Isolation, Characterization and Development of Novel Bioactive Compounds from Selected Medicinal Plants Used in Ayurveda

Thesis Submitted to AcSIR for the Award of the Degree of
DOCTOR OF PHILOSOPHY
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July 2016

Dedicated to my beloved parents

26th July, 2016

DECLARATION

I hereby declare that the Ph.D. thesis entitled "**Isolation, Characterization and Development of Novel Bioactive Compounds from Selected Medicinal Plants Used in Ayurveda"** is an independent work carried out by me under the supervision of Dr. Mangalam S. Nair at the Organic chemistry Section, CSTD, CSIR-NIIST, Thiruvananthapuram and it has not been submitted anywhere else for any other degree or diploma.

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CERTIFICATE

This is to certify that the work incorporated in this Ph.D. thesis entitled "**Isolation, Characterization and Development of Novel Bioactive Compounds from Selected Medicinal Plants Used in Ayurveda"** submitted by Ms. Dhanya S. R. to Academy of Scientific and Innovative Research (AcSIR) in fulfillment of the requirements for the award of the Degree of Doctor of Philosophy in Chemical Sciences, embodies original research work under my supervision/guidance. I further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources, have been duly cited and acknowledged.

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(Thesis Supervisor)

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ABBREVIATIONS

AAE	:	Ascorbic acid equivalent
ADHD	:	Attention deficit hyperactivity disorder
ADP	:	Adenosine diphosphate
Aq.	:	Aqueous
ATP	:	Adenosine triphosphate
A_0	:	Absorbance of control
A_t	:	Absorbance of sample
BC	:	Before Christ
BHA	:	Butylated hydroxy anisole
brs	:	Broad singlet
CC	:	Column chromatography
CCDC	:	The Cambridge Crystallographic Data Centre
$CDCl_3$	:	Deuterated chloroform
CD_3OD	:	Deuterated methanol
CfAE	:	*Chonemorpha fragrans* acetone extract
CfEE	:	*Chonemorpha fragrans* ethanol extract
CFU	:	Colony-forming unit
cm	:	Centimetre
CoA	:	Coenzyme A
CXCR	:	Chemokine receptor
°C	:	Degree Celsius
DCM	:	Dichloromethane
DEPT	:	Distortionless enhancement by polarization transfer
DMSO	:	Dimethyl sulphoxide
DNA	:	Deoxyribonucleic acid
DPPH	:	1,1'-diphenyl-2-picrylhydrazyl
$DPPH^{\bullet}$	:	1,1'-diphenyl-2-picrylhydrazyl radical
EDTA	:	Ethylenediaminetetraacetic acid
ERK	:	Extracellular signal–regulated kinases
ESI	:	Electronspray ionization
EtOAc	:	Ethyl acetate
d	:	Doublet

dd	:	Doublet of doublet
FDA	:	Food and Drug Administration
Fig.	:	Figure
FITC	:	Fluorescein isothiocyanate
Fr.	:	Fractions
g	:	Gram
GABA	:	Gamma-aminobutyric acid
GAE	:	Gallic acid equivalent
h	:	Hour
HIV	:	Human immunodeficiency virus
HMBC	:	Heteronuclear multiple bond correlation
HMQC	:	Heteronuclear multiple-quantum correlation
HPLC	:	High performance liquid chromatography
HRMS	:	High-resolution mass spectrometry
HTS	:	High throughput screening
Hz	:	Hertz
ICAM	:	Intercellular Adhesion Molecule
IC_{50}	:	Concentration required for 50% inhibition
IKK	:	IκB kinase
IR	:	Infrared
J	:	Coupling constant
JNK	:	c-Jun N-terminal kinases
KBr	:	Potassium bromide
kg	:	Kilogram
L	:	Litre
M	:	Molar
m	:	Multiplet
M^+	:	Molecular ion
MAPK	:	Mitogen-activated protein kinases
MeOH	:	Methanol
mg	:	Milligram
MHz	:	Mega Hertz
mL	:	Millilitre
mM	:	Millimolar

mmol	:	Millimoles
MMP	:	Matrix metalloproteinases
m.p.	:	Melting point
MPA	:	Mycophenolic acid
mRNA	:	Messenger RNA
MTT	:	3-(4,5-Dimethylthiazal-2-yl)-2,5-diphenyl terazolium bromide
$NaBH_4$	:	Sodium borohydride
$NADP^+$	:	Nicotinamide adenine dinucleotide phosphate
NADPH	:	Reduced form of $NADP^+$
NF-κB	:	Nuclear Factor kappa-light-chain-enhancer of activated B cells
nm	:	Nanometre
NMR	:	Nuclear Magnetic Resonance
NOESY	:	Nuclear overhauser effect spectroscopy
NP	:	Natural Product
ORTEP	:	Oak ridge thermal ellipsoid plot
p	:	para
PARP	:	Poly (ADP-ribose) polymerase
PBS	:	Phosphate-buffered saline
PCC	:	Pyridinium chlorochromate
PCNA	:	Proliferating cell nuclear antigen
PEG	:	Polyethylene glycol
ppm	:	Parts per million
Prep.	:	Preparative
QE	:	Quercetin equivalent
RNA	:	Ribonucleic acid
ROS	:	Reactive Oxygen Species
r.t.	:	Room temperature
s	:	Singlet
SD	:	Standard deviation
sh.	:	Shoulder peak
STAT	:	Signal transducer and activator of transcription
syn.	:	Synonym
t	:	Triplet
THF	:	Tetrahydrofuran

TIMP	:	Tissue inhibitor of metalloproteinase
TLC	:	Thin layer chromatography
TMS	:	Tetramethylsilane
TNF	:	Tumor necrosis factor
UV	:	Ultraviolet
V	:	Vascular endothelial growth factor
WHO	:	World Health Organization
α	:	Alpha
β	:	Beta
δ	:	Delta
γ	:	Gamma
λ_{max}	:	The wavelength at which absorbance is maximum
μg	:	Microgram
μl	:	Microlitre
μM	:	Micromolar
υ_{max}	:	Maximum frequency

Chapter 1

Role of Natural Products in Modern Drug Discovery- with Special Emphasis to Plant Derived Anticancer Agents

1.1. Introduction

Natural products (NPs), the secondary metabolites derived from plants, animals and micro-organisms have been the basis of medicaments for the management of various diseases all over the world since time immemorial. Before the advancement of high throughput screening (HTS) and molecular modelling, around 80% of the available drugs were either derived from natural products or inspired by them.[1,2] Almost half of the drugs approved since 1994 are based on NPs, but the interest in natural product based drug discovery was reduced considerably during the last few decades due to the intricacies of NP structures, scarcity of raw materials and the difficulties in obtaining the patent (especially in the case of plant derived NPs).[3,4,5] Another prominent factor which led to the decline in the pace of NP research was the rapid growth of other modern drug discovery processes such as combinatorial chemistry, advanced synthetic chemistry, molecular modelling and HTS which were expected to lead to many new drugs.[3,6] Despite these problems, NPs still manage to provide their fair share in gifting new chemical entities with lead potential and are the source of most successful drug candidates.[7,8]

A revival of interest in NP drugs occurred in last decade due to several reasons. One reason is the widespread belief that NPs are healthier and have less side effects than synthetic drugs.[9] Second reason for the renewed interest in NPs is that, even though the much celebrated combinatorial chemistry produced a plethora of compounds for evaluation, only three novel molecules viz., sorafenib, ataluren and vemurafenib (fig. 1.1) got approved since 2014.[10]

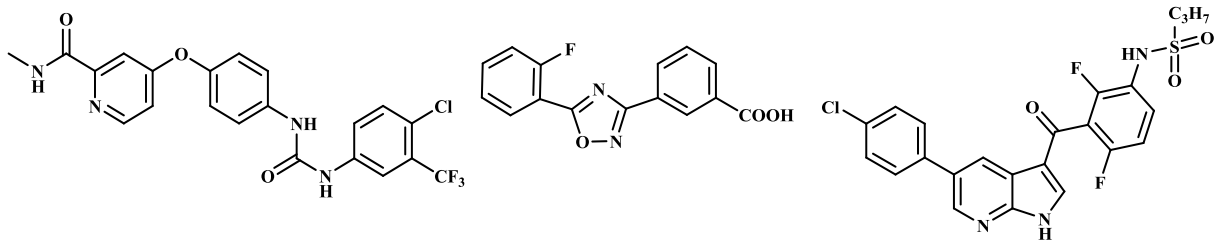


Figure 1.1: Sorafenib Ataluren Vemurafenib

Compounds derived from natural sources offer large structural diversity and desired chirality, owing to their unique biosynthetic pathway which is otherwise very tough to achieve through synthetic methodologies. Pure bioactive compounds isolated from plants and marine organisms play a significant role in modern drug discovery process, especially in the anticancer and antihypertensive therapeutic areas.[11,12,13] Nowadays, the synthesis of natural product-like libraries is also gaining a lot of interest and the percentage of synthetic derivatives of NPs as drugs are increasing, thus augmenting the value of NP research.[14] In order to gain the correct perspective into this area, a literature survey of recent NP and NP derived/inspired drugs have been carried out and the details are given in the proceeding sections.

From a total of 1562 new drugs approved in between 1985-2014, approximately 22% (351 drugs) were vaccines and other biologic drugs ("usually a large, >45 residues peptide or protein, either isolated from an organism/cell line or produced by biotechnological means in a surrogate host"[15] such as hormones), 26% (296 drugs) were NP and NP derived compounds whereas 25% (395 drugs) were NP mimics and synthetic drugs inspired/mimicked from NPs (fig. 1.2). Only 27% were found to be purely synthetic drugs. Among all these drugs only 67 molecules were found to be unmodified NPs.[10]

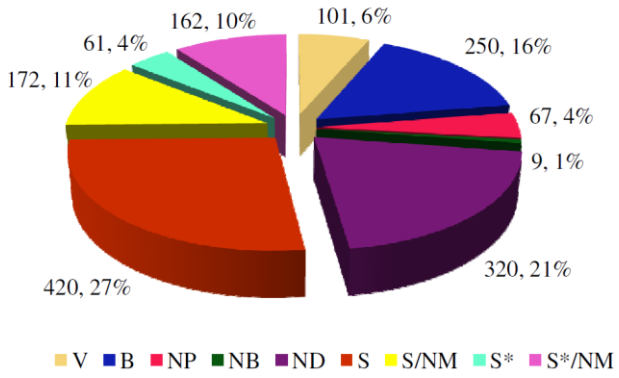


Figure 1.2: Type of new drugs approved in between 1985-2014

Source: Newman & Cragg, *J. Nat. Prod.* 2016, 79, 629.

Abbreviations used: V-Vaccine, B- Biologics, NP- Natural product (secondary metabolites), NB- Botanical drug (defined mixtures), ND- NP derivative, S- Purely synthetic, S*- Synthetic drug with NP pharmacophore, S/NM- synthetic compounds showing competitive inhibition of the NP substrate, S*/NM- synthetic compounds with natural product pharmacophore showing competitive inhibition of the natural product substrate

Some important unmodified natural product drugs which were approved in between 2005 to 2012[16] along with their source and disease areas where they are used are given in table 1.1. Biologic drugs, vaccines etc., are excluded from the list. The structures of these drugs are given later in respective sections where they are discussed in detail.

Table 1.1: FDA approved unmodified NP drugs launched in between 2005-2012

Year	Generic name	Source	Disease area
2005	Dronabinol/cannabidiol	*Cannabis s*p.(p)	Pain
2005	Fumagillin	*Aspergillus fumigatus* (f)	Antiparasitic
2005	Ziconotide	*Conus magus* (ssn)	Pain
2006	Polyphenon E Ointment (Green tea polyphenols)	*Camellia sinensis* (p)	Genital and perianal warts
2007	Trabectedin	*Ecteinascidia turbinata* (ssq)	Cancer
2009	Romidepsin	*Chromobacterium violaceum* (b)	Cancer
2009	Colchicine	*Colchicum autumnale* (p)	Acute gout
2009	Capsaicin	*Capsicum* sp. (P)	Pain
2011	Spinosyn A : D 5 : 1	*Saccharopolyspora spinosa* (b)	Antiparasitic
2012	Omacetaxine mepesuccinate	*Cephalotaxus harringtonia* (p)	Cancer
2012	Ingenol mebutate	*Euphorbia peplus* (p)	Actinic keratosis

Abbreviations used: p-plant, b-bacteria, f-fungus, ssn-sea snail, ssq-sea squirt

A large number of NP inspired and NP derived (semi-synthetic natural products) drugs as compared to unmodified NP drugs have been approved in recent years. Such important natural product derived/inspired drugs that were approved in between 2005 and 2015[16,17,18] along with their lead compounds, source and diseases area in which they are used are given in table 1.2. Once again, the structures of the important lead compounds are provided in respective sections, where they are discussed in detail.

Table 1.2: FDA approved NP inspired/derived drugs launched in between 2005-2015

Sl. No.	Year	Generic name	Lead compound	Source	Disease area
1	2005	Doripenem	Thienamycin	Microbial	Antibacterial
2	2005	Tigecycline	Tetracycline	Microbial	Antibacterial
3	2005	Zotarolimus	Sirolimus	Microbial	Cardiovascular surgery
4	2005	Tamibarotene	Retinoic acid	Plant	Acute myelogenous leukemia
5	2005	Abraxane	Paclitaxel	Plant	Breast cancer
6	2005	Exenatide	Exenadin-4	Lizard	Diabetes
7	2006	Anidulafungin	Echinocandin B	Microbial	Antifungal
8	2006	Varenicline	Cytisine	Plant	Nicotine dependence
9	2006	Hycamtin	Camptothecin	Plant	Cervical cancer
10	2006	Cesamet	Tetrahydro-cannabinol	Plant	Chemotherapy, nausea and vomiting
11	2007	Lisdexamfetamine	Amphetamine	Plant	ADHD
12	2007	Retapamulin	Pleuromutilin	Microbial	Antibacterial
13	2007	Ixabepilone	Epothilone B	Microbial	Cancer
14	2008	Ceftobiprole medocaril	Cephalosporin C	Microbial	Antibacterial
15	2008	Umirolimus	Sirolimus	Microbial	Cardiovascular surgery
16	2008	Methylnaltrexone	Morphine	Plant	Opioid-induced constipation
17	2009	Tebipenem pivoxil	Thienamycin	Microbial	Antibacterial
18	2009	Telavancin	Vancomycin	Microbial	Antibacterial
19	2009	Vinflunine	Vinorelbine/ vinblastine	Plant	Cancer
20	2009	Nalfurafine	Morphine	Plant	Pruritus
21	2010	Cabazitaxel	Paclitaxel	Plant	Cancer
22	2010	Fingolimod	Myriocin	Microbial	Multiple sclerosis
23	2010	Ceftaroline fosamil	Cephalosporin	Microbial	Antibacterial

24	2010	Eribulin	Halichondrin B	Marine	Cancer
25	2010	Mifamurtide	Muramyl dipeptide	Microbial	Cancer
26	2010	Zucapsaicin	Capsaicin	Plant	Pain
27	2011	Brentuximab vedotin	Dolastatin 10	Marine	Cancer
28	2012	Arterolane /piperaquine	Artemisinin	Plant	Antimalarial
29	2012	Dapagliflozin	Phlorizin	Plant	Type II diabetes
30	2012	Carfilzomib	Epoxomicin	Microbial	Cancer
31	2012	Novolimus	Sirolimus	Microbial	Cardiovascular surgery
32	2013	Canagliflozin	Phlorizin	Plant	Type II diabetes
33	2013	Trastuzumab emtansine	Maytansine	Plant	Cancer
34	2014	Naloxigol	Thebaine (paramorphine)	Plant	Opioid-induced constipation
35	2014	Empagliflozin	Phlorizin (p)	Plant	Type II diabetes
36	2014	Oritavancin	Vancomycin	Microbial	Skin infection
37	2014	Vorapaxar	Himbacine	Plant	To reduce the risk of heart attack
38	2015	Ceftazidime/ avibactam	Cephalosporin	Microbial	Intra-abdominal infections

The above list clearly suggests that secondary metabolites from terrestrial plants, microbes and marine organisms play a vital role in the development of new drugs and drug leads. Major source of these NP drugs were plants followed by microorganisms. A brief discussion on the drugs developed from marine sources, terrestrial microbial sources, terrestrial animal sources and a detailed description of terrestrial plant-derived drugs giving particular importance to plant-derived anticancer agents are portrayed in the following sections.

1.2. Drugs from marine organisms

Oceans, which cover more than 70% of earth's surface, are the endless reservoir of structurally unique natural products. In the past few decades, marine natural sources

including marine plants, microorganisms and sponges have attracted much attention from the scientific community in their search for the development of new drugs or molecular entities with lead potential. Biosynthetic pathways of secondary metabolites in marine organisms are quite different from that of terrestrial organisms[19] and in contrast to the terrestrial environment, the number of bioactive compounds from marine animals such as sponges, tunicates, molluscs etc., exceed aquatic plants (algae) by a large margin.[20,21]

The nucleosides viz., spongouridine and spongothymidine (fig. 1.3), isolated from the Caribbean sponge *Tethya crypta* were considered to be the earliest two compounds obtained from the marine world. These two compounds further led to the synthesis of important anticancer drugs cytarabine (fig. 1.3, used especially for the treatment of myeloid leukemia and non-Hodgkin's lymphoma) and the antiviral drug vidarabine (fig. 1.3) which are in clinical use since 1974.[22,23] It took approximately 30 years to launch another marine NP into the market. The compound which was approved by FDA in 2005 was ziconotide (a complex peptide with molecular formula- $C_{102}H_{172}N_{36}O_{32}S_7$), which is the synthetic correspondent of a naturally occurring ω-conotoxin peptide, isolated from the venom of marine snail *Conus magus*.[24] The compound is used to alleviate neuropathic pain.[25]

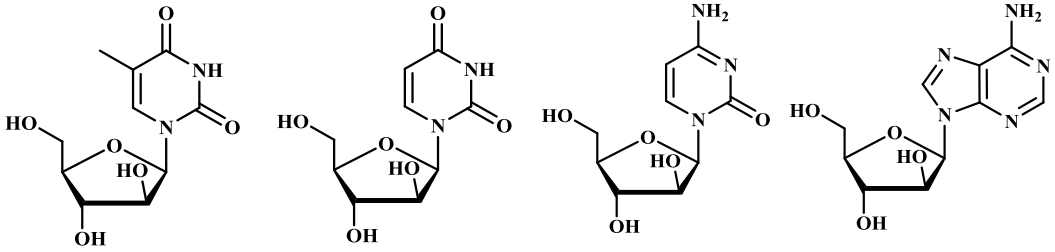


Figure 1.3: Spongouridine Spongothymidine Cytarabine Vidarabine

One of the thrust areas where marine natural products contributed largely is in anticancer drug development.[25b] The latest compound from the marine source, which came into the market for the treatment cancer is the alkaloid, trabectedin (also known as ecteinascidin 743 or ET-743, trade name- yondelis, fig. 1.4), which got approved in 2007. The compound is used for the treatment specific soft tissue sarcomas that cannot be removed by surgery or is advanced.[18c] Trabectedin was isolated from the sea squirt *Ecteinascidia turbinata*, the total synthesis of which was reported by Corey *et al*., in 1996.[26,27] The compound acts through alkylating the N2 of guanines in the minor groove of DNA and by poisoning transcription-coupled nucleotide excision repair.[28]

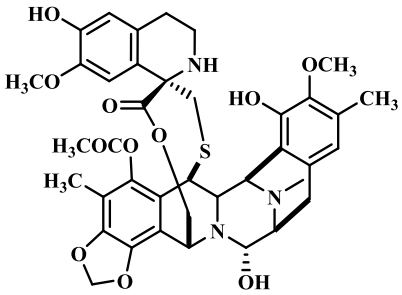


Figure 1. 4: Trabectedin

Another important anticancer drug is eribulin (eribulin mesylate is marketed under the trade name halaven)[29] which was derived from the compound halichondrin B (fig. 1.5) isolated from the marine sponge *Halichondria okadai.*[30] The compound was found be very effective against breast cancer and is available in the market since 2010. Eribulin acts through inhibiting the microtubule growth by binding with it (one molecule of the compound binds with two microtubules).[31]

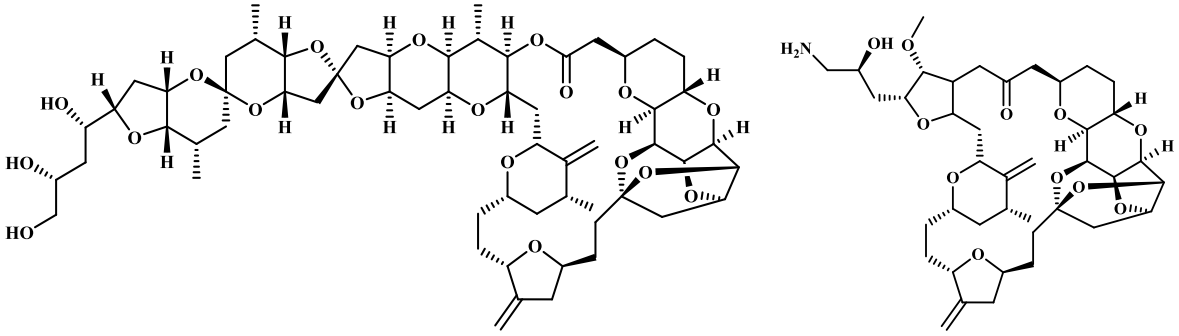


Figure 1.5: Halichondrin B Eribulin

A good number of compounds such as bryostatin 1 (isolated from the bryozoan *Bugula neritina*), marizomib (isolated from the marine actinomycete *Salinispora tropica*), plitidepsin (isolated from the tunicate *Aplidium albicans*) etc., (fig. 1.6) are now under various stages of clinical trials for the treatment of cancer.[25]

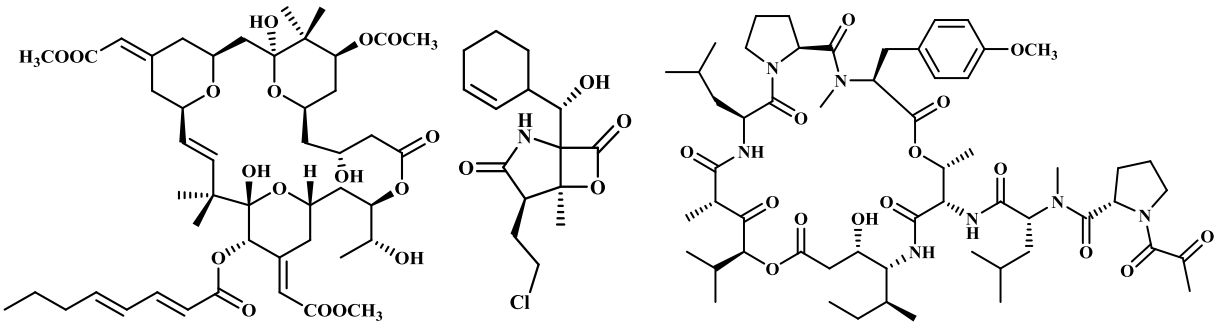


Figure 1.6: Bryostatin 1 Marizomib Plitidepsin

1.3. Drugs from terrestrial microorganisms

Exploration of microorganisms for providing new drug candidates flourished after the pioneering discovery of the antibiotic, penicillin from the fungus *Penicillium chrysogenum* (previously known as *P. notatum*, fig. 1.7) by Alexander Fleming in the year 1928.

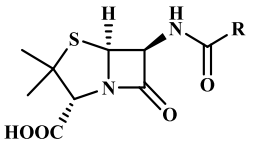
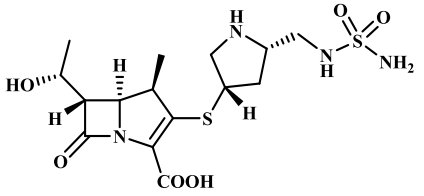


Figure 1.7: Penicillin

After the success of penicillin, many antibacterial compounds belonging to various classes viz., β-lactams, cephalosporins, aminoglycosides, tetracyclines, and polyketides were developed from microorganisms. Actinomycetes were found to be the primary source of β-lactam antibiotics (latest ones include doripenem, tebipenem pivoxil etc. fig. 1.8) where as fungi are the major source of cephalosporin antibiotics (a class of β-lactam antibiotics isolated from fungus, latest ones include ceftobiprole, ceftaroline fosamil etc. fig. 1.9).[32]



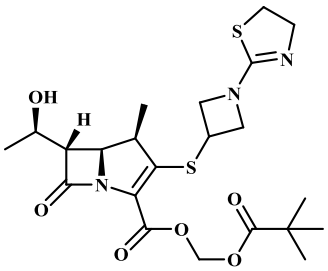
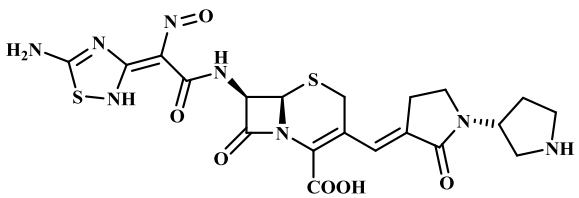


Figure 1.8: Doripenem Tebipenem pivoxil



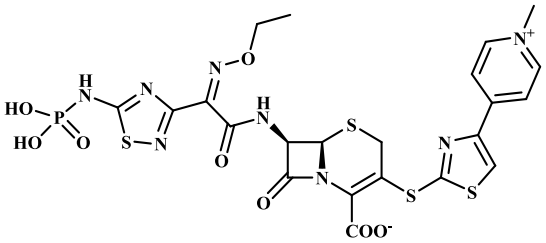


Figure 1.9: Ceftobiprole Ceftaroline fosamil

Microorganisms also provided drug leads for the treatment of fungal diseases. One compound which obtained FDA approval recently for the treatment of fungal infections caused by *Candida* sp. is micafungin (fig. 1.10).[33,34]

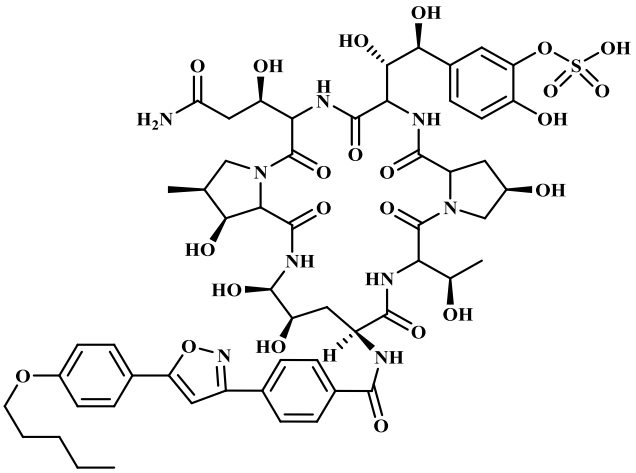


Figure 1.10: Micafungin

Terrestrial microorganisms were found to be very effective against parasitic diseases as well. Ivermectin,[35] obtained by the synthetic modification of avermectin (fig. 1.11)[36] (identified from the soil bacteria *Streptomyces avermitilis*) was found to be a highly efficient drug against an array of parasites. Ivermectin (fig. 1.11) proved to be a magical drug, especially for the people in African regions, as it was effective in the treatment of river blindness and lymphatic filariasis.[37] For this revolutionary discovery Satoshi Ōmura, who isolated avermectin-producing *S. avermitilis* from the soil samples and William C. Campbell, who discovered the chemical structure and modified avermectins to more potent ivermectin, received the Nobel Prize in physiology in the year 2015.[38]

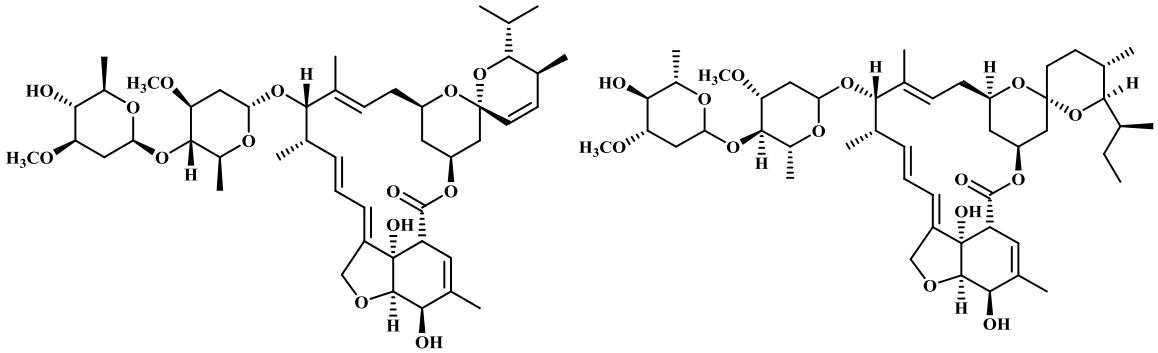
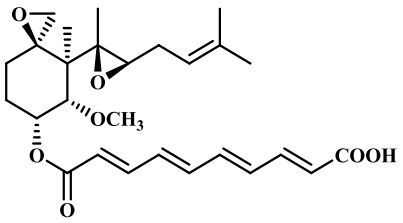


Figure 1.11: Avermectin B1 Ivermectin

Fumagillin (isolated from the fungus *Aspergillus fumigatus*) and spinosyn A and D (5:1 mixture, isolated from the bacteria *Saccharopolyspora spinosa*) are two recently introduced antiparasitic agents (fig. 1.12) approved in 2005 and 2011 respectively.



