synthesis. In this sense, we believe that the structure and functionality of germacrone may well serve as a versatile building block for the efficient preparation of a wide variety of bioactive molecules. In this context, we attempted to utilize germacrone as a useful synthon and herein, we describe a convenient methodology for the semi synthesis of indole appended germacrone. Though we have a number of nucleophiles having different properties, we preferably chose the indole heterocycles for our present

study, since they are present in a wide variety of natural compounds with diverse physiological activities.

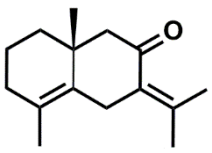
5B.3.2. Results & Discussions

We initiated our work by exploring the reaction of nucleophilic substitution on germacrone (**49)** in the presence of 10 mol % of $In(OTf)_3$ in CH_3CN at room temperature. The reaction afforded two products: cyclized product of germacrone Eudesma-4,7(11),dien-8-one (**3a)** and indole substituted eudesmane skeleton (**4a**) were obtained in 27 and 22 % of yield (**Scheme 5B.1**).



Scheme 5B.1

The structure of the products was assigned based on ^1H, ^{13}C and 2D NMR techniques. The ^1H NMR spectrum (**Fig. 5B.11**) of **3a** showed two doublets at δ 3.41 (d, J = 18.0 Hz, 1H) and 3.06 (d, J = 17.5 Hz, 1H) ppm corresponded to the -CH_2 protons in the C-6th position. The methylene protons in the C-9th position appeared as doublets at δ 2.32 (d, J = 15.5 Hz, 1H) and 2.25 (d, J = 15.5 Hz, 1H) ppm. Four methyl groups resonated at δ 2.09, 1.85, 1.66 and 1.06 ppm, respectively. The ^{13}C NMR spectrum (**Fig. 5B.12**) displayed the presence of 15 carbons with four methyl (δ 26.0, 23.4, 19.1 & 14.1 ppm), five methylene (δ 55.7, 38.3, 32.4, 29.9 & 22.6 ppm), and six quaternary carbons (δ 203.2, 144.1, 131.0, 130.5, 126.1 & 35.8 ppm), which includes a carbonyl carbon. All the peaks were in good agreement with the desired product **3a**. Final evidence for the structure was obtained from the high resolution mass spectral analysis, which displayed a peak at m/z 217.1594 $(M-H)^+$. The product **3a** was identified as **Eudesma-4,7(11),dien-8-one,** a natural product reported from the soft coral *Nephthea* species.[12]



Eudesma-4,7(11),dien-8-one

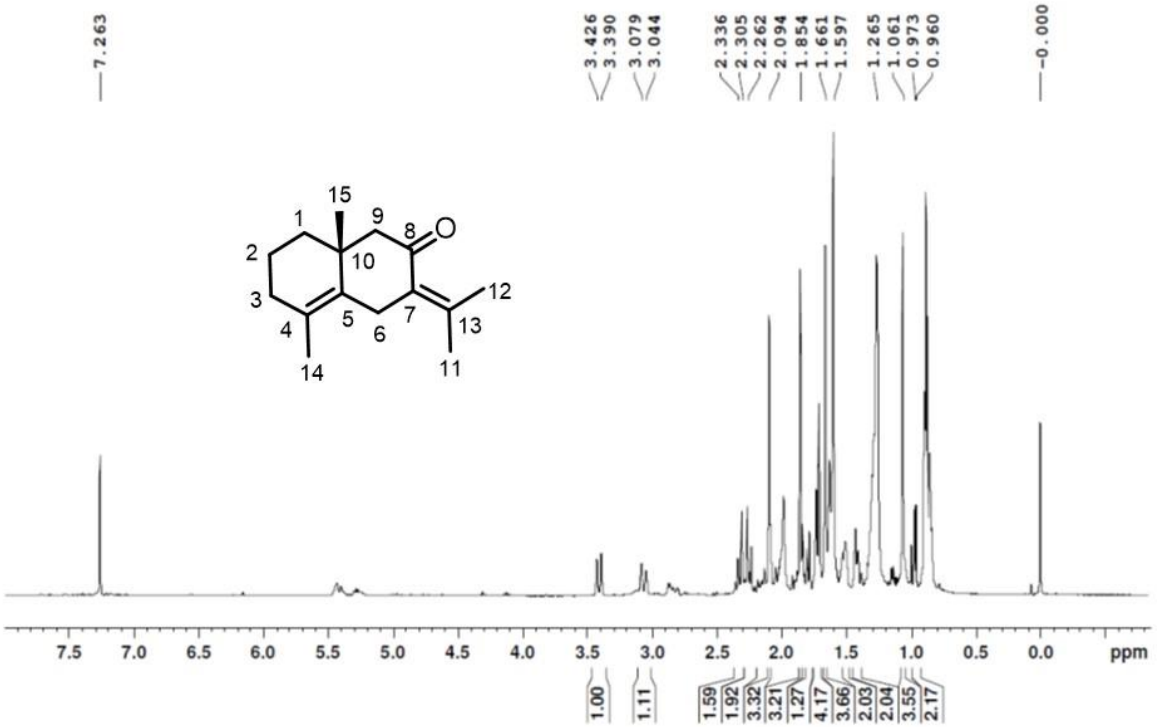


Figure 5B.11. ^{1}H NMR spectrum of **3a** in $CDCl_3$

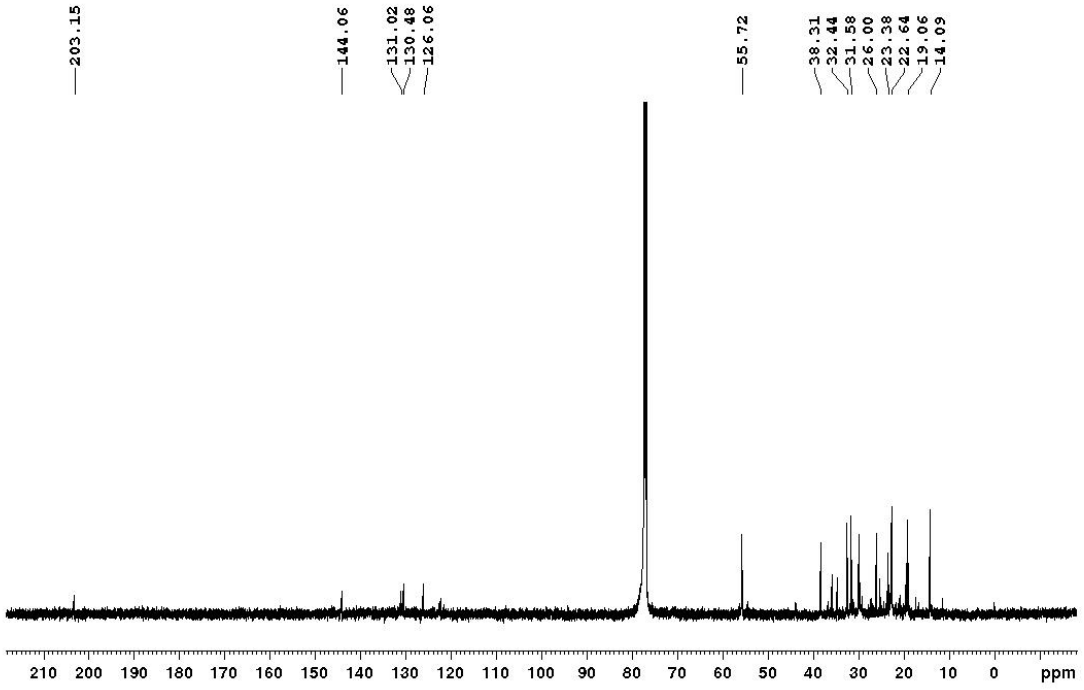


Figure 5B.12. ^{13}C NMR spectrum of **3a** in $CDCl_3$

In the ^{1}H NMR spectrum of **4a** the aromatic protons resonated between *δ 7.96*-6.98 ppm and the proton at the bridge appeared as doublets of doublets at *δ* 2.98 (H-5) ppm. The methyl group attached to the carbon bearing an indolyl group resonated as a singlet at *δ* 1.63 (H-14) ppm. The ^{13}C NMR spectrum showed 23 carbons and DEPT-135 revealed the presence of four methyl, five methylene, six methine and seven quaternary carbons. The carbonyl carbon resonated at *δ* 212.6 ppm. The HMBC correlation of the methyl group at *δ* 1.63 (H-14) with the C-3 carbon of indole (*δ* 124.8) confirms the attachment of nucleophile at the C-4th position. The stereochemistry of the product was established from the NOE spectrum. Correlations were observed between H-5 and H-14, thus confirming that both are in α-configuration. Finally, the mass spectra showed the molecular ion peak at *m/z* 358.2149 $(M+Na)^{+}$ supported the molecular formula $C_{23}H_{29}NO$. All the spectral data are shown in **Figure 5C.13-20**. Spectral assignments are given in **Table 5B.3**.