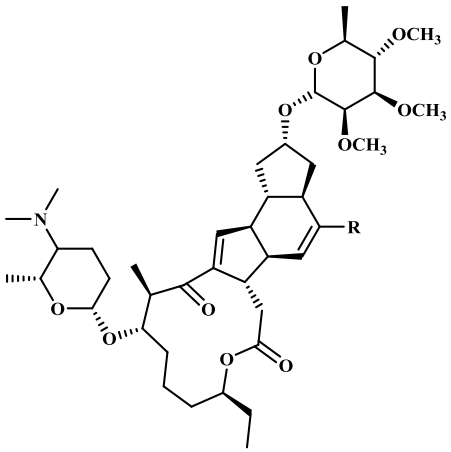
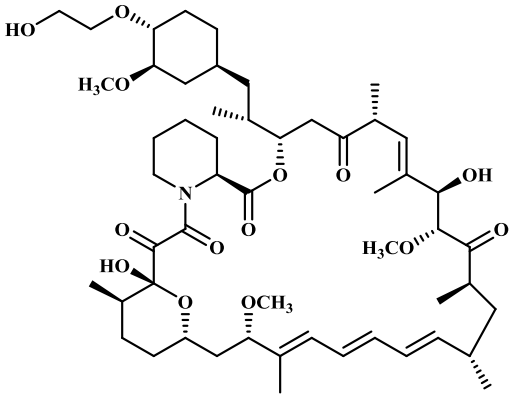


Figure 1.12: Fumagillin R = H; Spinosyn A, R = CH_3; Spinosyn D

Apart from providing novel antibiotic and antiparasitic agents, microorganisms are now emerging as promising sources of immunosuppressant and anticancer drugs too.[39] Everolimus (fig. 1.13), a derivative of rapamycin (also known as sirolimus, an immunosuppressant macrolide isolated from the bacteria *Streptomyces hygroscopicus*) is one of the latest immunosuppressant drugs that came into the market for preventing the rejection of transplant after surgery. It is used mainly during the surgical treatment of renal cell carcinoma.[40] Mycophenolate mofetil, (fig. 1.13), the prodrug of mycophenolic acid (MPA), isolated from the fungus *Penicillium brevicompactum*, is another important FDA approved immunosuppressant drug, especially used in patients after heart, kidney and liver transplantation. After oral administration, the prodrug gets hydrolysed to the active MPA. MPA acts by inhibiting the proliferation of immune cells that attack the transplanted organ and thus prevent transplant rejection.[41]



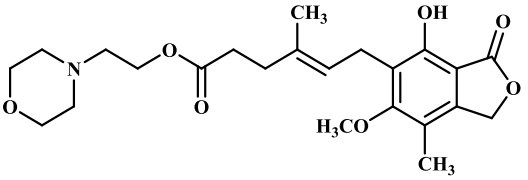


Figure 1.13: Everolimus Mycophenolate mofetil

One of the most popular and extensively used chemotherapeutic drugs now in clinical use is the anthracycline compound doxorubicin (fig. 1.14, trade name adriamycin) which was isolated from the bacterial species *Streptomyces peucetius* var. *Caesius* (produced by the mutagenic treatment of the soil bacteria *S. peucetius*). Doxorubicin is the 14-hydroxy derivative of daunorubicin (fig. 1.14), which is abundantly available in several species of *Streptomyces*.[42,43] Doxorubicin hydrochloride is used for the treatment of solid tumors such as breast, lung, ovarian cancers etc., and for haematological cancers such as Hodgkin's lymphoma and leukemias.[44] The compound acts through binding to the DNA associated enzymes such as topoisomerases and through delaying the DNA repairing. It is also capable of generating free radicals, which can further bring about DNA damage.[45,46] Major side effect associated with doxorubicin treatment is the cardiotoxicity, which can lead to heart failure.[47]

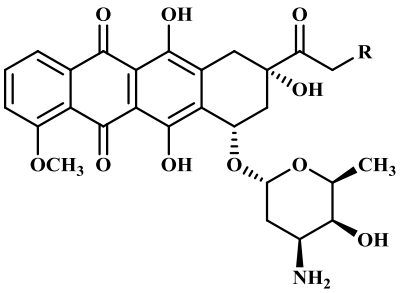
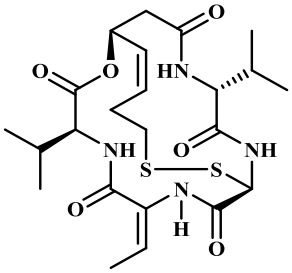


Figure 1.14: R = OH; Doxorubicin, R = H; Daunorubicin

Two recent anticancer drugs obtained from microorganisms are romidepsin and amrubicin hydrochloride (fig.1.15). Romidepsin was isolated from the Gram-negative soil bacteria *Chromobacterium violaceum* and it is used for the treatment of cutaneous T-cell lymphoma. The compound acts as a novel histone deacetylase (HDAC) inhibitor.[48] Amrubicin hydrochloride is the first totally synthetic doxorubicin derivative.[49] The compound is used to treat advanced stages of small cell lung cancer.[50]



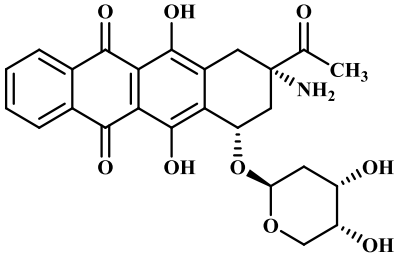


Figure 1.15: Romidepsin Amrubicin

1.4. Drugs from terrestrial animals

The majority of the protein-based biologic drugs (monoclonal antibodies, enzyme modulators, receptor modulators etc.)[51] were developed from human or other animal cells and since they are developed by recombinant DNA technologies, they have been excluded from the category of biochemical NPs in this report. Other than the biologic drugs, many biochemical (secondary metabolites) drugs were also developed from animals belonging to bovine, porcine, equine, canine species. For example, heparin (fig. 1.16), the anticoagulant drug was first isolated from canine liver cells and is pharmaceutically prepared from porcine or bovine source.

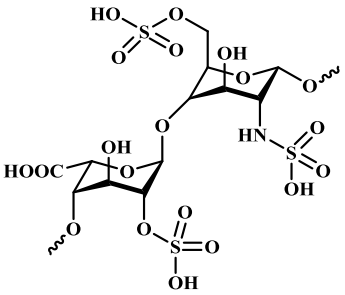
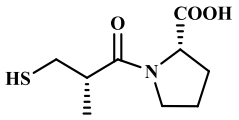
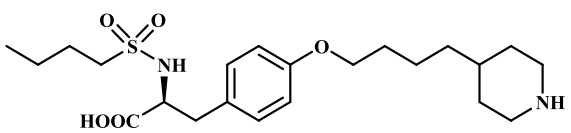


Figure 1.16: Structure of heparin

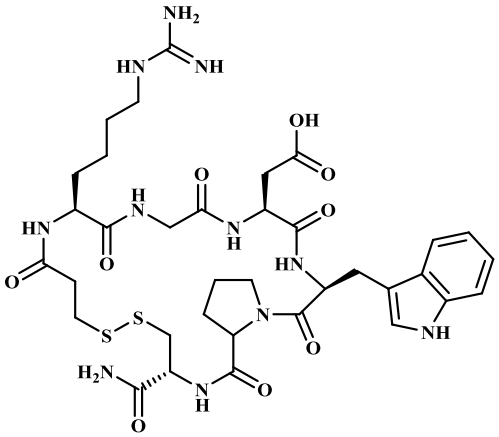
Apart from mammals, secondary metabolites from other vertebrates and invertebrates also served as leads for the development of various drugs. For example captopril (fig. 1.17), an angiotensin-converting enzyme (ACE) inhibitor, is used for the treatment of hypertension. It is the first drug developed based on the bradykinin-potentiating peptides (BPPs) isolated from the venom of a Brazillian viper *Bothrops jararaca*.[52] The two important antiplatelet drugs viz., tirofiban and eptifibatide (fig. 1.17), were derived from proteins isolated from the venom of poisonous snakes *Echis carinatus* and *Sistrurus miliarius barbouri* respectively.[53]



Captopril



Tirofiban



Eptifibatide

Figure 1.17:

Exenatide, the first insulin mimetic protein, used for the treatment of type II diabetes mellitus is the synthetic analogue of exenadin-4,[54] isolated from the saliva of venomous lizard Gila monster (*Heloderma suspectum*).[34,55] The thrombin inhibitor protein bivalirudin, developed from hirudin (isolated from the medicinal leech *Hirudo medicinalis*) is another example of a drug developed from terrestrial animal sources.[34]

1.5. Drugs from terrestrial plants

Isolation of bioactive compounds from medicinal plants attracted the attention of mankind much earlier than that of animal, microbial and marine sources since plants were readily available and were already used in various traditional systems of medicine.[56] The therapeutic use of plants can be traced back to time immemorial. The earliest record of the use of medicinal plants dates back to 2600 BC, which is by the Mesopotamians. The use of medicinal plants by Egyptians is recorded in "*Ebers Papyrus*" which was written around 1500 BC. In this, the therapeutic applications of more than 700 drugs were documented. The history of Chinese medicine can be obtained from *Chinese Materia Medica* (dated from about 1100 BC).[57,58] The earliest mention of the use of medicinal plants in India has been found in '*Rig-Veda'* which was written in between 4000 and 1600 B.C. In the *'Atharvaveda'* we find the more varied use of drugs. Specific properties of drugs and their uses are mentioned in detail in the *Ayurveda,* which is considered as an 'upa veda'. Documentation of the use medicinal plants in India can also be obtained from *Charaka Samhita* and *Susrutha Samhitha* which explain about 341 and 516 drugs respectively.[59]

Ayurveda, the long-established system of medicine practiced in India for thousands of years is now gaining worldwide attention as an alternative system of medicine.[60] It is considered as the science of longevity. *Ayurveda* is a Sanskrit word derived from two roots: '*Ayus*' which means life, '*Veda*' which means knowledge. Thus *Ayurveda* roughly translates as "Knowledge for life". *Ayurveda* deals not only with the treatment of diseases, but it is, in fact, a complete way of life. It is a unique holistic system based on the interaction of body, mind and spirit.

In Ayurvedic treatment, herbal formulations are utilized in different forms such as kashayas/kwadhams (decoctions of raw medicines), choornas (prepared by grinding herbs into a fine powder), pills, balms etc. In each of these, several ingredients are carefully balanced according to individual needs. Even though *Ayurveda* and other traditional systems of medicine do not concentrate directly upon the active principle of

herbal formulations, they have become the basis for the discovery of new drugs/drug leads in modern medicine. Several promising molecules have been developed by taking the advantage of Ayurvedic knowledge basis. Examples include, *Rauwolfia* alkaloids (to reduce hypertension), psoralens (in vitiligo), *Holarrhena* alkaloids (in amoebiasis), L-dopa from *Mucuna pruriens* (Parkinson's disease), piperidines (bioavailability enhancer), picrosides (hepatic protection), phyllanthins (antivirals), curcumine (inflammation), withanolides, steroidal lactones and glycosides (immunomodulators) etc.[61]

A plethora of bioactive compounds with lead potential (especially against malaria and the chronic diseases such as cancer, Alzheimer's etc.) were isolated from plants. The majority of these active ingredients were isolated from plants which are used in various traditional systems of medicine or used in folklore medicine. Important plant-derived compounds which are used as drugs or drug leads against various disease conditions are discussed below.

1.5.1. Antimalarial drugs from plants

Malaria is one of the most dreadful infectious diseases caused by the protozoan parasite, *Plasmodium falciparum*. It is transmitted by the female *Anopheles* mosquitoes and affects nearly 40% of the global population. Malaria is responsible for approximately one million deaths, worldwide per annum and among them, more than 90% are known to occur in Africa.[62]

The alkaloid quinine, isolated from the bark of *Cinchona officinalis* belonging to Rubiaceae family (fig. 1.18) was one of the earliest antimalarial drugs obtained from plants. The bark of the plant had been used by indigenous people in Amazon region for the treatment of fever and later the Europeans used it to treat malaria. The aryl amino alcohol, quinine, isolated in 1820 by Pierre Joseph Pelletier and Joseph Caventou, was the mainstay as an antimalarial agent for about hundred years, until more effective drugs were introduced.[63,64]

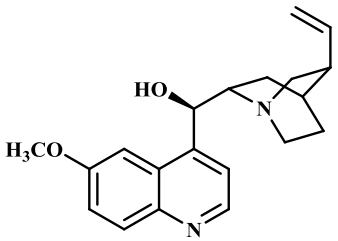


Figure 1.18: Quinine

Quinine served as a proto-type for the development of synthetic antimalarial agents such as chloroquine (belonging to 4-amino quinoline class) and primaquine (belonging to 8-amino quinoline class) (fig. 1.19) with superior activity. However, during 1960's malaria bounced back strongly due to the emergence of resistance by *P. falciparum* to chloroquine.

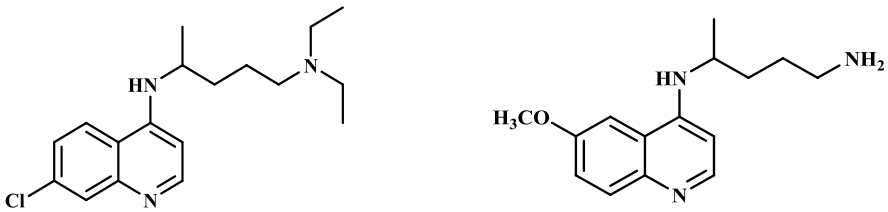


Figure 1.19: Chloroquine Primaquine

The path-breaking discovery of antimalarial agents was achieved, when Youyou Tu isolated artemisinin in 1972 from the Chinese medicinal plant *Artemisia annua* (sweet wormwood), belonging to Asteraceae family (fig. 1.20).[65] The compound was isolated as colourless crystals from the diethyl ether extract of *A. annua*, extracted at low temperatures. The plant was selected for the study based on the knowledge accessed from the ancient Chinese medicinal record ''*Handbook of Prescriptions for Emergencies''*. For the revolutionary discovery of artemisinin, which significantly reduced the mortality rates of patients suffering from malaria, Youyou Tu was awarded the Nobel Prize in physiology for the year 2015.[66,67]

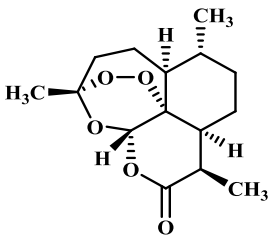
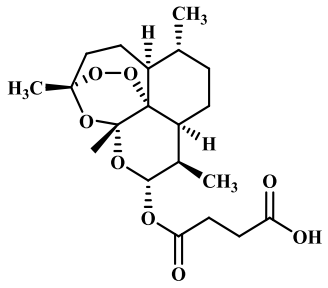


Figure 1.20: *Artemisia annua* Artemisinin

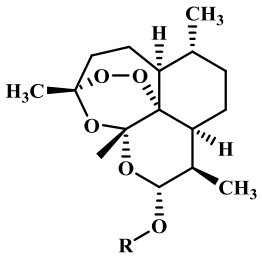
Artemisinin is a highly oxygenated sesquiterpene lactone containing a trioxane ring system with an endoperoxide bridging. The compound was found be very effective against chloroquine resistant malarial parasite and it kills the parasites during early stage of its development, which is responsible for the unprecedented success of the compound. Artemisinin acts through intraparasitic heam-mediated cleavage of peroxide linkage to form oxygen centred radical, which then converts to carbon free radical. This carbon free radicals damage some proteins of the parasite through alkylating to it,

leading to the death of parasite.[68] In malarial parasites haem-iron is derived from the proteolysis of host cell haemoglobin. It has also been found that haem derives from the parasite's haem biosynthetic pathway at the early stages and from haemoglobin digestion during the latter stages.[69] Even though artemisinin was found be very effective, it is insoluble in water which lowers the bio-availability of the compound. Semi-synthetic derivatives of artemisinin such as artesunate, artemether, dihydroartemisinin, artelinic acid and artemotil were found to be much more potent and bio-available than the parent compound. Other derivatives like artemisone[70] are now under clinical trials for the treatment of malaria. Nowadays to minimize resistance, combination therapy of artemisinin/artemisinin derivatives with other drugs such as lumefantrine, piperaquine, and pyronaridine is gaining more interest. One such example is arterolane (artemisinin inspired synthetic drug)/piperaquine combination which got approved in 2012.[66] Artemisinin and its derivatives are still serving as the most potent molecules for the treatment of malaria all over the world. Structures of some artemisinin derivatives are given in chart 1.1.

Chart 1.1: Structures of artemisinin derivatives

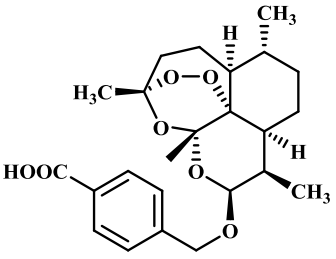


Artesunate

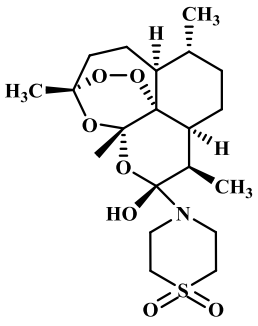


R = H; Dihydroartemisinin, R = CH_3; Artemether

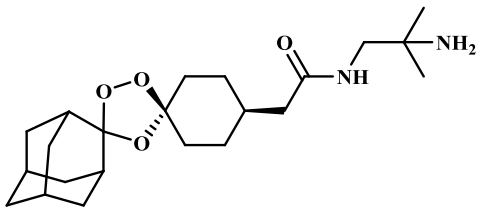
R = C_2H_5; Artemotil



Artelinic acid



Artemisone



Arterolane

1.5.2. Anti-inflammatory drugs from plants

Inflammation is one of the biological processes which help to maintain the homeostasis of the body and in a controlled manner it is beneficial to the body to fight diseases. Inflammation can arise due to physical damage, microbial attack and immune responses. Inflammation becomes dangerous when regulation of inflammation by the body becomes dysfunctional.

Colchicine, an alkaloid isolated from the plant *Colchicum autumnale* (meadow saffron) belonging to Colchicaceae family is an important anti-inflammatory compound used for the treatment of gout (fig. 1.21). Extract of *C. autumnale* was used for the treatment of gout since ancient times, and oral colchicine has been in use as an unapproved drug for many years. However, colchicine was approved by US FDA for the treatment of acute gout and familial Mediterranean fever only in 2009. Colchicine prevents microtubule assembly and acts as a mitotic poison. The compound also changes the expression of genes involved in inflammatory processes.[71] Colchicine has a narrow therapeutic index and its use has been limited as a result of toxic effects produced even after using the prescribed doses. Colchicine toxicity can lead to gastrointestinal problems such as abdominal pain, diarrhoea and vomiting.[72]

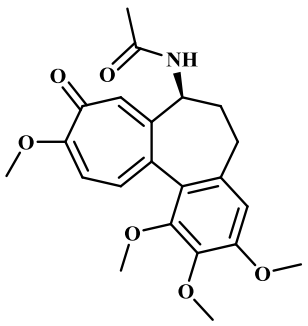


Figure 1.21: Colchicine

Other plant-derived compounds such as curcumin, resveratrol, capsaicin, epigallocatechin-3-gallate, quercetin etc., are now under various phases of clinical trials for the treatment of inflammatory diseases.[73]

1.5.3. Cardiovascular drugs from plants

Cardiovascular diseases (diseases related to heart and blood vessels) are the primary cause of death worldwide and it accounts for nearly 30% of all death across the globe. Digoxin (fig. 1.22), a cardiac glycoside isolated from the foxglove plant *Digitalis lanata* (belonging to Plantaginaceae family) is used for the treatment of mild

to moderate heart failures in adults. Digoxin is administrated as intravenous injection or used in the form of tablet. Another compound similar to digoxin is digitoxin (fig. 1.22) isolated from *Digitalis purpurea*. Even though both these compounds were structurally similar, only digoxin is currently used and was found to be much more effective than digitoxin.[74]

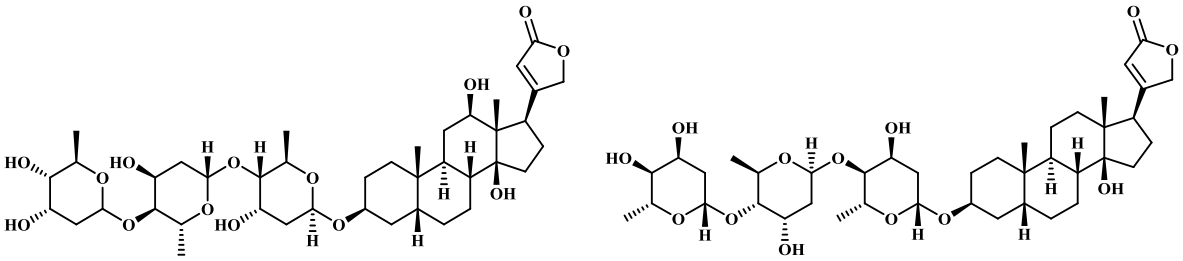


Figure 1.22: Digoxin Digitoxin

Another cardioactive glycoside ouabain (also known as g-strophanthin, fig. 1.23), isolated from the seeds of *Strophanthus gratus*, belonging to Apocynaceae family can be used in small quantities for the treatment of hypotension and cardiac arrhythmias. The compound acts through the inhibition of Na+/K+-ATPase sodium-potassium ion pump.[75]

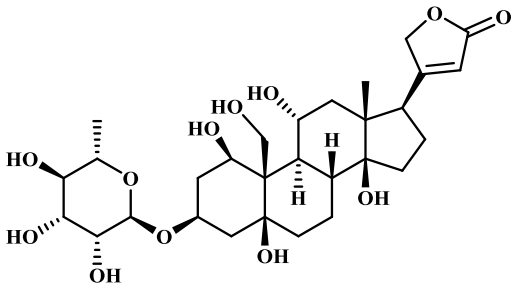
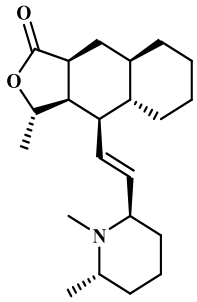


Figure 1.23: Ouabain

Vorapaxar (brand name Zontivity, fig. 1.24), synthesised based on the alkaloid himbacine[76] (fig. 1.24, isolated from the bark of the Australian plant *Galbulimima baccata* belonging to Himantandraceae family) got FDA approval in 2014 to decrease the risk of heart attack, stroke, and to restore the blood flow to the heart in patients with a previous heart attack. Vorpraxar is an orally active protease-activated receptor 1 (PAR-1) antagonist that blocks thrombin-mediated platelet activation.[77]



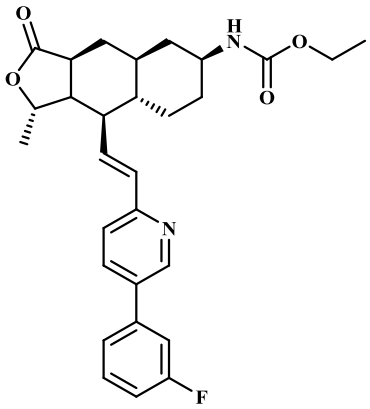


Figure 1.24: Himbacine Vorapaxar

Hypertension is considered to be the most prominent cause leading to an increase in cardiovascular diseases. Reserpine (fig. 1.25), an indole alkaloid isolated from the dried root of *Rauwolfia serpentina* (Indian snakeroot)[78] belonging to Apocynaceae family, was used as a medication to treat blood pressure. *R. serpentina* is regarded as one of the earliest Ayurvedic drug which attracted international attention. In India, the plant has been used in traditional systems of medicine for the treatment of snake bites, feverish illness, insanity etc. The powdered roots of the plant are used as sedative and to treat rheumatism, epilepsy and eczema.[79] Apart from being an antihypertensive agent the compound is also used as a tranquilizer. However, the compound is seldom used today due its adverse side effects.

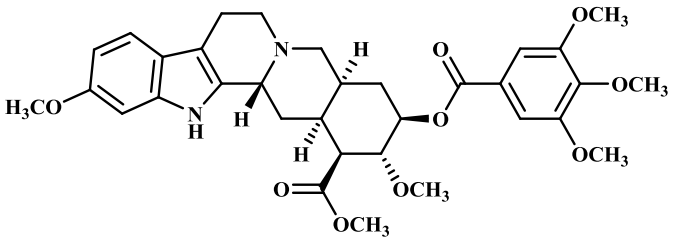


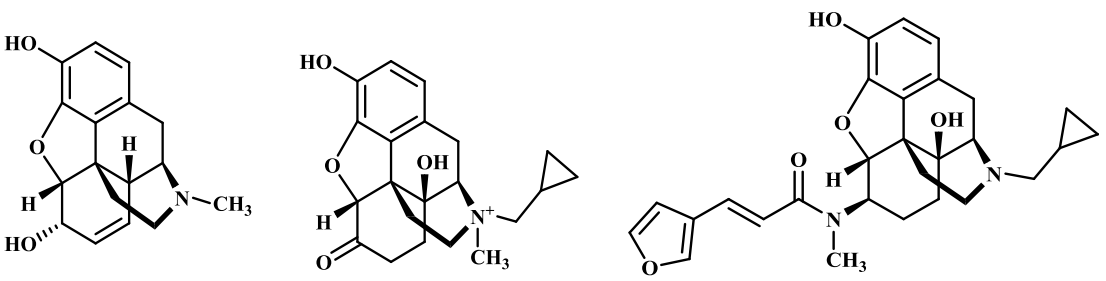
Figure 1.25: Reserpine

1.5.4. Analgesic and neurological drugs from plants

Secondary metabolites isolated from plants and their derivatives have been in the mainstay for the treatment of severe pain. The alkaloid morphine, isolated in the beginning of the nineteenth century (in between 1803-1806) by Friedrich Wilhelm Sertürner from the seeds of Opium poppy (*Papaver somniferum* belonging to family Papaveraceae) is considered to be the first active ingredient isolated from plants.[80] Morphine and its more potent derivatives such as codeine (3-methylmorphine), hydromorphone, heroine etc., are still used to alleviate pain in modern medicine.

Morphine derivatives are now finding use in the treatment of other diseases as well. For example, the derivatives viz., methylnaltrexone has been approved in 2008 for the treatment of opioid-induced constipation and nalfurafine is used for the treatment of pruritus (severe itching of the skin).[16b] Structures of morphine and its derivatives are given in chart 1.2. The abuse of opioid drugs can lead to addiction. For example, heroine is a highly addictive drug and a large dose of it depresses heart rate and breathing which can become fatal. Regular use of the compound in small quantities also affects the physical and mental condition of the addicted person and can lead to depression as well as antisocial personality disorder.

Chart 1.2: Structures of morphine and its derivatives



Other important analgesic alkaloids include thebaine (fig. 1.26, paramorphine), isolated from Iranian poppy (*Papaver bracteatum*) and cocaine (fig. 1.26), isolated from *Erythroxylum coca* belonging to Erythroxylaceae family.[16a] Oxycodone, a semi-synthetic derivative of thebaine, is used as an analgesic as well used for the treatment of cough. Naloxigol (fig. 1.26, PEGylated naloxol), another derivative of thebaine, is used to treat opioid-induced constipation.[18b]

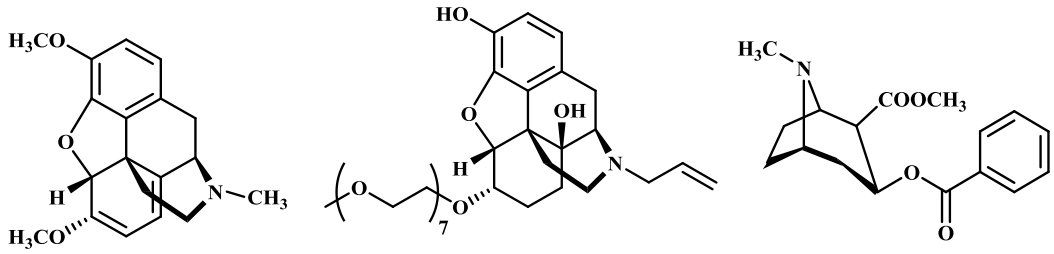


Figure 1.26: Thebaine Naloxigol Cocaine

Cannabinoids, a class of secondary metabolites isolated from the plant *Cannabis sativa* shows exquisite analgesic activity. A mixture of dronabinol and cannabidiol (fig. 1.27, marketed as Sativex) was approved in 2006 to alleviate neuropathic pain.

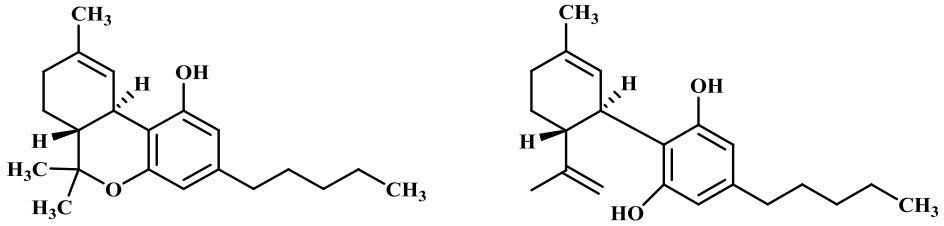


Figure 1.27: Dronabinol Cannabidiol

Another important plant derived analgesic compound is capsaicin isolated from plants belonging to *Capsicum* sp. The Capsaicin 8% Patch (marketed as Qutenza) was approved by the FDA for the management of neuropathic pain associated with postherpetic neuralgia in 2009. Zucapsaicin, the *cis*-isomer of capsaicin (fig. 1.28, manufactured by Winston Pharmaceuticals), got approval as an analgesic agent in 2010.