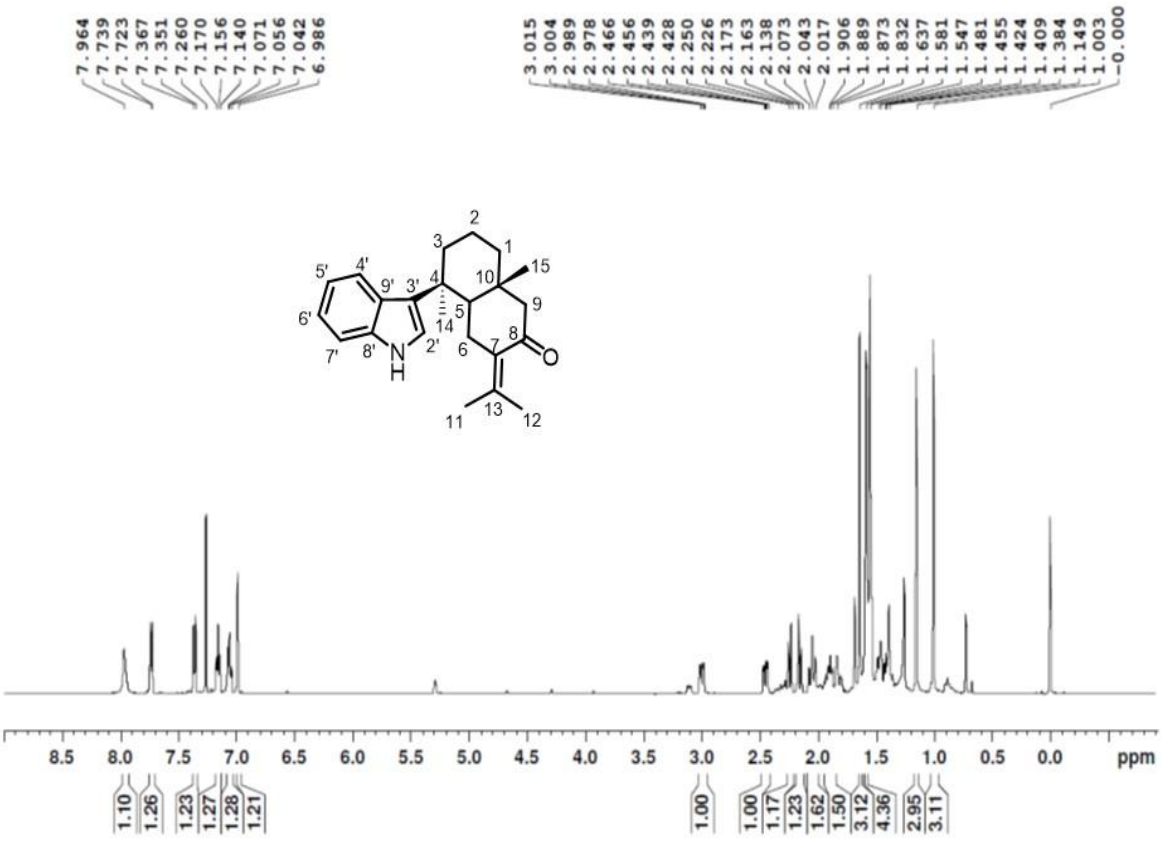


Figure 5B.13. ^{1}H NMR spectrum of **4a** in $CDCl_3$

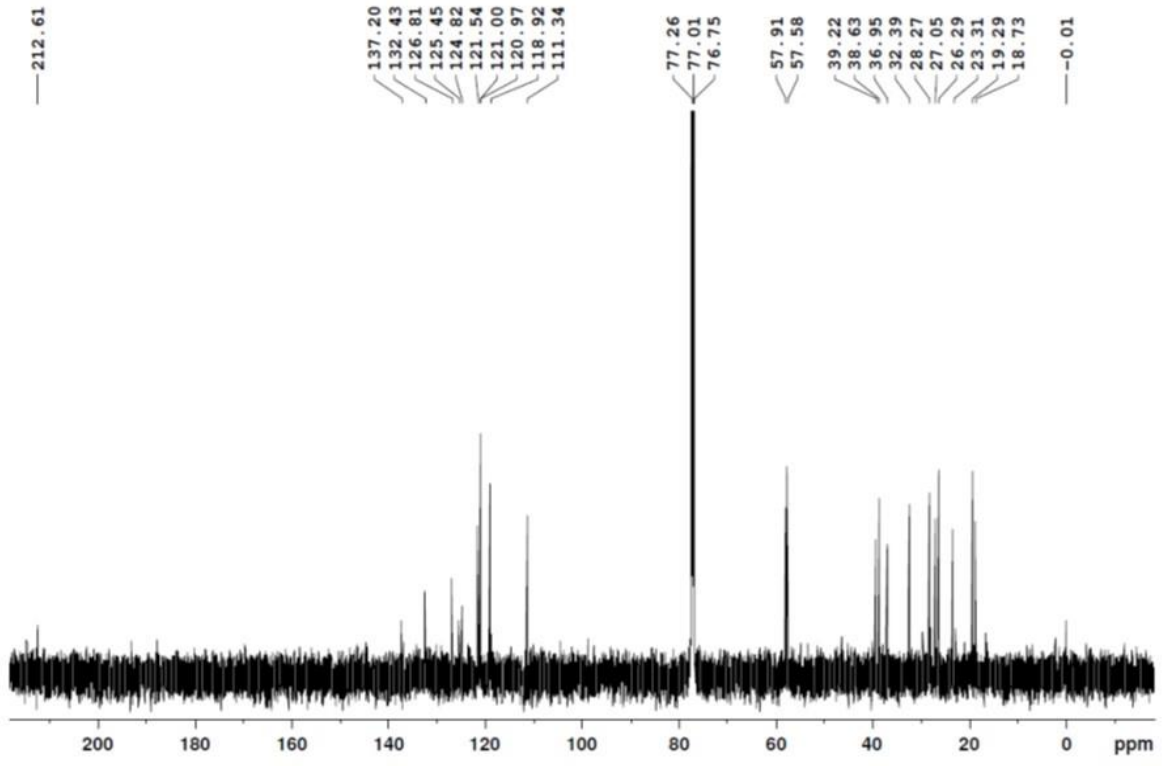


Figure 5B.14. ^{13}C NMR spectrum of **4a** in $CDCl_3$

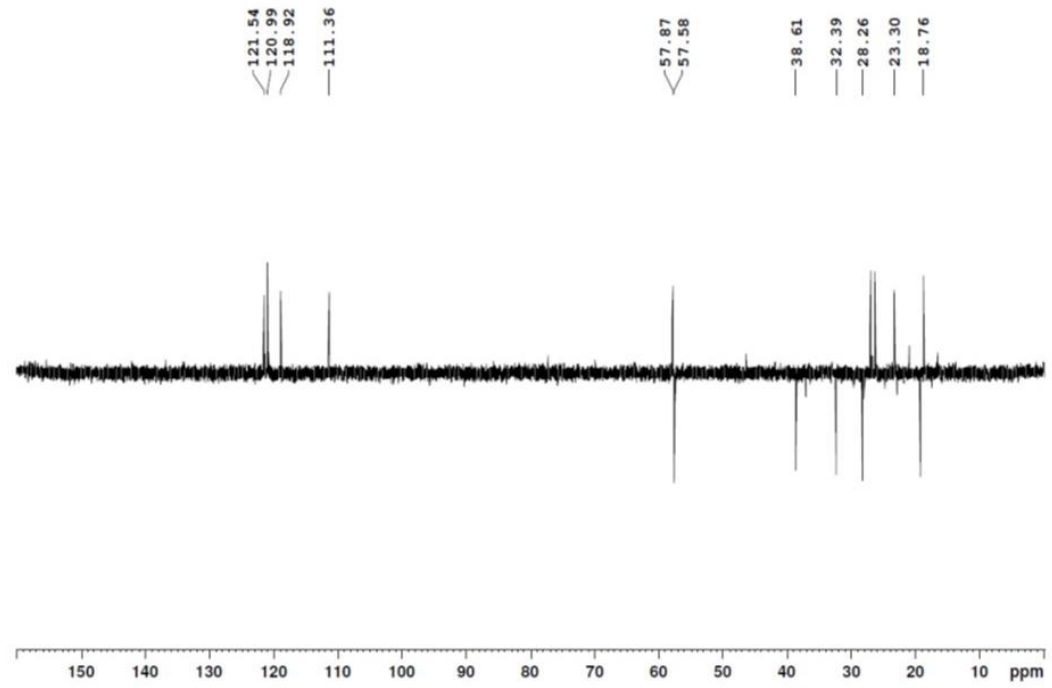


Figure 5B.15. DEPT-135 spectrum of **4a** in $CDCl_3$

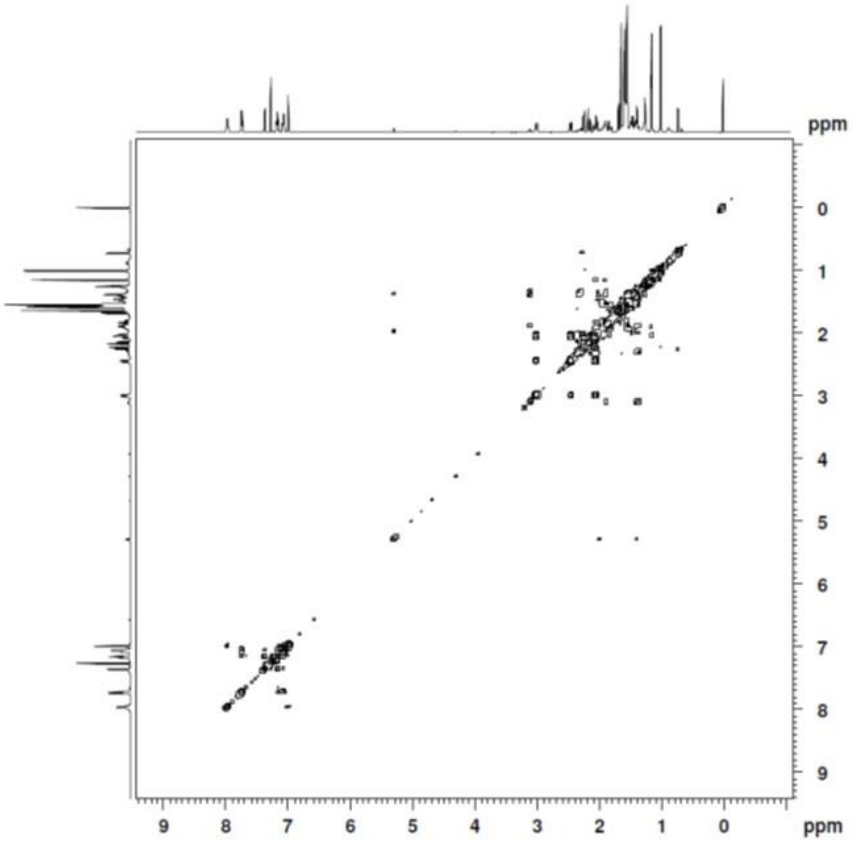


Figure 5B.16. COSY spectrum of **4a** in $CDCl_3$

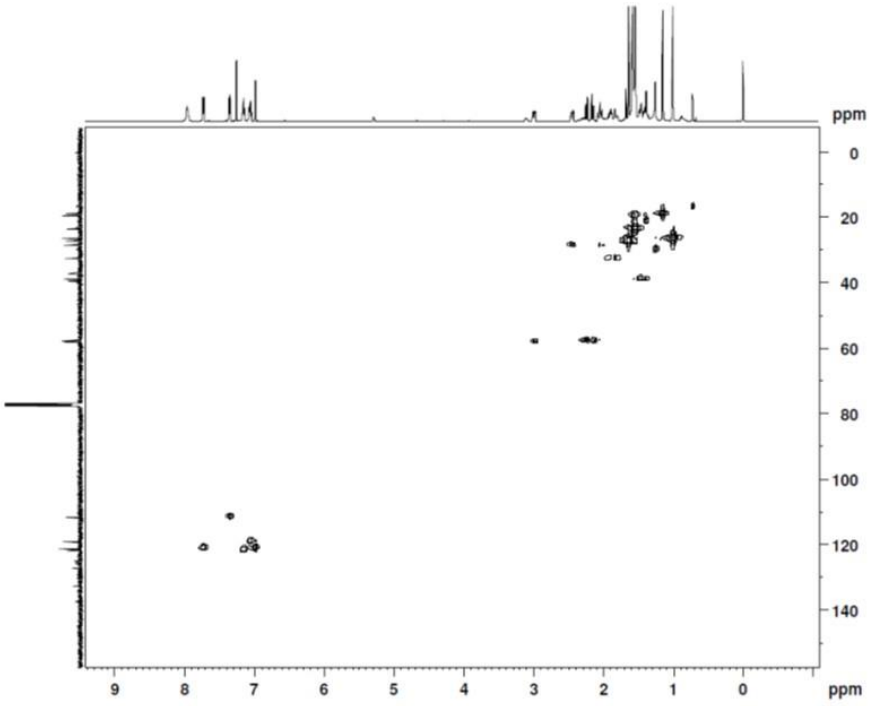


Figure 5B.17. HMQC spectrum of **4a** in $CDCl_3$

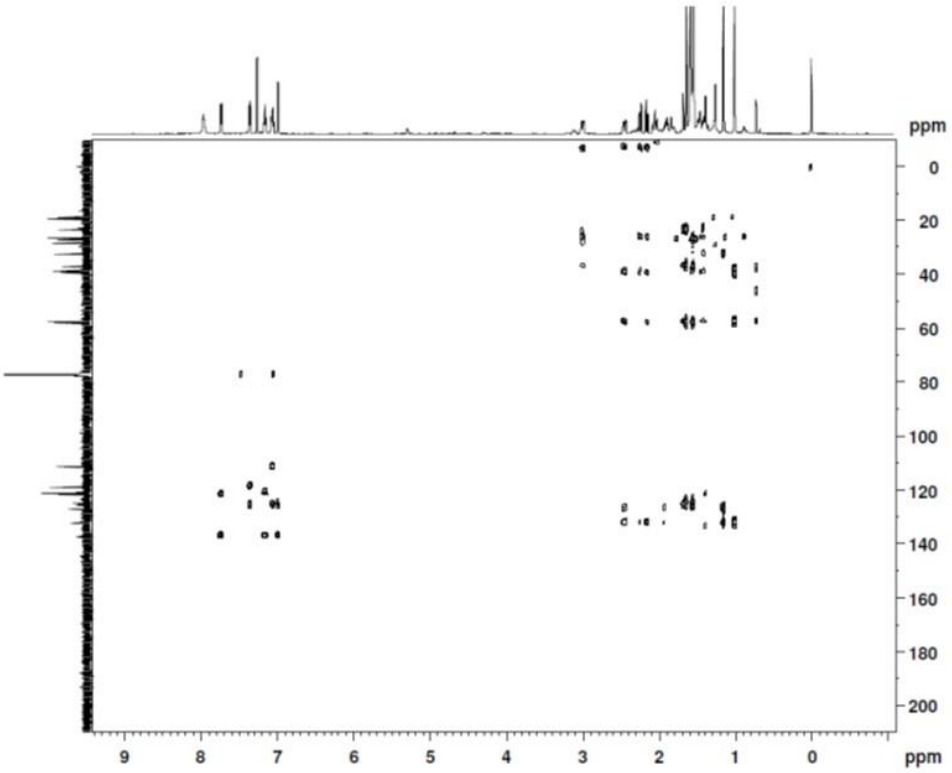


Figure 5B.18. HMBC spectrum of **4a** in $CDCl_3$

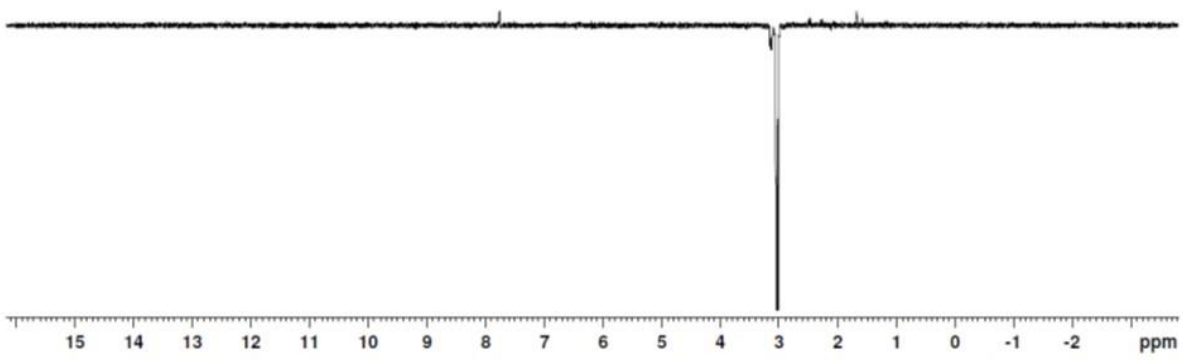


Figure 5B.19. NOE spectrum of **4a** in $CDCl_3$

Table 5B.3. Spectroscopic evidence for assigned stereochemistry

No.	^{1}H	^{13}C	HMBC
1	1.89/1.86	32.4	H-2
2	1.50/1.40	19.3	
3	1.47	38.6	
4		36.9	H-14, H-15
5	2.98	57.9	H-14, H-15
6	2.46/2.08	28.3	
7		126.8	H-6, H-12, H-13
8		212	
9	2.24/2.14	57.6	
10		39.2	
11	1.00	26.3	
12	1.15	18.7	
13		132.4	H-6, H-9, H-13, H-14
14	1.63	27.0	
15	1.54	23.3	
1’			
2’	6.98	121.5	
3’		124.8	H-14
4’	7.73	121.0	
5’	7.05	118.9	
6’	7.15	121.5	
7’	7.35	111.3	
8’		137.2	H-4’, H-6’, H-2’
9’		125.4	

5B.3.3. Optimisation Studies

Detailed optimization studies were carried out for the cyclization reaction of germacrone with lewis acid. The catalytic activity of different Lewis acids such as Sc(OTf)3, $Yb(OTf)_3$, $Cu(OTf)_2$, $Eu(OTf)_3$, $Ag(OTf)_3$, $Zn(OTf)_2$, $FeCl_3$, $In(OTf)_3$, *etc* were studied, out of which the best transformation was obtained with 10 mol % $In(OTf)_3$

(**Table 5B.4**., entry 2). Among the several solvents screened, CH_3CN was found to be the ideal medium for the reaction. From the detailed optimization studies, 1 equivalent of germacrone and 1 equivalent of indole in the presence of 10 mol % of $In(OTf)_3$ in CH_3CN at room temperature was found to be the best condition for the reaction. The results are summarized in **Table 5B.4**.

Table 5B.4. Optimisation studies for a suitable catalyst system for product **4a**

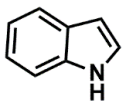
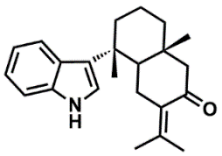
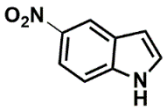
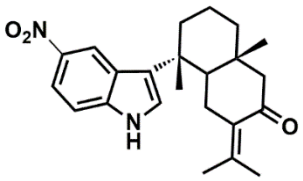
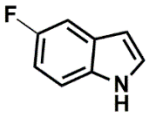
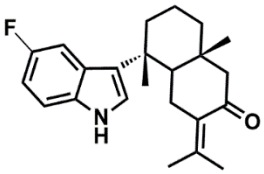
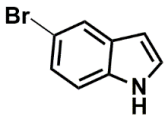
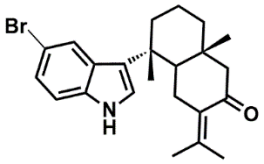
Entry[a]	Lewis acid	Solvent	Temp	Product yield[b] 4a
1	$Sc(OTf)_3$	CH_3CN	RT	15
2	**$In(OTf)_3$**	**CH_3CN**	**RT**	**22**
3	$Yb(OTf)_3$	CH_3CN	RT	NR
4	$Zn(OTf)_3$	CH_3CN	RT	NR
5	$Fe(OTf)_3$	CH_3CN	RT	14
6	$Cu(OTf)_3$	CH_3CN	RT	18
7	$Ag(OTf)_3$	CH_3CN	RT	NR
8	$Eu(OTf)_3$	CH_3CN	RT	NR
9	Bronsted acid	CH_3CN	RT	NR
10	AlCl3	CH_3CN	RT	trace
11	$In(OTf)_3$	DCM	RT	NR
12	$In(OTf)_3$	CH_3OH	RT	NR
13	$In(OTf)_3$	CHCl3	RT	19
14	$In(OTf)_3$	THF	RT	NR
15	$In(OTf)_3$	Toluene	RT	NR

16	$In(OTf)_3$	CH_3CN	80° C	NR

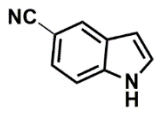
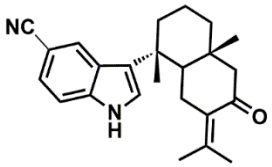
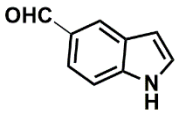
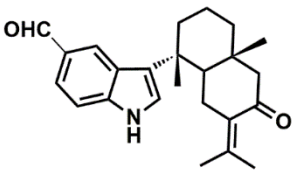
[a]Reaction conditions: Germacrone and indole (1.0 equiv.), Lewis acid (10 mol %), Solvent 2 mL, room temperature, 12 h, [b]recovered yield.

The generality of the reaction was studied under the optimized conditions and the results are depicted in **Table.5B.5**.

Table 5B.5. Generality of the reaction

Entry	Indole	Product	% of Yield (Recovered) 3	4
1		 **4a**	27	22
2		 **4b**	36	25
3		 **4c**	28	28
4		 **4d**	25	13

Continued…

5		 **4e**	60	18
6		 **4f**	47	4.00

Reaction conditions: Germacrone and indole (1.0 equiv.), Lewis acid (10 mol %), CH_3CN: 2 mL, room temperature, 12 h.

5B.3.4. Plausible Mechanism

According to theoretical studies from literature reports, germacrone presents in two major conformations in solution (**Fig. 5B.20**). In the predominant conformation (1a), the distance between C-1 and C-4 is quite short (2.80 Å), which favours a transannular cyclization process. Theoretical calculations at the DFT level have been carried out on the activation and reaction energies from the major conformer **1a** to the bicyclic carbocation intermediates **II** and **III**, resulting from the initial protonation–cyclization of the double bonds 1,10 and 4,5, respectively (**Fig. 5B.21**). The eudesmanic carbocation II originated through 1,10- protonation, involves the initial production of carbocation I, which subsequently cyclizes to II. [13]

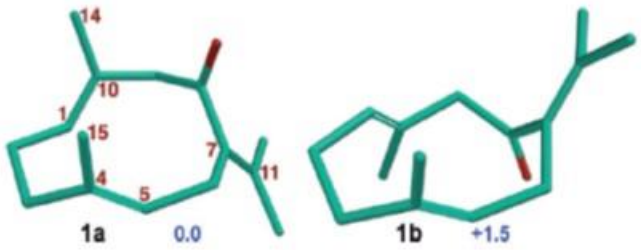


Figure 5B.20. Major (1a) and minor (1b) solution conformations of germacrone

The mechanism for the formation of the transannular cyclization product involves the initial coordination of Lewis acid with the double bond in the C-1 position, which triggers the subsequent cyclization to provide an intermediate

carbocation. The subsequent migration of the adjacent bond to this cationic centre furnishes the bicyclic skeleton with a more stable carbocationic intermediate. Finally, either the elimination of a proton occurs providing the bicyclic product **3a** or the nucleophilic attack of the nucleophiles on II gave the product **4a** (**Fig. 5B.22**).

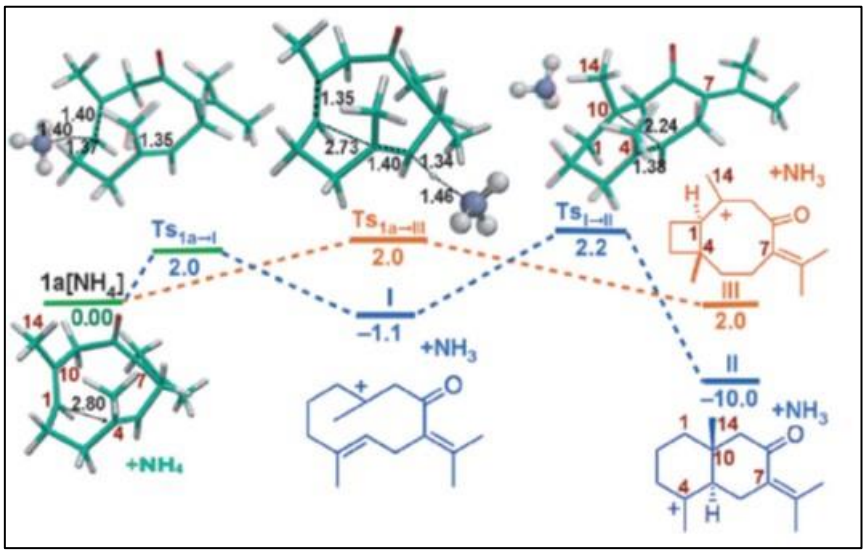


Figure 5B.21. Energy diagram for the formation of carbocations **I–III**

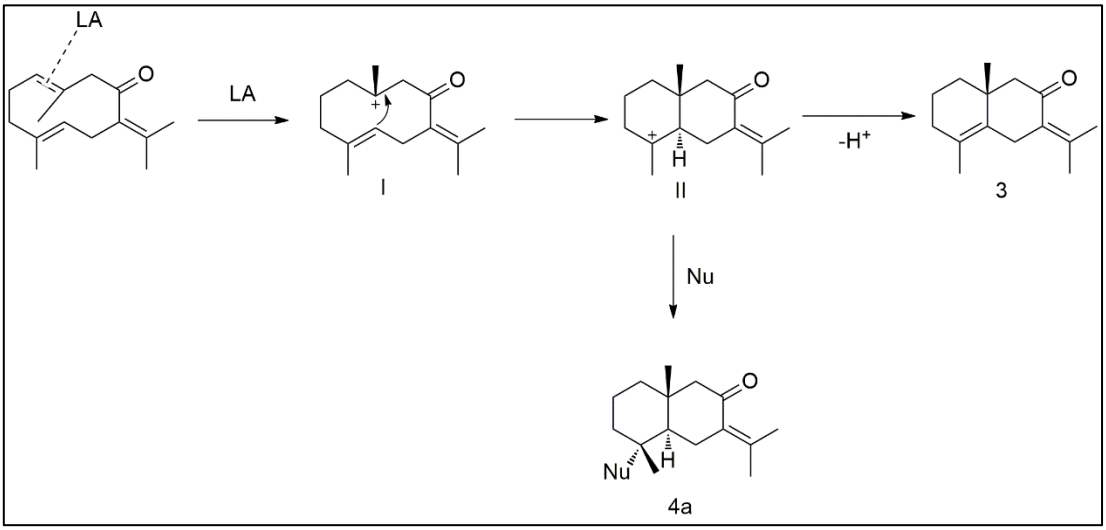


Figure 5B.22. Plausible mechanism

5B.4. Conclusion

Chemoprofiling of the medicinally important plant *C. raktakanta* was hitherto uninvestigated. We have successfully isolated and characterized seven major compounds from the acetone extract of *Curcuma raktakanta,* which includes five sesquiterpenes: furanodiene, germacrone, gweicurculactone, zerumbone epoxide,

comosone II and two sterols: *β*–sitosterol and stigmasterol. We have developed a method for the Lewis acid catalysed annulation reactions of germacrone-indole derivatives to access a natural product Eudesma-4,7(11),dien-8-one and an indole appended cyclised product. Under Lewis acid catalysis, we could synthesize indole substituted eudesmane moiety from germacrone-indole adduct.

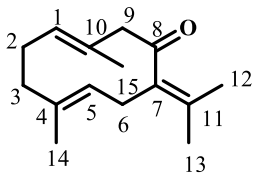
5B.5. Experimental data

Same as in Chapter 2A.

5B.6. Spectra data

Compound 49 (Germacrone)

Fraction pool 3 (fr.12-15) on silica gel CC afforded three compounds. Compound **49** was isolated as a colourless crystalline solid (1.0 g). A detailed spectral analysis and on comparison with literature, the compound was identified as **Germacrone**.