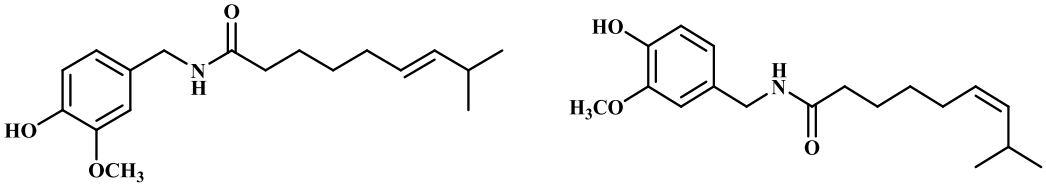
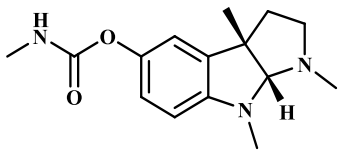


Figure 1.28: Capsaicin Zucapsaicin

Plant-based NPs, as well as their derivatives are also finding application in the management of Alzheimer's disease. Physostigmine (fig.1.29), isolated from *Physostigma venenosum* belonging to Fabaceae family has been used for years to relieve pain and to treat central nervous system (CNS) diseases. The compound obtained approval for the treatment of Alzheimer's disease and in 2006. It is now approved for the treatment of Parkinson's disease also. Galantamine (fig. 1.29), an alkaloid isolated from the bulb of *Galanthus woronowii* belonging to Amaryllidaceae family, is also used for the treatment of Alzheimer's disease (used as galantamine

hydrobromide). The compound acts by inhibiting the acetylcholinesterase (AChE) as well as by modulating the nicotinic acetylcholine receptor (nAChR). Many more plant-based acetylcholinesterase inhibitors are now in various stages of clinical trials.[17,81]



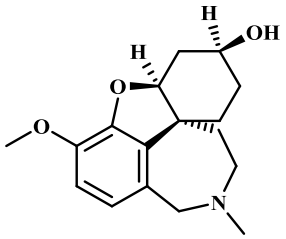
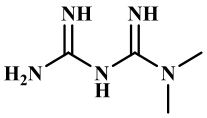


Figure 1.29: Physostigmine Galantamine

1.5.5. Antidiabetic drugs from plants

Diabetes is a metabolic disease in which the body does not produce or does not respond properly to the hormone insulin. There are two major types of diabetes; type I and type II. Type I diabetes results from body's failure to produce sufficient insulin, whereas type II diabetes (considered as a lifestyle disease) arises due to the inability of the cells to properly use insulin. Herbal medicine has played a significant role in managing diabetes in Asia and Africa for centuries. Standardized herbal extracts such as Glucosol (an extract from the leaves of *Lagerstroemia speciosa* standardized to 1% corosolic acid**)** and BGR-34 [a mixture of ingredients from four plants described in *Ayurveda*, launched by the Council for Scientific and Industrial Research (CSIR), India in 2016] are used to manage type II diabetes. Apart from the herbal mixtures, large number of individual phytochemicals have undergone clinical trials and a few are already in clinical use.[82]

Metformin (fig. 1.30, dimethylbiguanide), the most widely used oral antidiabetic agent all over the world, was developed from the natural compound galegine. Galegine (fig. 1.30, isoamylene guanidine) was isolated from the plant *Galega officinalis* (French lilac, goat's rue; Fabaceae family) which is known as a folk medicine in Europe, for the treatment of diabetes. The compound galegine was also found to lower the blood glucose level and served as a model for the synthesis of metformin. Metformin significantly reduces the blood glucose level and it is found to be less toxic compared to the parent compound. The compound was given FDA approval in 1994 for the treatment of type II diabetes mellitus. The precise mechanism of action of the compound is still unclear.[83]



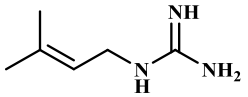


Figure 1.30: Metformin Galegine

Phlorizin (fig. 1.31) is an important dihydrochalcone glycoside isolated from the bark of apple tree (*Malus domestica* belonging to Rocaseae family).[84] The compound lowers glucose plasma levels and improves insulin resistance levels through inhibition of sodium glucose co-transporters (SGLT-2).[85] However, the compound was halted from becoming a drug, due to the drawbacks such as poor intestinal absorption and inactivation by lactase-phlorizin hydrolase.[81]

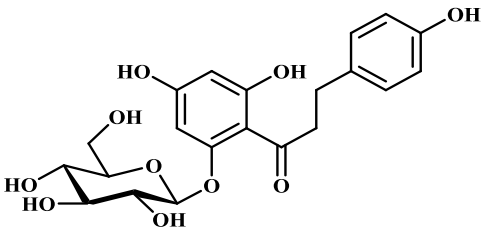
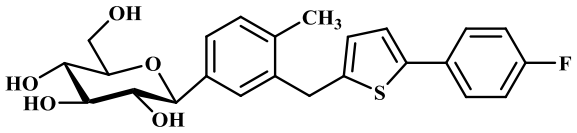


Figure 1.31: Phlorizin

Even though phlorizin did not advance into clinical development, it served as lead for the development of new class of SGLT-2 inhibitors. Two new advanced phlorizin analogues viz., canagliflozin and empagliflozin (fig. 1.32) got FDA approval in 2013 and 2014 respectively for the treatment of type II diabetes.[18a,b]



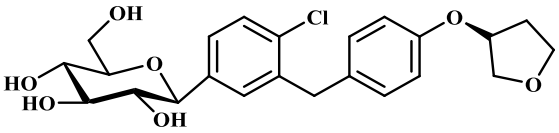


Figure 1.32: Canagliflozin Empagliflozin

1.5.6. Anticancer drugs from plants

1.5.6.1. Cancer

Cancer is the most dreadful disease of the twenty-first century which claims millions of lives worldwide per annum. It stands second to cardiovascular diseases as a leading cause of death in developing countries. It is the single cause of nearly 12.5% of overall mortality rates. It is estimated that by 2030, the number of cases and deaths caused by cancer would rise above 21.4 million and 13.1 million respectively.[86]

Cancer is not a single disease but a group of related malignant diseases characterized by the uncontrollable and abnormal growth of cells in the body. During the normal growth and development of an organism, cells die in an ordered and controlled manner upon receiving specific signals, generally known as apoptosis. In the case of cancer, there will be too little apoptosis and the cells will ignore the normal cellular signals, which results in their uncontrollable growth and proliferation. Cancer begins with the division of a single abnormal cell, which continues to divide without stopping. Accumulation of these abnormal cells in the body results in the formation of tumors. These abnormal cells can also spread to other parts of the body through the blood stream, known as metastasis and can become fatal. Tumors which cannot metastasise are known as benign tumors and are not generally harmful to health.[87]

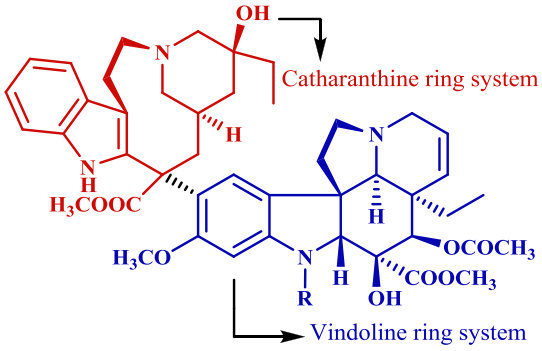
The major causes of cancer include unhealthy life style and other environmental factors such as usage of tobacco, exposure to chemicals, ionizing radiations etc. Tobacco smoking is the prime reason for lung cancer and cancers associated with other respiratory organs and mouth. Even though, ethanol is not carcinogenic, over consumption of alcohol may also lead to cancer. Alcohol consumption along with smoking can synergistically increase the risk of cancer. Prolonged exposure to carcinogens such as aromatic amines, benzene, asbestos, heavy metals, pesticides and other organic chemicals can initiate cancer. Another prominent reason for cancer is chronic viral infections, which accounts for nearly 18% of the cancer cases worldwide. *Hepatitis* B and C viruses, *Human papilloma* viruses, *Helicobacter pylori*, *Schistosoma haematobium* etc., are the pathogens responsible for virus-induced cancers. Obesity, unhealthy diet, lack of exercise, excess use of salt, inherited mutation in genes etc., can also initiate the risk of cancer in human.[88,89]

Common treatment options available for cancer include surgery, chemotherapy and radiation therapy, but in most of the cases, combinations of these three are used. Nowadays, many methods such as immunotherapy, targeted therapy, hormone therapy, stem cell therapy and photodynamic therapy are emerging as new tools for the treatment of cancer. Even then, chemotherapy is the most promising treatment option for patients with advanced cancer. Chemotherapy or ‘chemo’ is referred to the use of medicines/drugs to treat cancer. These drugs enter into the body through the blood stream and work by killing, preventing or slowing down the growth of cancer cells. Sometimes, only chemotherapy is sufficient to cure cancer (especially for haematological cancers) but in many cases, the combination of chemotherapy with

radiotherapy or surgery is found to be more effective. There are two types of chemotherapy. Chemotherapy before surgery/radiation to reduce the tumor volume is known as neo-adjuvant chemotherapy whereas the use of drugs after surgery/radiation, to destroy the remaining tumor, is termed as adjuvant chemotherapy.[90] Plants are the most fruitful source of chemotherapeutic agents due to their unique mode of action. Some of the important chemotherapeutic agents developed from plants and their mechanism of action are explained in the following section.

1.5.6.2. Vincristine and vinblastine

The well-known vinca alkaloids, vincristine and vinblastine, are the first natural compounds that advanced into clinical use for the treatment of cancer. Vincristine (previously known as leurocristine)[91] and vinblastine (earlier known as vincaleukoblastine)[92] were isolated from Madagascar periwinkle *Catharanthus roseus* (L.) G. Don (*Vinca rosea* L.) belonging to Apocynaceae family (fig. 1.33). *C. roseus* was widely used for the treatment of diabetes in various traditional systems of medicine and during the course of investigation of hypoglycemic agents from the *C. roseus*, it was observed that extracts of the plant possess very good antileukemic activity. This serendipitous observation paved the path for the isolation and structural elucidation of the most promising antitumor vinca alkaloids viz., vincristine and vinblastine.[93,94]



R = CHO; Vincristine
R = CH_3; Vinblastine

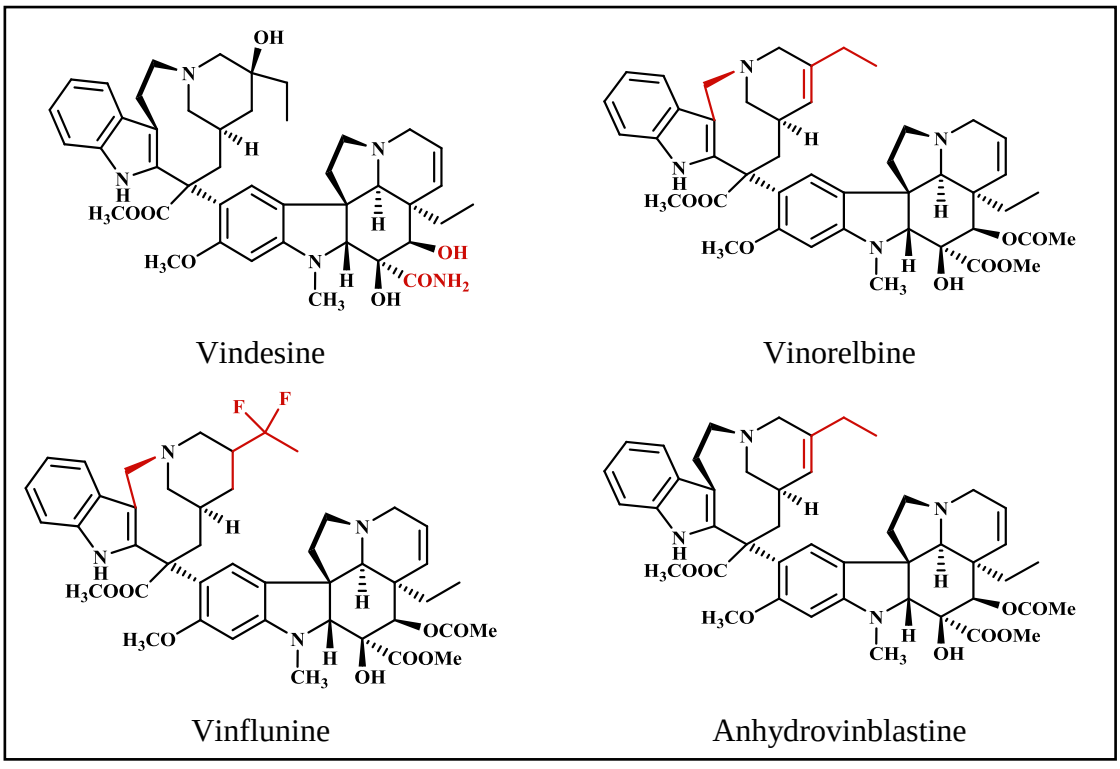
Figure 1.33: *Catharanthus roseus*

Both vincristine and vinblastine are dimeric indole-indoline alkaloids in which a catharanthine ring system is connected to a vindoline ring system by a single bond. Even though vinblastine was isolated before vincristine, the latter got FDA approval first. Vincristine was approved by US FDA in 1963 and is used for the treatment of acute lymphoblastic leukemias and lymphomas. Vinblastine got FDA approval in 1965 and is used for the treatment of bladder and breast cancers. Both these compound were

proven to be very effective against Hodgkin's lymphoma (a type of blood cell tumor that develops from lymphatic cells). Cytotoxicity of these alkaloids could be attributed to their interaction with tubulin proteins. These compounds inhibit tubulin polymerization by binding to the *β*-tubulin heterodimers and hence prevent the formation of microtubulin polymers which in turn halt cell division.[93,95]

After the success of vinblastine, many analogues of the alkaloid were prepared and evaluated for their antitumor activity. Vindesine[96] was the first semi-synthetic analogue of vinblastine to enter into the market. The compound is used to treat acute lymphocytic leukemia, lung carcinomas, breast cancer and chronic myelogenous leukemia. 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.[97] Fluorinated derivative, vinflunine,[98] entered the market in 2009 and is used in the metastatic treatment of bladder cancer. Another derivative anhydrovinblastine is under clinical trials for the treatment of advanced solid tumors.[94] Structures of important vinblastine derivatives are given in chart 1.3. Differences from basic vinblastine structure are indicated using red colour.

Chart 1.3: Structures of vinblastine derivatives



1.5.6.3. Camptothecin and its derivatives

The ground-breaking discovery of plant-derived anticancer compounds was made by the research team lead by Monroe E. Wall and Mansukh C. Wani of Research Triangle Institute (RTI) in North Carolina as part of their search for novel anticancer agents from natural sources, an initiative put forward by Cancer Chemotherapy National Service Center (CCNSC) formed by National Cancer Institute (NCI), USA. Two novel compounds viz., camptothecin and taxol having the unique mechanism of action were discovered by them. Both these compounds were later proved to be life-saving drugs for millions suffering from cancer.[99]

Camptothecin (CPT) is a pentacyclic quinoline based alkaloid isolated from the wood bark of *Camptotheca acuminata,* a native of China (belonging to Nyssaceae family, fig. 1.34). The compound was isolated by Wall and Wani in 1958 through the method of bioactivity-guided fractionation. The structure of the compound was established in 1960 using various spectroscopic techniques and X-ray analysis; however these findings were published only in 1966.[100] Two natural derivatives of CPT with potent antileukemic activity viz., 10-hydroxycamptothecin (HCPT) and 10-methoxycamptothecin were also isolated from the plant by the same group in 1969.[101] HCPT was found to be more potent than CPT in terms of its cytotoxic activity.

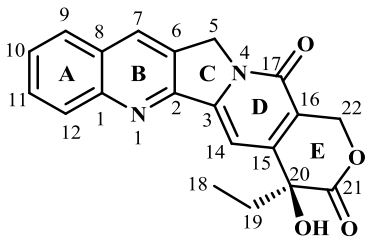


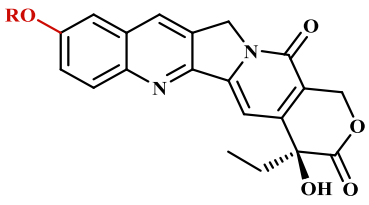
Figure 1.34: *Camptotheca acuminata* Camptothecin

CPT acts through inhibiting the topoisomerase I (T-I) enzyme which is involved in the winding and unwinding of DNA. CPT blocks the reanneling step, resulting in the accumulation of cleavable complex and it has been proposed that the compound interacts with DNA topoisomerase I or a topoisomerase I-DNA complex in a noncovalent manner.[102] From the structure-activity relationship (SAR) studies, it has been found that the hydroxylation at the A ring, especially at position C-10, will increase the activity whereas the oxidation of B ring nitrogen will reduce the activity.

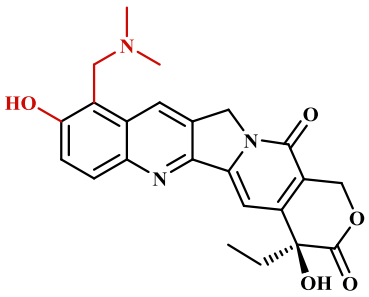
α-Hydroxyl group at position C-20 and the lactone ring at the E ring were found to be crucial in providing the anticancer activity. Acetylation of the compound resulted in complete loss of the activity.[99b]

Even though CPT was found to be a very potent antileukemic and antitumor agent, the reduced water solubility and undesirable side effects in patients resulted in a long delay in the development of CPT as a drug. Topotecan (hycamtin) and irinotecan (camptosar), the semi-synthetic derivatives of CPT[103] with good water solubility and improved activity, are now in clinical use. These compounds were approved by FDA in 1996. Topotecan is used for the treatment of ovarian and lung cancers where as irinotecan finds application in the treatment of colon and rectal cancers. These derivatives contain amino group in them which can be easily converted into hydrochloride salts and thus increase the solubility in water. Similar to CPT, these derivatives also act through inhibiting the DNA topoisomerase I.[104] A combination of hycamtin and *cis*-platin got FDA approval in 2006 for the treatment of cervical carcinoma. Several other derivatives such as karenitecin, gimatecan, diflomotecan, elomotecan etc., are now under various stages of clinical trials for the treatment of different types of cancers. Structures of CPT derivatives are given in chart 1.4. Differences from the primary structure are indicated using red colour.

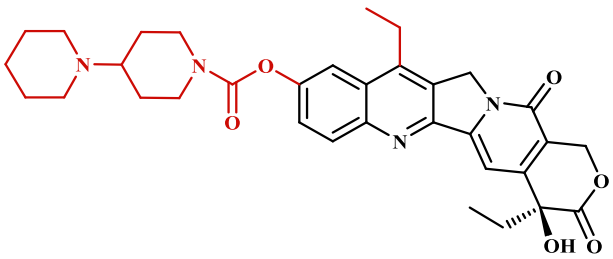
Chart 1.4: Structures of camptothecin derivatives



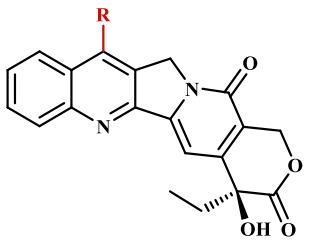
R = H; 10-hydroxycamptothecin
R = CH_3; 10-methoxycamptothecin



Topotecan

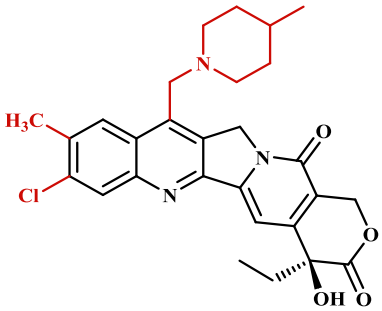


Irinotecan

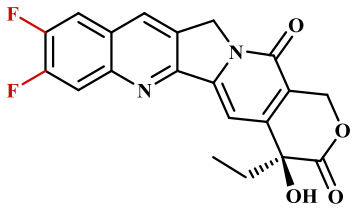


R = $–C_2H_4Si(CH_3)_3$; Karenitecin
R = $–CH=N-O-C(CH_3)_3$; Gimatecan

Chart 1.4 continues...



Elomotecan



Diflomotecan

1.5.6.4. Taxol and its derivatives

Taxol (generic name paclitaxel) is a complex diterpene isolated from the bark of slow growing Pacific yew tree, *Taxus brevifolia* (fig. 1.35) belonging to Taxaceae family. The compound was isolated in 1966 by Wall, Wani and co-workers. The complete structural characterization of the compound was achieved in 1971[105] and it got FDA approval in 1992 for the treatment of refractory ovarian cancer. The compound is currently employed for the treatment of breast cancer, lung cancer and AIDS-related Kaposi's sarcoma. The compound was given the name 'taxol' much before unravelling its complete structure. The name was given based on the facts that it was obtained from the genus *Taxus* and contained an alcoholic functional group. Later it has been established that taxol possesses a basic [9.3.1.$0^{3,8}$] pentadecane tetracyclic ring system with *N*-benzoyl-*β*-phenylisoserine side chain attached to the C-13 hydroxyl as an ester linkage. Other three ester groups were found to be at C-2, C-4 and C-10 positions. The free alcoholic groups were attached to C-1 and C-7 positions. One of the unique structural features of the compound is the presence of 4-membered oxide ring which is not commonly found in any of the other natural taxanes.[99b]

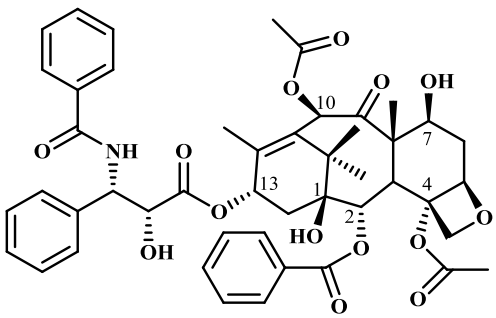


Figure 1.35: *Taxus brevifolia* Taxol

Despite its enormous cytotoxic potential, it took almost 20 years for the compound to enter into clinical use. The two major hurdles in the development of taxol were the supply of natural source for the compound and limited solubility. The yield of taxol from the bark was very low and the collection of the bark would also lead to the death of the slow growing tree. Supply crisis was solved by developing the semi-synthesis of taxol from a readily available natural product, 10-deacetyl baccatin III (fig. 1.36, 10-DAB) which possesses the complex tetracyclic ring system. 10-DAB can be isolated from the regenerating needles of European yew tree, *Taxus baccata* (also found in lower Himalayas).[106] Synthesis of taxol was achieved from 10-DAB by attaching the synthetic version of the side chain at position 13. Nowadays taxol is also commercially produced (by Phyton Biotech) through the method of plant cell fermentation/plant tissue culture.[107]

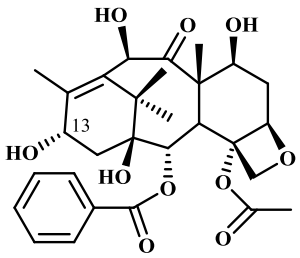


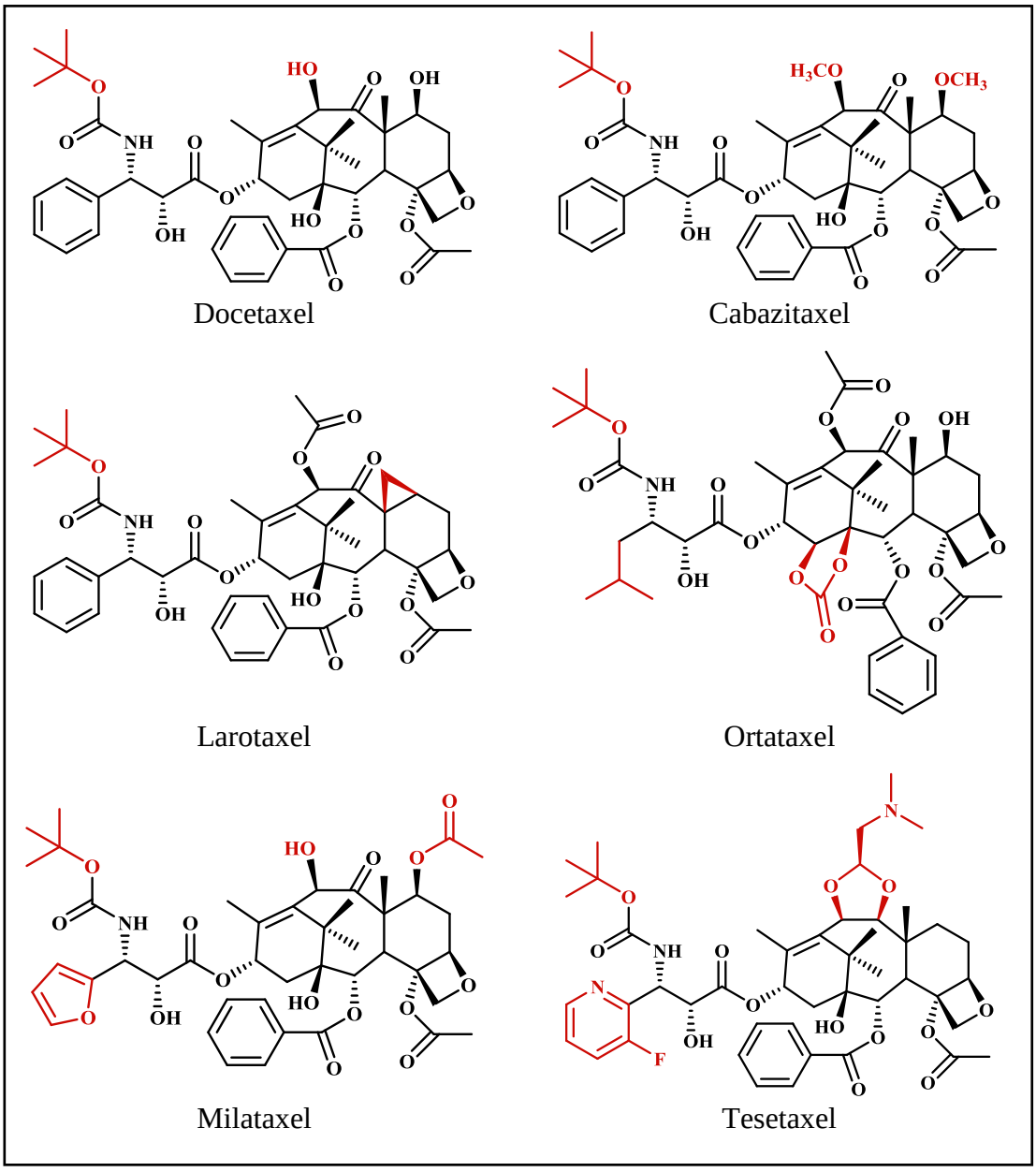
Figure 1.36: 10-deacetyl baccatin III

A significant breakthrough in the development of taxol happened when Susan Horwitz and co-workers at Albert Einstein College of Medicine, Yeshiva University, New York, revealed the unprecedented mechanism of action of the compound.[108] In contrast to many of the natural compounds which inhibit the microtubule formation, taxol acts through the stabilization of microtubules. The compound promotes the assembly of tubulin into microtubules and thus disturbs the equilibrium between the soluble and polymeric forms of tubulin. The compound induces cell cycle arrest at the G2/M phase and leads to cell death through apoptosis.[94] SAR studies of the compound were reported by Kingston in 1991, which suggested that the oxetane ring and phenylisoserine side chain were essential for the activity and modification on the taxane ring had remarked effect on the activity of the compound.[109]

Taxol served as lead for the development of other taxane type anticancer compounds with similar mechanism of action. The first analogue of taxol which was introduced for clinical use is docetaxel (taxotere).[110] The compound was approved in 1995 and is used as a single drug or in combination with other anticancer drugs for the

treatment of prostate, breast, head, neck, stomach, and ovarian cancers. Abraxane, the albumin-bound nanoparticle of paclitaxel, got FDA approval in 2005 for the treatment of advanced breast cancer. Another derivative, cabazitaxel was approved in 2010. Derivatives such as larotaxel, ortataxel, milataxel, tesetaxel etc., are now in the pipeline for the development as new drugs.[16a] Structures of taxol derivatives are given in chart 1.5. Differences from the primary structure are indicated using red colour.

Chart 1.5: Structures of taxol derivatives



1.5.6.5. Podophyllotoxin and its derivatives

Podophyllotoxin is an aryl tetralin lignan isolated from the resin of *Podophyllum peltatum* roots (Devil's apple or Mayapple), belonging to Berberidaceae family (fig. 1.37). The compound was isolated from the plant in 1880 by Podwyssotzki, but the exact structure of the compound was established only in 1950s.[93] The compound is also present in other *Podophyllum* species such as *P. emodi* and *P. pleianthum*. Even though the compound showed promising anticancer activity, it did not enter into the clinical use due to its high toxicity. The compound is a mitotic inhibitor acting on tubulin. It prevents the polymerization of tubulin to microtubule and thus arrests the cell cycle.[111]

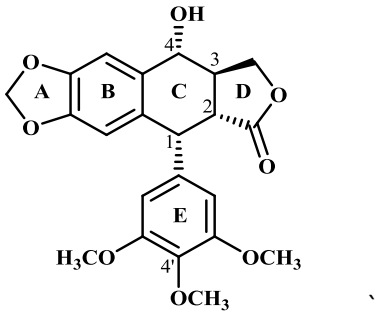
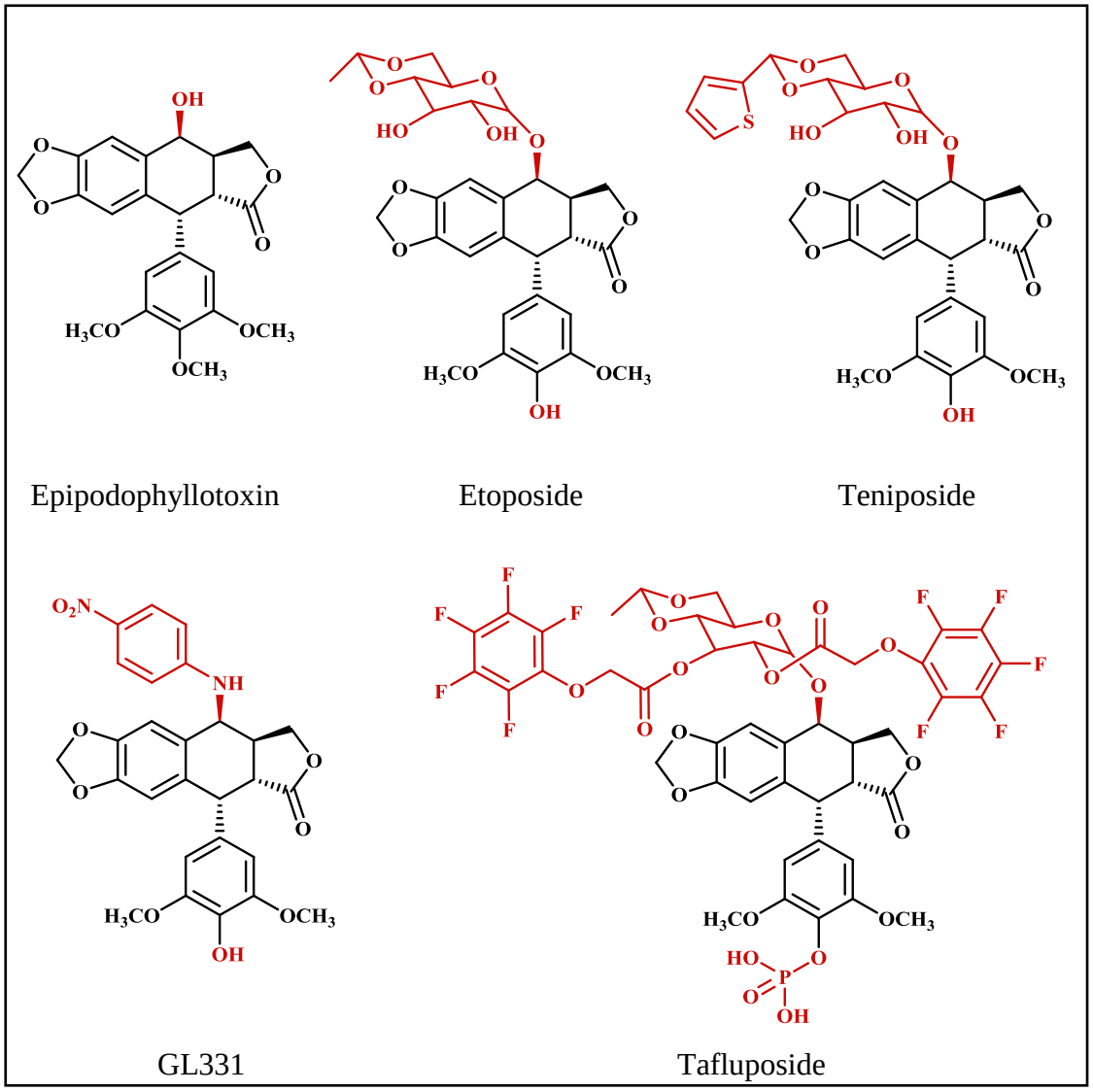


Figure 1.37: *Podophyllum peltatum* Podophyllotoxin

Two important anticancer drugs viz., etoposide and teniposide were later obtained through semi-synthesis of the natural compound, epipodophyllotoxin (C-1 epimer of podophyllotoxin which can be synthesized from podophyllotoxin[112]). In etoposide and teniposide, the α-C-4-hydroxyl group in the C ring was replaced by β-C-4-glucosyl group and the methoxy group at C-4' in the E ring has been changed to hydroxyl group. These glycoside derivatives were more soluble and less toxic compared to the parent compound and are now used for the treatment of small-cell lung cancer, testicular cancer, leukemias, lymphomas and other cancers. Mechanism of these derivatives was found to be different from that of podophyllotoxin. Etoposide, teniposide as well as epipodophyllotoxin do not affect mitosis, but inhibit cell cycle progression prior to mitosis in the late S or early G2 phase. They inhibit the essential enzyme DNA topoisomerase II which results in the cellular double-strand DNA breaks.[113,114] SAR studies have revealed that 4β-configuration at the C ring, *trans* lactone (D ring), free rotation of ring E, 4'-OH etc., are crucial in providing the anticancer activity.[115]

Etopophos, a highly water-soluble prodrug of etoposide, which contains a phosphate group at C-4' position was approved for intravenous use by FDA in 1996. Other derivatives such as tafluposid, GL331 etc., are now under clinical trials.[16a,104] Structures of podophyllotoxin derivatives are given in chart 1.6. Differences from the basic structure are indicated using red colour.

Chart 1.6: Structures of podophyllotoxin derivatives



1.5.6.6. Homoharringtonine

Homoharringtonine (fig. 1.38) is a cephalotaxus alkaloid ester isolated from the ever green tree *Cephalotaxus harringtonia* (Japanese plum yew, fig. 1.39), belonging to Cephalotaxaceae family. Homoharringtonine was isolated by Powell *et al.*, in 1970.[116] The compound is also obtained from other *Cephalotaxus* species such as *C. fortunei*, *C.*

hainanensis etc. Homoharringtonine (Omacetaxine mepesuccinate) got FDA approval in the year 2012 for the treatment of acute myeloid leukemia.

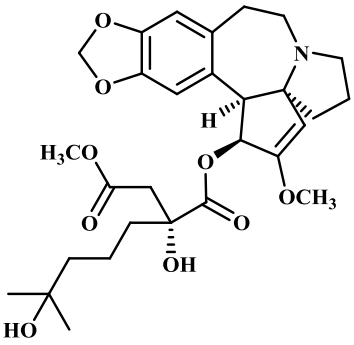
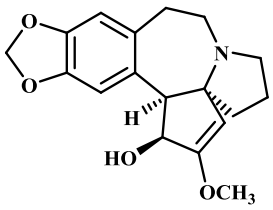


Figure 1.38: *Cephalotaxus harringtonia* Homoharringtonine

The racemic mixture of homoharringtonine and harringtonine[117] (isolated from *C. harringtonia*) has been used in China for the treatment of acute and chronic myelogenous leukemia.[93] Both homoharringtonine and harringtonine are esters of cephalotoxine[118] (fig. 1.39) which differ only in the number of methylene carbons in the ester side chain. Homoharringtonine acts by inhibiting the protein synthesis at the ribosomal level and blocks the cell-cycle progression.[12,119]



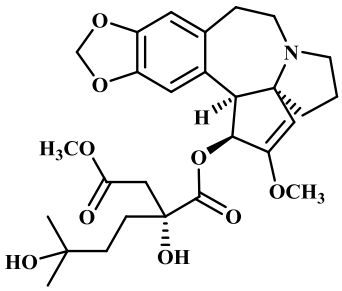
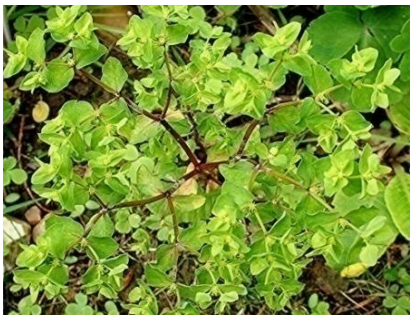


Figure 1.39: Cephalotoxine Harringtonine

1.5.6.7. Ingenol mebutate

Ingenol mebutate (fig. 1.40) is a tricyclic diterpene ester isolated from the sap of *Euphorbia peplus* (milkweed) belonging to Euphorbiaceae family.[120]



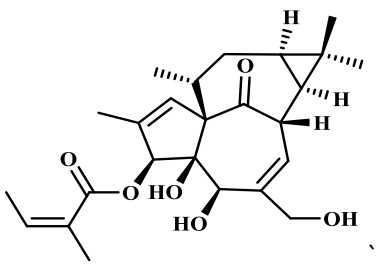


Figure 1.40: *Euphorbia peplus* Ingenol mebutate

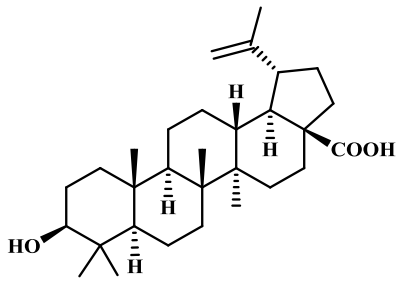
Extracts of the *E. peplus* have been used for treating various skin diseases in traditional Thai and Australian medicines. Gel formulation of ingenol mebutate got FDA approval in 2012 for the treatment of actinic keratosis. Actinic keratosis (also known as solar keratosis) is a type of precancerous dermatological condition that can progress to invasive squamous cell carcinoma. Increased exposure to UV radiation can lead to actinic keratosis. Even though the exact mechanism of action of the compound is poorly understood, it has been reported that the topical application of ingenol mebutate resulted in local induction of necrosis and inflammation.[121]

Apart from the above-mentioned anticancer agents, many more plant derived NPs and their derivatives are now under various stages of clinical trials.[16,122] Important plant derived unmodified natural compounds which are under different phases of clinical trials are listed in table 1.3. Structures of the compounds are shown in chart 1.7.

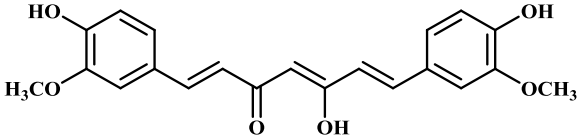
Table 1.3: Important plant derived unmodified anticancer NPs in clinical trials

Sl. No.	Compound	Major source	Family
1	Betulinic acid	*Ziziphus mauritiana*	Rhamnaceae
2	Curcumin	*Curcuma longa*	Zingiberaceae
3	Genistein	*Glycine max*	Fabaceae
4	Gossypol	*Gossypium* sp.	Malvaceae
5	Lycopene	*Solanum lycopersicum*	Solanaceae
6	Perillyl alcohol	*Lavendula x intermedia*	Lamiaceae
7	Picropodophyllotoxin	*Podophyllum peltatum*	Berberidaceae
8	Protopanaxadiol	*Panax ginseng*	Araliaceae
9	Resveratrol	*Vitis vinifera*	Vitaceae
10	Silybin	*Silybum marianum*	Asteraceae

Chart 1.7: Structures of plant derived unmodified anticancer NPs in clinical trials

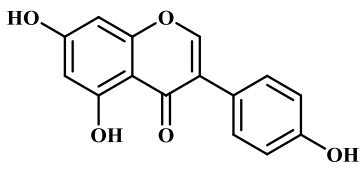


Betulinic Acid

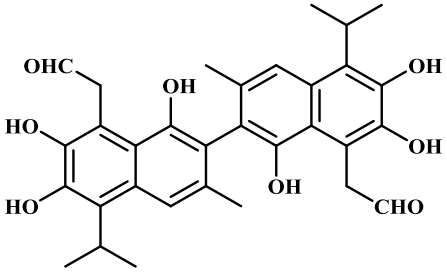


Curcumin

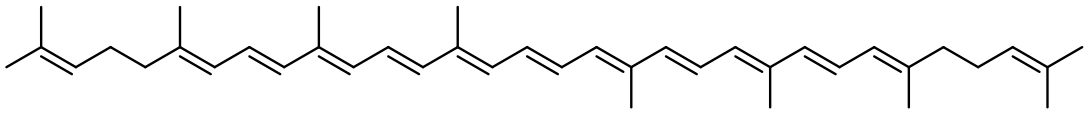
Chart 1.7 continues...



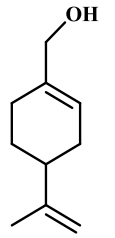
Genistein



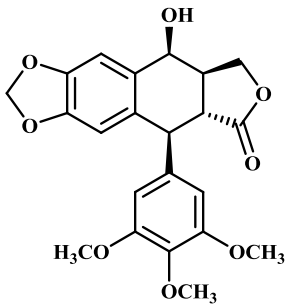
Gossypol



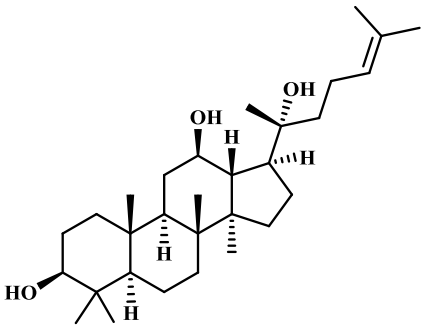
Lycopene



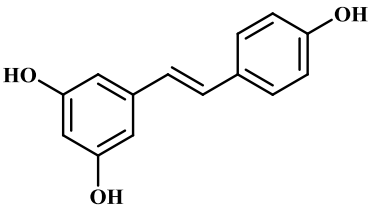
Perillyl alcohol



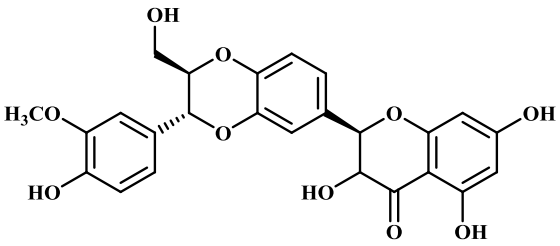
Picropodophyllotoxin



Protopanaxadiol



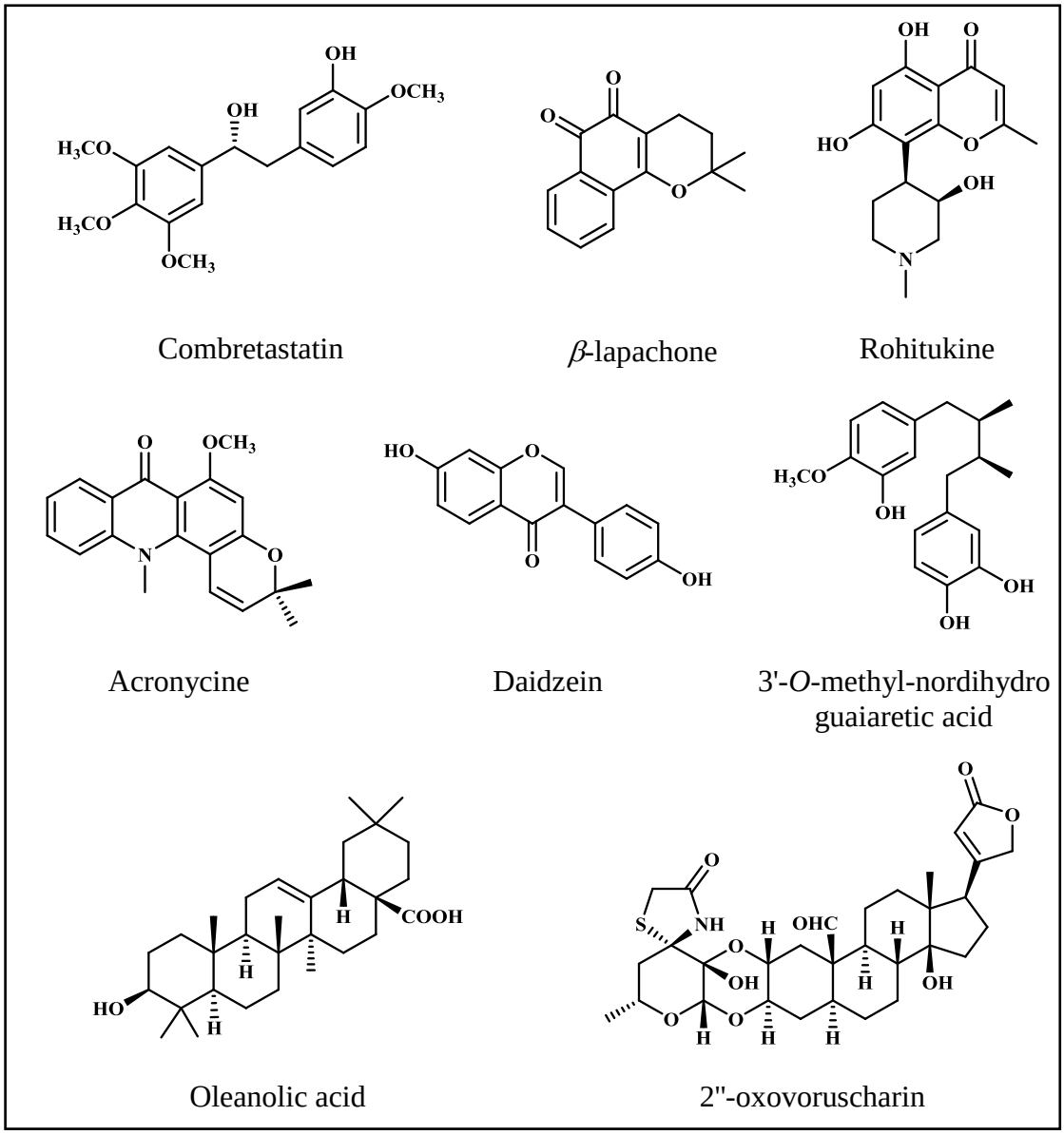
Resveratrol



Silybin

Apart from the single chemical entities from plants mentioned above, different synthetic derivatives of natural compounds such as combretastatin, *β*-lapachone, rohitukine, acronycine, daidzein, 3'-*O*-methyl-nordihydroguaiaretic acid, oleanolic acid, 2″-oxovoruscharin etc., are also now under various stages of clinical trials.[16,122] Structures of the lead compounds named above are given in chart 1.8.

Chart 1.8: Structures of important anticancer lead compounds isolated from plants



1.6. Outline and organisation of the thesis

The preceding discussion clearly depicts the pivotal role of natural products and their analogues in modern drug discovery process. Plants have been in the mainstay as the source of new molecular entities with lead potential. Bioprospecting of medicinal plants, which are used in various traditional systems of medicine have provided many successful drugs with unique mechanism of action. Drugs such as artemisinin, quinine, metformin etc., were all developed based on the knowledge accessed from traditional medicine. India is considered as the "Botanical Garden of World" with approximately 45,000 plant species, of which several thousand of them have been found to be of medicinal use. *Ayurveda*, the traditional system of medicine practiced in India has been

the basis of many modern drugs, including reserpine and has the potential to provide numerous other molecules with lead potential. 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 used in *Ayurveda* during the Ph.D. programme.

Chapter 1 gives a brief introduction to the role of natural products in 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. Special emphasis was given to plant-derived drugs, giving predominance to anticancer agents developed from terrestrial plants.

Phytochemical investigation and bioactivity studies on the medicinal plant *Chonemorpha fragrans* belonging to Apocynaceae family is the subject matter of chapter 2. Results obtained during the antioxidant activity studies of C. *fragrans* and antibacterial activities of the alkaloids isolated are also presented in this chapter.

Chapter 3 of the thesis is divided into two parts. Part A deals with the isolation and characterization of bioactive compounds from the seeds of *Piper cubeba* belonging to Piperaceae family. Part B of this chapter explains the synthetic transformation and anticancer activity studies on cubebin, the major compound isolated from *P. cubeba.*

Isolation of nimbolide from *Azadirachta indica* (belonging to Meliaceae family) leaves as well as the *in vitro* and *in vivo* anticancer activity studies of nimbolide against human colon cancer cell lines is discussed in chapter 4. The antimicrobial activities of nimbolide, desacetylnimbin and amide derivatives of nimbolide have been studied. Further, the activity enhancement when used in combination with antimicrobial agents was also studied and the results obtained are explained in this chapter.

Elephantopus scaber is an important medicinal plant belonging to Asteraceae family. Isolation of major sesquiterpene lactones viz., isodeoxyelephantopin and deoxyelephantopin from *E. scaber* has been carried out for studying their anticancer activities. The anticancer activity studies of these compounds against different cancer cell lines are described in chapter 5.

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Chapter 2

Phytochemical Investigation and Biological Activity Studies on the Medicinal Plant *Chonemorpha fragrans*

2.1. Introduction

As described in the introduction chapter, there is a renewed interest in the exploration of medicinal plants used in traditional systems of medicine since they have the higher chance of containing novel bio-active molecules in them. One such family of plants which enjoys great reputation in traditional systems of medicine all over the world is Apocynaceae. It is commonly known as the 'Dogbane' family, comprising of approximately 424 genera and ~5,100 species of tropical trees, shrubs, woody climbers and herbs, wherein most of them produce milky latex.[1,2]

Important medicinal plants such as *Alstonia scholaris, Catharanthus roseus, Holarrhena antidysenterica, Nerium oleander, Rauwolfia serpentina, Tabernaemontana heyneana, Wrightia tinctoria* etc., belong to this family. Plants belonging to Apocynaceae family are a rich source of important biologically active molecules and since many of them find extensive application in traditional systems of medicine, they have been well explored for their phytochemistry and pharmacology. Many Apocynaceae plants contain cardiotonic glycosides, steroidal alkaloids and iridoids in them.[3]

The alkaloid vinblastine isolated from *Catharanthus roseus* is useful in treating Hodgkin's disease and vincristine sulphate is used to treat acute leukemia in children. Many synthetic derivatives of these compounds were also marketed drugs and more are under clinical trials. An indole alkaloid, reserpine isolated from the plant *Rauwolfia serpentina* has the twin effect of lowering blood pressure and can act as a tranquilizer (details are given in chapter 1).

The genus *Chonemorpha,* belonging to Apocynaceae family is one of the least explored genus in the family and it consists of nearly 15 species of large, evergreen woody vines distributed in tropical and subtropical Asia. The name and distribution of ten most important plant species belonging to the genus *Chonemorpha* are given in table 2.1.[4,5]

Table 2.1: Name and distribution of plants belonging to the genus *Chonemorpha*

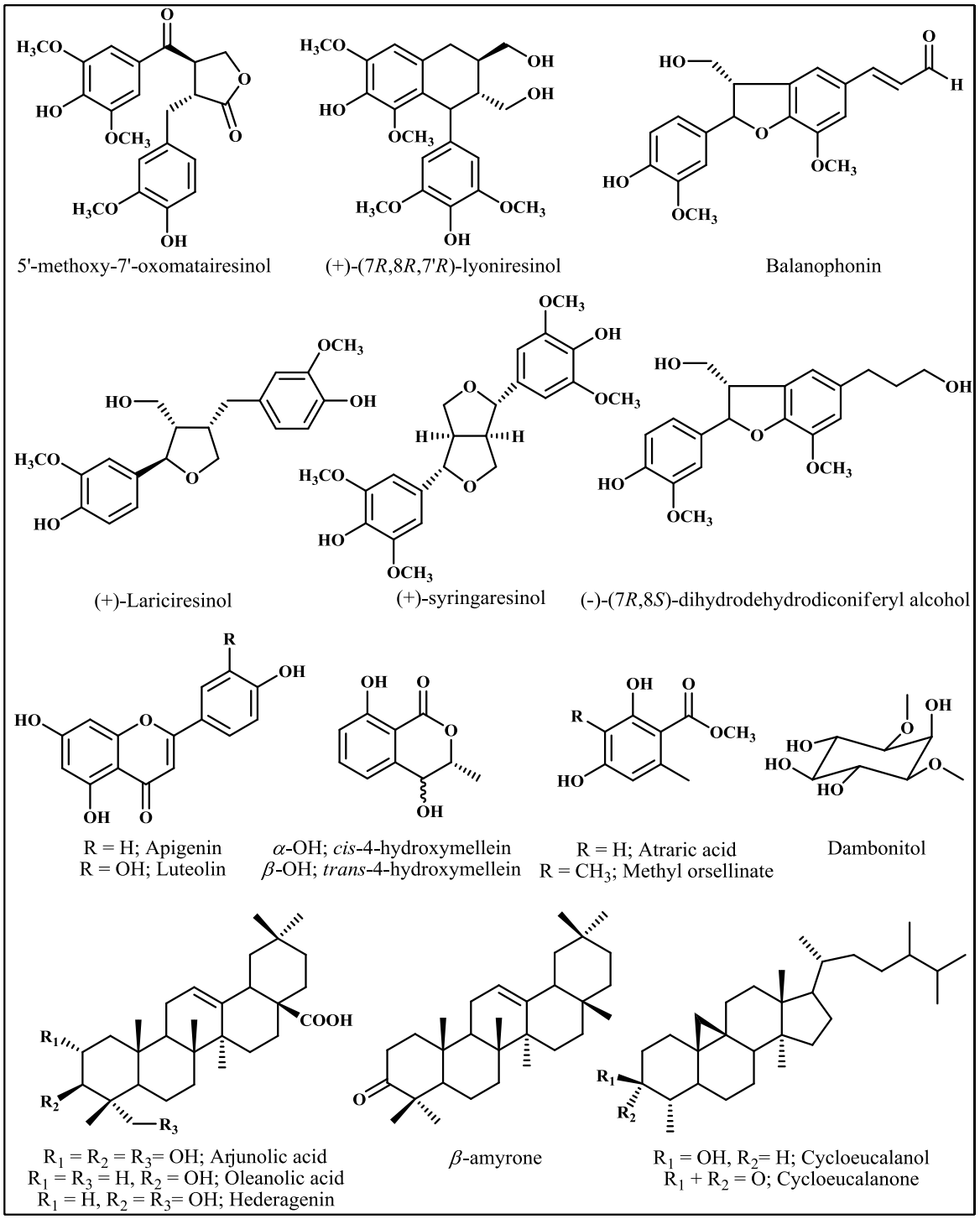
Sl. No.	Plant	Distribution
1	*Chonemorpha assamensis*	India, Bangladesh
2	*Chonemorpha eriostylis*	China, Vietnam
3	*Chonemorpha floccosa*	China
4	*Chonemorpha fragrans*	China, India, Indonesia, Malaysia, Myanmar, Sri Lanka, Thailand
5	*Chonemorpha griffithii*	China, India, Myanmar, Nepal, Thailand
6	*Chonemorpha megacalyx*	China, Laos, Thailand
7	*Chonemorpha mollis*	Java island
8	*Chonemorpha parviflora*	China, India, Myanmar, Nepal, Thailand
9	*Chonemorpha splendens*	China
10	*Chonemorpha verrucosa*	China, Bhutan, India, Indonesia, Laos, Malaysia, Myanmar, Thailand, Vietnam

There are limited reports available on the phytochemistry of the genus *Chonemorpha.* Only two species viz., *C. griffithii* and *C. fragrans* have been studied earlier for their chemical constituents.

2.1.1. *Chonemorpha griffithii*

Chonemorpha griffithii, a woody climber, was earlier considered as a synonym of *Chonemorpha fragrans* but it was recorded as a parallel species of *C. fragrans* by Li *et al*., in the Flora of China.[4] This was later supported by the result obtained during the phytochemical investigation of *C .griffithii* by Bai *et al*., in 2013 who found that there were no overlap of constituents between *C. griffithii* and *C. fragrans.* Around 20 compounds, including six lignans, six triterpenoids, four phenolic acids, two flavonoids, one cyclitol and one aliphatic acid were isolated from the aerial parts of *C. griffithii.* Two triterpenoids viz., cycloeucalanol and cycloeucalanone were identified as the chemotaxonomic markers for the species *C. griffithii.*[6] The name and structure of the compounds isolated from *C. griffithii* are given in chart 2.1.

Chart 2.1: Name and structure of the compounds isolated from *C. griffithii*



2.1.2. *Chonemorpha fragrans*

Chonemorpha fragrans (Moon) Alston [synonym: *C. macrophylla* G. Don, *C. grandiflora*] is an important medicinal plant belonging to the genus *Chonemorpha.* It is commonly known as 'Wood vine' in English, 'Perukurumba' in Malayalam and 'Murva' in Sanskrit.[7,8] The plant is distributed in the moist forests throughout India including Andaman islands.[9] *C. fragrans* is a stout lactiferous climber with large sweet-

scented flowers. The roots of the plant are sweet, bitter, astringent, laxative, digestive, expectorant and febrifuge, which are useful in vitiated conditions of vata and kapha, skin diseases, leprosy, scabies, syphilis, constipation, hyper acidity, diabetes, jaundice etc.[8] Alcoholic extract of *C. fragrans* shows antihyperglycemic effect in alloxan induced diabetic rats, oral glucose tolerant rats, and also shows hypoglycemic effect in fasted rats.[10] The alcoholic extract also possesses skeletal muscle relaxant property. It produces depolarizing type of muscle paralysis similar to that produced by succinylcholine (the drug used for muscle relaxation).[11]

Figure 2.1: Picture of *Chonemorpha fragrans* plant, leaves, flower and root

C. fragrans extract has been screened for its activity against the parasites *Entamoeba hystolica* and *Trichomonas vaginalis*. Selection of *C. fragrans* for carrying out these studies by Shah *et al.*, was based on two aspects viz., (i) knowledge accessed from *Ayurveda* about the anti parasitic action of the species and (ii) phylogenetic relationship of the genus to *Holarrhena antidysentrica* (‘Tellicherry bark’ in English), which is the source of antiamoebic steroidal alkaloid conessine (fig. 2.2).[12] Studies have also revealed that chonemorphine (a steroidal alkaloid isolated from *C. fragrans*) acts as the antiamoebic principle of *C. fragrans*.

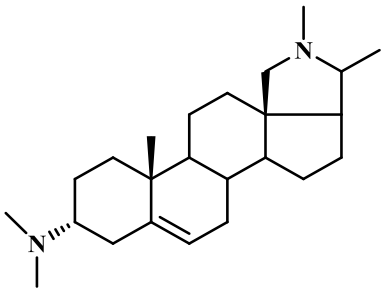
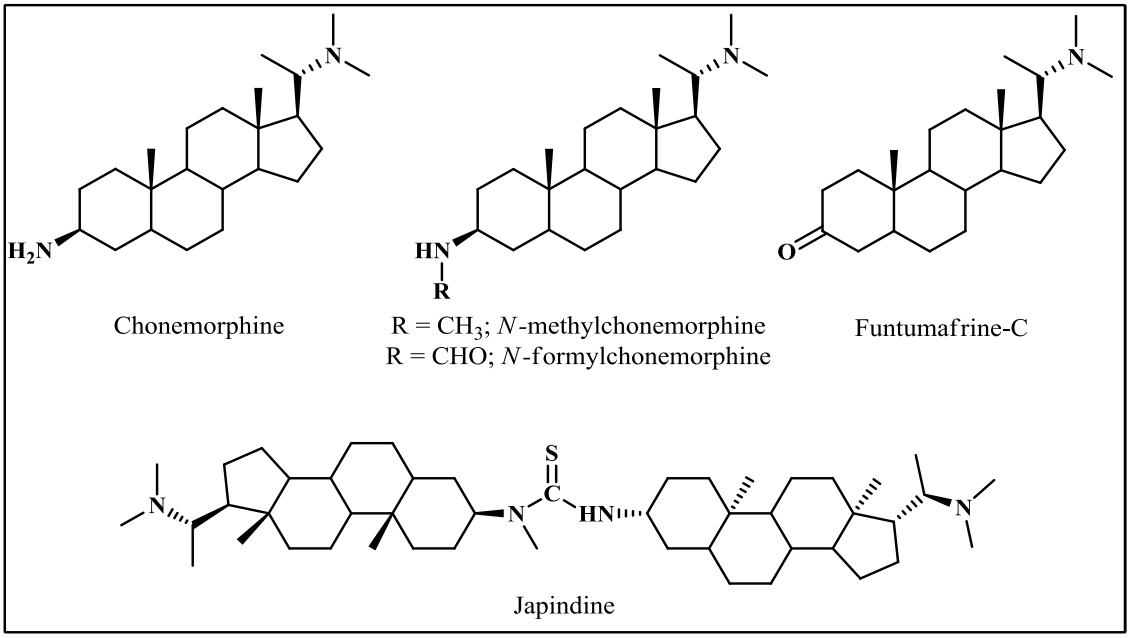


Figure 2.2: Structure of conessine

C. fragrans roots have been reported to contain steroidal alkaloids as the major constituents. Root bark of *C. fragrans* contains 3.03% of total alkaloids.[13] Apart from the alkaloid constituents, non-alkaloids such as borneol acetate and *β*-sitosterol were isolated from the leaves of *C. fragrans* and preliminary phytochemical analysis of root powder suggested the presence of sterols, saponins and flavonoids in it.[14] Phytochemical investigation of *C. fragrans* root has been carried out by Ashima Chatterjee and her group, which resulted in many publications in between 1965-1975. They have isolated pregnane type steroidal alkaloids such as chonemorphine,[15] funtumafrine,[16] *N*-methylchonemorphine, japindine and *N*-formylchonemorphine[17] from the root bark of *C. fragrans*. Structures of these alkaloids are given in chart 2.2.

Chart 2.2: Structures of steroidal alkaloids isolated from *C. fragrans*



The major alkaloid, chonemorphine was established as a promising antiamoebic agent using both *in vitro* and *in vivo* methods. The compound was found to be very effective against the parasite *Entamoeba histolytica*[18] and was also found to have very good antitrichomonal activity against *Trichomonas vaginalis*.[12,19]

From the earlier reports, it is clear that *C. fragrans* roots contain pregnane type steroidal alkaloids as its principal constituent. Structure and stereochemistry of these compounds mainly depend upon the biogenetic pathway from which they are obtained. Since the later sections in this chapter deal with the isolation and characterization of some pregnane type steroidal alkaloids, a detailed discussion of biosynthesis of such alkaloids is included here.

2.2. Biosynthesis of pregnane-type steroidal alkaloids

Pregnane type steroidal alkaloids are defined as compounds possessing a basic pregnane skeleton (fig. 2.3) with amino substitution at either C-3 or C-20 or at both positions. Pregnane type steroidal alkaloids are synthesized from cholesterol, which is made from acetyl-CoA via the formation of three different intermediates viz., isopentenyl pyrophosphate, squalene and pregnenolone.