Molecular formula: $C_{15}H_{22}O$

FT-IR (Neat, υ_{max} cm^{-1}): 1678, 2854, 2922 cm^{-1}.

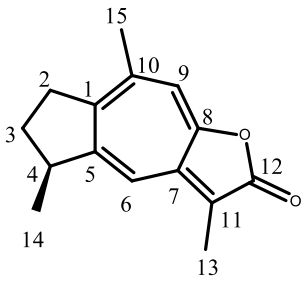
^{1}H NMR (500 MHz, $CDCl_3$, TMS): *δ* 4.98 (br d, *J* = 11.0 Hz, 1H, H-1), 4.71 (br d, *J* = 13.7 Hz, 1H, H-5), 3.41 (d, *J* = 10.5 Hz, 1H, H-9*β*), 2.95 (dd, 1H, H-9*α*), 2.93 (br d, 1H, H-6*β*), 2.85 (br d, 1H, H-6*α*), 2.35 (m, 1H, H-*2α)*, 2.14 (m, 1H, H-3*β*), 1.77 (s, 3H, H-12), 1.72 (s, 3H, H-13), 1.44 (s, 3H, H-14), 1.63 (s, 3H, H-15) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): *δ* 15.6 (C-14), 16.7 (C-15), 19.9 (C-12), 22.4 (C-13), 24.1 (C-2), 29.2 (C-6), 38.1 (C-3), 56.0 (C-9), 125.4 (C-5), 126.7 (C-4), 129.6 (C-7), 132.7 (C-1), 135.0 (C-10), 137.2 (C-11), 208.0 (C-8) ppm.

HRMS (ESI): *m/z* calcld for $C_{15}H_{22}O$ is 218.1670. Found 219.1722 $(M+H)^+$.

Compound 50 (Gweicurculactone)

The second compound from fraction pool 2 was obtained as orange crystalline solid (42 mg). The structure of compound **50** was assigned as **Gweicurculactone** based on various spectral analysis and on comparison with literature reports.



Molecular formula: $C_{15}H_{16}O_2$

FT-IR (Neat, υ_{max} cm^{-1}): 2950, 2379, 1725, 1630, 1600, 1545, 1505, 1268, 1121, 898 cm^{-1}.

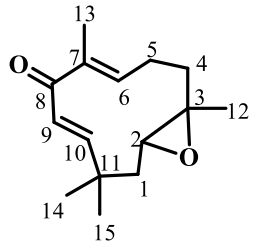
^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 6.90 (s, 1H, H-6), 6.75 (s, 1H, H-9), 3.10 (m, 1H, H-4), 2.86-2.67 (m, 2H, H-2), 2.25 (s, 3H, H-14), 2.13 (m, 1H, H-3a), 2.00 (s, 3H, H-13), 1.53 (m, 1H, H-3b), 1.26 (s, 3H, H-15) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): *δ* 170.6 (C-12), 156.6 (C-5), 154.8 (C-8), 146.2 (C-7), 144.2 (C-1), 136.5 (C-10), 117.9 (C-6), 116.3 (C-9), 103.6 (C-11), 43.8 (C-4), 33.6 (C-2), 31.9 (C-3), 24.7 (C-15), 20.0 (C-14), 7.6 (C-13) ppm.

HRMS (ESI): *m/z* calcld for $C_{15}H_{16}O_2$ is 228.1150. Found 251.1050 $(M+Na)^+$.

Compound 51 (Zerumbone epoxide)

Compound **51** isolated from fraction pool 2 as colourless crystalline solid (8 mg). The spectral data were similar to that of **Zerumbone epoxide** previously reported. The spectral assignments were carried out based on 2D NMR and literature reports.



Molecular formula: $C_{15}H_{22}O_2$

FT-IR (Neat, υ_{max} cm^{-1}): 1630, 1250, 880, 761 cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$, TMS): *δ* 6.12 (s, 3H, H-10, H-9, H-6), 2.74 (d, *J* = 11.5 Hz, 1H, H-2), 2.40 (m, 2H, H-5), 2.29 (d, *J* = 14.0 Hz, 1H, H-4), 1.94 (d, *J* = 14.0 Hz, 1H, H-1), 1.86 (s, 3H, H-13), 1.46 (t, 1H, *J* = 11.5 Hz, H-

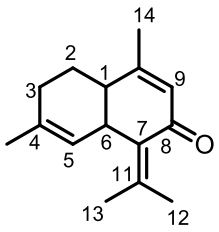
1), 1.37-1.33 (m, 1H, H-4), 1.30 (s, 3H, H-14), 1.22 (s, 3H, H-12), 1.09 (s, 3H, H-15) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 203.0 (C-8), 159.5 (C-10), 147.8 (C-6), 139.5 (C-7), 128.3 (C-9), 62.8 (C-2), 61.4 (C-3), 42.6 (C-1), 38.2 (C-4), 36.0 (C-11), 29.8 (C-15), 24.7 (C-5), 24.0 (C-14), 15.6 (C-12), 12.1 (C-13) ppm.

HRMS (ESI): *m/z* calcld for $C_{15}H_{16}O_2$ is 234.1619. Found 257.1521 $(M+Na)^+$.

Compound 52 (Comosone II)

Compound **52** was isolated from fraction pool 2 as a yellowish oily substance (8 mg). The structure of the compound was assigned as **Comosone II** based on the analysis of various spectral data and literature reports.



Molecular formula: $C_{15}H_{20}O$

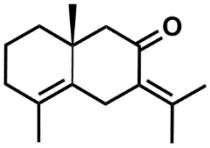
FT-IR (Neat, υ_{max} cm^{-1}): 1665, 1651, 1615, 1439, 1379, 754 cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 5.90 (1H, s, H-9), 4.92 (1H, s, H-5), 3.75 (1H, d, J = 2.0 Hz, H-6), 2.74 (1H, s, H-1), 2.20 (1H, m, H-2), 2.06 (3H, s, H-12), 1.93 (3H, s, H-15), 1.87 (3H, s, H-13), 1.82 (2H, m, H-3), 1.78 (1H, m, H-2), 1.57 (3H, s, H-14) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 191.9 (C-8), 158.6 (C-10), 141.8 (C-11), 135.1 (C-4), 133.4 (C-7), 130.8 (C-9), 122.0 (C-5), 39.8 (C-6), 38.3 (C-1), 26.0 (C-3), 25.3 (C-2), 23.5 (C-15), 23.0 (C-12), 21.9 (C-13), 21.0 (C-14) ppm.

HRMS (ESI): *m/z* calcld for $C_{15}H_{20}O$ is 216.1514. Found 217.1598 $(M+H)^+$.

Eudesma-4,7(11),dien-8-one (3a): Following the general experimental procedure, germacrone 1 (15 mg, 0.0687 mmol), indole (8.04 mg, 0.0687 mmol), $In(OTf)_3$ (3.86 mg, 0.00687 mmol), in 2 mL acetonitrile at room temperature for 12 h gave the product **3a** as colourless solid (3.2 mg, 27 %).

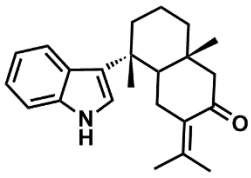


^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 3.41 (d, *J* = 18.0 Hz, 1H), 3.06 (d, *J* = 17.5 Hz, 1H), 2.32 (d, *J* = 15.5 Hz, 1H), 2.25 (d, *J* = 15.5 Hz, 1H), 2.09 (s, 3H), 1.85 (s, 3H), 1.98 (m, 2H), 1.83-1.78 (m, 2H), 1.66 (s, 3H), 1.53 (m, 1H), 1.40 (m, 1H), 1.06 (s, 3H) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 203.2, 144.1, 131.0, 130.5, 126.1, 55.7, 38.3, 35.8, 32.4, 31.6, 29.9, 26.0, 23.4, 22.6, 19.1, 14.1 ppm.

HRMS (ESI): *m/z* calcd for $C_{15}H_{22}O$ is 218.1671. Found 217.1594 $(M-H)^+$.

(5R,8aR)-5-(1H-indol-3-yl)-5,8a-dimethyl-3-(propan-2 ylidene)octahydronaphthalen -2(1H)-one (4a): Following the general experimental procedure, germacrone 1 (15 mg, 0.0687 mmol), indole (8.04 mg, 0.0687 mmol), $In(OTf)_3$ (3.86 mg, 0.00687 mmol), in 2 mL acetonitrile at room temperature for 12 h gave the product **4a** as colourless solid (4 mg, 22 %).



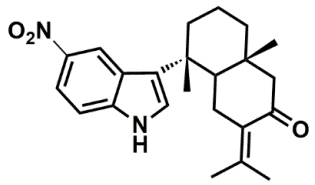
^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 7.96 (s, 1H), 7.73 (d, *J* = 8.0 Hz, 1H), 7.36 (d, *J* = 8.0 Hz, 1H), 7.15 (t, *J* = 7.0 Hz, 1H), 7.05 (t, *J* = 7.0 Hz, 1H), 6.98 (s, 1H), 2.98 (dd, *J* = 5.5, 13.0 Hz, 1H), 2.46 (dd, *J* = 5.0, 13.5 Hz, 1H), 2.24 (d, *J* = 12.0 Hz, 1H), 2.14 (d, *J* = 12.5 Hz, 1H), 2.08 (t, *J* = 15.0 Hz, 1H, 1.89-1.86 (m, 2H), 1.63 (s, 3H), 1.54 (s, 3H), 1.50 (m, 1H), 1.47 (m, 2H), 1.40 (m, 1H), 1.15 (s, 3H), 1.00 (s, 3H) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 212.6, 137.2, 132.4, 126.8, 125.4, 124.8, 121.5, 121.0, 118.9, 111.3, 57.9, 57.5, 39.2, 38.6, 36.9, 32.4, 28.3, 27.0, 26.3, 23.3, 19.3, 18.7 ppm.

HRMS (ESI): *m/z* calcd for $C_{23}H_{29}NO$ is 335.2249. Found 358.2149 $(M+Na)^+$.

(5R,8aR)-5,8a-dimethyl-5-(5-nitro-1H-indol-3-yl)-3-(propan-2ylidene)octa hydronaphthalen -2(1H)-one (4b): Following the general experimental procedure, germacrone (15 mg, 0.0687 mmol), 5-nitroindole (11.13 mg, 0.0687 mmol), $In(OTf)_3$

(3.86 mg, 0.00687 mmol), in 2 mL acetonitrile at room temperature for 12 h gave the product **4b** as colourless solid (5.5 mg, 25 %).

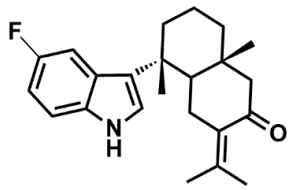


^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 8.71 (s, 1H), 8.42 (s, 1H), 8.09 (d, J = 9.0 Hz, 1H), 7.39 (d, J = 9.0 Hz, 1H), 7.17 (s, 1H), 2.96 (dd, J = 5.0, 8.0, 13.0 Hz, 1H), 2.55 (dd, J = 5.0, 9.0, 14.0 Hz, 1H), 2.30 (m, 1H), 2.10 (m, 1H), 2.02-1.93 (m, 2H), 1.86 (m, 1H), 1.66 (s, 3H), 1.57 (s, 3H), 1.50-1.41 (m, 3H), 1.24 (s, 3H) 1.00 (s, 3H) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 211.6, 140.1, 131.8, 127.6, 127.4, 124.6, 118.1, 117.4, 111.3, 57.8, 57.6, 39.5, 38.8, 36.8, 32.5, 28.4, 26.4, 26.2, 24.1, 19.2, 18.9 ppm.

HRMS (ESI): *m/z* calcd for $C_{23}H_{28}N_2O_3$ is 380.2100. Found 403.2007 $(M+Na)^+$.

(5R,8aR)-5-(5-fluoro-1H-indol-3-yl)-5,8a-dimethyl-3-(propan-2-ylidene)octa hydro-naphthalen-2(1H)-one (4c): Following the general experimental procedure, germacrone (15 mg, 0.0687 mmol), 5-fluoroindole (9.27 mg, 0.0687 mmol), $In(OTf)_3$ (3.86 mg, 0.00687 Mmol), in 2 mL acetonitrile at room temperature for 12 h gave the product **4c** as colourless solid (5 mg, 28 %).



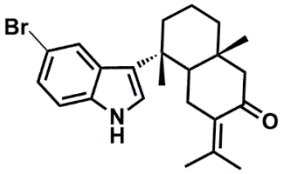
^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 7.98 (s, 1H), 7.37 (d, J = 10.5 Hz, 1H), 7.04 (s, 1H), 6.92 (t, J = 8.5 Hz, 1H), 2.91 (dd, J = 5.0, 8.0, 13.0 Hz, 1H), 2.46 (dd, J = 5.0, 9.0, 14.0 Hz, 1H), 2.26 (d, J = 13.0 Hz, 1H), 2.13 (d, J = 12.5 Hz, 1H), 2.04 (m, 1H), 1.92 (m, 1H),1.85 (m, 1H), 1.61(s, 3H), 1.53 (s, 3H), 1.44 (m, 3H), 1.19 (s, 3H), 1.00 (s, 3H) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 212.2, 158.0, 133.6, 132.2, 127.0, 122.9, 111.9, 110.0, 106.0, 57.6, 57.6, 39.4, 38.7, 36.7, 32.4, 28.3, 26.6, 26.2, 23.3, 19.3, 18.8 ppm.

HRMS (ESI): *m/z* calcd for $C_{23}H_{28}FNO$ is 353.2155. Found 376.2060 $(M+Na)^+$.

(5R,8aR)-5-(5-bromo-1H-indol-3-yl)-5,8a-dimethyl-3-(propan-2ylidene)octa hydronaphthalen-2(1H)-one (4d): Following the general experimental procedure, germacrone (15 mg, 0.0687 mmol), 5-bromoindole (13.39 mg, 0.0687 mmol),

$In(OTf)_3$ (3.86 mg, 0.00687 mmol), in 2 mL acetonitrile at room temperature for 12 h gave the product **4d** as colourless solid (3 mg, 13 %).

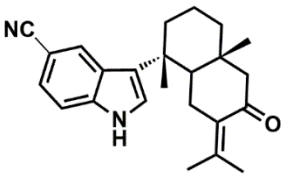


^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 8.02 (s, 1H), 7.84 (s, 1H), 7.24 (s, 2H), 7.01 (s, 1H), 2.89 (dd, *J* = 5.0, 8.0, 13.0 Hz, 1H), 2.46 (dd, *J* = 5.0, 8.5, 13.5 Hz, 1H), 2.28 (d, *J* = 12.5 Hz, 1H), 2.15 (d, *J* = 12.5 Hz, 1H), 2.05 (t, *J* = 13.5 Hz, 1H), 1.94 (m, 1H), 1.86 (m, 1H), 1.65 (s, 3H), 1.52 (s, 3H), 1.43 (m, 3H), 1.23 (s, 3H), 1.01(s, 3H) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 212.2, 135.7, 132.1, 127.1, 124.7, 124.4, 123.3, 122.4, 112.8, 112.2, 57.7, 57.5, 39.3, 38.6, 36.8, 32.4, 28.3, 26.8, 26.3, 23.5, 19.3, 18.8 ppm.

HRMS (ESI): *m/z* calcd for $C_{23}H_{28}BrNO$ is 413.1354. Found 436.1266 $(M+Na)^+$.

3-((1R,4aR)-1,4a-dimethyl-6-oxo-7-(propan-2-ylidene)decahydronaphthalen-1-yl)-1H-indole-5-carbonitrile (4e): Following the general experimental procedure, germacrone (15 mg, 0.0687 mmol), 5-cyanoindole (9.75 mg, 0.0687 mmol), $In(OTf)_3$ (3.86 mg, 0.00687 mmol), in 2 mL acetonitrile at room temperature for 12 h gave the product **4e** as colourless solid (3 mg, 18 %).



^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 8.09 (s, 1H), 8.01 (s, 1H), 7.44 (m, 1H), 7.34 (s, H), 7.14 (s, 1H), 6.63 (s, 1H), 2.92 (dd, *J* = 5.0, 8.0, 13.0 Hz, 1H), 2.51 (dd, *J* = 5.0, 9.0, 14.0 Hz, 1H), 2.29 (m, 2H), 2.11 - 1.95 (m, 3H), 1.86 (m, 1H), 1.62 (s, 3H), 1.55 (s, 3H), 1.46 (m, 3H), 1.26 (s, 3H), 1.00 (s, 3H) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 211.4, 131.9, 127.7, 126.5, 125.0, 124.3, 123.3, 112.3, 111.9, 103.6, 57.8, 57.6, 39.6, 38.8, 36.7, 32.5, 28.3, 26.3, 26.2, 23.9, 19.3, 18.9 ppm.

HRMS (ESI): *m/z* calcd for $C_{24}H_{28}N_2O$ is 360.2202. Found 383.2106 $(M+Na)^+$.

5B.7. References

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Chapter 5C: Isolation and Anticancer Studies of the Rhizomes of *Hedychium Flavescens* Carey Ex Roscoe

5C.1. Introduction

Hedychium flavescens Carey ex Roscoe commonly known as yellow ginger, cream ginger lily or cream garland lily, is native to the eastern Himalayas, including northeast India and Nepal. The genus name *Hedychium* is derived from two ancient Greek words 'hedys' means sweet of pleasant taste or smell and 'chio/ chion', meaning snow, which refers to the fragrant white flowers. The name of the species 'flavescens' refers to the pale yellow, cream yellow or turning yellow colour of flowers. Worldwide, the *Hedychium* genus consists of 80 species.[1] There are 29 species distributed in the tropical and sub-tropical regions of China and 40 species growing in Indonesia. It is well documented that the essential oils of the plants in this genus have many medicinal efficacies, including cercaricidal properties. Innumerable species were used in traditional medicines for the treatment of asthma, blood purification, bronchitis, gastric diseases and anti-emetics, especially among the hill tribes of Uttarakhand, as well as for eye diseases in Nagaland. *Hedychium* species were widely cultivated for their perfume essences and the aerial stems constitute valuable raw material for manufacturing paper. Moreover, the flowers are edible in some of the species.

Yellow ginger (*H. flavescens*) has been extensively cropped around the world as a garden ornamental due to its large, attractive flowers and fragrance. In addition, many Asian Buddhist shrines use yellow ginger as a garden ornamental. The species has a confront distribution in India; recently, *H. flavescens* has been recorded in the semi-evergreen forests of Niyamgiri hills in Odisha.[2] The phytochemical and pharmacological study of *H. flavesence* is insufficiently explored. So far, the antibacterial activity of the essential oil of this species has been reported.[3] The essential oil contains linalool (35 %), β-pinene (27 %), and 1,8-cineole (13 %) as the principal compounds.[4] Therefore, in the present study, we have attempted to isolate

the marker compounds from the acetone extract of the rhizomes of *H. flavesence*, and cytotoxic studies. To the best of our knowledge, the chemo-profiling of *H. flavescens* is reported for the first time.