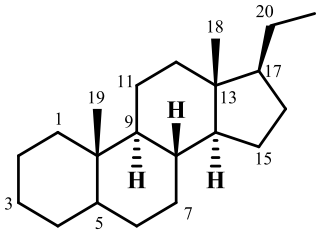
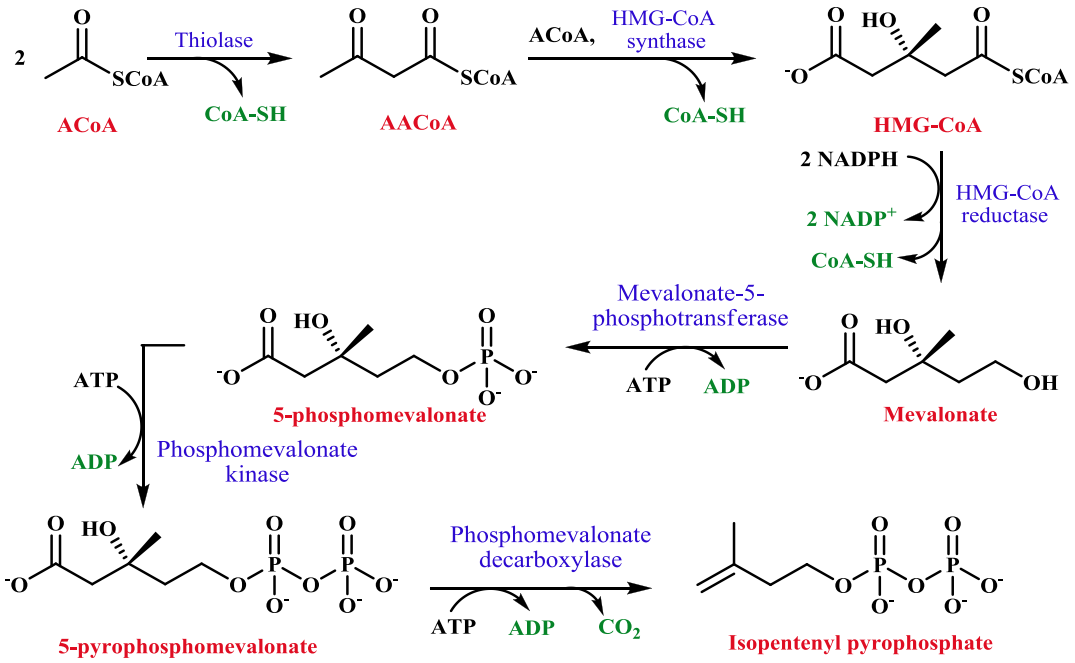


Figure 2.3: Basic pregnane skeleton

2.2.1. Biosynthesis of isopentenyl pyrophosphate (IPP)

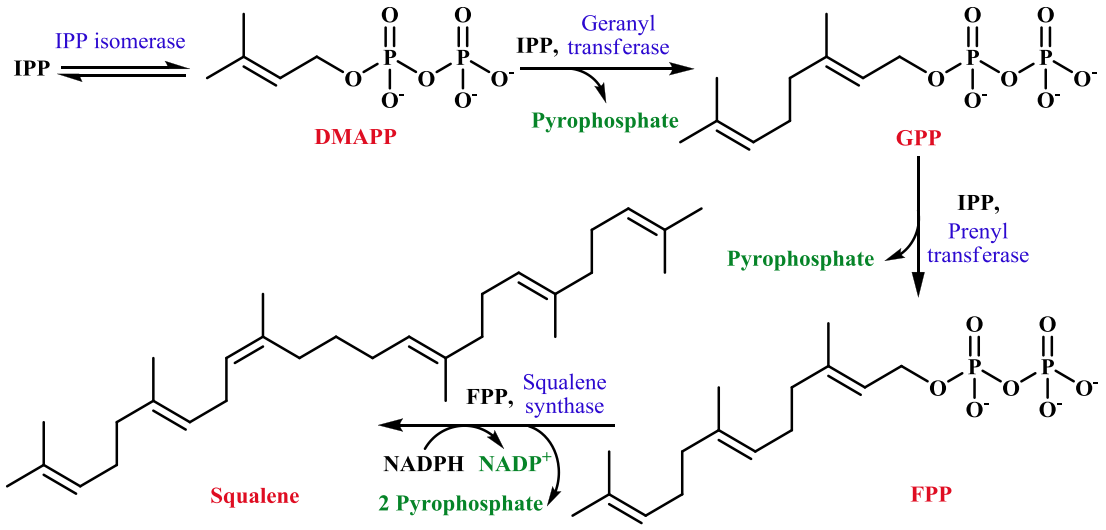
Two molecules of acetyl-CoA (ACoA) condense in presence of thiolase enzyme to form acetoacetyl-CoA (AACoA), which again condenses with one more molecule of ACoA in presence of HMG-CoA synthase to give *β*-hydroxy-*β*-methylglutaryl-CoA (HMG-CoA). In the next step HMG-CoA is reduced by two molecules of NADH in presence of HMG-CoA reductase to form mevalonate. Mevalonate is converted to isopentenyl pyrophosphate (IPP) by utilizing a total of three ATP molecules. Mevalonate first gets converted into 5-phosphomevalonate and then to 5-pyrophosphomevalonate in presence of mevalonate-5-phosphotransferase and phosphomevalonate kinase enzymes respectively by utilizing two molecules of ATP. This 5-pyrophosphomevalonate is then converted into 3-phospho-5-pyrophospho mevalonate utilizing another molecule of ATP. In the next step, phosphate at position 3 and the nearby carboxyl group leave the molecule in presence of phosphomevalonate decarboxylase giving isopentenyl pyrophosphate (scheme 2.1).[20]



Scheme 2.1: Biosynthetic pathway of isopentenyl pyrophosphate

2.2.2. Biosynthesis of squalene

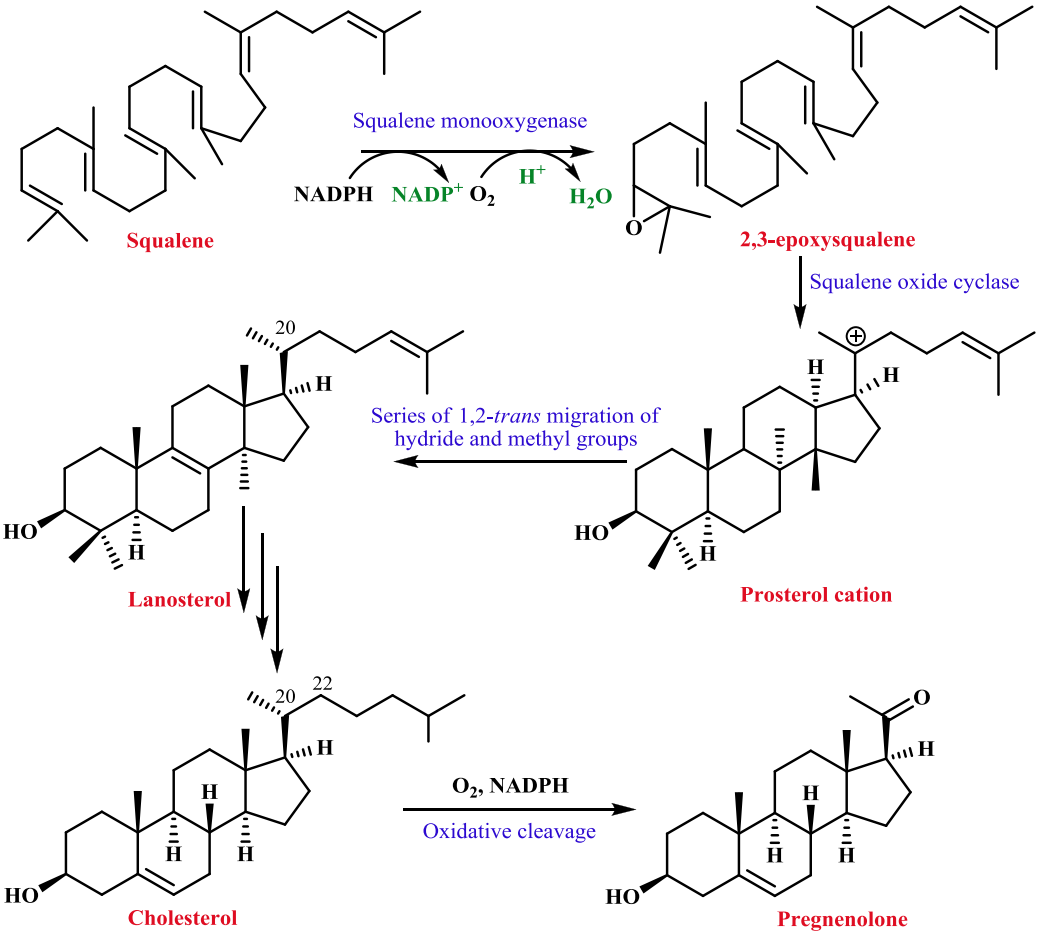
Squalene is synthesized from isopentenyl pyrophosphate (IPP) through a series of enzyme catalyzed reactions. First, IPP undergoes head to tail condensation with dimethylallyl pyrophosphate (formed by the isomerisation of IPP) resulting in the formation of geranyl pyrophosphate (GPP). GPP then undergoes condensation in a head to tail manner with one molecule IPP to yield farnesyl pyrophosphate (FPP). Finally, two molecules of FPP are joined together in a head to head fashion, leading to the formation of squalene (scheme 2.2).[20]



Scheme 2.2: Biosynthetic pathway for squalene from IPP

2.2.3. Biosynthesis of pregnenolone

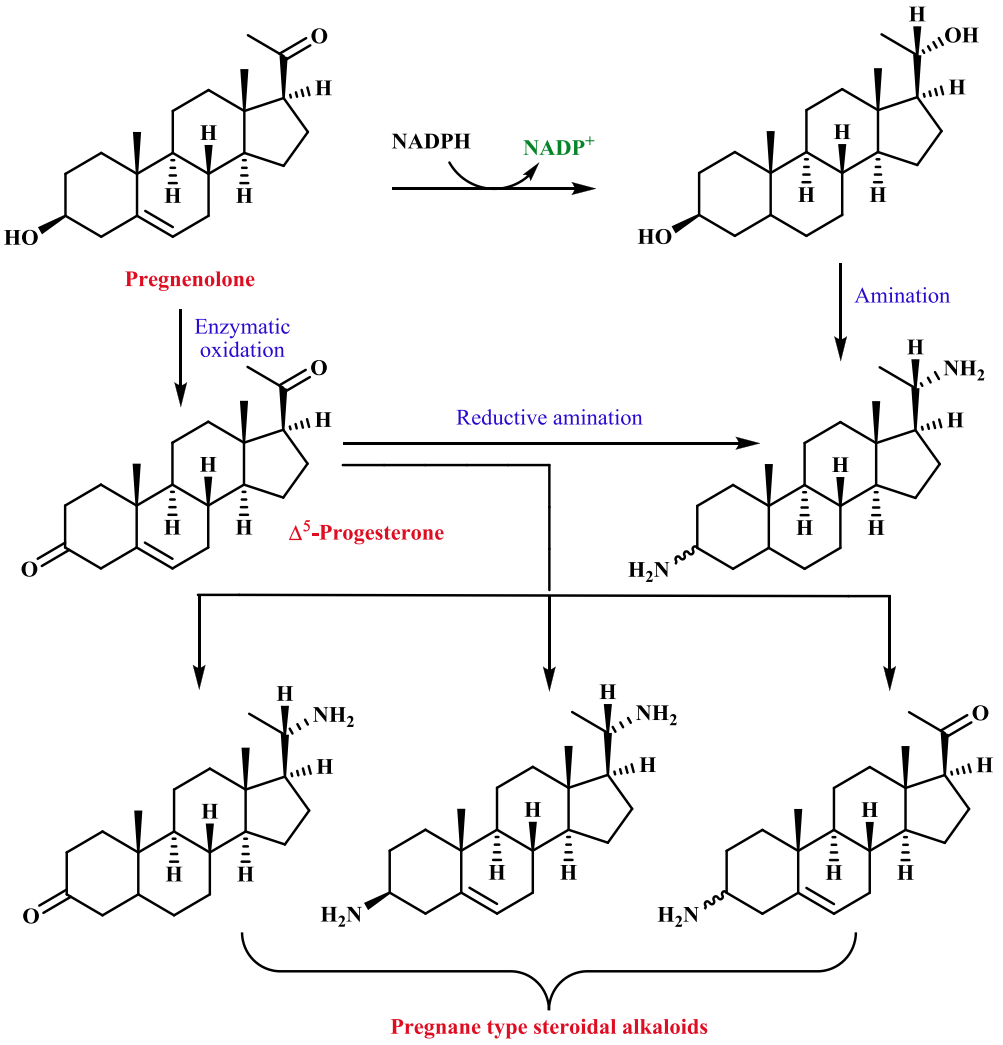
Squalene gets converted into squalene-2,3-epoxide by the action of squalene monooxygenase enzyme in presence of NADH and oxygen. Linear squalene epoxide then gets cyclised by the enzyme squalene oxide cyclase to form prosterol cation, which contains the positive charge at C-20. This prosterol is then converted into lanosterol through a series of 1,2-*trans* migrations of hydride and methyl groups. Lanosterol is then converted to cholesterol through a series of reactions involving nineteen steps, where three methyl groups from lanosterol are oxidized and then removed.[20,21] Cholesterol is then converted into pregnenolone via the oxidative cleavage of the side chain between C-20 and C-22 of cholesterol.[22]



Scheme 2.3: Biosynthetic pathway for pregnenolone from squalene

2.2.4. Biosynthesis of pregnane type steroidal alkaloids

Pregnane-type steroidal alkaloids are obtained from pregnenolone by oxidation of followed by reductive amination. Oxidation of pregnenolone gives Δ^5-progesterone which on reductive amination gives a variety of steroidal alkaloids (scheme 2.4).



Scheme 2.4: Biosynthetic pathway for steroidal alkaloids from pregnenolone

2.3. Aim and scope of the present work

C. fragrans is an important medicinal plant, the roots of which is extensively used in the preparation of many Ayurvedic formulations such as 'Varunadi khwatham', 'Yogaraja guggulu', 'Kumaryasava', Manjishtadi kashayam' etc. Even though the preliminary phytochemical analysis of *C. fragrans* roots revealed the presence of sterols, saponins and flavonoids in them, there has been no detailed report on the phytochemical investigation of the non-alkaloid constituents. As mentioned earlier, five alkaloids have been reported from the root bark of the plant; however after 1970's no attempts were made to investigate the phytochemistry of the plant further. Therefore it appeared timely and relevant to carry out the phytochemical investigation of *C. fragrans.* So as part of this Ph.D. program, a detailed study of *C. fragrans* root has been undertaken. Detailed investigation of the root of the plant has been carried for identifying its alkaloid and non-alkaloid constituents. Evaluation of chemical indices of

two different extracts of *C. fragrans* roots, preliminary *in vitro* antioxidant potentials of the extracts and the isolated phenolic compounds as well as *in vitro* antimicrobial potential of the alkaloids isolated have been carried out and the results are described in this chapter.

2.4. Isolation and characterization of compounds from *Chonemorpha fragrans* roots

2.4.1. Extraction

C. fragrans roots were collected from a medicinal plant garden in Thiruvananthapuram and a voucher specimen was deposited in JNTBGRI, Palode, Thiruvananthapuram, Kerala (JN TBGRI 83447). The material was dried, powdered and then subjected to extraction with acetone at room temperature (5 L × 3) to yield 36 g of the acetone extract (CfAE) from 3 kg of dried roots. The residue obtained after extraction using acetone was further extracted using ethanol at room temperature (5 L × 3) to yield 38 g of the ethanol extract (CfEE). These two extracts were tested for the presence of alkaloids in them, using Dragendorff's (DD) reagent. Ethanol extract gave an orange red precipitate on addition of DD reagent, whereas the acetone extract did not show the presence of any alkaloids. TLC profile of acetone and ethanol extracts on visualization using DD reagent again revealed that acetone extract contained non-alkaloids whereas ethanol extract contained alkaloids as major constituents in them.

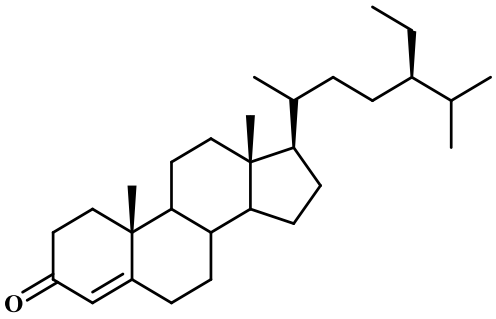
Since the non-alkaloid components in this plant have not been explored earlier, our primary attempt was to isolate non-alkaloid constituents and hence purification of acetone extract was undertaken at first.

2.4.2. Isolation and characterization of non-alkaloid constituents

After studying the TLC extensively, 28 g of the acetone extract was subjected to column chromatographic purification using silica gel (100-200 mesh). Column elution was started using 100% hexane and increase in polarity was carried out by increasing the amount of ethyl acetate. Final elution was carried out using 5% methanol in ethyl acetate. A total of 301 fractions of approximately 150 mL each were collected. According to the similarity in TLC they were pooled into nineteen major fraction pools.

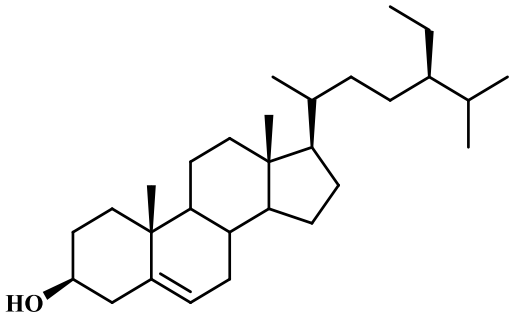
Fraction pool three (Fr. 16-38), obtained by eluting the column with 10% ethyl acetate in hexane showed the presence of a UV active compound with some minor impurities. It was again subjected to purification using alumina column chromatography. Subfractions 3-10 obtained by eluting the column with 3% ethyl

acetate in hexane yielded 100 mg of colourless crystals, which was labelled as compound **1**. Compound **1** was characterized as **sitostenone** (**stigma-4-en-3-one**), based on various spectral data (IR, ^{1}H NMR, ^{13}C NMR and HRMS) obtained for the compound and on comparison with literature reports.[23,24] The compound is being reported for the first time from any part of *C. fragrans.* The structure of the compound is shown below.



Compound **1 - *β*-sitostenone**

Fraction pool four (Fr. 39-70), obtained by eluting the column with 15% ethyl acetate in hexane on crystallization using the same solvent yielded 140 mg of colourless needle like crystals of compound **2**, which was characterized as the common phytosterol, ***β*-sitosterol**, based on various spectral data (IR, ^{1}H NMR, ^{13}C NMR and HRMS) obtained. The compound structure was further confirmed by TLC co-spotting with an authentic sample of *β*-sitosterol. The structure of the compound is shown below.



Compound **2 - *β*-sitosterol**

Fraction pool seven (Fr. 105-117), obtained by eluting the column with 25% ethyl acetate in hexane on crystallization using the same solvent yielded 30 mg of pale yellow crystals of compound **3**. Alcoholic solution of compound **3** responded positively towards Shinoda test, indicating that the compound is a flavonoid derivative.

One of the important spectral tools used for the structural elucidation of flavonoids is the UV-visible spectroscopy. Generally, flavonoids will give two major

absorptions, known as band I and band II in the UV spectrum. Band I appears in the range 300-400 nm, which could be attributed to the absorption due to B ring cinnamoyl system and band II (in the range 240-285 nm) absorption arising due to the A ring benzoyl system (fig. 2.4).

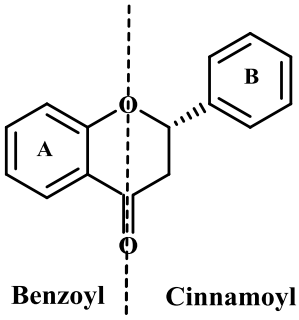
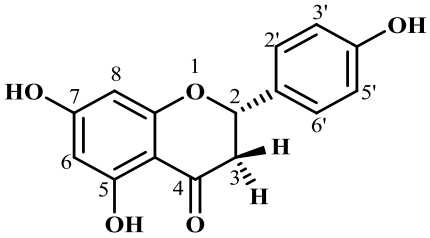


Figure 2.4: Benzoyl and cinnamoyl systems in basic flavonoid skeleton

UV spectrum of compound **3** (fig. 2.5) showed a low intensity peak at 336.3 nm appearing as a shoulder to the peak at 296 nm. This UV spectral pattern suggested that the compound contains a flavanone or flavanonol skeleton (usually in flavanone and flavanonol, low intensity band I absorption will appear as a shoulder to band II).[25] IR spectrum of the compound showed broad absorption at 3440 cm^{-1} suggesting the presence of a hydroxyl group and absorption at 1640 cm^{-1} suggested the presence of a carbonyl group. In the ^{1}H NMR spectrum (fig. 2.6), the aromatic protons of B ring appeared as two pairs of *ortho* coupled doublets, each integrating for two protons, centred at δ 7.26 (J = 8.7 Hz) and 6.83 (J = 8.7 Hz) i.e., the H-3′,5′ doublet appeared upfield from H-2′,6′ doublet. Singlet at δ 5.93 integrating for two protons could be ascribed to the H-8 and H-6 protons. The H-2 proton appeared as doublet of doublet at δ 5.28. Two sets of doublet of doublets centred at δ 3.05 and 2.69 could be attributed to the two protons at position 3 adjacent to the carbonyl group. ^{13}C NMR spectrum (fig. 2.7) clearly indicated that the compound contained 15 carbon atoms. The signal at δ 195.9 confirmed the presence of a carbonyl carbon. The peaks at δ 78.8 and 42.8 could be attributed to the C-2 and C-3 carbons. The mass spectrum showed molecular ion peak at m/z 273.0765, which is the $[M+H]^{+}$ peak. From all the above spectral details and on comparison with the literature[26,27] reports compound **3** was confirmed as 4′,5,7-trihydroxyflavanone–commonly known as **naringenin**. The structure of the compound is shown below.



Compound **3 - Naringenin**

Naringenin is abundantly available in citrus fruits,[28] especially grapes and oranges. However; this is the first report on the isolation of naringenin from any part of *C. fragrans*. Naringenin is known to possess antioxidant, anti-inflammatory, antidiabetic and anticancer activities.[29,30]

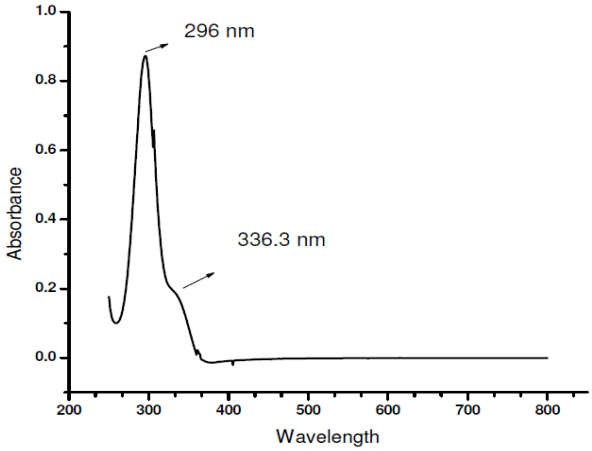


Figure 2.5: UV spectrum of naringenin

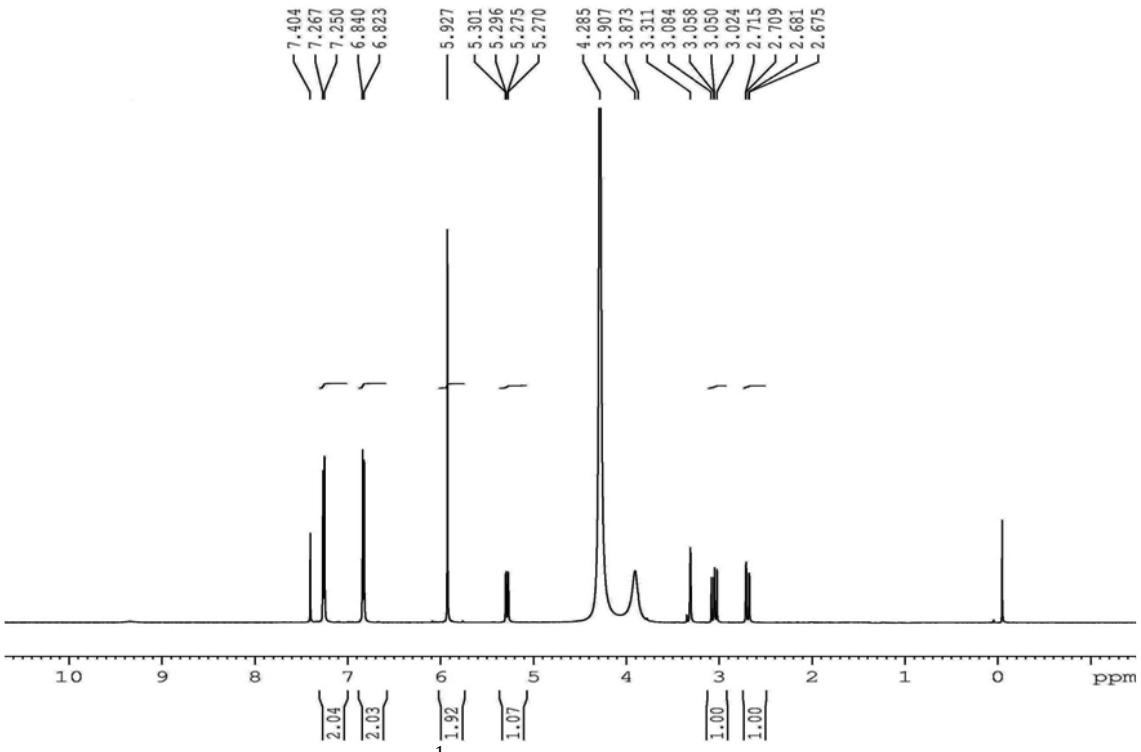


Figure 2.6: ^{1}H NMR spectrum of naringenin

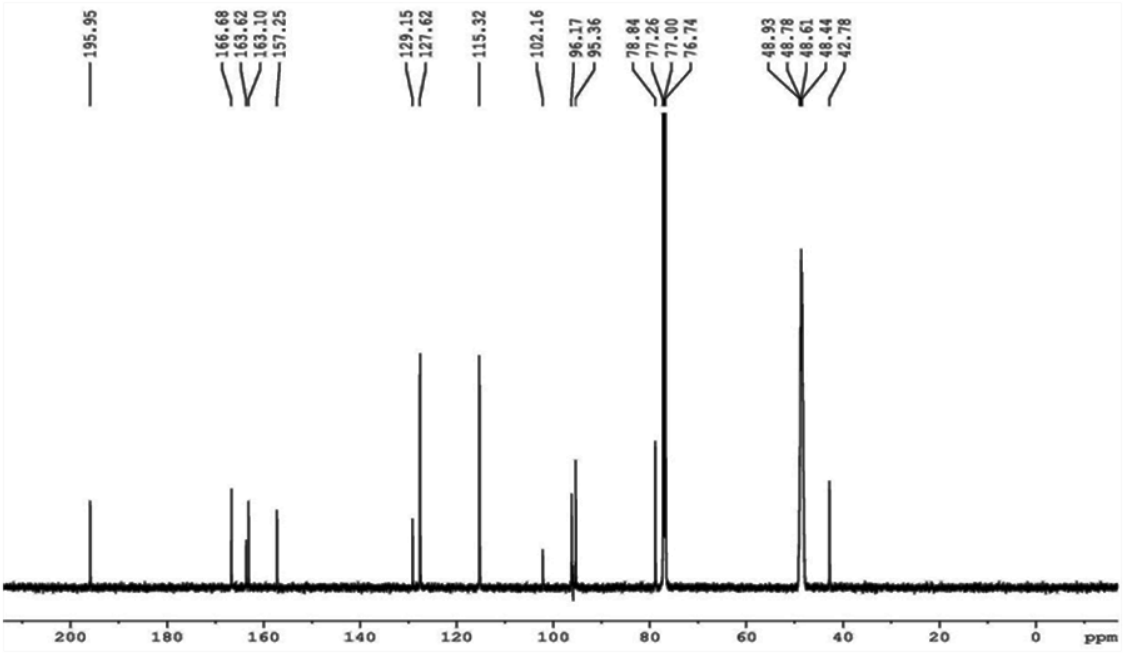
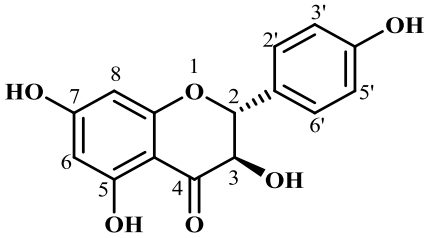


Figure 2.7: ^{13}C NMR spectrum of naringenin

Fraction pool eight (Fr. 118-125), obtained by eluting the column with 30% ethyl acetate in hexane yielded 6 mg of white amorphous powder of compound **4**. Alcoholic solution of the compound also responded positively towards Shinoda test, indicating that the compound is a flavonoid derivative. UV spectrum (fig. 2.8) of the compound showed similar absorption pattern as that of compound **3** with a low intensity peak at 324.8 nm appearing as a shoulder to the peak at 290.4 nm, suggesting the presence of a flavonone or flavanonol. IR spectrum of the compound showed broad absorption at 3447 cm^{-1} indicating the presence of a hydroxyl group and absorption at 1642 cm^{-1} suggested the presence of a carbonyl group. ^{1}H NMR spectrum (fig. 2.9) of the compound showed two pairs of *ortho* coupled doublets, integrating for two protons each, centred at δ 7.37 and 6.90, which could be attributed to the B ring aromatic protons. The H-2 and H-3 protons appeared as two separate doublets δ 5.01 and 4.54 respectively. ^{13}C NMR spectrum (fig. 2.10) clearly indicated that the compound contained 15 carbon atoms. The signal at δ 196.5 confirmed the presence of a carbonyl carbon. The peaks at δ 83.6 and 72.5 could be attributed to the C-2 and C-3 carbons. The mass spectrum of the compound showed molecular ion peak at m/z 287.0563, which is the $[M-H]^+$ peak. From all the above spectral details and on comparison with the literature[31,27] reports, the compound was confirmed as 3,4′,5,7-tetrahydroxyflavanonol commonly known as **aromadendrin** or **dihydrokaempferol**. The structure of the compound is shown below.



Compound **4 - Aromadendrin**

Aromadendrin has been isolated previously from the plant *Pinus sibirica*, belonging to Pinaceae family[32] and its glycosides are available in many citrus fruits; the compound is being reported for the first time from any part of *C. fragrans*. Aromadendrin shows inhibitory activity against of monoamine oxidase and inhibit the multidrug transporter MRP1.[33]

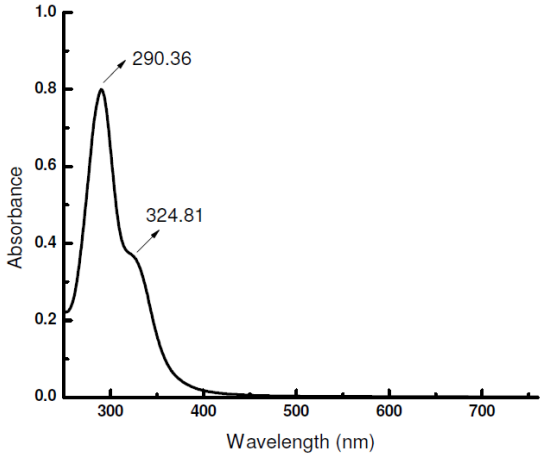


Figure 2.8: UV spectrum of aromadendrin

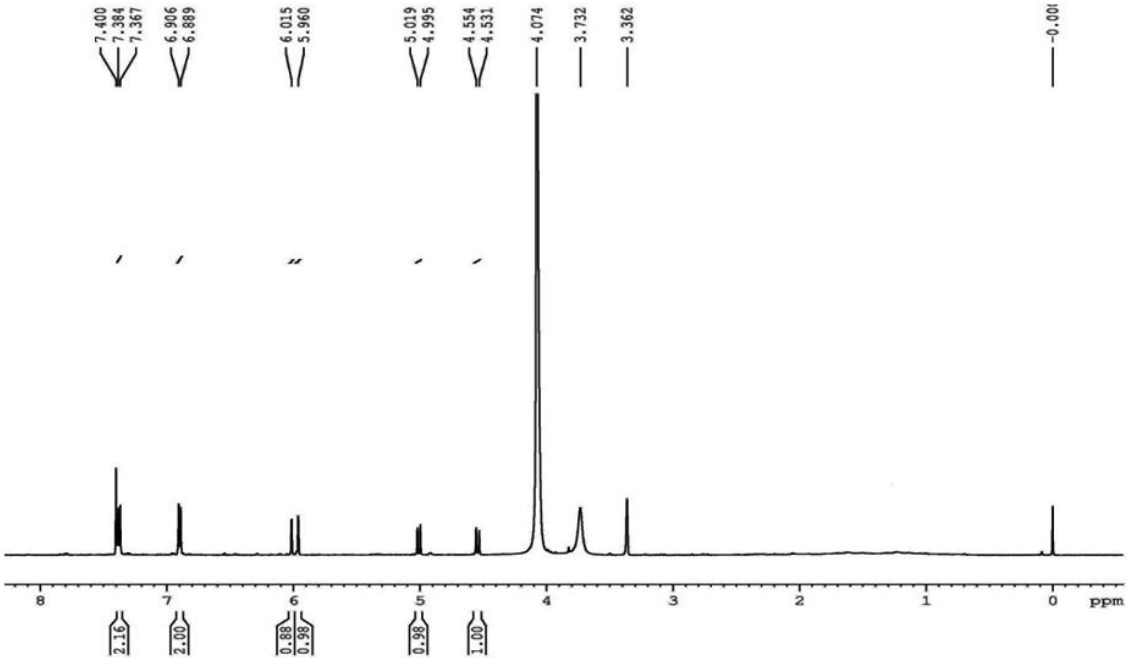


Figure 2.9: ^{1}H NMR spectrum of aromadendrin

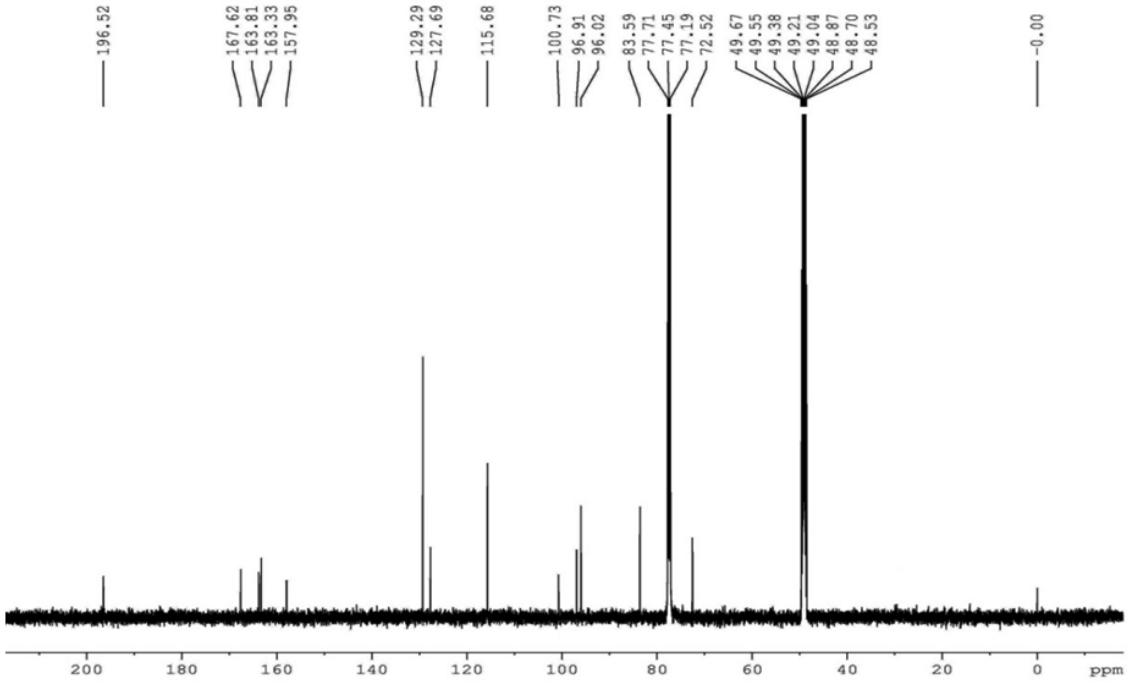
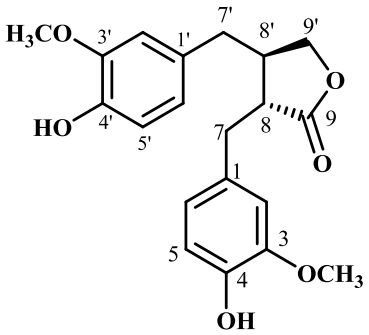


Figure 2.10: ^{13}C NMR spectrum of aromadendrin

Twelfth fraction pool (Fr. 157-184) obtained by eluting the column with 40% ethyl acetate in hexane showed the presence of two UV active compounds with some impurities. This was further purified using silica gel column chromatography to yield 280 mg of a viscous liquid, which was labeled as compound **5** and 15 mg of white amorphous powder, labeled as compound **6.**

IR spectrum of the compound **5** showed broad absorption at 3425 cm^{-1} indicating the presence of hydroxyl group. Absorption observed at 1761 cm^{-1} suggested the presence of a carbonyl group in lactone ring. ^{1}H NMR spectrum (fig. 2.11) of the compound suggested the presence of six aromatic protons (2H multiplets, each at δ 6.81 and 6.61, 1H doublet of doublet at δ 6.51 and 1H doublet at δ 6.42). Broad singlet at δ 5.61, integrating for two protons, could be attributed to two hydroxyl groups in the aromatic ring. Doublet of doublets at δ 4.16 and 3.89 integrating for one proton each suggested the presence of deshielding groups such as hydroxy or ether linkage. Two singlets seen at δ 3.822 and 3.816, each integrating for three protons was indicative of two aromatic methoxy groups. Doublet of doublets at δ 2.96 and 2.88 each integrating for one proton indicated the presence of benzylic methylene at position 7. Multiplet in between δ 2.64-2.45 integrating for four protons could be attributed to benzylic protons at 7′ and the protons at 8 and 8′. ^{13}C NMR spectrum (fig. 2.12) of the compound showed the presence of 20 carbons. The peak at δ 178.9 ppm confirmed the presence of ester/lactone carbonyl. The presence of two methoxy groups was confirmed by the two

peaks at δ 55.83 and 55.77 ppm. DEPT-135 spectrum of the compound clearly indicated that the compound contains three $-CH_2$ groups. Mass spectrum of the compound gave molecular ion peak at m/z 381.1306, which is the $[M+Na]^+$ peak. From all these spectral data and on comparison with the literature,[34,35] the compound was identified as the lignan lactone **matairesinol.** The structure of the compound is shown below.



Compound **5 - Matairesinol**

Matairesinol is usually found in whole grains, oil seeds, fruits, vegetables etc.[36] However; this is the first report on the isolation of matairesinol from *C. fragrans.* The compound possesses very good anticancer,[37] antioxidant,[38] anti-inflammatory,[39] anti HIV,[40] antirheumatoid[41] and immunomodulatory[42] activities.

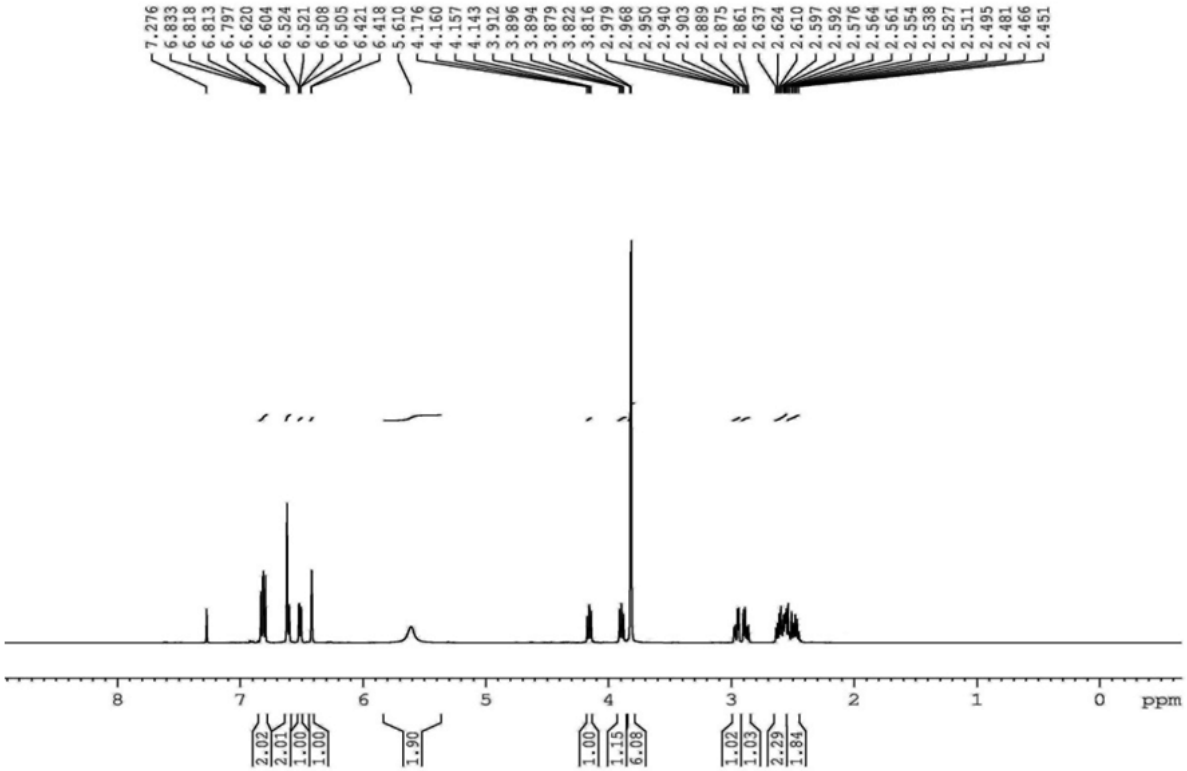


Figure 2.11: ^{1}H NMR spectrum of matairesinol

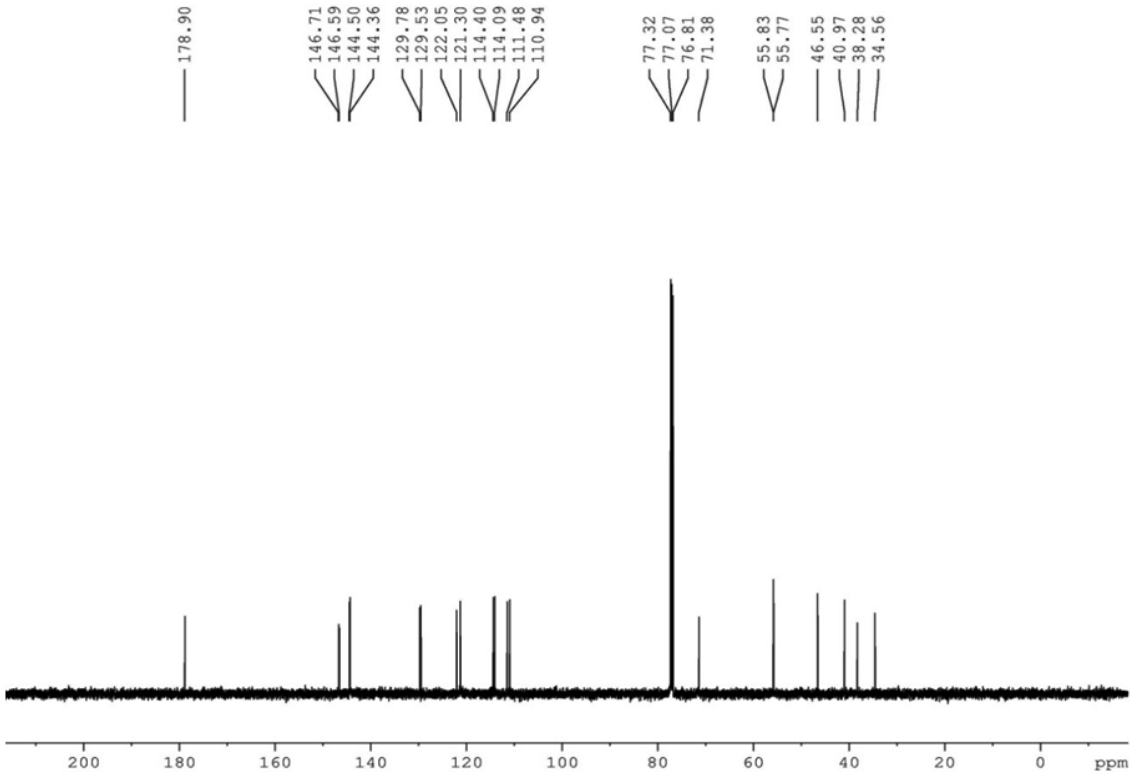
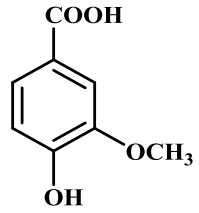


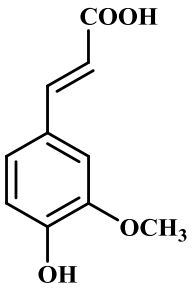
Figure 2.12: ^{13}C NMR spectrum of matairesinol

Compound **6**, obtained as a white amorphous powder, was characterized as the phenolic acid, **vanillic acid** based on the spectral data (IR, ^{1}H NMR, ^{13}C NMR, HRMS) obtained and on comparison with literature reports.[43] The compound identity was further confirmed by TLC co-spotting with authentic sample of vanillic acid. The structure of the compound is shown below.

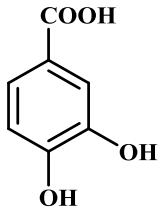


Compound **6 - Vanillic acid**

Thirteenth (Fr. 185-195) and fourteenth (Fr. 196-220) fraction pools obtained by eluting the column with 45% and 50% ethyl acetate in hexane yielded 12 mg of compound **7** and 10 mg of compound **8** respectively. These compounds were characterized as the phenolic acids, ferulic acid and protocatechuic acid respectively, based on various spectral data (IR, ^{1}H NMR, ^{13}C NMR, HRMS) obtained and comparison with the literature reports.[44,45] The structures of the compounds are given below.

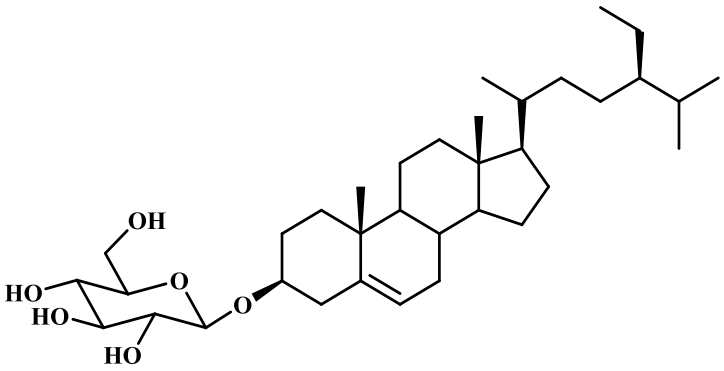


Compound **7 - Ferulic acid**



Compound **8 - Protocatechuic acid**

Nineteenth fraction pool (Fr. 275-301) obtained by eluting the column with 100% ethyl acetate followed by 5% methanol in ethyl acetate, gave 25 mg of compound **9** as a white amorphous powder. The compound responded positively towards Liebermann-Burchard (test for steroids), Keller-Killiani and Molish's tests (test for glycosides) suggesting that the compound is a steroidal glycoside. The compound was successfully characterized as **sitosterol-3-*O*-*β*-D-glucopyranoside** based on the spectral data obtained (IR, ^{1}H NMR, ^{13}C NMR and mass) and on comparison with the literature reports.[46] The structure of the compound is shown below.



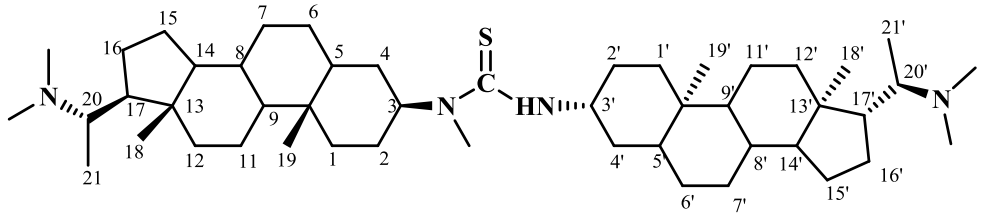
Compound **9 - Sitosterol-3-*O*-*β*-D-glucopyranoside**

The above mentioned three phenolic acids and the glycoside are being reported for the first time from any part of *C. fragrans*.

2.4.3. Isolation and characterization of alkaloid constituents

Ethanol extract (CfEE, 36 g) was used for the isolation of alkaloid constituents from *C. fragrans* roots. Alkaloid mixture was extracted from CfEE using standard acid–base extraction technique.[47] Alkaloid extract thus obtained (4.2 g) was purified using column chromatography using basic alumina as the adsorbent and mixtures of hexane–ethyl acetate as the eluent. Column elution was started using 100% hexane and increase in polarity was carried out by increasing the amount of ethyl acetate. A total of 38 fractions of approximately 50 mL each were collected.

Fractions 9 and 10 obtained by eluting the column with 5% ethyl acetate in hexane gave 6 mg of compound **10** as white amorphous powder. The TLC was visualized using Dragendorff's reagent, which gave orange colour spot indicating the presence of an alkaloid. IR spectrum of the compound gave absorptions at 3377 and 1528 cm^{-1} suggesting the presence of an –NH group connected to >C=S functionality.[17] ^{1}H NMR spectrum (fig. 2.13) and ^{13}C NMR spectrum (fig. 2.14) of the compound clearly indicated that the compound is a pregnane type steroidal alkaloid dimer. Available literature on the phytochemistry of *C. fragrans* suggested that the compound may be the steroidal alkaloid dimer, japindine. ^{1}H NMR spectrum showed singlets at δ 0.64 and 0.80, both integrating for six protons each, suggesting the presence of a total of four tertiary methyl groups in the steroidal skeleton (H-18, 18' and H-19, 19' respectively). Doublet at δ 0.87 integrating for six protons could be attributed to the secondary methyl groups at C-21 and C-21'. Singlet at δ 2.17 integrating for twelve protons could be attributed to the two *N*,*N*-dimethyl groups in the molecule (2 × -$N(CH_3)_2$). These spectral values and integration again confirmed the dimeric nature of the compound. A singlet resonating at δ 2.89 integrating for three protons was assigned to the *N*-methyl group in the thiourea moiety. Multiplet at δ 2.42, integrating for two protons corresponded to the methine protons at C-20 and 20'. Multiplet at δ 4.36 may be ascribed to the proton at C-3', which showed HOMO COSY correlation with the –NH proton of the thiourea group which appeared as a doublet at δ 5.07 (J = 7.5 Hz). All other aliphatic protons resonated in between δ 2.02 and 0.70. In the ^{13}C NMR spectrum the thiocarbonyl group (>C=S) resonated at δ 179.9. Methine carbon near to the *N*,*N*-dimethyl group resonated at δ 61.2, the *N*,*N*-dimethyl carbons at δ 39.8 and *N*-methyl carbon resonated at δ 31.6. Even though IR and ^{1}H NMR spectral details of the compound were reported earlier by Banerji *et al.*, to the best of our knowledge, the ^{13}C NMR spectral details of the compound is being reported for the first time. Mass spectrum of the compound gave molecular ion peak at m/z 749.6507 which is the $[M+H]^{+}$ peak. Collecting all these spectral data and comparing with that reported in literature[17] structure of compound **10** was confirmed to be same as **japindine**. Japindine is the only sulphur containing dimeric steroidal alkaloid known to date. *C. fragrans* has been found to be the sole source of the compound and so far the compound has not been reported from any other plant species. The structure of the compound is given below.



Compound **10 - Japindine**

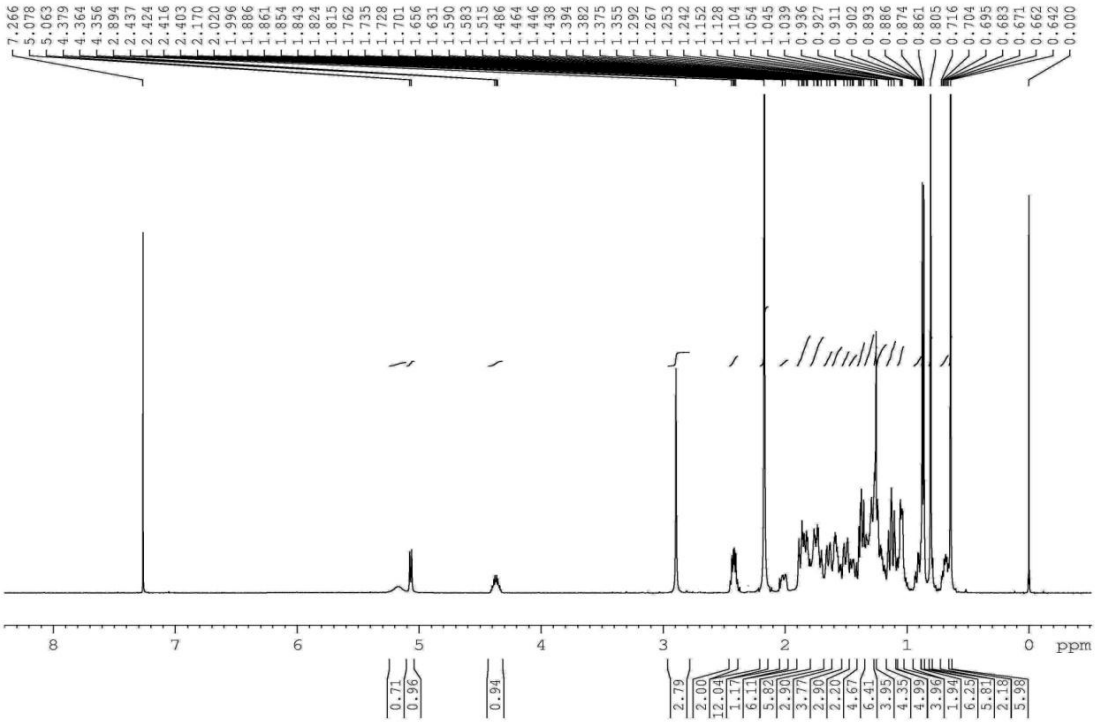


Figure 2.13: ^{1}H NMR spectrum of japindine

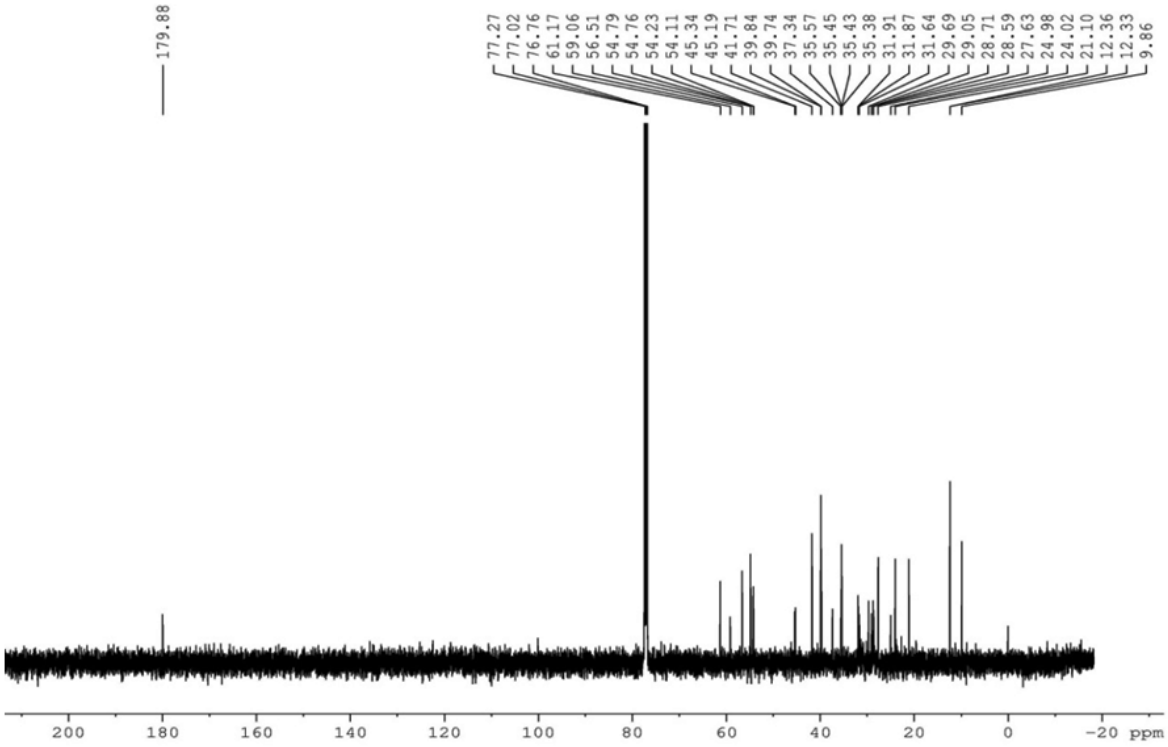


Figure 2.14: ^{13}C NMR spectrum of japindine

Compound **11** (21 mg) obtained as colourless solid from fractions 13-15, obtained by eluting the column with 10% ethyl acetate in hexane, was found to be UV inactive, suggesting that the compound does not contain any chromophore in it. The TLC was visualized using Dragendorff's reagent, which gave orange colour spot indicating the presence of an alkaloid. IR spectrum of the compound showed absorption at 3311 cm^{-1} suggesting the presence of a hydroxyl group. ^{1}H NMR (fig. 2.16) and ^{13}C NMR spectra (fig. 2.17) of the compound was suggestive of a pregnane type steroidal alkaloid, which were the type of compounds reported earlier from this plant.

Singlets at δ 0.65 and 0.80, both integrating for three protons each in the ^{1}H NMR spectrum suggested the presence of two tertiary methyl groups in the steroidal skeleton. Doublet at δ 0.91 integrating for three protons suggested the presence of a secondary methyl group too. Singlet at δ 2.22 integrating for six protons could be attributed to the two methyl groups attached to nitrogen (-*N*$(CH_3)_2$). Multiplet at δ 2.51 could be assigned to the methine proton next to the *N*,*N*-dimethyl group. Proton attached to the carbon bearing hydroxyl group resonated as a multiplet at δ 3.59. By considering the biosynthetic pathway and literature reports, it has been concluded that the –OH group is *β* oriented. All other aliphatic protons appeared as multiplets in between δ 1.99 to 0.63. ^{13}C NMR spectrum of the compound showed the presence of twenty three carbon atoms. Comparison of the ^{13}C NMR spectral values, and DEPT-135 as well as HMQC spectra suggested that there are five methyl groups, nine methylene groups, seven methine groups and two quaternary carbons in the molecule. Carbon bearing the hydroxyl group resonated at δ 71.3. Methine carbon near to the *N*,*N*-dimethyl group resonated at δ 61.5 and the *N*,*N*-dimethyl carbons resonated at δ 39.8. In the HMBC spectrum, C-20 which resonated at δ 61.5 showed correlation with *N*,*N*-dimethyl protons at δ 2.22 and with H-20 at δ 0.91. Both C-17 and C-14 showed HMBC correlation with H-18 whereas C-5 (44.8) and C-9 (54.6) showed HMBC correlation with H-19.

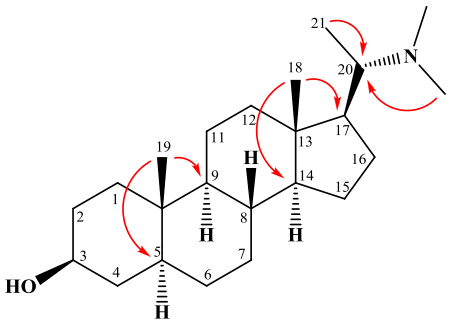
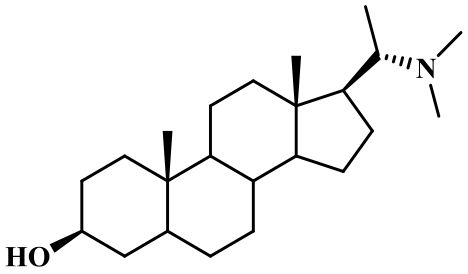


Figure 2.15: Selected HMBC correlations in compound **11**

Mass spectrum of the compound gave molecular ion peak at m/z 348.3271 which is the $[M+H]^+$ peak. By incorporating all the spectral details and on comparison with the literature, the compound was found to be **deaminooxochonemorphine** or **sarcorucinine D**. Structure and stereochemistry of the compound was assigned on the basis of spectral analysis, literature reports[48] and considering the biosynthetic pathway of pregnane type steroidal alkaloids. The structure of the compound is shown below.



Compound **11 - Sarcorucinine D**

The compound has been isolated earlier from *Sarcococca rucifolia* belonging to Buxaceae family.[48] and it has also been synthesized from the steroidal alkaloid, chonemorphine.[49] To the best of our knowledge this is the first report on the isolation of **sarcorucinine D** from any part of *C. fragrans* and the compound is being reported for the first time from Apocynaceae family.

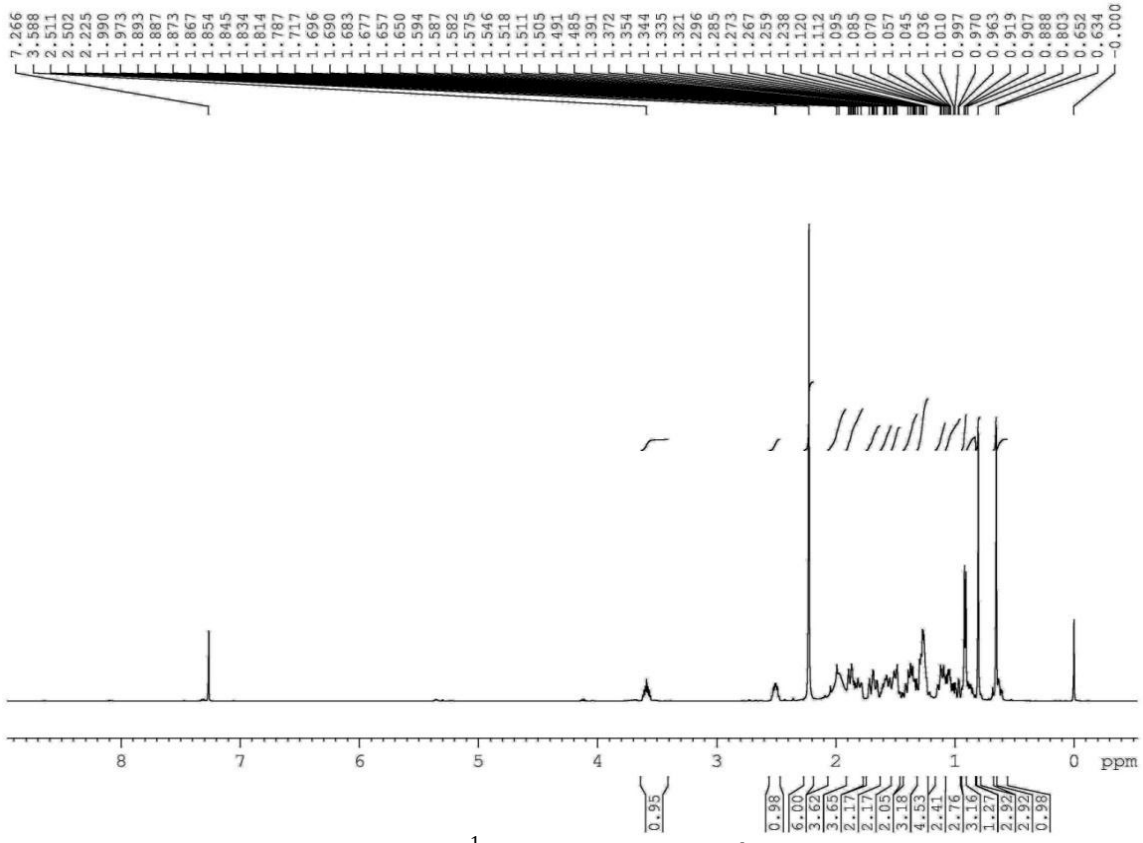


Figure 2.16: ^{1}H NMR spectrum of sarcorucinine D

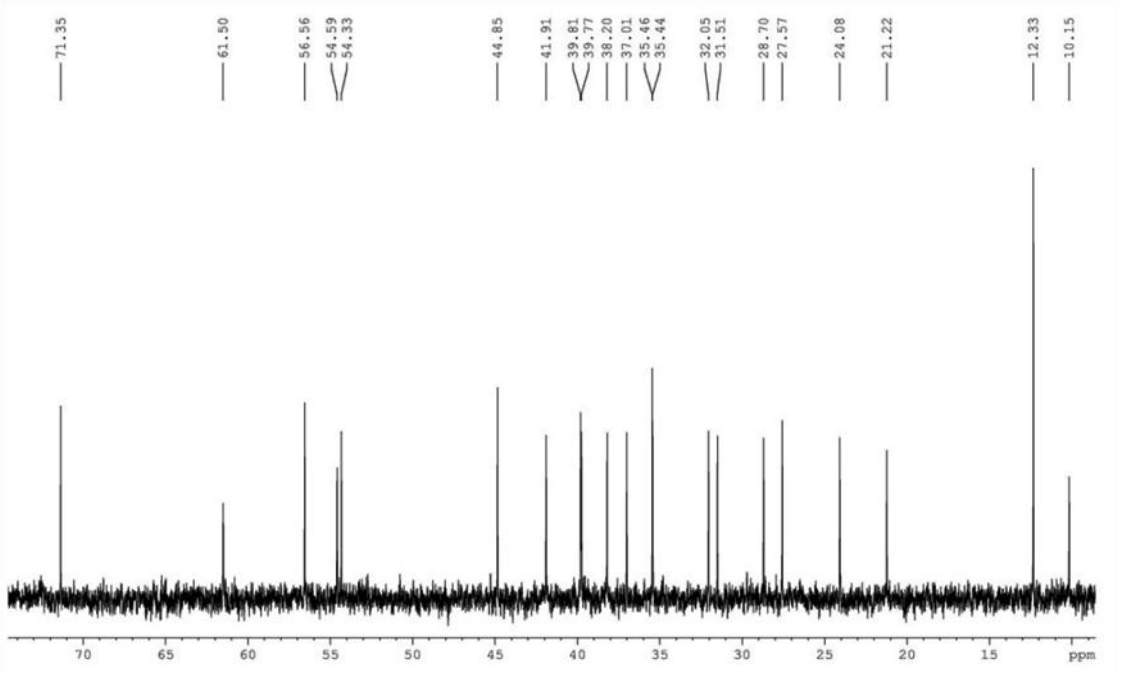
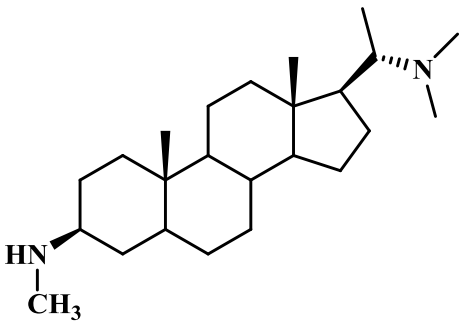


Figure 2.17: ^{13}C NMR spectrum of sarcorucinine D (expanded from 10-75 ppm)

Fractions 16-17 obtained by eluting the column with 15% ethyl acetate in hexane yielded 11 mg of compound **12** as white amorphous powder. The compound was also UV inactive and gave orange red precipitate on addition of Dragendorff's reagent. IR spectrum of the compound showed absorption at 3270 cm^{-1}, suggesting the presence of an –NH group. Information obtained from ^{1}H NMR (fig. 2.18) and ^{13}C NMR (fig. 2.19) spectra of the compound was again suggestive of a pregnane type steroidal alkaloid. Singlets observed at δ 0.66 and 0.83, both integrating for three protons each in the ^{1}H NMR spectrum suggested the presence of two tertiary methyl groups in the steroidal skeleton. Doublet at δ 0.93 integrating for three protons suggested the presence of a secondary methyl group. Singlet seen at δ 2.25 integrating for six protons could be attributed to the two methyl groups attached to nitrogen (–*N*$(CH_3)_2$). Multiplet appearing at δ 2.53 could be assigned to the methine proton adjacent to the *N*,*N*-dimethyl group. –*N*CH_3 protons resonated as singlet at δ 2.58. A multiplet centred at δ 2.77 could be ascribed to the proton attached to carbon bearing –*N*HCH_3 group. All other aliphatic protons appeared as multiplets in between δ 1.98 and 0.66. Comparison of the ^{13}C NMR spectral values together with DEPT-135 as well as HMQC spectrum suggested that there are six methyl groups, nine methylene groups, seven methine groups and two quaternary carbons in the molecule. Methine carbon near to the –*N*$(CH_3)_2$ group resonated at δ 61.7, carbon bearing –*N*HCH_3 group resonated at δ 58.8, the *N*,*N*-dimethyl carbons at δ 39.8 and *N*-methyl carbon resonated at δ 31.9.