5C.1.1. Morphology

H. flavescens phenotypically resembles *H. flavum* and *H. coronarium* in respect of specific diagnostic characters such as more or less sessile leaves and dense imbricated bracts, each subtending 2-5 flowers. However, it differs in having leaves pubescent adaxially with dense pubescent on midrib beneath, leaf apex caudate-acuminate and pubescent leaf sheath. Stamen is longer than or sub-equaling the labellum. The colour of the labellum is creamy yellow to pale yellow with a deep yellow patch at the base, labellum longer than wide and apex 2-cleft. Besides, *H. flavescens* has wider bract, dark green in colour and autumn flowering, whereas *Hedychium flavum* has distinctly narrow bracts, medium green in color and flowers earlier than *H. flavescens*. *H. coronarium* has a milky white labellum with pale yellow to greenish-yellow patch at the base and the stamen is shorter than the labellum.

Figure 5C.1. *H. flavescens*

5C.2. Phytochemistry

Hedychium plants mainly contain labdane type diterpenes**.** Itokawa *et al*., reported the isolation of four labdane diterpenes coronarin A, B, C and E -labda-8(17), 12-diene-15,16-dial from the rhizomes of *H. coronarium*.[5] Nakatani *et al.,* reported (E)-labda-8(17),12-diene-l & 16-dial, coronarin D, isocoronarin D, labda-8(17),11,13-trien-15(16)-olide, an ester of labda-8(17),11,13-trien-15-al-16-oic acid and 7-*β*-hydroxycoronarin B.[6] Coronarin D methyl ether, 6-oxo-7,11,13-labdatri-ene-16,15-olide, cryptomeridiol, hedychenone, pivocatin A, 4-hydroxy-3-methoxy cinnamaldehyde, 4-hydroxy-3-methoxyethylcinnamate were also reported from the

same species.[7] Rao *et al.,* reported 6-oxo-7,11,13-labdatrien-16,15-olide, spicatanol, spicatanol methylether, hedychenone, 7-hydroxy hedychenone, yunnacoronarin D, 7-acetoxyhedychenone, 8(12)-Drimene and hedychialactone B from the rhizomes of *H. spicatum.*[8] Songsri *et al*., reported coronarin E, villosin, (E)-15,16-bisnorlabda-8(17),11-dien-13-one, (E)-labda-8(17),12-dien-15,16-dial, (E)-14,15,16-trinorlabda-8(17),11-dien-13-oic acid, 16-hydroxylabda-8(17),11,13-trien-15,16-olide, 15-methoxylabda-8(17),11,13-trien-15,16-olide, coronarin D, zerumin A and zerumin B from *H. ellipticum*.[9]

The essential oil of *H. coronium* contain *β*-pinene (24.8 %) and 1,8-cineole (40.2 %) as the major constituents.[10] About 175 compounds were identified from the essential oil of the flowers of *H. coronarium* which includes, linalool, cis jasmone, methyl benzoate, eugenol, (E)-isoeugenol, methyl jasmonate, jasmin lactone, methyl *epi*-jasmonate, nitriles, indole and oximes, were found to make a significant contribution to the scent of the flowers.[11] A detailed investigation of the essential oil was carried out by Sakhanokho and co-workers in several *Hedychium* species.[4] The essential oil of *H. spicatum* rhizome is used in perfumery and medicines. The rhizomes were reported to be stomachic, carminative, a bronchodilator stimulant and a tonic. Koundal *et al.,* analysed the essential oil of this plant by GC-MS analysis and identified about 22 compounds from the rhizome oil with major components as 1,8-cineole, *β*-eudesmol, *β*-pinene and 10-epi–*γ*-eudesmol.[12] The essential oil from the rhizomes of *Hedychium gardnerianum* Roscoe (`Kahili ginger') contains about 30 % of sesquiterpenes, mainly cadinane derivatives. The sesquiterpenes, *α*-corocalene epoxide, 6,7;7,10-bisepoxy-6,7-*seco*-calamenene, 7-*epi*-*trans*- and 7-*epi*-*cis*-sesquisabinene hydrate, 1,10;7,10-bisepoxy-1,10-*seco*-calamenene, 10-*epi*-cubenol, and *ar*-curcumen-1,10-diol were reported from this plant.[13]

5C.3. Pharmacology

The rhizomes of *Hedychium coronarium* have been used in traditional medicine to treat headaches, skin diseases, tonsillitis, infected nostrils, fever and rheumatism. The plant has remarkable medicinal properties and is useful in both tradition as well as modern medicines. The reported activity of the plant includes potent inhibitory action, antimicrobial activities, antifungal, anti-inflammatory,

antibacterial, analgesic effects, neuropharmacological, anti-inflammatory and cytotoxic activities.[14] Dash *et al*., reported a preliminary cytotoxic analysis of the methanolic extract of *H. coronarium* in the brine shrimp lethality bioassay, showed potent cytotoxic activity.[15] The essential of *Hedychium* species exhibit significant insecticidal activities.[16]

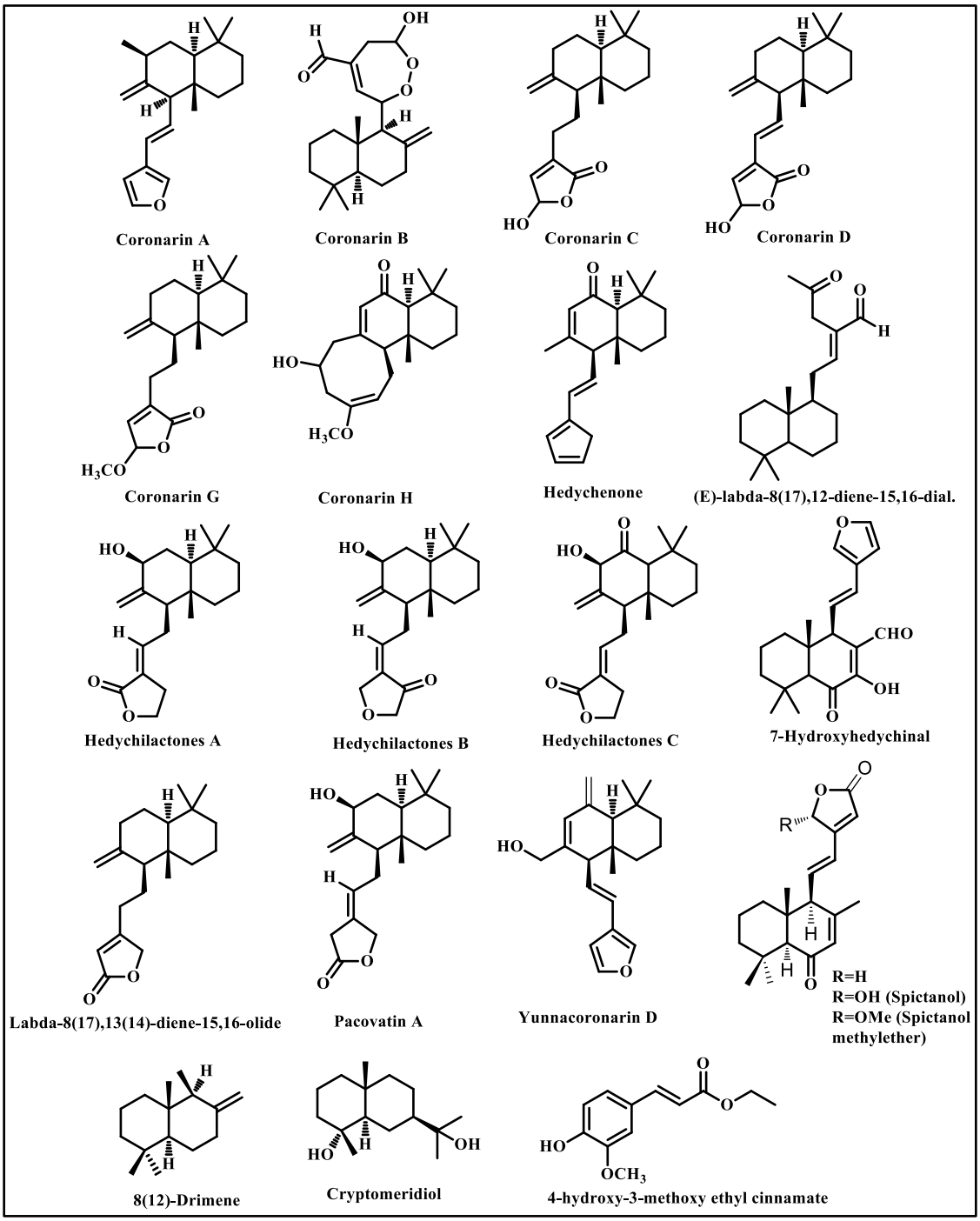


Figure 5C.2. Compounds previously reported from *Hedychium* genus

The labdane diterpenes isolated from the rhizomes of *H. coronarium* hedyforrestin C, coronarin H and 15-methoxylabda-8(17),13-dien-16,15-olide showed good anti-inflammatory activity against LPS-stimulated TNF-α, IL-6, and IL-12 p40 productions with IC_{50} ranging from 0.19 ± 0.11 to 10.38 ± 2.34 M.[17] Labdane diterpenes from *H. ellipticum* exhibited antimycobacterial activity against *Mycobacterium tuberculosis* and cytotoxicity against KB, MCF7, NCI-H187 and vero cells.[9] Joy *et al*., evaluated the antimicrobial activity of the essential oil from fresh and dried rhizomes of *H. coronarium* against four microorganisms, including two bacteria and two fungi. From the results, it was found that the activity was higher in the fresh sample than the dried. Both samples showed better activity against *Trichoderma sp*. and *Candida albicans* than against the bacteria *Bacillus subtilis* and *Pseudomonas aeruginosa*.[18] Sabulal *et al*., reported the antimicrobial activity of four *Hedychium* species *H. spicatum, H. venustum, H. coronarium* and *H. flavescens*. *H. flavescens* rhizome oil also showed strong activity against the fungi *Candida albicans* and *C. glabrata*.[19]

5C.4. Extraction, isolation and characterization of compounds from the rhizomes of *H. flavescens*

5C.4.1. Collection and extraction of plant material

The rhizomes of *H. flavescens* were collected from Tropical Botanical Garden and Research Institute Herbarium (JNTBGRI) - Palode, Thiruvananthapuram, Kerala, India. The taxonomist of JNTBGRI authenticated the plant material and a voucher specimen (voucher number TBGRI 83466) was deposited in the herbarium repository of the institute. Air-dried, powdered rhizome of *H. flavescens* (500 g) was extracted with hexane (3 L) for two days at room temperature and then filtered. The process was repeated thrice. The whole filtrate was then concentrated at 50˚C under reduced pressure to yield 10 g of the hexane extract. Next, the extraction process was repeated with acetone and ethanol to yield 12 g and 3.8 g of the crude extracts.

5C.4.2. Preliminary cytotoxic studies of the extracts

The extracts were evaluated for cytotoxicity using MTT assay. The extracts were examined for cytotoxicity against lung adenocarcinoma (A549). Screening of the extracts of *H. flavescens* revealed both hexane and acetone extracts showed potent

antiproliferative activity (< 5 µg/mL). Ethanol extract showed the least activity against A549 cells with an IC_{50} of 88.61 µg/mL at 48 h. The results are shown in **Table 5C.1** and the effect of various concentrations are represented in **Figure 5C.3**.

Table 5C.1. Cytotoxicity data for extracts in A549 over a time of 24 and 48 h.

Extracts	Cytotoxicity (IC_{50}) in µg/mL	
	A549	
	24 h	48 h
Hexane	<5	< 5
Acetone	< 5	< 5
Ethanol	>100	88.61 ± 0.045

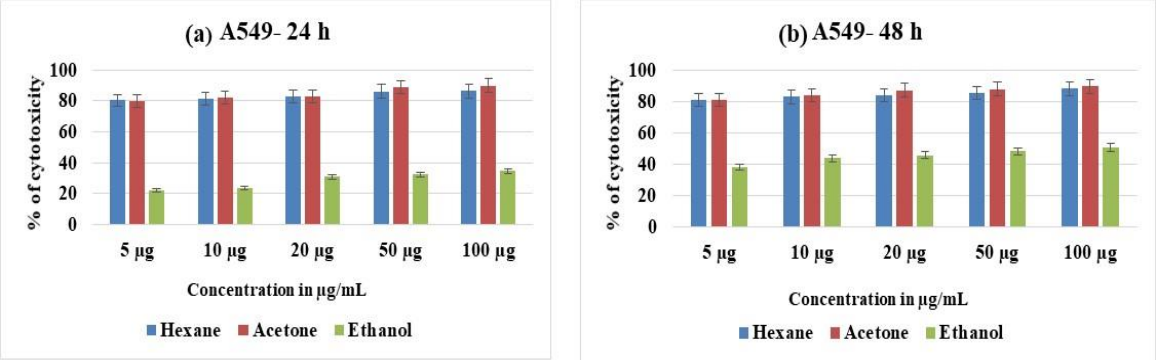


Figure 5C.3. MTT assay of extracts on A549 cells (a) at 24 h (b) cells at 48 h

5C.4.3. Isolation and characterisation of the compounds

After examining the TLC of the hexane and acetone extracts, both showed similar TLC profile. Hence we mixed both the extracts to do the fractionation. 20 g of the crude extract was then subjected to silica gel (100-200 mesh) column chromatography (CC) and eluted with *n*-hexane/ethyl acetate gradient with increasing the amount of ethyl acetate afforded 50 fractions. According to their TLC results, the fractions were further pooled into seven fraction pools (F1-F7) and each fraction pool was successively subjected to silica gel CC separation. The isolation procedure is represented in **Figure 5C.4**.

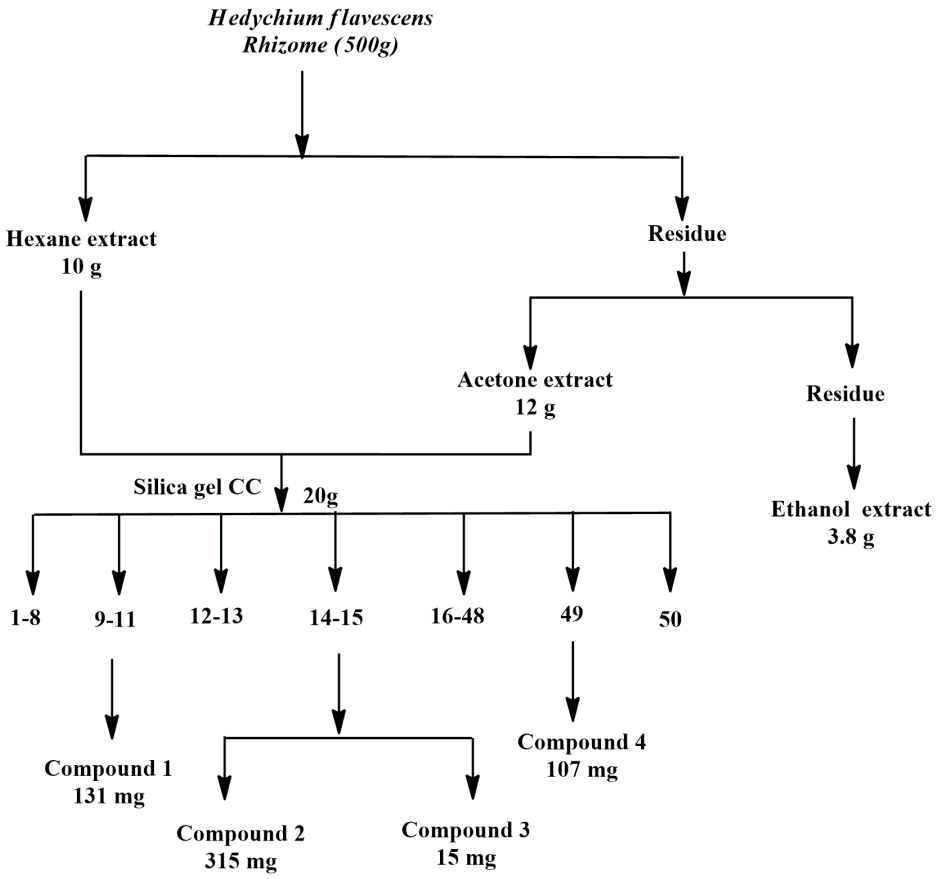


Figure 5C.4. Extraction process

Compound **53** was obtained as a colourless viscous oil (131 mg) from fraction pool 2 (Fr. 9-11). The IR spectra showed the presence of an *exo*-methylene group (3080, 1643 and 865 cm^{-1}). The ^{1}H NMR spectrum (**Fig. 5C.5**) showed that the compound has a labdane type skeleton with three tertiary methyl group resonated at δ 0.90, 0.85 & 0.84 ppm and *exo*-methylene group at δ 4.76 (1H, d, J = 1.5 Hz) and δ 4.53 ppm (1H, d, J = 2.0 Hz) and a β–substituted furan ring at δ 7.35 (2H, s) & 6.54 (1H, br s) and a *trans* olefinic double bond at δ 6.20 (1H, d, J = 16.0 Hz) & 5.97 (1H, dd, J = 15.5, 9.5 Hz), respectively. The ^{13}C NMR (**Fig. 5C.6**) and DEPT-135 revealed the presence of 20 carbon atoms. The methyl groups resonated at δ 33.6, 22.0 and 15.0 ppm, and the olefinic carbons at δ 150.3, 128.3, 121.7 & 108.0 ppm. The HRMS analysis also supported the data with a parent molecular ion peak at m/z 285.2209 $(M+H)^{+}$. Hence, from the spectral data and comparison with literature reports compound **53** was identified as **Coronarin E.**[20] Coronarin E was previously reported from the rhizomes of *Hedychium coronarium, Hedychium ellipticum,* [21] *Alpinia malaccensis* [22] and *Hedychium roxburghii* Blume [23] of the Zingiberaceae family.

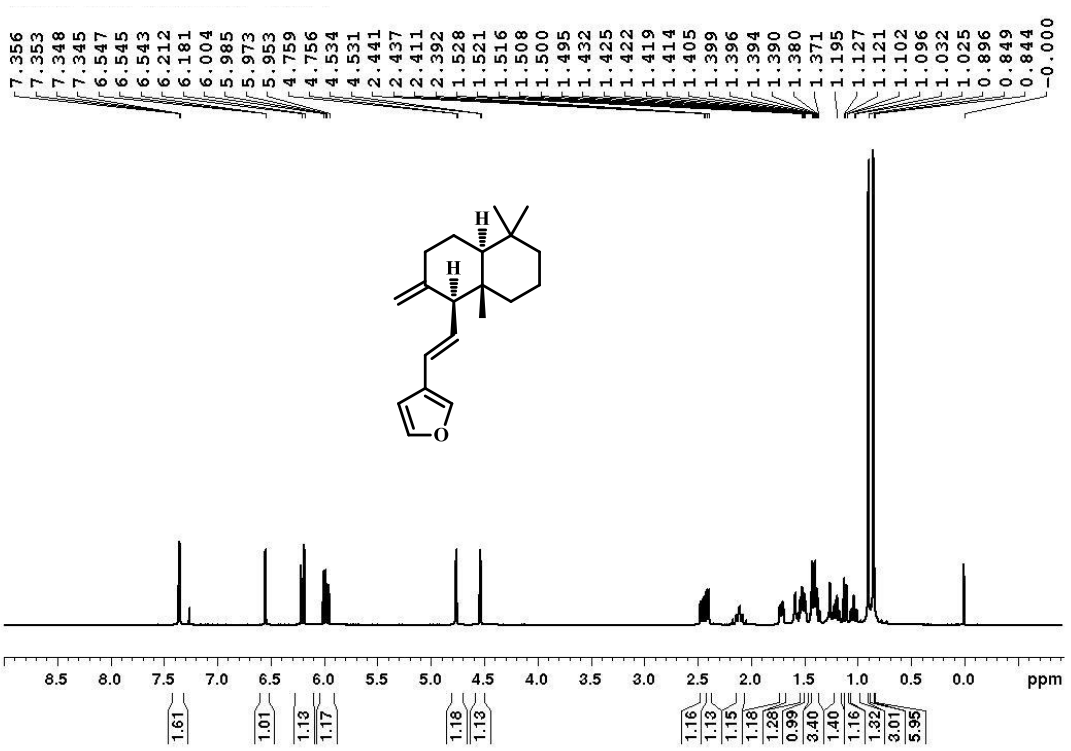


Figure 5C.5. ^{1}H NMR spectrum of compound **53** in $CDCl_3$

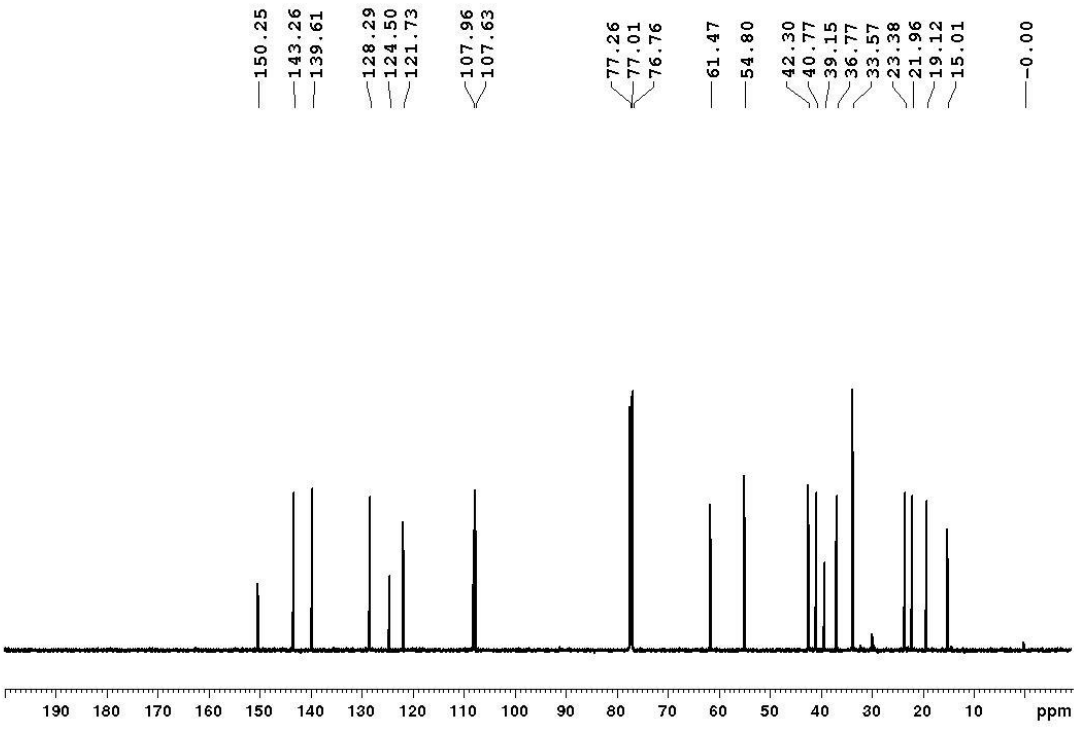
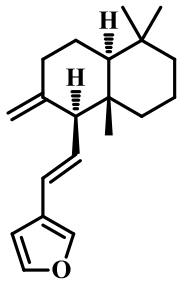


Figure 5C.6. ^{13}C NMR spectrum of compound **53** in $CDCl_3$



Compound **53- Coronarin E**

Fraction pool 4 (Fr. 14-15) on repeated CC separation yielded two molecules designated as compound **54** and compound **55**. The compound **54** (315 mg) was obtained as colourless needle-like crystal with melting point 103-105˚ C. The IR absorption bands showed the characteristic peak of a hydroxyl group (3400 cm^{-1}), an α,β-unsaturated γ-lactone ring (1720 cm^{-1}) and an *exo*-methylene group (3100, 1680, 980, 850 cm^{-1}). In addition, the ^{1}H NMR spectrum (**Fig. 5C.7**) of compound **54** showed characteristic peaks of labdane diterpenoids, *viz*. three methyl groups (δ 0.74, 0.82 & 0.89 ppm, 3H each) and *exo*- methylene hydrogens (δ 4.87, 4.82, 4.57 & 4.46 ppm). The ^{13}C NMR spectrum (**Fig. 5C.8**) displayed the signals for 20 carbons. Comparison of the spectral data obtained for **54** with literature records for labdane diterpenes indicated that the compound is similar to the previously reported isocoronarin D.[24] The HRESIMS analysis also supported the data with a parent peak *m/z* at 319.2271$(M+H)^{+}$. However, its ^{1}H and ^{13}C NMR spectra presented some resonances as pairs of signals, with nearly identical values of chemical shifts, suggesting that **54** was a mixture of compounds structurally related. This was especially evidenced by the signals attributed to H-14 (δ 5.06/5.03, d, J = 5.5 Hz), H-15 [(δ 4.27 ppm, dd, J = 5.5, 2.0 Hz); δ 4.45 ppm (dd, J = 6.0, 10.5 Hz), and δ 4.25 ppm (dd, J = 5.5, 2.5 Hz), δ 4.45 ppm (dd, J = 6.0, 10.5 Hz)] and H-17 [(δ 4.87 ppm, *d*, J = 1.0 Hz; δ 4.57, d, J = 1.0 Hz and δ 4.82, d, J= 1.5 Hz; δ 4.35, br d, J = 1.0 Hz)], that indicated a 1:1 epimeric mixture of **54** at C-14. Similarly, it was also possible to identify the duplicated signals in the ^{13}C NMR spectrum, especially for the carbons of the lactone ring (127.9/127.8 (C-13) & 66.6/66.2 (C-14)). The complete ^{1}H and ^{13}C assignments for the epimers were obtained from HMQC and HMBC experiments. Hence the compound was identified as **C14-epimers of Isocoronarin D**.[25]

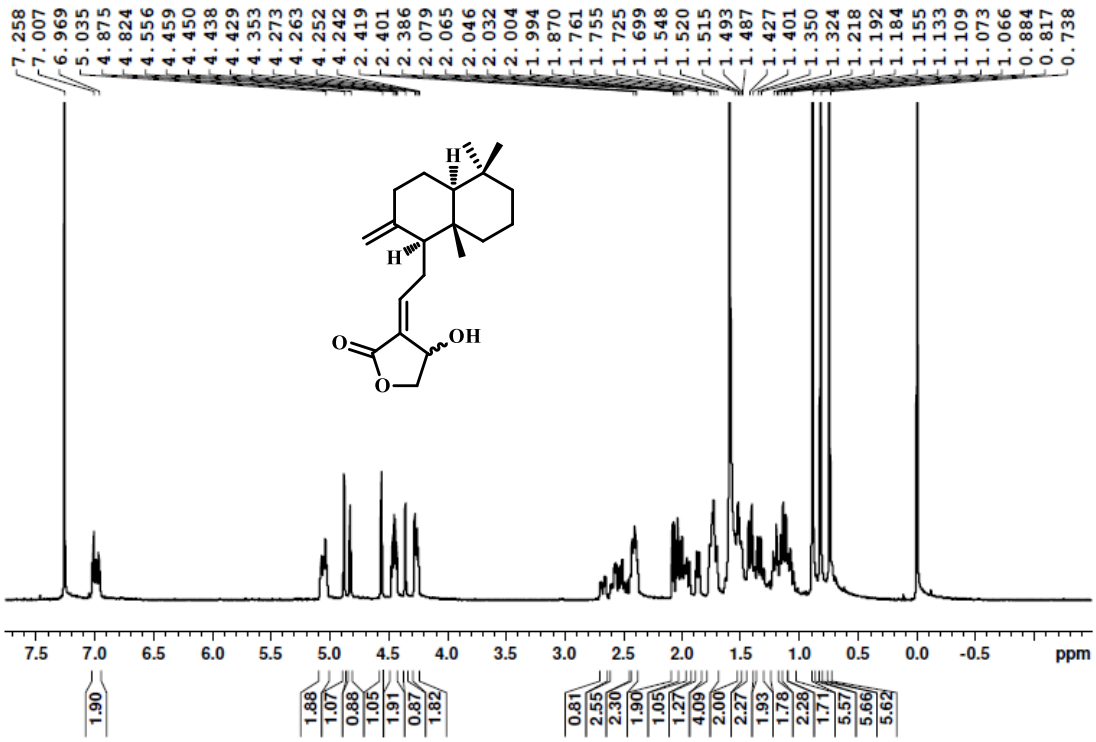


Figure 5C.7.^{1}H NMR spectrum of compound **54** in $CDCl_3$

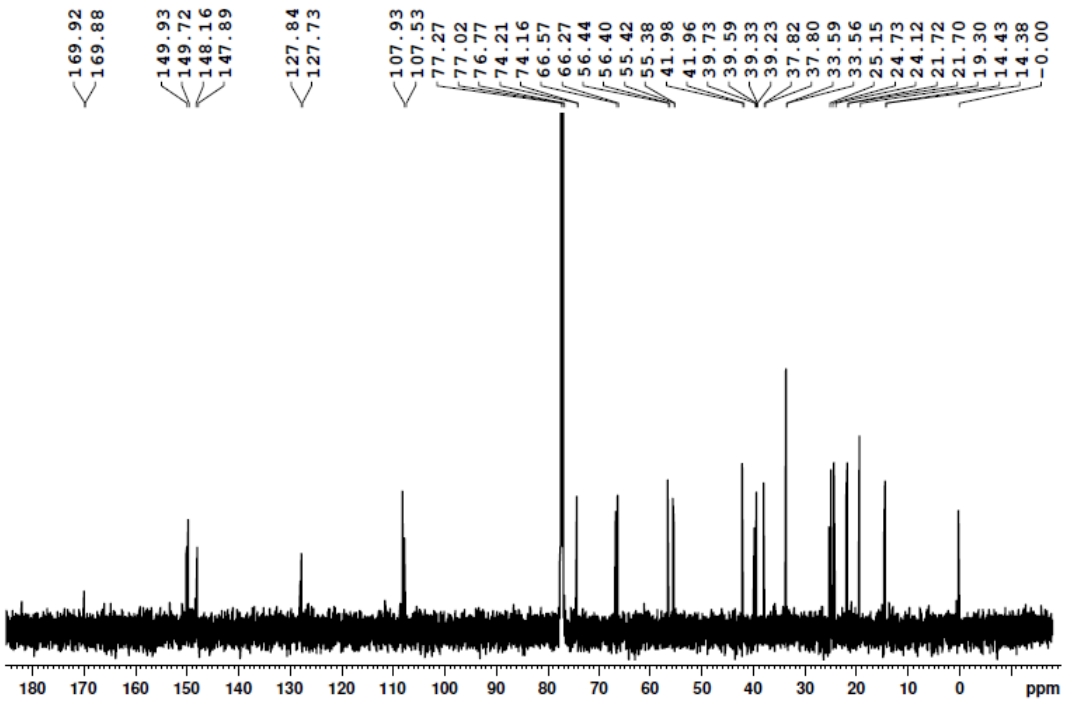
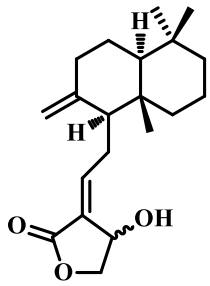
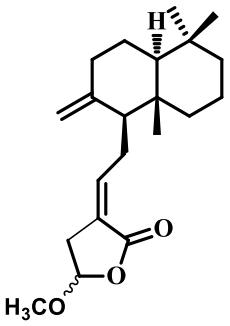


Figure 5C.8. ^{13}C NMR spectrum of compound **54** in $CDCl_3$



Compound **54 - C14-epimers of Isocoronarin D**

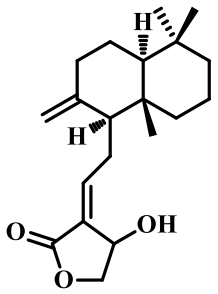
Compound **55** was obtained as a yellowish oily compound (15 mg) from the same fraction pool on silica gel CC. The IR absorption spectrum showed the presence of a hydroxyl group (3400 cm^{-1}), an α, β-unsaturated γ-lactone ring (1760 cm^{-1}) and an exomethylene group (3100, 1680, 940, 880 cm^{-1}). ^{1}H NMR spectrum (**Fig. 5C.9**) evidenced the labdane nature of the previous compounds, which revealed three signals of methyl groups (δ 0.72, 0.81 & 0.88 ppm, 3H each) and *exo*-methylene hydrogens (δ 4.40, 4.35, 4.81 & 4.83 ppm). Another characteristic peak was the presence of two methoxy groups displayed by the six proton singlet at δ 3.52 ppm. The ^{1}H and ^{13}C spectra of compound **55** consist of duplicated signals, similar to that of compound **54** suggested it could be an epimeric mixture with a ratio of 1:1. The use of 2D NMR techniques such as HMQC and HMBC spectroscopy allowed the assignment of peaks for the epimeric mixture. The mass spectrum of the compound showed a molecular ion peak at *m/z* 355.2249, which is the $(M+Na)^{+}$ peak. Hence from the spectral data and literature reports compound **55** was identified as **C-15 epimers of methoxycoronarin D.** [25]



Compound **55 - C-15 epimers of methoxycoronarin D**

Fraction pool 6 (Fr. 49) on repeated CC separation on silica gel, we could isolate a colourless crystalline solid (107 mg) as compound **56**, with a sharp melting point at 178-180° C. As in the previous compounds, the IR spectrum exhibited a hydroxyl group (3385 cm^{-1}), an *α*, *β*-unsaturated γ-lactone ring (1725 cm^{-1}), and an

exo-methylene group (3085, 1675, 880 cm^{-1}). The ^{1}H and ^{13}C spectra (**Fig. 5C.11 & 12**) have a similar pattern as that of compound **54**, with no duplicate signals**.** Based on the spectral data and comparing with literature reports, the compound was identified as **Isocoronarin D,** which was well supported by a molecular ion peak at *m/z* 319.2271, which is the $(M+H)^{+}$ peak.[6]



Compound **56 - Isocoronarin D**

It is worth mentioning that all the compounds isolated from *H. flavescens* were previously reported from *H. coronarium* [26], but this is the first report from this species. Singh *et al*., first reported Isocoronarin D and the relative stereochemistry of the chiral center at C-14 was assigned based on X-ray studies which indicated *β*-configuration for the hydroxyl group.[27] The *α*-isomer of isocoronarin D was reported from the plant species *Alpinia calcarata* (Zingiberaceae), named as calcaratarin D.[28] In the present study, after analyzing the spectral data we got a 1:1 mixture of isocoronarin D and calcaratarin D, so it's difficult to assign the relative stereochemistry by NMR data. Similarly compound **55** is also a 1:1 mixture of C-15 epimer of coronarin D methyl ether. Isocoronarin D and C-14 epimeric mixture of isocoronarin D serves as the chemomarkers of this species. Botanically, the species *Hedychium flavum* and *H. flavescens* have very similar morphological characteristics, which may leads to confusion in identification of the species.[29] Therefore, these chemotaxonomic findings have great significance in distinguishing the two *Hedychium* species.

5C.5. Biological studies

5C.5.1. *In vitro* anticancer activity

We evaluated all the isolated compounds against lung adenocarcinoma (A549) and normal lung fibroblast (WI-38) using MTT assay. Doxorubicin was used as the standard. The results of the cytotoxic studies are shown in **Table 5C.2**. The effect of various concentrations of compounds **53-56** on A549 and WI-38 cell lines at 24 and 48 h are shown in **Figure 5C.13**.