Mass spectrum of the compound gave molecular ion peak at m/z 361.3220, which is the $[M+H]^+$ peak. Deducing from all the spectral details and on comparison with the literature, the compound was found to be ***N*-methylchonemorphine** or **dictyophlebine**, which was previously reported from the root bark of *Chonemorpha fragrans*.[17] The structure and stereochemistry of the compound is assigned on the basis of spectral analysis, literature reports and by considering the biosynthetic pathway of the compound. The structure of the compound is depicted below.



Compound **12 - Dictyophlebine**

The compound was also reported from other plants such as *Dictyophlebia lucida*,[50] *Sarcococca saligna*,[51] *S. hookeriana*[52] and *S. wallichii*.[53] It possesses acetylcholinesterase inhibitory activity, antiplasmodial,[51-52,54] anti-inflammatory[53] and antileishmanial[55] activities.

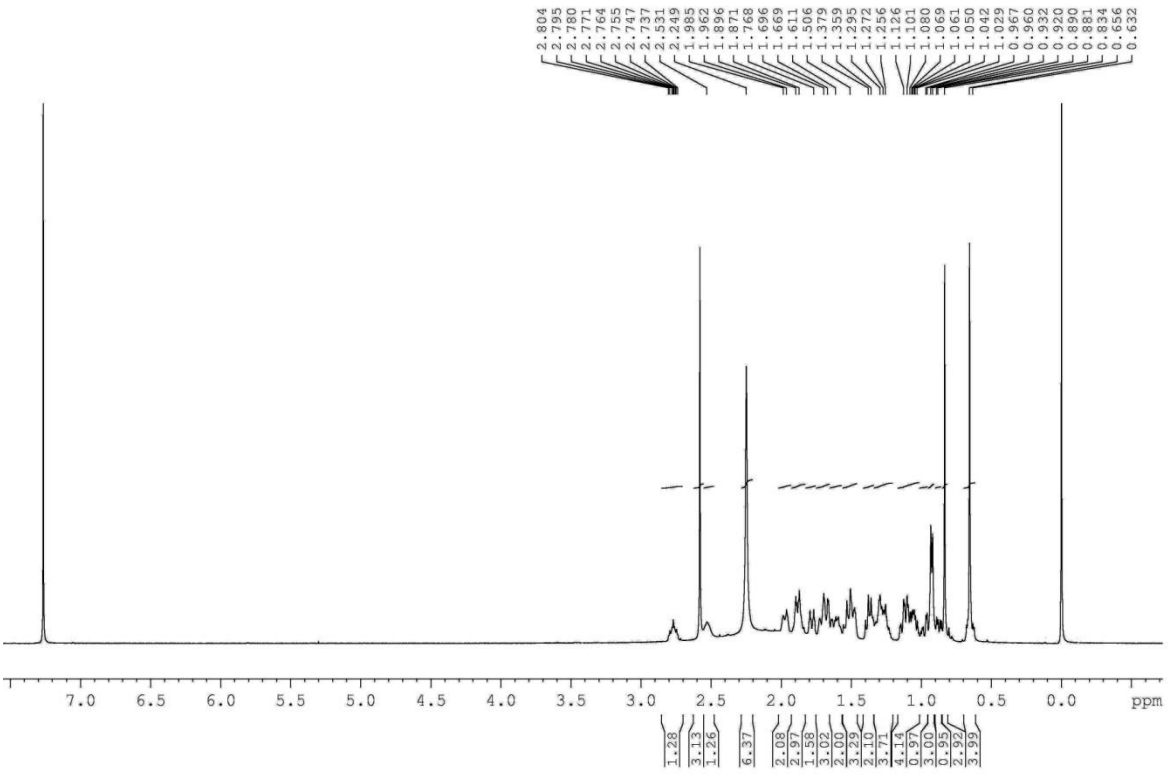


Figure 2.18: ^{1}H NMR spectrum of dictyophlebine

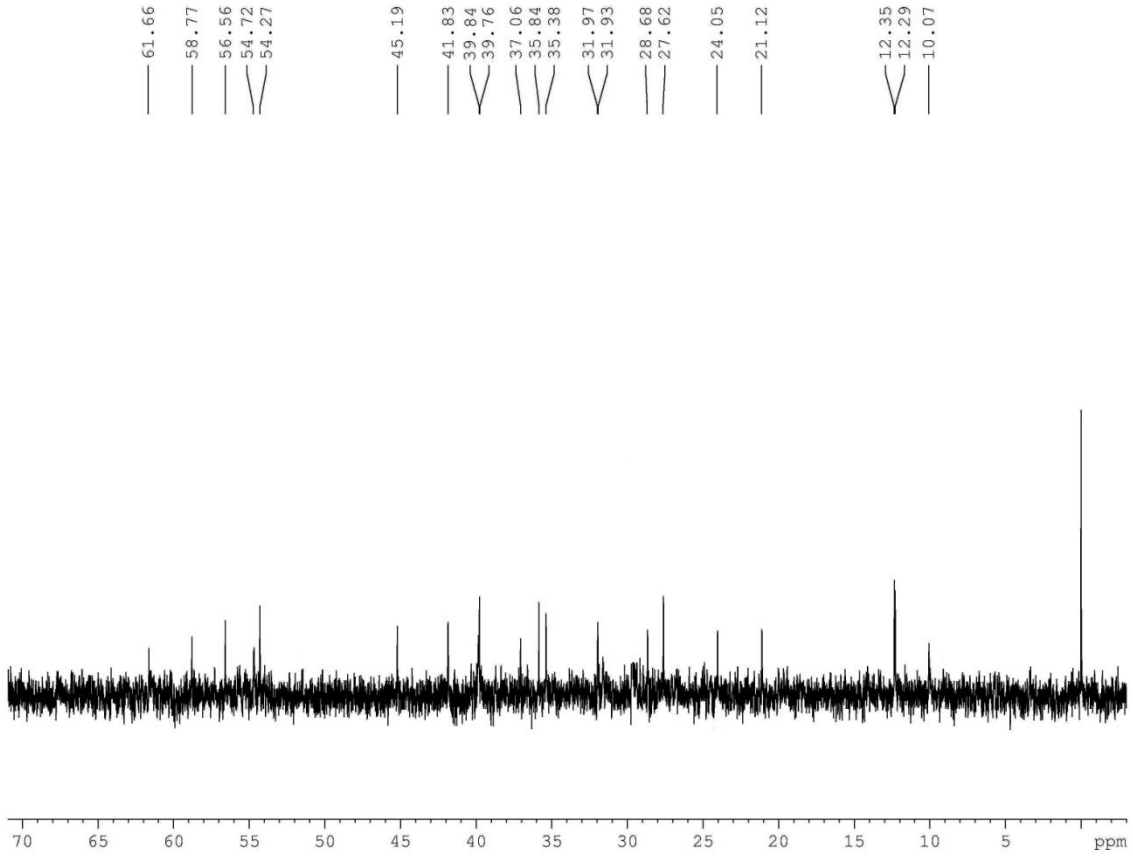
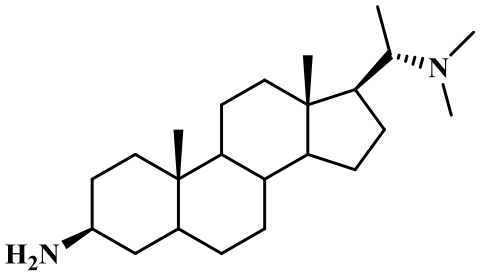


Figure 2.19: ^{13}C NMR spectrum of dictyophlebine (expanded from 0-70 ppm)

Compound **13** (6 mg) obtained as a white amorphous powder from fractions 22-23, obtained by eluting the column with 25% ethyl acetate in hexane, was found to be UV inactive but gave an orange red precipitate on addition of Dragendorff's reagent. Compound **13** was found to be the steroidal alkaloid amine, chonemorphine on the basis of various spectral data obtained and on comparison with the literature reports.[15] Chonemorphine, isolated by Chatterjee *et al*., in 1967, is the first alkaloid reported from the root bark of *C. fragrans*. The compound was also reported from *Malouetia bequaertiana* belonging to Apocynaceae family[56] and *Sarcococca hookeriana* belonging to Buxaceae family.[55] The structure of the compound is shown below.



Compound **13 - Chonemorphine**

Compound **14** (32 mg) was isolated as pale yellow solid from fractions 29-31 obtained by eluting the column with 50% ethyl acetate in hexane. The compound gave positive result when tested using Dragendorff's reagent, suggesting the presence of an alkaloid. IR spectrum of the compound showed absorption at 3282 and 1661 cm^{-1} suggesting the presence of amidic –*N*H and –C=O groups respectively. Singlets at δ 0.64 and 0.79, both integrating for three protons each in the ^{1}H NMR spectrum (fig. 2.21) suggested the presence of tertiary methyl groups. Doublet at δ 0.88 integrating for three protons suggested the presence of a secondary methyl group. Singlet seen at δ 2.19 integrating for six protons could be attributed to the protons of *N*,*N*-dimethyl group. Multiplet observed at δ 2.45 could be assigned to the methine proton adjacent to *N*,*N*-dimethyl group. Proton attached to the carbon bearing –*N*HCHO group appeared as a multiplet at δ 3.86. The –*N*H proton of the amidic group appeared as a doublet at δ 5.40. A singlet seen at δ 8.09 suggested the presence of an aldehydic group. This peak was accompanied by another low intensity singlet at δ 8.10, which may have resulted due to rotational isomerism.[57] All other aliphatic protons appeared as multiplets in between δ 1.86 to 0.65. The ^{13}C NMR spectrum (fig. 2.22) of the compound showed the presence of twenty four carbon atoms in the molecule and again suggested that the compound is a pregnane type steroidal alkaloid. Aldehydic carbon resonation was observed at δ 160.4 accompanied by a lower intensity peak at δ 163.4 (which may be due to rotational isomerism). Comparison of the ^{13}C NMR spectral values along with DEPT-135 and HMQC spectra suggested that there are five methyl groups, nine methylene groups, eight methine groups and two quaternary carbons in the molecule. In the HMBC spectrum, C-20 appearing at δ 61.3 showed correlation with *N*-methyl protons at δ 2.22 and with H-20 at δ 0.91. Both C-17 and C-14 showed HMBC correlation with H-18 whereas C-5 and C-9 showed HMBC correlation with H-19. Selected HMBC and NOESY correlations are depicted in fig. 2.20.

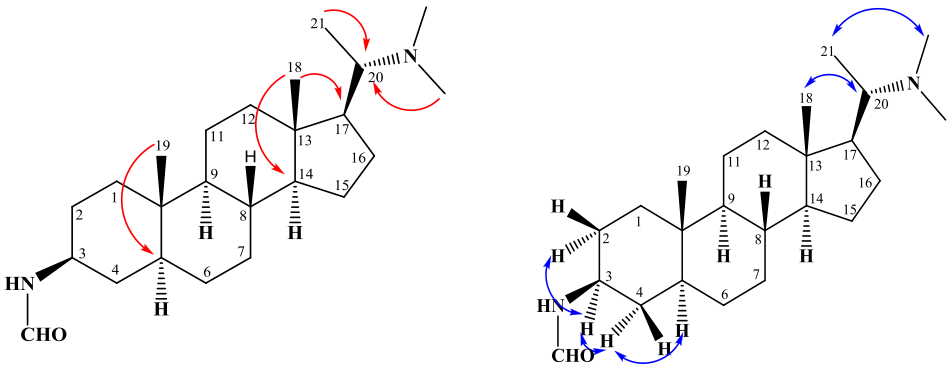
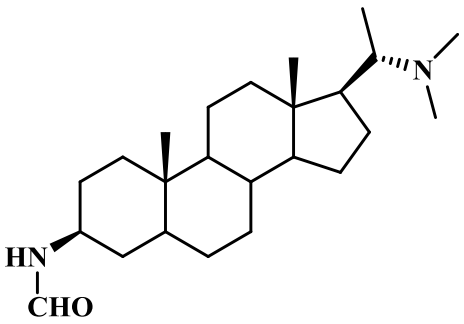


Figure 2.20: Selected HMBC correlations Selected NOESY correlations

Mass spectrum of the compound showed molecular ion peak at m/z 375.3381, which is the $[(M+H)^+]$ peak. From all these spectral details and on comparison with the literature reports[58] the compound was identified as ***N*-formylchonemorphine**, which was previously reported from the root bark of *Chonemorpha fragrans.*[17] The structure and stereochemistry of the compound as shown below was assigned on the basis of literature reports and considering the biosynthetic pathway of pregnane type steroidal alkaloid.



Compound **14 - *N*-formylchonemorphine**

The compound was earlier reported from the plants *Sarcococca saligna*[58] and *S. hookeriana*.[59] *N*-formylchonemorphine have been reported to show very good antileishmanial activity and moderate antibacterial activity.[59]

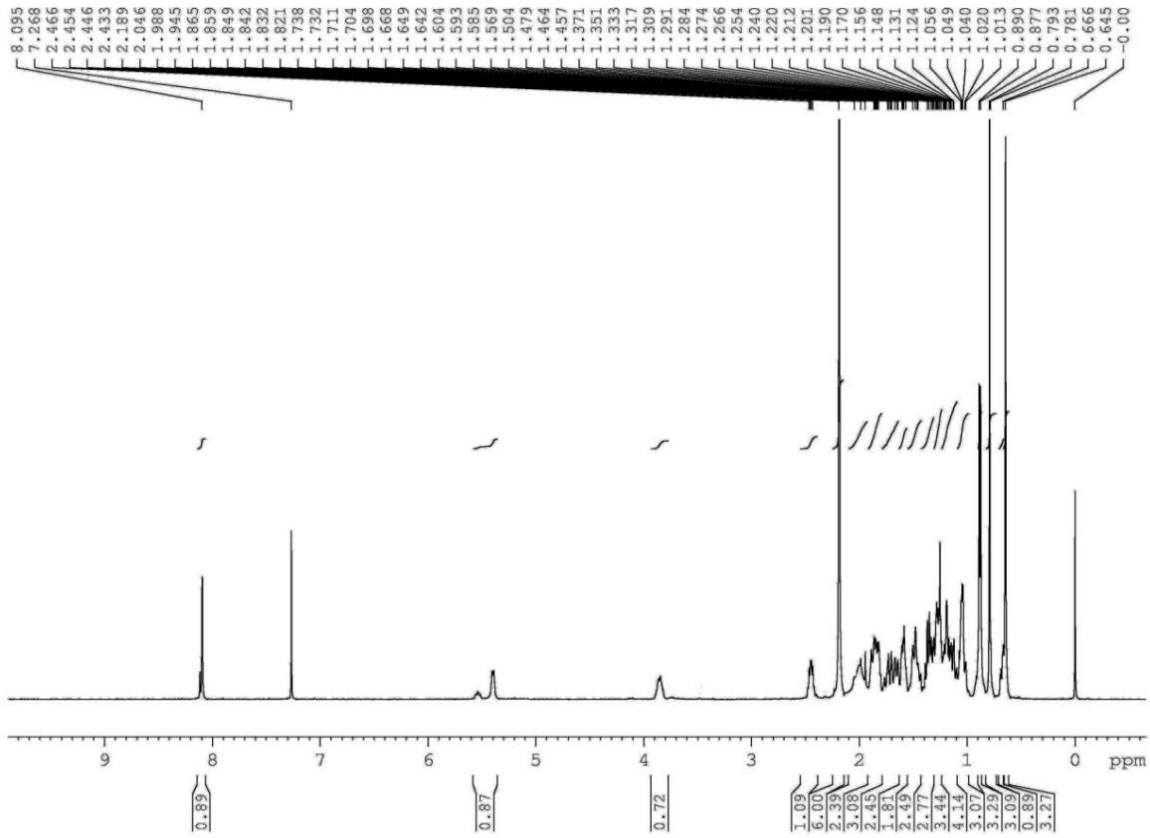


Figure 2.21: ^{1}H NMR spectrum of *N*-formylchonemorphine

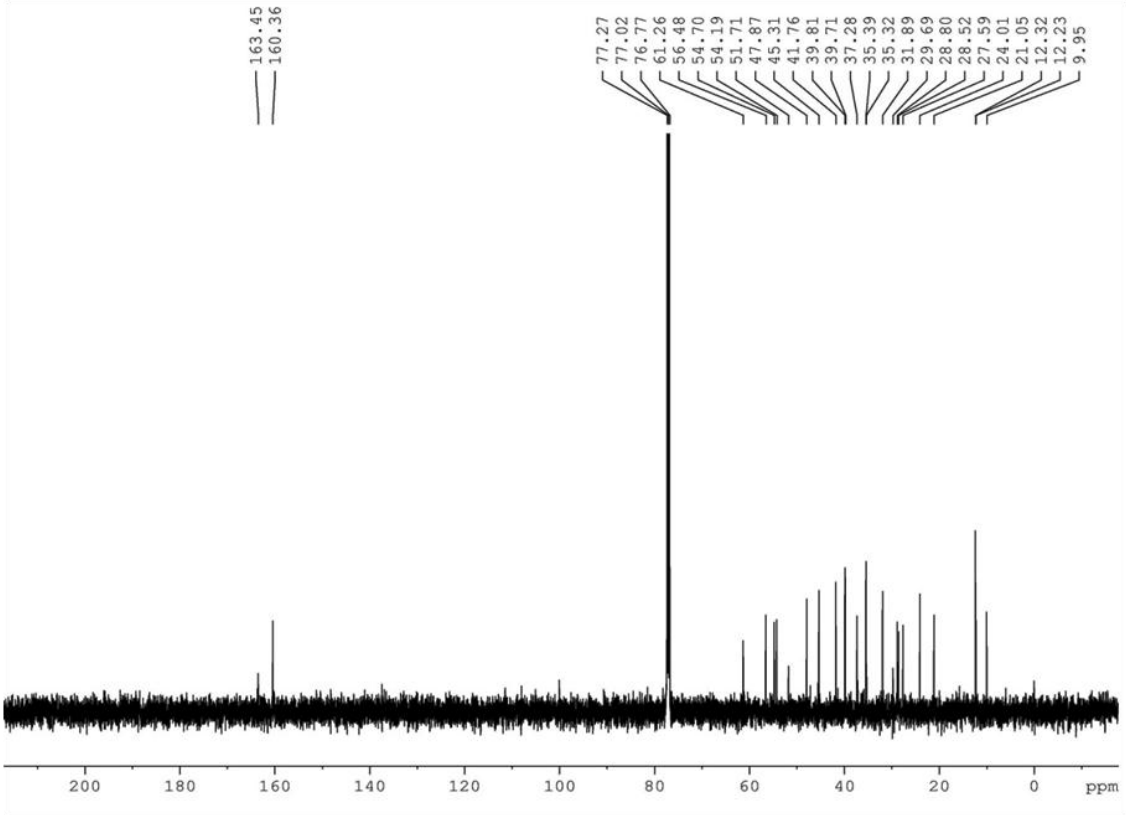


Figure 2.22: ^{13}C NMR spectrum of *N*-formylchonemorphine

2.5. Antioxidant activity studies on *C. fragrans* and its phenolic constituents

2.5.1. Reactive oxygen species and plant derived antioxidants

Reactive oxygen species (ROS) including free radicals such as hydroxyl radical ($OH^{\cdot}$), superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) etc., are introduced in the body through both endogenous and exogenous sources. Endogenously ROS are produced in the cells during ATP production and exogenous sources include radiation, tobacco smoke, pollutants etc.[60] Deleterious ROS produced via normal physiological processes are removed from the body through its own antioxidant defence mechanisms.[61] When there is an imbalance between ROS generation and oxidant removal system, ROS will be produced in excess which can damage cellular lipids, proteins, DNA etc., and inhibit their normal function.[60] These ROS are also responsible for oxidative stress, which in turn results in various neuro-degenerative diseases, ageing, cardiovascular diseases, cancer etc.[62,63,64]

According to Halliwell and Gutteridge, an antioxidant is defined as "any substance that when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate."[65] Dietary

antioxidants such as vitamin C, vitamin E, carotenoids and phenolic compounds will reduce oxidative stress and protect cells against the damaging effects caused by ROS.[66] Fresh fruits, vegetables, spices, tea, coffee, soyabeans, legumes etc., are the major sources of exogenous dietary antioxidants. Major antioxidant compounds and their sources are given in table 2.2.

Table 2.2: Major antioxidant compounds and their sources

Antioxidant compounds	Sources
Vitamin C (Ascorbic acid)	Lemon, oranges, tomatoes, gooseberries, green vegetables etc.
Vitamin E (Tocopherols)	Vegetable oils, wheat germ oil, whole grains, nuts, cereals, fruits, eggs, meat etc.
β-carotene	Fruits, grains, oils, vegetables like carrots, spinach etc.
Lycopene	Tomatoes
Phenolic compounds • Flavonoids – quercetin, apigenin, hesperitin, catechin, gallocatechin etc. • Resrveratrol and its derivatives • Phenolic acids – ferulic acid, caffeic acid, protocatechuic acid, vanillic acid, syringic acid etc. • Other polyphenols	Fresh fruits, vegetables, soybean, legumes, tea, chocolate, spices, red wine etc.

Since plant derived antioxidants play a vital role in human health care system, in the present study we have evaluated the antioxidant capacities of acetone and ethanol extracts of *C. fragrans* using *in vitro* methods. Preliminary radical scavenging efficiency of the extracts as well as the isolated phenolic compounds was also carried out using DPPH radical scavenging method.

2.5.2. Total phenolic content (TPC)

Since phenolic compounds are one of the important antioxidants, total phenolic content (TPC) of *C. fragrans* root acetone extract (CfAE) and *C. fragrans* root ethanol extract (CfEE) were estimated using Folin-Ciocalteu (FC) colorimetric method[67] by expressing the results as equivalents of gallic acid. FC method is considered as one of the simplest, reproducible and best methods for the estimation of total phenolic content. FC reagent is a mixture of heteropolytungstates and molybdates, which is yellow in colour. Under basic condition FC reagent reacts with phenolic compounds and undergoes reduction to form a blue species, possibly $(PMoW_{11}O_{40})^{4-}$, that can be detected spectrophotometrically at 760 nm.[68]

Using the aforesaid method, TPC of CfAE and CfEE were estimated. TPC of CfAE was found to be 115.64 ± 7.22 mg gallic acid equivalents/100 g of dry roots and for CfEE it has been found to be 102.77 ± 3.30 mg gallic acid equivalents/100 g of dry roots. From the results obtained it is clear that the acetone extract contains slightly more phenolics in it compared to ethanol extract. Presence of high phenolic content in the case CfAE was consistent with the results obtained during the isolation of components from CfAE.

2.5.3. Total flavonoid content (TFC)

Total flavonoid content (TFC) was estimated using aluminium chloride ($AlCl_3$) colorimetric method[69] and the results are expressed as equivalents of quercetin. Aluminium chloride forms acid stable complexes with the C-4 keto group and either C-3 or C-5 hydroxyl group of the flavones and flavonols. In addition, aluminium chloride forms acid labile complexes with the *ortho* dihydroxyl groups in the A- and B-ring of flavonoids.[70] Binding of Al^{3+} ion with quercetin is shown in fig. 2.23.

The total flavonoid content of CfAE was found to be 78.82 ± 1.31 mg of quercetin equivalents/100 g dry roots and CfEE contained 61.19 ± 3.79 mg of quercetin equivalents/100 g dry roots. The study revealed that flavonoid content also is higher in CfAE as compared to CfEE.

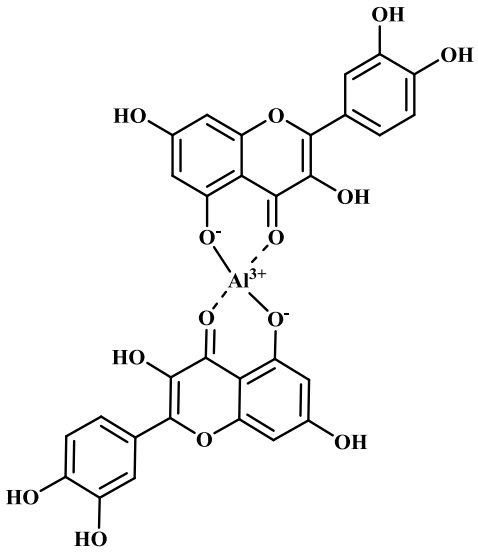


Figure 2.23: Binding of Al^{3+} ion with quercetin

2.5.4. Total antioxidant capacity (TAC)

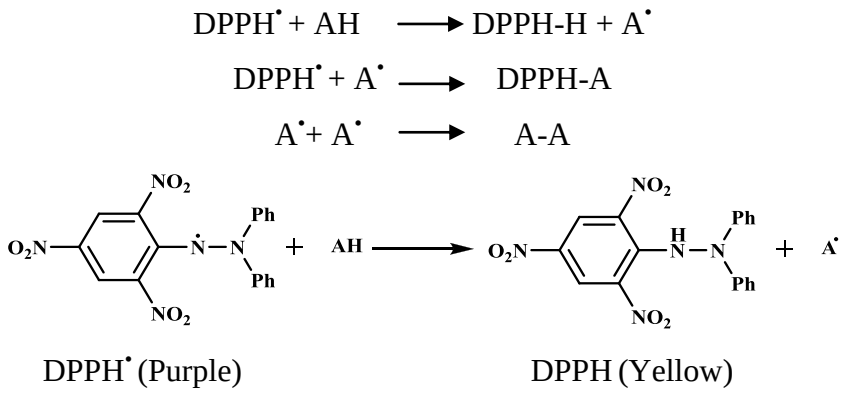
Total antioxidant capacity (TAC) of *C. fragrans* extracts were estimated using phosphomolybdenum method[71] by expressing the results as equivalents of ascorbic acid. The method is based upon the reduction of Mo (VI) to Mo (V) by the antioxidant compound and followed by the formation of a green phosphate / Mo (V) complex with absorption at 695 nm. The antioxidant capacity CfAE was found to be 498.03 ± 13.73 mg of ascorbic acid equivalents / 100 g dry roots and in the case of CfEE, TAC was found to be 307.29 ± 5.20 mg of ascorbic acid equivalents / 100 g dry roots. The study revealed that antioxidant capacity also is higher for CfAE as compared to CfEE. High antioxidant activity of CfAE could be attributed to the presence of high phenolic and flavonoid content in it.

2.5.5. Free radical scavenging activity

Free radical scavenging capacity of *C. fragrans* extracts as well as the isolated phenolic compounds was evaluated using $DPPH^{\bullet}$ scavenging activity. $DPPH^{\bullet}$ (1,1'-diphenyl-2-picrylhydrazyl radical) is a stable free radical which is purple in colour. Antioxidant compound reduces $DPPH^{\bullet}$ to yellow coloured diphenylpicrylhydrazine. The extent of the reaction depends upon the hydrogen donating ability of the antioxidant.[72] The scavenging action of antioxidants can be expressed as shown in scheme 2.5. The absorbance is read at 517 nm and the percentage of inhibition is calculated using the equation:

$$\text{Percentage of radical scavenging activity} = [(A_0-A_t)/A_0] \times 100$$

Where, A_0 and A_t are the absorbance of control and sample respectively.



Scheme 2.5: DPPH radical scavenging by antioxidant

IC_{50} values (the concentration of the sample required to scavenge 50% of free radicals) of each sample were calculated from the graph plotted with concentration of the samples against percentage inhibition of the free radical. Butylated hydroxyanisole (BHA) was used as the standard for comparison. Results obtained are tabulated in table 2.3.

Table 2.3: IC_{50} values of extracts and isolated phenolic compounds

Sl. No.	Sample Name	IC_{50} values (μg/mL)
1	CfAE	318.51
2	CfEE	381.80
3	Naringenin	>1000
4	Aromadendrin	>1000
5	Matairesinol (MIR)	47.05
6	Vanillic acid	225.81
7	Ferulic acid (FA)	65.53
8	Protocatechuic acid (PA)	**26.9**
9	BHA	33.1

Results indicated that CfAE showed higher radical scavenging activity compared to CfEE. This may be due to the presence of high phenolic and flavonoid content in it which are capable of donating hydrogen radical and can form stable phenoxyl radical. Among the various phenolic compounds tested, protocatechuic acid showed maximum radical scavenging activity with an IC_{50} value of 26.9 μg/mL, which has been found to be better than that of the standard (BHA) used in the study. Matairesinol and ferulic acid showed comparable activity with that of BHA. $DPPH^{\bullet}$ scavenging capacity of vanillic acid was found to be lower when compared to the other

two phenolic acids. The two flavonoids viz., naringenin and aromadendrin did not show any radical scavenging activity in the tested concentration range (100 to 1000 μg/mL). The plot of percentage of DPPH radical scavenging versus concentration of sample is given in fig. 2.24.