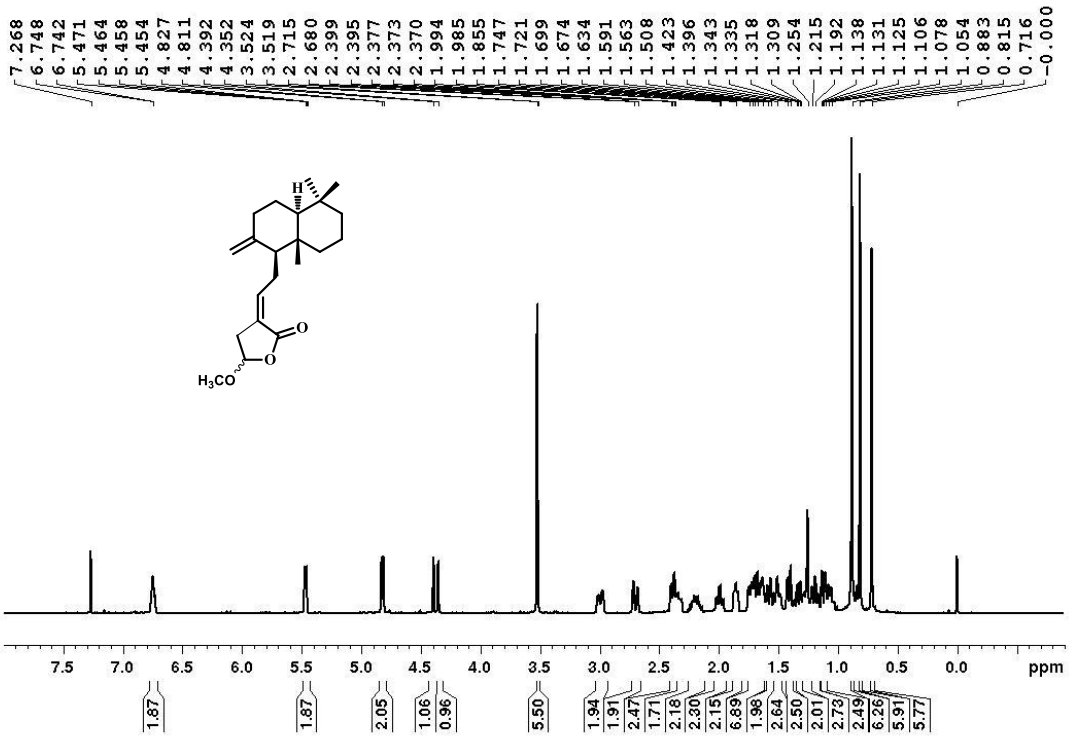


Figure 5C.9. ^{1}H NMR spectrum of compound **55** in $CDCl_3$

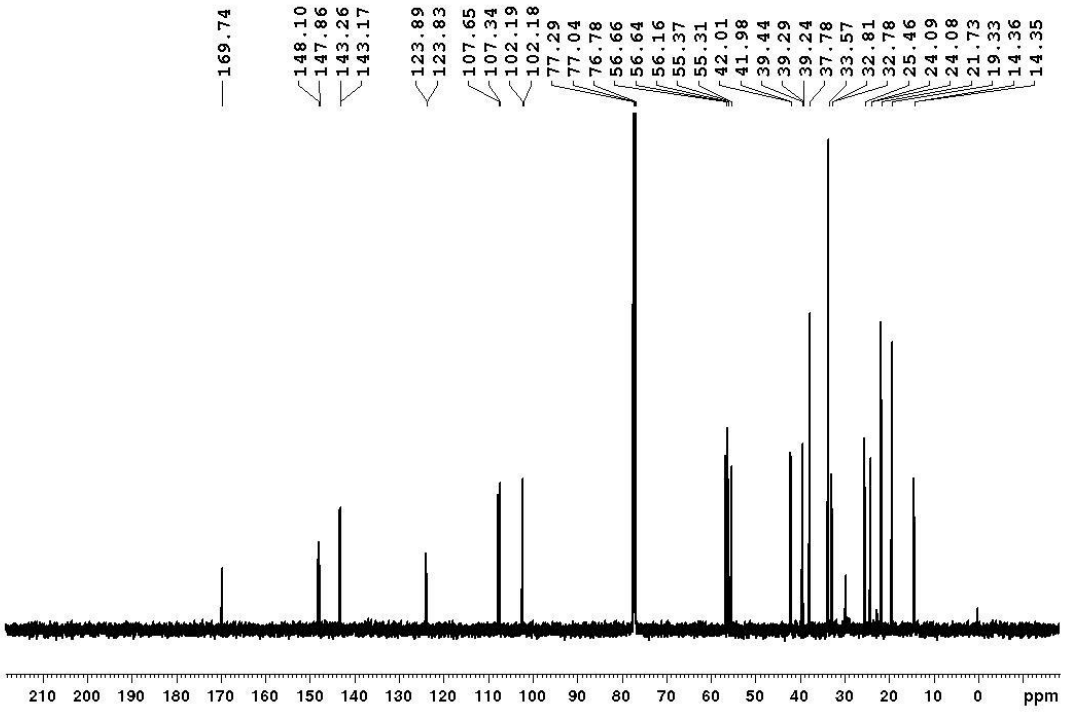


Figure 5C.10. ^{13}C NMR spectrum of compound **55** in $CDCl_3$

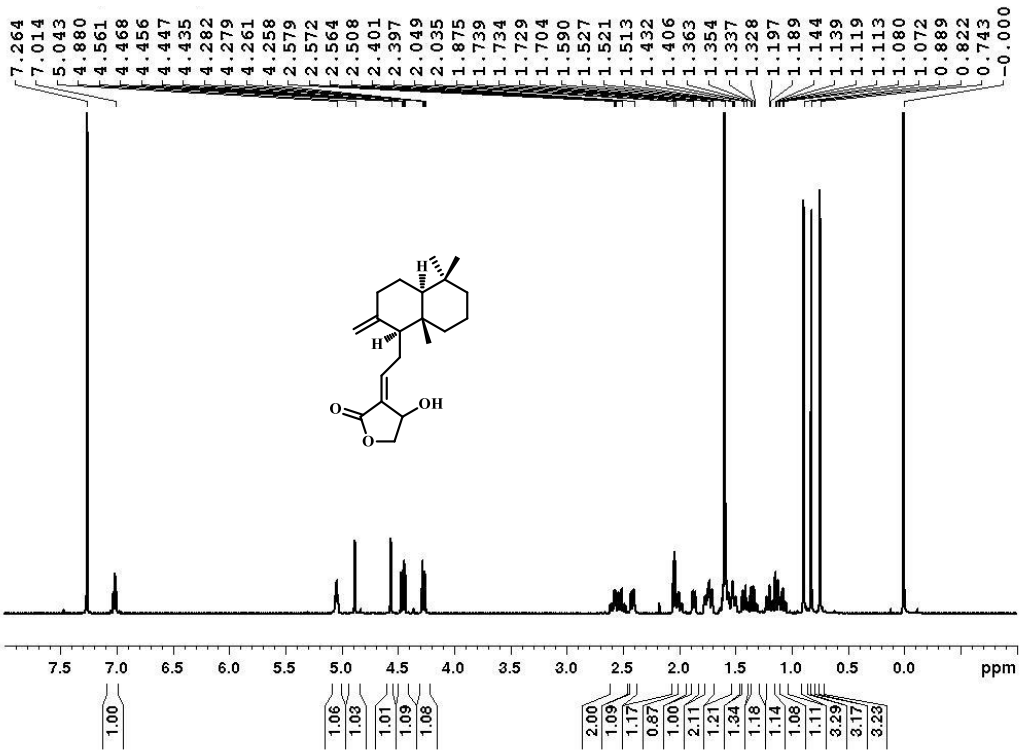


Figure 5C.11. ^{1}H NMR spectrum of compound **56** in $CDCl_3$

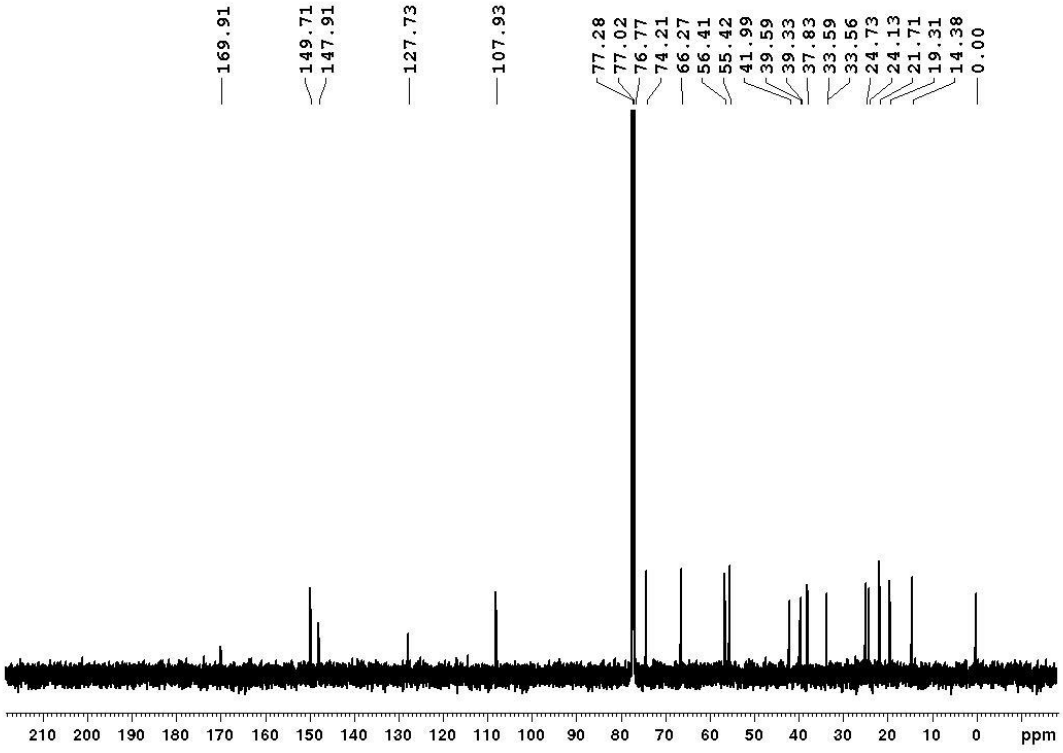


Figure 5C.12. ^{13}C NMR spectrum of compound **56** in $CDCl_3$

Table 5C.2. Cytotoxicity data for compounds **52-56** in A549 and WI-38 cells over a time of 24 and 48 h. Results are expressed as mean ± SD.

Compounds	Cytotoxicity (IC_{50}) in µM			
	A549		WI-38	
	24 h	48 h	24 h	48 h
53	0.59 ± 0.001[b]	0.52 ± 0.003[b]	>20[c]	>20[c]
54	17.78 ± 0.02[c]	0.59 ± 0.0015[b]	>20[c]	>20[c]
55	>20[c]	0.68 ± 0.0015[b]	>20[c]	>20[c]
56	1.23 ± 0.063[b]	1.22 ± 0.004[c]	>20[c]	>20[c]
Doxorubicin[a]	2.16 ± 0.01	0.92 ± 0.0025	18.2 ± 0.01	5.6 ± 0.005

[a]Standard drug; [b]Statistical significance: DOX vs Compounds $p < 0.001$; [c]Not tested; A549 - human lung adenocarcinoma; WI-38 - normal lung fibroblast.

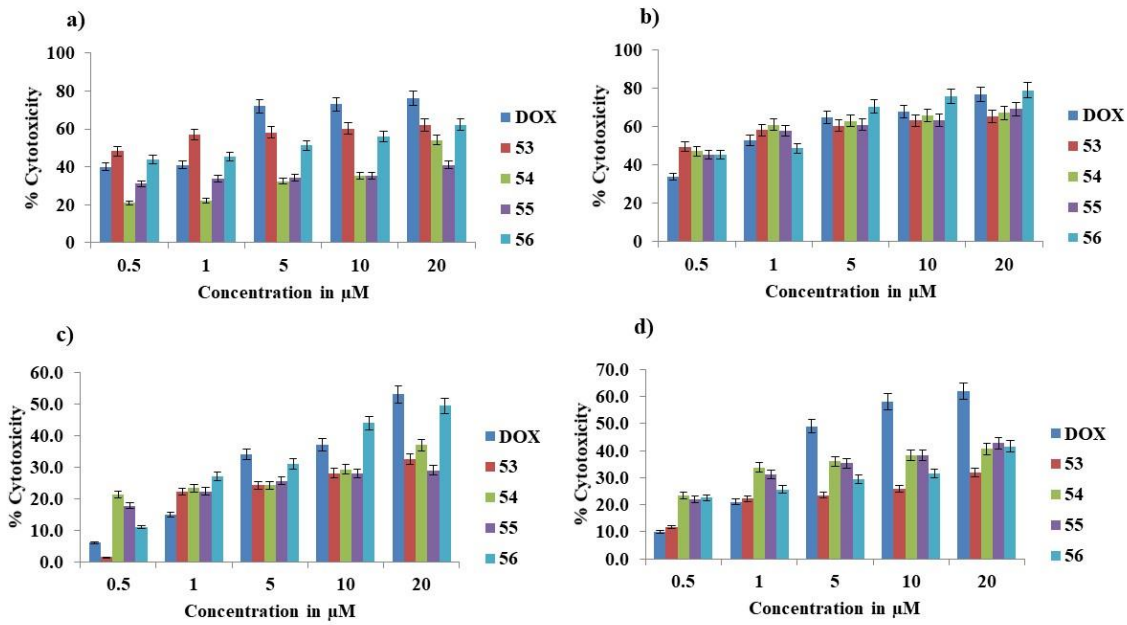


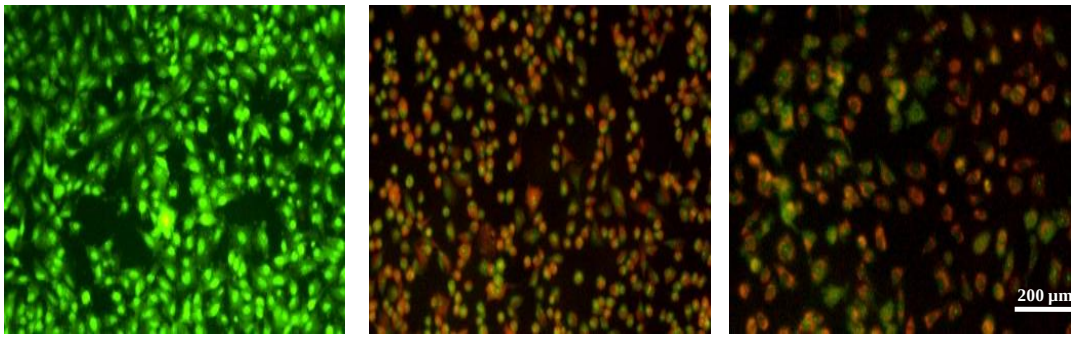
Figure 5C.13. MTT assay of compound **53-56** on (a) A549 cells at 24 h(b) on A549 cells at 48 h (c) WI-38 cells at 24 h (d) WI-38 cells at 48 h.

Results of MTT assay indicated that all the tested compounds have reasonably good cytotoxic effects towards A549 cells with IC_{50} values falling below 20 µg/mL. At the same time, these compounds showed least cytotoxicity towards WI-38, the normal lung fibroblast cells. With 24 h incubation, 20 µM of compound **53** showed 61.9 % inhibition on cell proliferation whereas DOX, compound **56**, **54**, and **55** showed 76, 62.1, 54.2 and 41 % inhibition. The IC_{50} values for 24 h treatment were 2.16, 0.59,

17.78, > 20, and 1.23 μM, respectively, for DOX and compound **53-56**. Upon 48 h of treatment the IC_{50} values of DOX, and compounds **53-56** showed 0.92, 0.52, 0.59, 0.68, and 1.22 μM respectively. In the case of WI-38 cells, 20 μM concentration of DOX showed 53 and 62 % cytotoxicity, respectively with 24 and 48 h of treatment, at the same time, the IC_{50} values of all the compounds were above 20 μM. Compound **53** was found to be more cytotoxic than DOX towards A549 cells but not to the normal lung fibroblast cells. The studies revealed the cell-specific cytotoxic effect of the compounds, where the standard cytotoxic drug doxorubicin inhibited the cell proliferation of both normal and cancer cells. From the results, compound **53** was exhibiting an appreciable apoptotic effect towards A549 cells even at a concentration of 0.52 μM at 48 h, which showed a negligible cytotoxic effect in WI-38 cells.

5C.5.2. Live dead assay (Acridine orange/ethidium bromide staining)

In acridine orange-ethidium bromide dual staining procedure (live dead assay), acridine orange is a nonfluorescent membrane-permeant dye which will be turned into fluorescent upon conversion by cellular esterase enzymes; thus, all the viable cells will be appeared green in colour with FITC filter, whereas, ethidium bromide being impermeant to cell membrane, enters only into dead cells with compromised cell membrane and gives red fluorescence upon binding with DNA. Fluorescent images of the live dead assay showed a reduced number of cells in compound **53** treated group, with most of the cells exhibiting yellow/red fluorescence when compared to the untreated cells. Thus, the result indicated the apoptosis-inducing ability of compound **53** even at a concentration of 0.52 μM (**Fig.5C.14**).



(a) (**b**) (**c**)

Figure 5C.3.14. Induction of apoptosis by live-dead assay (a) control (b) A549 cells treated with compound **53** at 0.59 μM (c) doxorubicin at 2.16 μM. Scale bar corresponds to 200 μm.

5C.6. Conclusion

Antiproliferative activity was confirmed in the various extracts of rhizomes of *Hedychium flavescens* (Zingiberaceae). The phytochemical investigation of the rhizomes of *H. flavescens* led to the isolation of four labdane diterpenes. Their structures were established as coronarin E, C-14 epimers of isocoronarin D, C-15 epimers of coronarin D methyl ether and isocoronarin D. All the isolated compounds were assessed for their cytotoxicity against the human lung adenocarcinoma (A549) cell line and showed significant cytotoxicity. Moreover, all the compounds were non-toxic towards the normal lung fibroblast (WI-38) cells. This is the first study to report the chemoprofiling of this plant species.

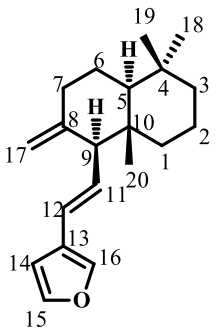
5C.7. Experimental section

Same as in Chapter 2A.

5C.8. Spectral data

Compound 53 (Coronarin E)

Compound **53** (131 mg) was obtained as colourless oily substance from fractions 9-11. The compound was successfully characterized as **Coronarin E** based on the spectral data are given below.



Molecular formula: $C_{20}H_{28}O$

FT-IR (Neat, υ_{max} cm^{-1}): 3080, 2922, 1643, 1156, 895 cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 7.35 (2H, s, H-15 & H-16), 6.54 (1H, br s, H-14), 6.20 (1H, d, J = 15.5 Hz, H-12), 5.98 (1H, dd, J = 15.5 Hz, 9.5 Hz, H-11), 4.76 (1H, d, J = 1.5 Hz, H-17b), 4.53 (1H, d, J = 1.5 Hz, H-17a), 2.45 (1H, m, H-7β), 2.40 (1H, br d, J = 9.5 Hz, H-9), 2.10 (1H, m, H-7α), 1.71 (1H, m, H-6β), 1.52 (1H, m, H-2α), 1.49 (1H, m, H-1β), 1.37-1.43 (3H, m, H-2α, H-6α, H-3β), 1.19 (1H, dt, J = 13.5, 4.0 Hz, H-3α), 1.11 (1H, dd, J = 12.5, 3.0 Hz, H-5), 1.03 (1H, dt, J = 14.0, 4.0 Hz, H-1α), 0.90 (3H, s, H-18), 0.85 (3H, s, H-19), 0.84 (3H, s, H-20) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 150.3 (C-8), 143.3 (C-15), 139.6 (C-16), 128.3 (C-11), 124.5 (C-13), 121.7 (C-12), 108.0

(C-17), 107.6 (C-14), 61.5 (C-9), 54.8 (C-5), 42.3 (C-3), 40.8 (C-1), 39.2 (C-10), 36.8 (C-7), 33.6 (C-4/18), 23.4 (C-6), 22.0 (C-19), 19.1(C-2), 15.0 (C-20) ppm.

HRMS (ESI): *m/z* cacld for $C_{20}H_{28}O$ is 284.2140. Found 285.2209 $(M+H)^+$.

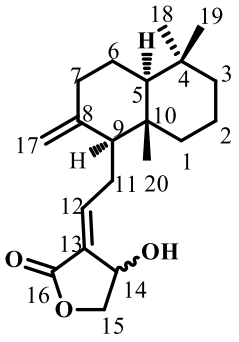
Compound 54 (C14-epimers of Isocoronarin D)

Compound **54** was obtained as colourless crystalline solid (315 mg) on silica gel CC separation of fraction pool 4. The compound was successfully characterized as **C14-epimers of Isocoronarin D**. NMR spectral assignments were made on the basis of 2D NMR and on comparison with literature reports.

Molecular formula: $C_{20}H_{30}O_3$

Mp: 103-105˚C

FT-IR (Neat, υ_{max} cm^{-1}): 3400, 3100, 1720, 1680, 980, 850 cm^{-1}



^{1}H NMR (500 MHz, TMS,$CDCl_3$): δ 7.00/6.95 (1H, m, H-12), 5.06/5.03 (1H, d, *J* = 5.5 Hz, H-14), 4.87 (1H, d, *J* = 1.0 Hz, H-17), 4.82 (1H, d, *J* = 1.0 Hz, H-17), 4.57 (1H, d, *J* = 1.0 Hz, H-17), 4.46 (1H, dd, *J* = 10.5, 6.0 Hz, H-15), 4.45 (1H, dd, *J* = 10.5, 6.0 Hz, H-15), 4.35 (1H, d, *J* =1.0 Hz, H-17), 4.27 (1H, dd, *J* = 5.5, 2.0 Hz, H-15), 4.25 (1H, dd, *J* = 5.5, 2.0 Hz, H-15), 2.66-2.70 (2H, m, H-11), 2.52-2.58 (2H, m, H-11), 2.37-2.43 (2H, m, H-7β), 2.01 (2H, m, H-7α), 1.95 (1H, br d, *J* = 11.5 Hz, H-9), 1.86 (1H, br d, *J* = 9.5 Hz, H-9), 1.73-1.75 (4H, m, H-6α, H-1β), 1.60/1.57 (1H, d, *J* = 2.5 Hz, H-2β), 1.52 (2H, m, H-2α) , 1.42 (2H, br d, *J* = 13.0 Hz, H-3β), 1.32-1.36 (2H, m, H-6β), 1.20 (2H, m, H-3α), 1.14 (2H, m, H-5), 1.07 (2H, m, H-1α), 0.89 (6H, s, H-18), 0.82 (6H, s, H-19), 0.74 (6H, s, H-20) ppm.

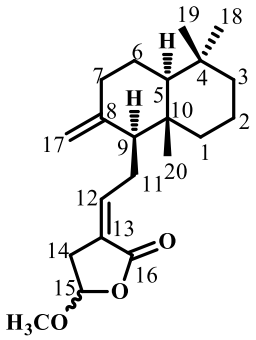
^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 170.1 (C-16), 149.9/149.7 (C-12), 148.2/147.9 (C-8), 127.9/127.8 (C-13), 108.0/107.5 (C-17), 74.3 (C-15), 66.6/66.2 (C-14), 56.5/56.4 (C-9), 55.5/55.4 (C-5), 42.0 (C-3), 39.8/39.6 (C-

10), 39.4/39.2 (C-1), 37.9 (C-7), 33.6 (C-4/C-18), 25.2/24.8 (C-11), 24.2 (C-6), 21.7 (C-19), 19.3 (C-2), 14.4 (C-20) ppm.

HRMS (ESI): *m/z* calcld for $C_{20}H_{30}O_3$ is 318.2194. Found 319.2271$(M+H)^+$.

Compound 55 (C15- Epimers of methoxycoronarin D)

Compound **55** (15 mg) was obtained along with compound two as a yellowish oily compound on silica gel CC separation of fraction pool 4. Compound **55** was characterized as **C15- Epimers of methoxycoronarin D** based on the spectral data and comparing the spectral data with literature reports. NMR spectral assignments were made on the basis of 2D NMR and literature reports.



Molecular formula: $C_{21}H_{32}O_3$

FT-IR (Neat, υ_{max} cm^{-1}): 3400, 1760, 1680, 940, 880 cm^{-1}.

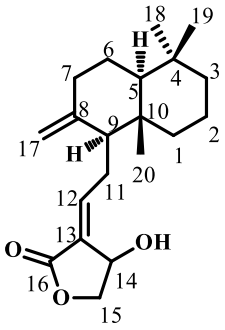
^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 6.74 (2H, m, H-12), 5.46 (2H, m, H-15), 4.83/4.81 (1H, s, H-17), 4.40/4.35 (1H, s, H-17), 3.52 (6H, s, H-21), 3.01/2.98 (1H, m, H-14), 2.72/2.68 (1H, m, H-14), 2.39/2.37 (1H, m, H-7β), 2.34/2.32 (1H, m, H-11), 2.19 (2H, m, H-11), 1.86 (2H, br t, J = 7.5 Hz, H-9), 1.99 (2H, m, H-7α), 1.73 (2H, br d, J = 13.0 Hz, H-6α), 1.69 (2H, br d, J = 12.5 Hz, H-1β), 1.58 (2H, m, H-2β), 1.49 (2H, m, H-2α), 1.41 (2H, br d, J = 13.5 Hz, H-3β), 1.33 (2H, dd, J = 12.5, 4.0 Hz, H-6β), 1.19 (2H, m, H-3α), 1.13/1.11 (1H, m, H-5), 1.06 (2H, m, H-1α), 0.88 (6H, s, H-18), 0.82 (6H, s, H-19), 0.72 (6H, s, H-20) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 169.7 (C-16), 148.1/147.9 (C-8), 143.3/143.2 (C-12), 123.9/123.8 (C-13), 107.6/107.3 (C-17), 102.2 (C-15), 56.6/56.7 (C-21), 56.2 (C-9), 55.4/55.3 (C-5), 42.0 (C-3), 39.4 (C-10), 39.3/39.2 (C-1), 37.8 (C-7), 33.6 (C-4/C-18), 32.8 (C-14), 25.5 (C-11), 24.1 (C-6), 21.7 (C-19), 19.3 (C-2), 14.4 (C-20) ppm.

HRMS (ESI): *m/z* calcld for $C_{21}H_{32}O_3$ is 332.2351. Found 355.2249 $(M+Na)^+$.

Compound 56 (Isocoronarin D)

Fraction 49 on silica gel CC yielded 107 mg of compound **56** as colourless crystalline solid. Compound **56** was identified as **Isocoronarin D** based on the spectral data and literature reports.



Molecular formula: $C_{20}H_{30}O_3$

Mp: 178-180˚C

FT-IR (Neat, υ_{max} cm^{-1}): 3385, 3085, 1725, 1675, 880 cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$, TMS):): δ 7.01 (1H, m, H-12), 5.04 (1H, t, *J* = 6.5 Hz, H-14), 4.88 (1H, s, H-17), 4.56 (1H, s, H-17), 4.45 (1H, dd, *J* = 10.5, 6.0 Hz, H-15b), 4.27 (1H, dd, *J* = 10.5, 1.5 Hz, H-15a), 2.47-2.61 (2H, m, H-11), 2.41 (1H, m, H-7β), 2.00 (1H, ddd, *J* = 13.5, 13, 5.5 Hz, H-7α), 1.86 (1H, br d, *J* = 9.5 Hz, H-9), 1.75 (1H, m, H-6α), 1.72 (1H, br d, *J* = 12.5 Hz, H-1β), 1.58 (1H, m, H-2β), 1.51 (1H, m, H-2α), 1.42 (1H, br d, *J* = 13.0 Hz, H-3β), 1.34 (1H,ddd, *J* = 13.0, 13.0, 4.5 Hz, H-6β), 1.19 (1H, ddd, *J* = 13.0, 13.0, 4.0 Hz, H-3α), 1.13 (1H, dd, *J* = 12.5, 2.5 Hz, H-5), 1.07 (1H, ddd, *J* = 12.5, 12.5, 4.0 Hz, H-1α), 0.89 (3H, s, H-18), 0.82 (3H, s, H-19), 0.74 (3H, s, H-20) ppm.

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 169.9 (C-16), 149.7 (C-12), 147.9 (C-8), 127.7 (C-13), 107.9 (C-17), 74.2 (C-15), 66.3 (C-14), 55.4 (C-5), 56.4 (C-9), 42.0 (C-3), 39.6 (C-10), 39.3 (C-1), 37.8 (C-7), 33.6 (C-4), 33.6 (C-18), 24.7 (C-11), 24.1 (C-6), 21.7 (C-19), 19.3 (C-2), 14.4 (C-20) ppm.

HRMS (ESI): *m/z* calcld for $C_{20}H_{30}O_3$ 318.2194. Found 319.2271$(M+H)^+$.

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ABSTRACT

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Title of the thesis: **Chemoprofiling and Biological Screening of Selected Medicinal Plants**

Natural products have played a key role in pharma research, as many medicines are either natural products or derivatives thereof. Natural products research continues to explore a variety of lead structures, which may be used as templates for the development of new drugs by the pharmaceutical industry. Considering the renewed interest in medicinal plants and traditional systems of medicine, we have focused our efforts on the phytochemical investigation and bioactivity studies of selected medicinal plants. **Chapter 1** gives a brief introduction to the role of natural products in modern drug discovery process. **Chapter 2** discusses the detailed phytochemical investigation of two cedar species, *Calocedrus decurrens* (Cupressaceae family) and *Cedrus deodara* (Pinaceae family). Anticancer studies of the extracts and isolated molecules were also presented in this Chapter. **Chapter 3** deals with the phytochemical investigation and biological study of two traditionally important species in *Bridelia* genus, *Bridelia retusa* (Chapter 3A) and *Bridelia stipularis* (Chapter 3B). Antibacterial and anticancer studies of the extracts and isolated molecules were also depicted in this chapters. **Chapter 4** is divided into two parts. Part A discuss the detailed phytochemical investigation of medicinally important species *Butea monosprema*. Part B explains the antiproliferative study of Amalaki Rasayana, a prominent rejuvenating Rasayana described in Indian traditional Ayurveda medicine. Since the Rasayana preparation makes use of the wood of *Butea monosperma*, the role of the same in the enhanced therapeutic property of Amla is validated here. **Chapter 5** divided into three parts, describes the isolation and pharmacology of three species from Zingiberaceae family. Part A deals with the isolation and characterisation of bioactives from *Curcuma aeruginosa*. Part B discuss the isolation of marker compounds from *Curcuma raktakanta* and synthetic modification of germacrone isolated from this species. Part C discuss the Phytochemical investigation and anticancer study *of Hedychium flavescens.*

Details of Publications, Emanating from the Thesis Work

Published

1. **Santhi Subramanyan**, Selvakumar Deepika, Anjitha Ajith, Anuja Joseph Gracy, Mathew Dan, Kaustabh Kumar Maiti, Ramavarma Luxmi Varma, Kokkuvayil Vasu Radhakrishnan, "Antiproliferative Labdane Diterpenes from the Rhizomes of *Hedychium flavescens* Carey ex Roscoe", **Chemical Biology and Drug Design**, 2021, 98:4,501-506, Doi: 10.1111/cbdd.13906.
2. **Santhi Subramanyan**, Varsha Karunakaran, S. Deepika, V. Sheeba, Kaustabh K. Maiti, R. Luxmi Varma, K.V. Radhakrishnan, Libocedroquinone: A promising anticancer lead against lung cancer from *Calocedrus decurrens* (Torr) Florin (**Accepted in Planta Medica International Open**)

Manuscript under preparation

1. **Santhi Subramanyan**, Selvakumar Deepika, Vishnu K. Omanakuttan, Kaustabh Kumar Maiti, Ramavarma Luxmi Varma, Kokkuvayil Vasu Radhakrishnan, Rajmohan Velayudhan Pillai, Antiproliferative potential of Amalaki Rasayana and effect of *Butea monosperma* (Lam.) Taub on the cytotoxicity (**Communicated to Journal of Drug Research in Ayurvedic Sciences**)
2. **Santhi. S**, R. Luxmi Varma, K.V. Radhakrishnan, *Butea monosperma*: A deep insight into the phytochemistry, pharmacology and Ayurvedic knowledge (**To be communicated to Phytotherapy Research**)

Posters Presented and Attended in Conferences

1. Anti-oxidant activity and phytochemical investigation from heart wood of *Calocedrus decurrans.* **S. Santhi**, P. Nisha, V. Sheeba and R. Luxmi Varma. Hisham Endowment Award Ceremony 2016 at Malabar Botanical Gardens, Calicut. [**Poster Presentation**]

2. Isolation and characterization of bioactives from heartwood of *Calocedrus decurrans*. **S. Santhi**, P. Nisha, V. Sheeba and R. Luxmi Varma. International conference on Tropical Plants and Molecular design at TKM, Kollam, 2017. [**Poster Presentation**]

3. Isolation, characterization and cytotoxic analysis of bioactives from the heartwood of *Calocedrus decurrans*. **S. Santhi**, T.R. Reshmitha, P. Nisha, V.Sheeba and R. Luxmi Varma. 2nd "International Conference on Nutraceuticals and Chronic Diseases" September 1-3,2017, Goa, India. [**Poster Presentation**]

4. Phytochemical investigation of the chemical constituents of *Butea monosperma* (Stem). **S. Santhi**, V. Rajmohan, K.V. Radhakrishnan, R. Luxmi Varma.International Seminar on Phytochemistry-2018, organized by Kerala Academy of Sciences (KAS) in association with Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), on 26th& 27th March 2018, at JNTBGRI, Palode, Thiruvananthapuram, Kerala. [**Poster Presentation**]

5. Antiproliferative labdane diterpenes from the rhizomes of *Hedychium flavescens* Carey ex. Roscoe. **S. Santhi**, S. Deepika, I. G. Shibi , M. Dan, Kaustabh K. Maiti, R. Luxmi Varma, K.V. Radhakrishnan. International Conference on Integrative Oncology, February 8 - 9, 2020, at Le Meridien, Kochi, Kerala. [**Oral**]

6. Phytochemical investigation of the chemical constituents of the rhizomes of *Curcuma aeruginosa* Roxb. **S. Santhi**, R. Luxmi Varma, K.V. Radhakrishnan**.** Progress and promises in Chemical Sciences (PPCS-2020) 1st International webinar series organized by Department of Chemistry, February, 2020, CHRIST (Deemed to be University), Bengaluru, India. [**Poster Presentation**]
7. Antiproliferative potential of Amalaki Rasayana on cervical carcinoma and influence of *Butea monosperma* (lam.) taub on the cytotoxicity. **Santhi S.**, Rajmohan V., R. Luxmi Varma, K.V. Radhakrishnan. International Ayurveda Seminar, March 12-19, 2021, Thiruvananthapuram 2021. **[Oral]**
8. Antiproliferative Labdane Diterpenes from the Rhizomes of Hedychium flavescens Carey ex. Roscoe. S. Santhi, S. Deepika, Anuja Joseph G., M. Dan, Kaustabh K. Maiti, R. Luxmi Varma, K.V. Radhakrishnan. International Webinar on 'Phytochemistry-Impacts and Applications', being organized by Kerala Academy of Sciences, Thiruvananthapuram, on September 27th and 28th, 2021. **[Oral]**

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RESEARCH ARTICLE

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Antiproliferative labdane diterpenes from the rhizomes of *Hedychium flavescens* Carey ex Roscoe

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Abstract

Antiproliferative activity was confirmed in the various extracts of rhizomes of *Hedychium flavescens* (Zingiberaceae). The phytochemical investigation of the rhizomes of *Hedychium flavescens* led to the isolation of four labdane diterpenes. Their structures were established as coronarin E (**1**), C-14 epimers of isocoronarin D (**2**), C-15 epimers of coronarin D methyl ether (**3**) and isocoronarin D (**4**). The structure of the compounds was identified based on spectroscopic analysis and on comparison with literature reports. All these compounds were assessed for their *in vitro* cytotoxicity against human lung adenocarcinoma (A549) cell line and showed significant cytotoxicity as reflected in IC_{50} value, *that is*, 0.52, 0.59, 0.68 and 1.22 μM compared with the control doxorubicin (IC_{50} 0.92 μM). Moreover, all the compounds were nontoxic towards the normal lung fibroblast (WI-38) cells. The chemo-profiling and cytotoxicity study of *Hedychium flavescens* is reported for the first time.

KEYWORDS

antiproliferative activity, coronarin E, *Hedychium flavescens*, labdane diterpenes, Zingiberaceae

1 | INTRODUCTION

The genus *Hedychium* J. Köenig. popularly known as 'ginger lilly' is the largest genus of the Zingiberaceae family in India. Worldwide, the *Hedychium* genus consists of approximately 80 species (Ray et al., 2017). Several species of *Hedychium* were used in traditional medicines for the treatment of inflammation, headache, rheumatic pain, asthma, blood purification, bronchitis, gastric diseases and anti-emetics (Chan & Wong, 2015; Ray et al., 2018). *Hedychium* plants mainly contain labdane-type diterpenes, sesquiterpenes and diarylheptanoids (Itokawa et al., 1988a, 1988b; Nakatani et al., 1994; Prabhakar Reddy et al., 2009; Suresh et al., 2010) with a broad spectrum of biological activities. It is well documented that the essential oils of the plants in this genus have many medicinal efficacies, including anti-inflammatory (Kiem et al., 2011), antimalarial (Martinez-Correa et al., 2017), antimicrobial (Joy et al., 2007), antihyperglycaemic (Prabhakar Reddy et al., 2009), antitumor (Oh et al., 2006), antifungal (Sakhanokho et al., 2013), chemopreventive (Endringer et al., 2014) and pediculicidal activity (Jadhav et al., 2007).

Hedychium flavescens Carey ex Roscoe, commonly known as yellow ginger, cream ginger lily or cream garland lily, is native to the eastern Himalayas, including northeast India and Nepal (Sarangthem et al., 2013). The species has a confront distribution in India; recently, the occurrence of *Hedychium flavescens* has been recorded in the semi-evergreen forests of Niyamgiri hills in Odisha (Misra & Sahoo, 2015). The phytochemical and pharmacological study of *H. flavescens* are insufficiently explored. So far, the antibacterial (Suksathan et al., 2013) and antimicrobial (Sabulal et al., 2007) activity of the essential oil of this species has been reported. The essential oil contains linalool (35%), *β*-pinene

(27%) and 1,8-cineole (13%) as the principal compounds (Sakhanokho et al., 2013). Therefore, in the present study, we have attempted to isolate the marker compounds from the hexane and acetone extract of the rhizomes of *H. flavescens*, and their cytotoxic studies. To the best of our knowledge, the chemo-profiling of *H. flavescens* is reported for the first time.

2 | MATERIALS AND METHODS

2.1 | General experimental procedure

The NMR experiments were recorded on Bruker Avance AMX 500 spectrometer; NMR solvent was $CDCl_3$ with 0.03% TMS as the internal standard. Mass spectra were recorded under ESI/HRMS at 60,000 resolution using Thermo Scientific Exactive mass spectrometer. Silica gel (230–400 mesh and 100–200 mesh) used for gravity column chromatography (CC). Analytical thin-layer chromatography (TLC) was carried out with Merck TLC silica gel 60 F_{254} coated on aluminium sheets. All the solvents used were purchased from Sigma-Aldrich and Merck.

2.2 | Plant material

The rhizomes of *H. flavescens* were collected from Jawaharlal Nehru Tropical Botanical Garden and Research Institute (JNTBGRI) Herbarium—Palode, Thiruvananthapuram, Kerala, India. The taxonomist of JNTBGRI authenticated the plant material, and a voucher specimen (voucher number 83466, JNTBGRI) was deposited in the herbarium repository of the institute.

2.3 | Extraction and isolation

Air-dried, powdered rhizome of *H. flavescens* (500 g) was extracted with hexane (3 L) for 2 days at room temperature and then filtered. The process was repeated thrice. The whole filtrate was then concentrated at 50°C under reduced pressure to yield 10 g of the hexane extract. The extraction process was repeated with acetone (12 g), followed by ethanol (3.8 g). The extracts were evaluated for cytotoxicity using the MTT assay. After examining the TLC of the hexane and acetone extracts, both showed a similar TLC profile. Hence, we mixed both the extracts to do the fractionation. 20 g of the crude extract was then subjected to silica gel (100–200 mesh) CC and eluted with *n*-hexane/ethyl acetate gradient with increasing the amount of ethyl acetate afforded 50 fractions. According to their TLC results, the fractions were further pooled into seven fraction pools (F1–F7) and each fraction pool was successively subjected to silica gel CC separation. Fraction pool F2 was eluted with 5% ethyl acetate-hexane to afford a pale yellow coloured oily substance counted as compound **1** (131 mg). Fraction pool F4 was subjected to repeated column chromatographic separation with 5% ethyl acetate-hexane yielded two compounds designated as compound **2** (315 mg) and compound **3** (15 mg). Compound **2** was obtained as colourless amorphous solid and compound **3** as a pale yellow coloured oily substance. Compound **4** was isolated from fraction pool F6 as colourless crystalline solid (124 mg), by precipitating with DCM. The structure of all the compounds was assigned by analysing these structures ^{1}H, ^{13}C, 2D NMR and HRMS data and also by comparison with reported values.

2.4 | Cell lines and cultural conditions

Human lung adenocarcinoma cell (A549) and human lung fibroblast cell (WI-38) were procured from National Centre for Cell Science, Pune, India. Short tandem repeat profiling was carried out to confirm the genetic identity of cell lines and the cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in an incubator at 37°C in a humidified atmosphere of 5% CO_2.

2.5 | Cell proliferation assay

Cell growth inhibitory effect of the extracts and isolated compounds from *H. flavescens* extract was analysed using 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) assay, as previously reported (Joseph et al., 2013). In this assay, the absorbance of formazan crystals formed by the enzymatic conversion of tetrazolium salt by mitochondrial dehydrogenase is measured at 570 nm using a microplate spectrophotometer (BioTek synergy/H1 microplate reader). The rate of inhibitory effect on cell proliferation was calculated using the formula: Proliferation rate (PR) % = [Abs sample/Abs control] × 100; Inhibitory rate (IR) % = 100 − PR. For this, A549 and WI-38 cells were seeded at a density of 8,000 cells/well in 96 well plate and incubated for 24 hr. Various concentrations of the test materials ranging from 0.5 to 20 μM were added to the cells for 24 and 48 hr. After the completion of the incubation period, 0.5 mg/ml MTT in HBSS was added to the cells and again kept for 2.5 hr and the so formed crystals were dissolved in DMSO and the absorbance was read at 570 nm. Concentrations giving 50% inhibition on cell growth (IC_{50}) were also calculated.

2.6 | Live–dead assay to determine apoptotic cells

From MTT assay, the most potent compound with the least IC_{50} value at 24 hr was selected for further screening. Dual staining with acridine orange and ethidium bromide is one of

the most commonly employed methods to detect apoptotic cells, where the differential uptake of two fluorescent dyes by viable and non-viable cells is monitored (Joseph et al., 2014). To perform the assay, A549 cells were seeded at a density of 5,000 cells/well and treated with or without compound **1**. Dual staining reagent was prepared by adding 1 µl each of acridine orange (from 5 mg/ml stock) and ethidium bromide (from 3 mg/ml stock) to 1 ml PBS and 100 µl from this was added to compound **1** treated and untreated cells. After 5 min of incubation, cells were washed with PBS and observed under the FITC filter of the fluorescence microscope (Nikon Eclipse TS 100, Japan).

2.7 | Statistical analysis

All the data were obtained from three independent experiments, and the results were presented as mean ± standard deviation. Statistical analysis was carried out using GraphPad instat 2 software, and the significant differences were evaluated using Student's *t* test.

3 | RESULTS AND DISCUSSION

About 500 g of the powdered rhizomes was extracted successively with hexane, acetone and ethanol. The extracts were examined for *in vitro* cytotoxicity against human lung adenocarcinoma (A549) cell line. Screening of the extracts of *H. flavescens* revealed both hexane and acetone extracts showed potent antiproliferative activity (˂5 µg/ml). Ethanol extract showed the least activity with an IC_{50} value of 88.6 µg/ml at 48 hr (Table S1).

The present work describes the successive isolation and purification of bioactive compounds from the rhizomes of *H. flavescens* and their antiproliferative activity against lung cancer and normal cell lines. The known isolates were identified based on spectroscopic analysis and comparison of their spectral and physical data with those in the literature (see Supporting Information). Compounds **2** and **3** were obtained as an epimeric mixture at C-14 and C-15. Hence, the structure of the compounds was identified as coronarin E (**1**) (Itokawa et al., 1988a, 1988b), C-14 epimers of isocoronarin D (**2**) (Taveira et al., 2005), C-15 epimers of coronarin D methyl ether (**3**) (Chimnoi et al., 2008) and isocoronarin D (**4**) (Nakatani et al., 1994; Figure 1).

It is worth mentioning that all the compounds isolated from *H. flavescens* were previously reported from *Hedychium coronarium* (Hartati et al., 2014), but this is the first report from this species. Apart from this, coronarin E was reported from the rhizomes of *Hedychium ellipticum* (Songsri & Nuntawong, 2016), *Alpinia malaccensis* (Nuntawong & Suksamrarn, 2008) and *Hedychium roxburghii* Blume

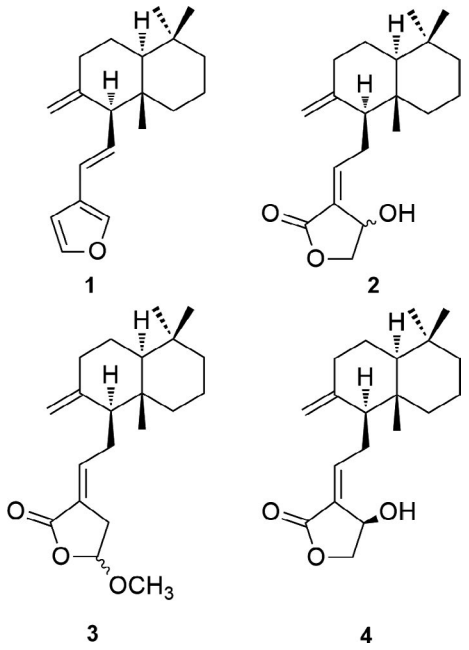

FIGURE 1 Chemical structures of compounds **1–4**

(Hartati et al., 2015) of the Zingiberaceae family. Singh *et al.* first reported Isocoronarin D and the relative stereochemistry of the chiral centre at C-14 was assigned based on X-ray studies, which indicated *β*-configuration for the hydroxyl group (Singh et al., 1991). The *α*-isomer of isocoronarin D was reported from the plant species *Alpinia calcarata* (Zingiberaceae), named as calcaratarin D (Kong et al., 2000). After analysing the spectral data, we got a 1:1 mixture of isocoronarin D and calcaratarin D, so it is difficult to assign the relative stereochemistry by the NMR data. Similarly, compound **3** is also a 1:1 mixture of C-15 epimers of coronarin D methyl ether. Coronarin E, isocoronarin D and C-14 epimers of isocoronarin D serve as the chemo-markers of this species. Botanically, the species *Hedychium flavum* and *H. flavescens* are close and are often confused due to similar morphological characteristics (Ray et al., 2018). Therefore, these chemotaxonomic findings have great significance in distinguishing the two *Hedychium* species (Tian et al., 2020).

From the literature reports, some of the compounds from *Hedychium* species are reported to have enhanced cytotoxic activities. Hence, we evaluated all these compounds against human lung adenocarcinoma (A549) and normal lung fibroblast (WI-38). Doxorubicin (DOX) was used as the standard drug. The results of the cytotoxic studies are shown in Table 1. The effect of various concentrations of compounds **1–4** on A549 and WI-38 cell lines at 24 and 48 hr is shown in Figure 2. The results showed that all the tested compounds possessed potent cytotoxic activity against A549 cells with an IC_{50} value falling below 20 µM. At the same time, these compounds showed the least cytotoxicity towards WI-38, the normal lung fibroblast cells. With 24 hr incubation, 20 µM of compound **1** showed 61.9%

TABLE 1 Cytotoxicity data for compounds **1–4** in A549 and WI-38 cells over a time of 24 and 48 hr

	Cytotoxicity (IC_{50}) in μM			
	A549		WI−38	
Compounds	**24 hr**	**48 hr**	**24 hr**	**48 hr**
Coronarin E (**1**)	0.59 ± 0.001[b]	0.52 ± 0.003[b]	>20[c]	>20[c]
C-14 epimers of Isocoronarin D (**2**)	17.78 ± 0.02[c]	0.59 ± 0.0015[b]	>20[c]	>20[c]
C-15 epimers of Coronarin D methyl ether (**3**)	>20[c]	0.68 ± 0.0015[b]	>20[c]	>20[c]
Isocoronarin D (**4**)	1.23 ± 0.063[b]	1.22 ± 0.004[c]	>20[c]	>20[c]
Doxorubicin[a]	2.16 ± 0.01	0.92 ± 0.0025	18.2 ± 0.01	5.6 ± 0.005

Note: Results are expressed as mean ± *SD*.

[a]Standard drug.

[b]Statistical significance: DOX versus Compounds $p < .001$.

[c]Not tested; A549—human lung adenocarcinoma; WI-38—normal lung fibroblast.

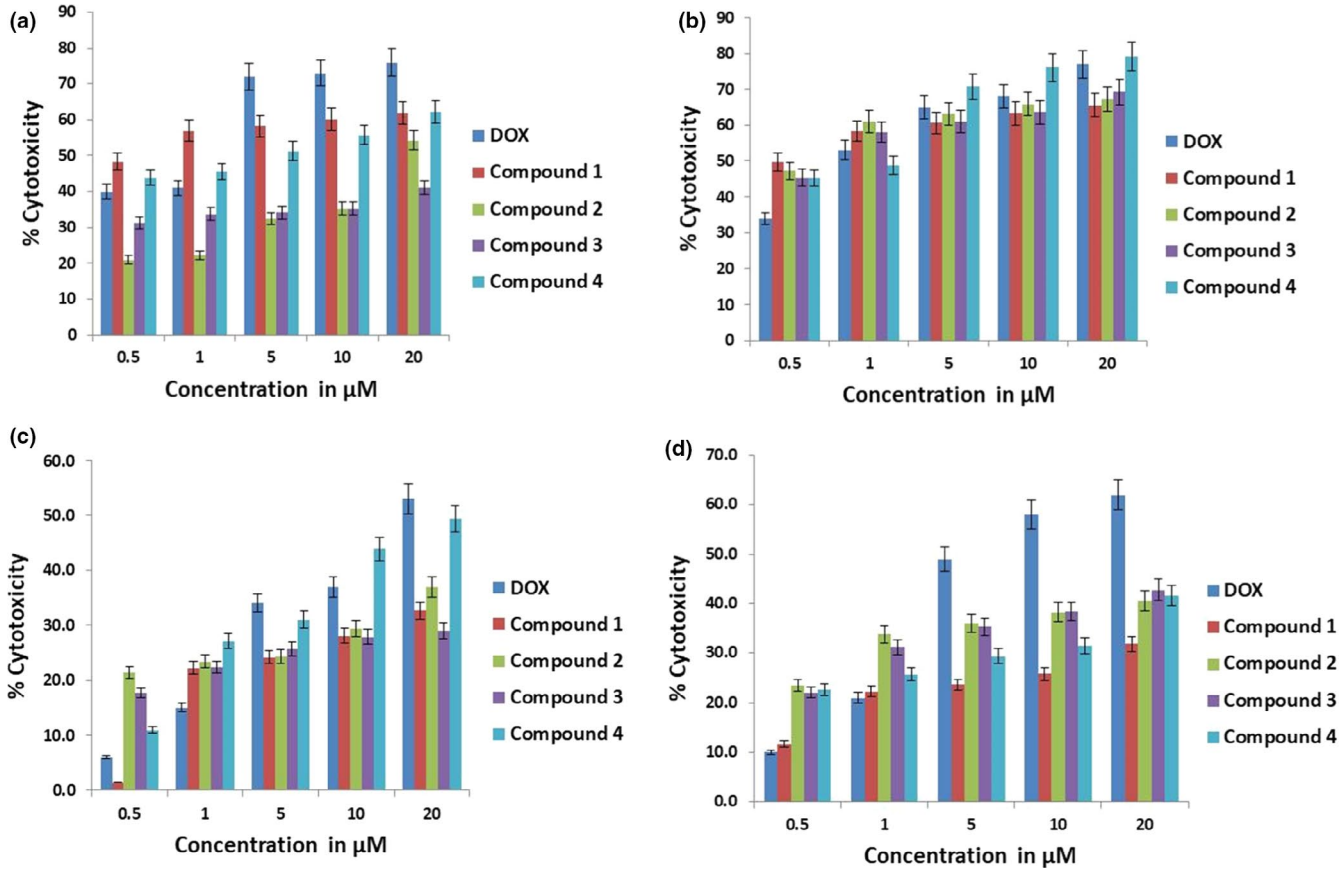

FIGURE 2 MTT assay of compounds **1–4** (a) MTT assay on A549 cells at 24 hr (b) MTT assay on A549 cells at 48 hr (c) MTT assay on WI-38 cells at 24 hr (d) MTT assay on WI-38 cells at 48 hr

inhibition on cell proliferation, whereas DOX and compounds **2–4** showed 76.0, 54.2, 41.0 and 62.1% inhibition. The IC_{50} values for 24 hr treatment were 2.16, 0.59, 17.78, >20 and 1.23 μM respectively for DOX and compounds **1–4**. Upon 48 hr of treatment, the IC_{50} values of DOX and compounds **1–4** were 0.92, 0.52, 0.59, 0.68, and 1.22 μM. In the case of WI-38 cells, 20 μM concentration of DOX showed 53 and 62% cytotoxicity, respectively, with 24 and 48 hr of treatment; at the same time, the IC_{50} values of all the compounds were above 20 μM. The studies revealed the cell-specific cytotoxic effect of the compounds, where the standard cytotoxic drug DOX was inhibiting the cell proliferation of both normal and cancer cells. The IC_{50} value for compounds **1–4** was less than that of DOX in A549 cells at 48 hr, and it was higher than 20 μM in WI-38 for all the compounds. From the results, compound **1** was exhibiting

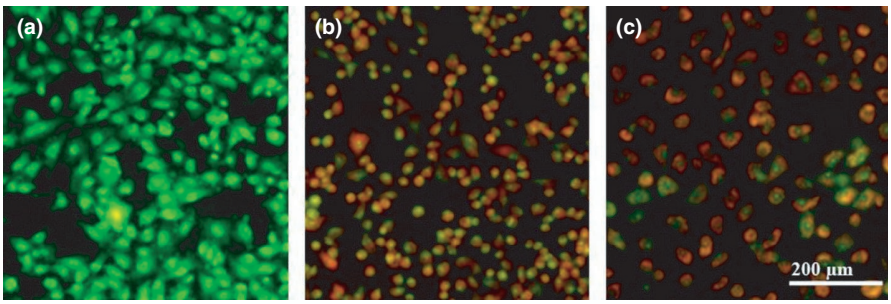

FIGURE 3 Induction of apoptosis by live–dead assay (a) control (b) A549 cells treated with compound **1** at 0.59 µM (c) doxorubicin at 2.16 µM. Scale bar corresponds to 200 µm

an appreciable apoptotic effect towards A549 cells even at a concentration of 0.52 µM at 48 hr, which showed a negligible cytotoxic effect in WI-38 cells.

In acridine orange–ethidium bromide dual staining procedure (live–dead assay), acridine orange is a nonfluorescent membrane–permeant dye which will be turned into fluorescent upon conversion by cellular esterase enzymes; thus, all the viable cells will be appeared green in colour with FITC filter, whereas ethidium bromide being impermeant to cell membrane enters only into dead cells with compromised cell membrane and gives red fluorescence upon binding with DNA. Fluorescent images of the live–dead assay showed a reduced number of cells in compound **1**-treated group, with most of the cells exhibiting yellow/red fluorescence when compared to the untreated cells. Thus, the result indicated the apoptosis-inducing ability of compound **1** even at a concentration of 0.59 µM at 24 hr (Figure 3).

4 | CONCLUSIONS

We have isolated and characterized four compounds from the rhizomes of *H. flavescens*, which can serve as the chemotaxonomic marker of this species. The *in vitro* cytotoxic analysis of compounds **1–4** was evaluated against human lung adenocarcinoma (A549) and normal lung fibroblast (WI-38). All the compounds showed excellent antiproliferative activity and are nontoxic towards normal cells compared with the standard drug DOX. To the best of our knowledge, this is the first phytochemical investigation and antiproliferative study of *H. flavescens*. The results indicated that coronarin E (**1**) could be a promising candidate as a potent antiproliferative agent against lung cancer. Moreover, further studies are needed to uncover the mechanism of inducing apoptosis by our compounds.

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CONFLICT OF INTEREST

The researchers claim no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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