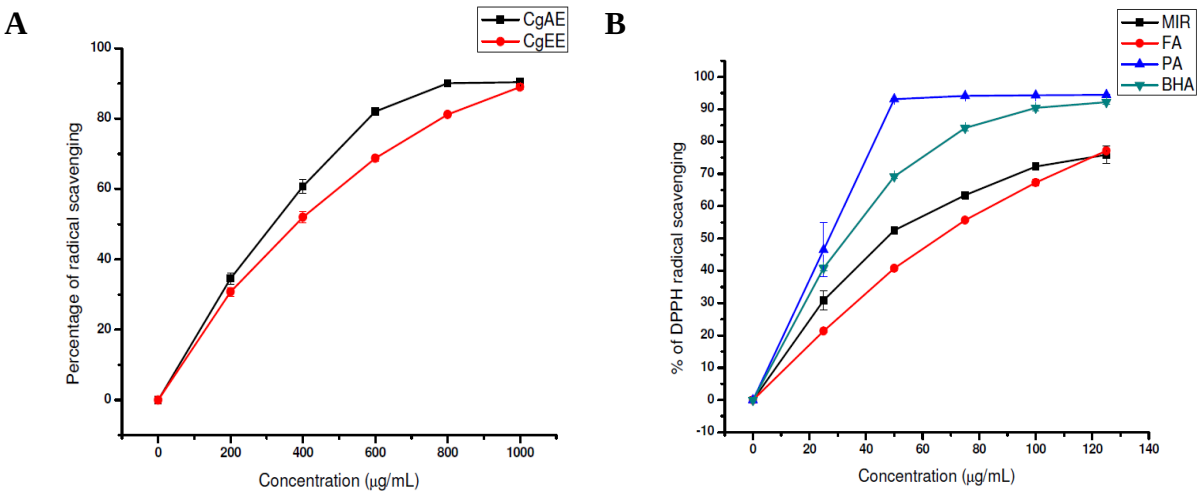


Figure 2.24: A) DPPH radical scavenging capacities of *C. fragrans* extracts B) DPPH radical scavenging capacities of phenolic compounds

One of the reasons for the poor radical scavenging activity of these two flavonoid compounds may be the absence of 3',4'-dihydroxy substitution pattern in the B ring, which appears to be a predominating factor in aiding the radical scavenging activity of flavonoids. Poor activity of naringenin could be again substantiated using the fact that it lacks a hydroxyl group in the C ring also. Even though there is a hydroxyl group in C-3 position of aromadendrin, the radical scavenging activity is low due to the absence double bond between C-2 and C-3 in the C ring, which prevents delocalization of electrons from the aryloxyl radical on the B ring to the A ring. The results obtained are in good agreement with the literature reports available on the structure activity studies on flavonoids.[73]

2.6. Antimicrobial studies of alkaloids isolated from *C. fragrans*

2.6.1. Need for new antimicrobial agents

Infectious diseases are one of the foremost reasons for death across the world, affecting people even in developed countries like US and UK.[74] Microbial species belonging to *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Clostridium* are the major Gram-positive pathogens and *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* etc., are the major Gram-negative pathogens, responsible for

serious infections in human beings. Till date, more than 70% of the antibiotics used are either NPs or their synthetic derivatives. Among them, microbial NPs are the chief source of antibiotics and microorganisms like actinomycetes, cyanobacteria, mycobacteria, fungi etc., are the most fruitful sources for the production of antibiotics.

The period between 1925-1950 was considered as the golden era of antibiotic drug discovery. As mentioned in chapter 1, effective treatment of infectious diseases became a reality after the serendipitous discovery of penicillin in 1928 by Alexander Fleming. Streptomycin was another important antimicrobial compound isolated from the soil-derived Streptomycetes by Selman Waksman. After that, several natural and semi-synthetic antibiotic classes including β-Lactams (eg: meropenem), aminoglycoside (eg: amikacin), macrolide (eg: azithromycin), tetracycline (eg: minocycline), rifamycin (eg: rifampicin), glycopeptide (eg: telavancin), quinolones (eg: balofloxacin), oxazolidinones (eg: linezolid) macrolactones (eg: fidaxomicin) etc., were introduced into the market to combat infectious diseases.[75,76,77]

One of the major concerns associated with antimicrobial therapy is the quickened emergence of antibiotic resistance related with existing antimicrobial agents which will limit their efficiency thus making the diseases which were under control, to fatal ones.[77-78,79] Emergence of multidrug-resistance of bacteria has increased steeply to such an extent in the twenty first century that WHO introduced the theme 'Antimicrobial resistance: no action today, no cure tomorrow' for the world health day celebrations in 2011.[80] Usually, resistance arises due to the mutation in microbes and the genes of resistance traits disseminate rapidly among bacteria of different taxonomic and ecological groups. Prolonged and excess use of antibiotics to control infectious diseases puts selective pressure on bacteria that provides a competitive advantage for mutated strains.[78,81]

Common multidrug resistant bacteria include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp., together known as 'ESKAPE' organisms.[75,82] To reduce the effect of antimicrobial resistance most of the pharmaceutical companies introduced synthetically modified molecules of the existing class of drugs since they have higher chances of success. Almost all of the antibiotic drugs marketed in between 1962 to 2000 were obtained by modifying the then existing class of drugs. Many new anti microbial agents, belonging to new chemical classes,

were introduced recently and even then the new classes of antibiotics were found be useful only for a short span of time due to emergence of resistance.[79,83]

Continuous emergence of antibiotic resistance demands development of new class of antibiotics with novel mechanisms of action. Scientific communities are now in search of new sources of antimicrobial agents apart from the microbial world. Widening the chemical diversity of drugs will be an effective way to provide new classes of antibiotics. Currently, much attention is given to medicinal plants since they are the reservoirs of molecules with wide structural diversity and novel modes of action. Even though steroidal alkaloids of *C. fragrans* were reported much earlier, they have not been tested for any bioactivity. Therefore, in the present study, we have investigated the antibacterial potentials of the alkaloids isolated from *C. fragrans* against a group of Gram-positive as well as Gram-negative bacteria. *These studies were done in collaboration with Dr. Nishanth Kumar S. and Dr. Dileep Kumar B. S., Agroprocessing and Natural Products Division, CSIR-NIIST.*

All the five alkaloids isolated viz., japindine (JPD), deaminooxo chonemorphine (DOC), *N*-formylchonemorphine (NFC), *N*-methylchonemorphine (NMC) and chonemorphine (CMN) were tested for their activity against four different Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermis* and *Staphylococcus simulans*) and five different Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Pseudomonas mirabilis* and *Salmonella typhi*). Tetracycline antibiotic minocycline (MCN) was used as the standard for comparison. Antimicrobial activity studies of these steroidal alkaloids are being reported for the first time.

2.6.2. Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs)

MIC (the lowest concentration of test compounds that inhibited the visual growth after incubating with the test bacteria for 18 h) and MBC (the lowest concentration of the compounds at which 99.99% or more of the initial inoculum was killed) ranges of alkaloids against various test bacteria are given in table 2.4. Among the various alkaloids tested, deaminooxochonemorphine (DOC) was found to be most effective against all the tested strains. Japindine, which is only found in *C. fragrans*, showed activity against *B. subtilis*, *S. epidermis*, *E. coli* and *K. pneumonia*. In the case of japindine best result was obtained against *S. epidermis*. Except chonemorphine all other

compounds were found to active against *B. subtilis*, with maximum activity being shown by DOC with MIC/MBC value of 32/64. All the tested alkaloids of *C. fragrans* except deaminooxochonemorphine were found to be ineffective against *S. aureus, S. simulans, P. aeruginosa, P. mirabilis* and *S. typhi*. While all these compounds were found to be effective against *K. pneumonia*, the best result was obtained for DOC which showed MIC/MBC value of 16/32. DOC showed maximum activity against the Gram-negative bacteria, *S. typhi* also, which is the causative agent of typhoid. The activity was comparable to that of minocycline (MCN), the positive control used in the study.

From the results obtained, it is clear that the activity of DOC is much higher compared to other alkaloids. The increased activity of the compound may be attributed to the presence of hydroxyl group in the C-3 position which is absent in all other alkaloids tested.

Table 2.4: MIC and MBC of test compounds against bacteria

Test bacteria	MIC/MBC (µg/mL)					
	JPD	**DOC**	**NMC**	**CMN**	**NFC**	**MCN**
	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC
B. subtilis	500/1000	32/64	500/1000	-	500/1000	4/4
S. aureus	-	16/32	1000/-	-	-	2/4
S. epidermis	125/250	32/64	125/125	500/1000	250/500	2/2
S. simulans	-	125/125	-		-	2/4
E. coli	500/500	32/64	-	-	-	1/2
K. pneumoniae	250/500	16/32	125/250	1000/1000	125/125	1/1
P. aeruginosa	-	64/125	-	-	-	1/1
P. mirabilis	-	64/125	-	-	-	4/8
S. typhi	-	8/16	-	1000/-	-	4/4

(-) Recorded no MIC up to 1000 µg/mL, Values represent mean of three replications

2.6.3. Antibacterial activity using disc diffusion method

Quantification of antibacterial activity was carried out using agar disc diffusion method and the results are shown in table 2.5. In all the bacterial strains tested maximum zone of inhibition was shown by DOC. Photograph of zone inhibition by DOC against the test bacteria is given in fig. 2.25.

Table 2.5: Zone of inhibition of the test compounds against bacteria

Test bacteria	Zone of inhibition (dia. in mm using 50 µg/mL of test compound)					
	JPD	**DOC**	**NMC**	**CMN**	**NFC**	**MCN**
B. subtilis	8 ± 0.57	24 ± 0.57	9 ± 1.17	-	13 ± 1.15	24 ± 1.00
S. aureus	-	26 ± 0.00	10 ± 0.78	-	-	23 ± 0.00
S. epidermis	16 ± 0.52	18 ± 1.00	11 ± 0.52	8 ± 1.12	12 ± 1.57	31 ± 0.52
S. simulans	-	13 ± 1.12	-	-	-	27 ± 0.77
E. coli	-	20 ± 0.72	-	-	-	25 ± 0.62
K. pneumoniae	12 ± 0.77	24 ± 0.52	12 ± 1.00	9 ± 1.52	16 ± 0.56	27 ± 0.00
P. aeruginosa	-	25 ± 2.22	-	-	-	29 ± 2.52
P. mirabilis	-	18 ± 0.78	-	-	-	23 ± 1.15
S. typhi	-	28 ± 0.00	-	7 ± 1.77	-	26 ± 1.71

(-) no zone of inhibition, Values represent mean of three replications

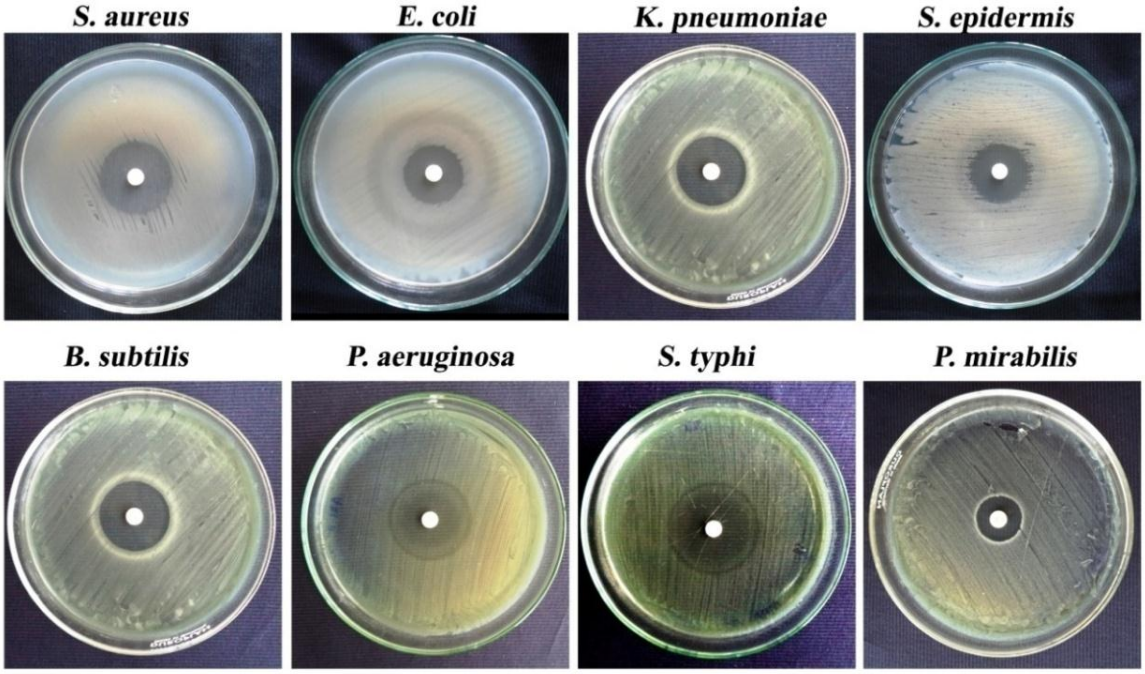


Figure 2.25: Photographs of zone of inhibition by DOC against various pathogens

In the case of Gram-positive pathogen *B. subtilis*, zone of inhibition for DOC was comparable with that of minocycline, the positive control used in the study and against *S. aureus* DOC showed higher zone of inhibition (26 ± 0.00) than that of minocycline. Most interestingly DOC showed zone of inhibition 28 ± 0.00 mm against the Gram-negative pathogen, *S. typhi* which was higher than that obtained for minocycline.

2.6.4. Cytotoxicity test against normal human cell lines

The cytotoxic activity of the most active alkaloid viz., deaminooxochonemorphine (DOC) was tested against H9c2 (rat embryonic cardiomyoblasts) cell line by MTT assay. Fig. 2.26 represents the confocal image of H9c2 cells after treatment with different concentrations of DOC (10, 50, 100 and 200 µM) and fig. 2.27 depicts the cytotoxicity of DOC expressed in bar graph (control, 10, 25, 50, 100 and 200 µM). The results showed that there is no significant cytotoxicity up to 100 µM. But at 200 µM concentration, the compound was found to be toxic against normal cells and at this concentration approximately 33% of cells were found to be dead. These results of the present study clearly indicate that the compound may be safe up to 100 µM concentration for the normal human cells. *These studies were done in collaboration with Dr. Vandana Sankar, Agroprocessing and Natural Products Division, CSIR-NIIST.*

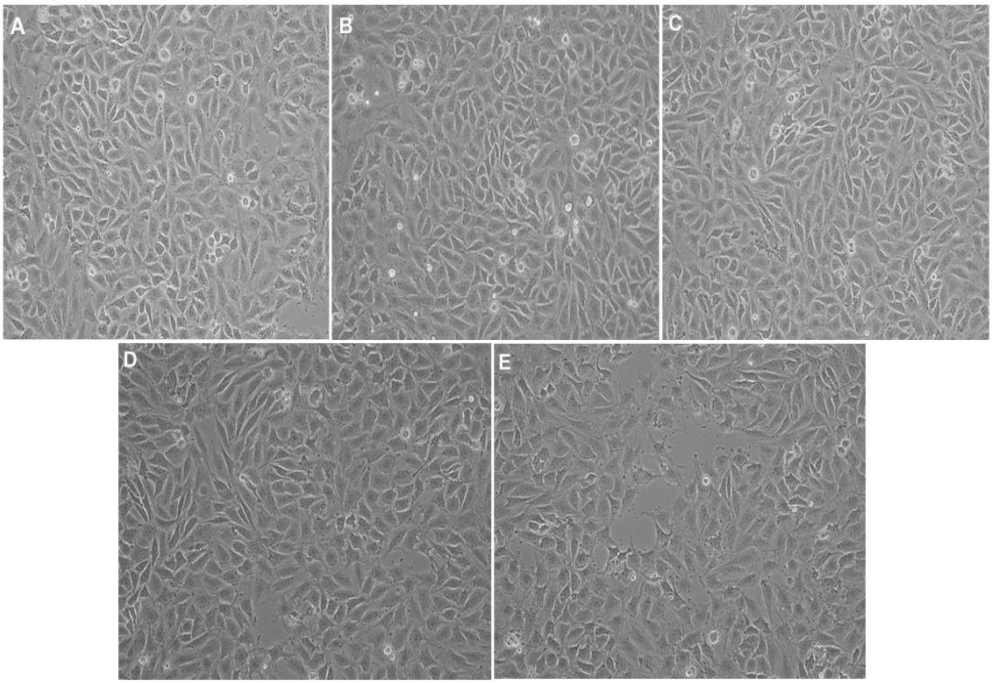


Figure 2.26: Confocal image of H9c2 cells when treated with different concentrations of DOC; A – control, B – treated with 10 µM DOC, C – treated with 50 µM DOC, D – treated with 100 µM DOC, E – treated with 200 µM DOC

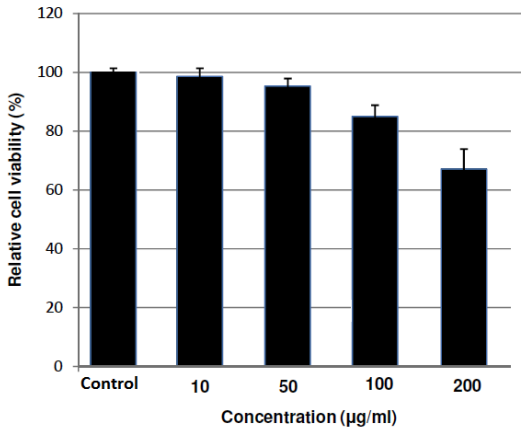


Figure 2.27: Relative cell viability after DOC treatment

2.7. Experimental

2.7.1. General Experimental details

Melting points are uncorrected and were determined using Fisher-Jones melting point apparatus. IR spectra were recorded on a Bruker Alpha FT-IR spectrometer. ^{1}H NMR spectra were recorded at 500 MHz and ^{13}C NMR at 125 MHz using deuterated chloroform ($CDCl_3$) or a mixture of $CDCl_3$ and deuterated methanol (CD_3OD) as the solvent on Bruker AMX 500 MHz spectrometer. Tetramethylsilane (TMS) was used as the internal standard and chemical shift values are expressed in δ-scale in units of parts per million (ppm) and coupling constants (*J*) in Hz. Abbreviations used in ^{1}H NMR are s - singlet, d - doublet, t - triplet, dd - doublet of doublet, brs - broad singlet and m - multiplet. Mass spectra were recorded using Thermo Scientific Exactive mass spectrometer under ESI/HRMS mode at 61800 resolution. The UV spectra were recorded on Shimadzu UV-1601 UV-visible spectrophotometer using spectroscopic grade methanol as solvent. Specific rotations were recorded on Rudolph Research Analytical Autopol I automatic polarimeter. The plant materials were dried in RRLT-NC natural convection air drier at 40-45 °C. Solvents used for chromatography were distilled prior to use. Column chromatography was carried out using silica gel (100-200 mesh) for non-alkaloid constituents and basic alumina was used for the separation of alkaloid constituents. The solvents were removed under reduced pressure using Büchi or Heidolph rotary evaporator. Analytical thin layer chromatography was performed on Merck silica gel 60 F_{254} aluminium sheets. Spots were first visualized under UV light (range 254-365 nm) and later using iodine chamber or by using Dragendorff's reagent.

2.7.2. Preparation of Dragendorff's (DD) reagent

Solution A: Dissolve 0.85 g of bismuth (III) subnitrate (Sigma-Aldrich, USA) in 10 mL glacial acetic acid (Merck, India) and dilute it with 40 mL distilled water.

Solution B: Dissolve 8 g of potassium iodide (Merck, India) in 20 mL distilled water

Stock solution: Mix equal volume of solutions A and B

Working solution: Mix 1 mL stock solution with 2 mL glacial acetic acid and 10 mL distilled water prior to use.

2.7.3. Isolation of compounds from *C. fragrans* roots

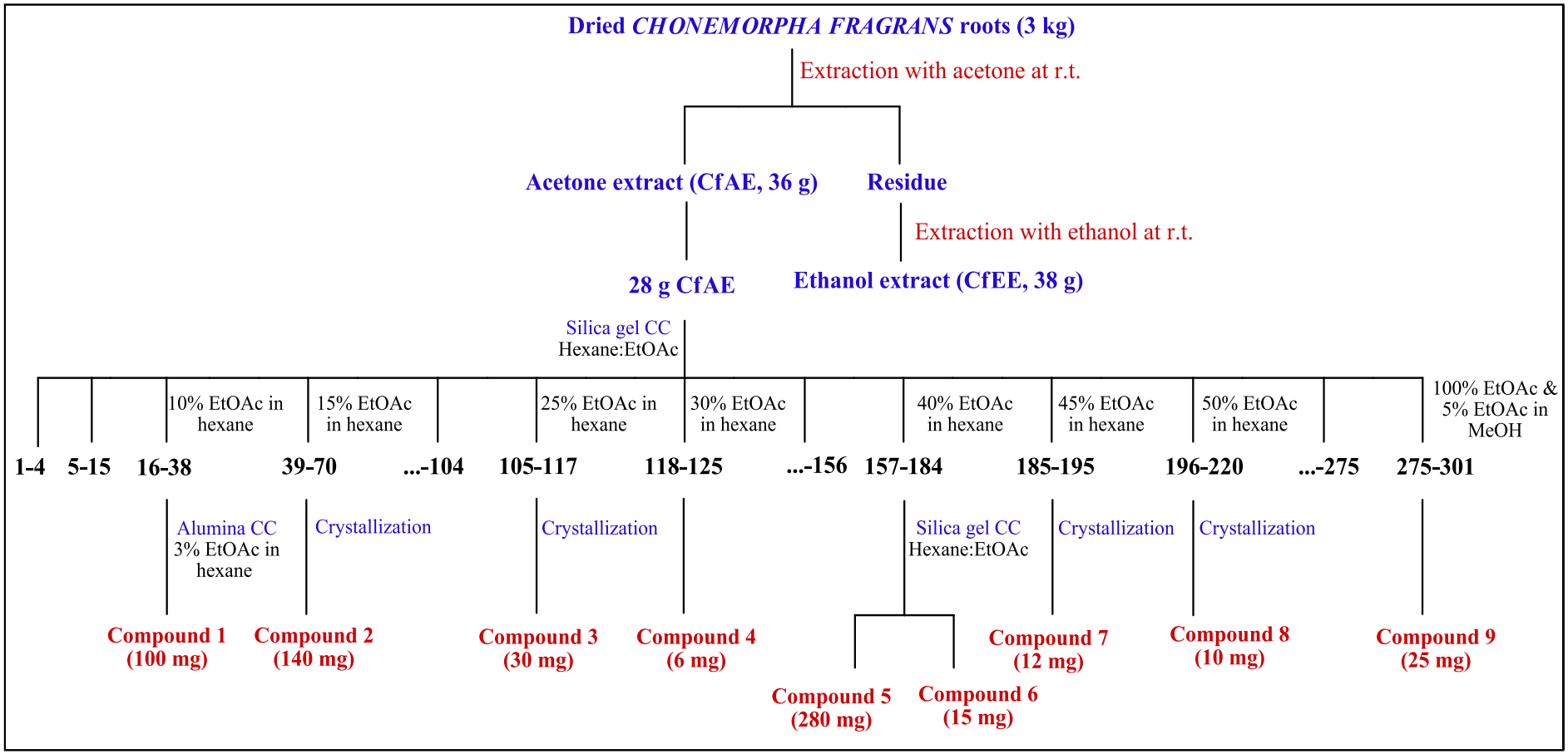
2.7.3.1. Collection of plant material and extraction

Chonemorpha fragrans roots were collected from a medicinal plant garden in Thiruvananthapuram and a voucher specimen was deposited in JNTBGRI, Palode, Thiruvananthapuram, Kerala (JN TBGRI 83447). The material was dried in an air oven maintained between 40-45 °C for three days. About 3 kg of the dried material was then powdered mechanically and subjected to further extraction with acetone at room temperature (5 L × 3). The total extract was then concentrated under reduced pressure using Heidolph rotary evaporator to yield approximately 36 g of the acetone extract (CfAE). The residue obtained after extraction using acetone was subjected to extraction using ethanol at room temperature (5 L × 3) to yield 38 g of the ethanol extract (CfEE) after removing the solvent. These two extracts were tested for the presence of alkaloid in them using Dragendorff's (DD) reagent. Ethanol extract gave orange red precipitate on addition of DD reagent whereas the acetone extract did not show the presence of any alkaloid.

2.7.3.2. Isolation of non-alkaloid constituents

C. fragrans acetone extract was investigated for the isolation of non-alkaloid constituents. After studying the TLC in various polarity of the solvent, 28 g of the acetone extract was subjected to column chromatographic purification using silica gel (500 g, 100-200 mesh). Column elution was started using 100% hexane and increase in polarity was carried out by increasing the amount of ethyl acetate. Final elution was carried out using 5% methanol in ethyl acetate. A total of 301 fractions of approximately 150 mL each were collected. According to the similarity in TLC, they were pooled into nineteen major fraction pools. Nine compounds were isolated from various fractions and the pictorial representation of the isolation of non-alkaloid phytochemicals from *C. fragrans* roots is given in chart 2.3.

Chart 2.3: Pictorial representation for the isolation of non-alkaloid constituents from *C. fragrans*



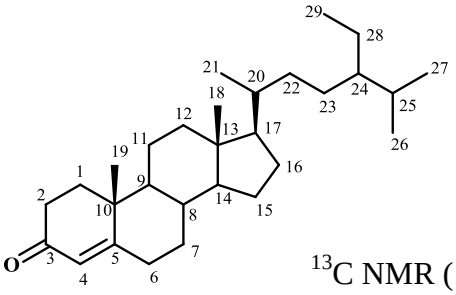
2.7.3.2.1. Isolation of compound 1

Fraction pool three (Fr. 16-38), obtained by eluting the column with 10% ethyl acetate in hexane showed the presence of an UV active compound along with some minor impurities. It was again subjected to column chromatographic purification using alumina by eluting with 3% ethyl acetate in hexane to yield 100 mg of colourless crystals. The compound was successfully characterized as **sitostenone** based on the spectral data obtained, as given below.

Colourless crystals; m.p. 96-98 °C, lit.[24b] 97-99 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 2939, 2868, 1677, 1617, 1459, 1377, 1268, 1231, 1184

^{1}H NMR (500 MHz, $CDCl_3$) : δ 5.74 (s, 1H, H-4), 1.19 (s, 3H, H-19), 0.93 (d, *J* = 6.0 Hz, 3H, H-21), 0.87-0.79 (m, 9H, H-26, H-27, H-29), 0.72 (s, 3H, H-18), 2.40-0.95 (m, 29H, other aliphatic hydrogens)



^{13}C NMR (125 MHz,$CDCl_3$) : δ 199.7 (C-3), 171.8 (C-5), 123.7 (C-4), 56.0, 55.9, 53.8, 45.8, 42.4 (C-13), 39.6 (C-10), 38.6, 36.1 (2C), 35.7, 35.6, 34.0, 33.9, 32.9, 29.1, 28.2, 26.0, 24.2, 23.0, 21.1, 21.0 (C-26), 19.8 (C-27), 19.0 (C-21), 18.7 (C-19), 11.9 (C-18), 11.2 (C-29)

HRMS (m/z) : 413.3772 $[(M+H)^+]$; $C_{29}H_{48}O$, requires 412.3705

NMR spectral assignments were made on the basis of DEPT-135 NMR analysis and on comparison with earlier literature reports.[24]

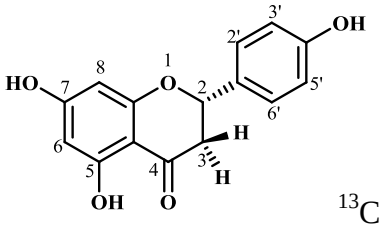
2.7.3.2.2. Isolation of compound 2

Fraction pool four (Fr. 39-70), obtained by eluting the column with 15% ethyl acetate in hexane on crystallization yielded 140 mg of colourless needle like crystals of

compound **2** with melting point 136-138 °C. The compound was successfully characterized as the common phytosterol, ***β*-sitosterol**, based on various spectral data, melting point[84] and on comparison with authentic sample using TLC.

2.7.3.2.3. Isolation of compound 3

Fraction pool seven (Fr. 105-117), obtained by eluting the column with 25% ethyl acetate in hexane on crystallization yielded 30 mg of pale yellow crystals. The compound was successfully characterized as the flavonoid **naringenin** based on the spectral data obtained, as given below.



Pale yellow crystals; m.p. 248-250 °C, lit.[85] 248-250 °C

UV (MeOH, λ_{max}, nm): 336.3 sh., 296

FT-IR (KBr, υ_{max}, cm^{-1})	:	3440, 2921, 2850, 2314, 1640, 1600, 1514, 1158, 1023
^1H NMR (500 MHz, $CDCl_3$ + CD_3OD)	:	δ 7.26 (d, *J* = 8.5 Hz, 2H, H-2', H-6'), 6.83 (d, *J* = 8.5 Hz, 2H, H-3', H-5'), 5.93 (s, 2H, H-6, H-8), 5.28 (dd, *J* = 13.0, 2.5 Hz, 1H, H-2), 3.05 (dd, *J* = 17.0, 13.0 Hz, 1H, H-3a), 2.69 (dd, *J* = 17.0, 13.0 Hz, 1H, H-3b)
^{13}C NMR (125 MHz, $CDCl_3$+ CD_3OD)	:	δ 195.9 (C-4), 166.7 (C-7), 163.6 (C-5), 163.1 (C-9), 157.2 (C-4'), 129.1 (C-1'), 127.6 (2C, C-2',6'), 115.3 (2C, C-3',5'), 102.2 (C-10), 96.2 (C-6), 95.4 (C-8), 78.8 (C-2), 42.8 (C-3)
HRMS (m/z)	:	273.0765 $[(M+H)^+]$; $C_{15}H_{12}O_5$, requires 272.0685

NMR spectral assignments were made on the basis of DEPT-135, 2D NMR analysis and on comparison with the literature reports.[26-27]

2.7.3.2.4. Isolation of compound 4

Fraction pool eight (Fr. 118-125), obtained by eluting the column with 30% ethyl acetate in hexane on crystallization yielded 6 mg of white amorphous powder, which was characterized as the flavonoid **aromadendrin** based on the spectral data obtained, as given below.

White amorphous powder

UV (MeOH, λ_{max}, nm): 324.8 sh., 290.4

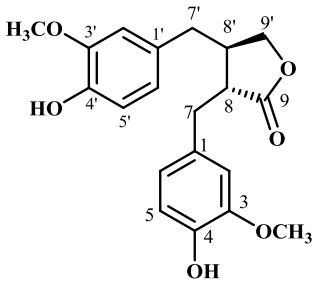
FT-IR (KBr, υ_{max}, cm^{-1})	:	3447, 2924, 2852, 1642, 1600, 1520, 1280, 1220, 1160
^{1}H NMR (500 MHz, $CDCl_3$ + CD_3OD)	:	δ 7.37 (d, *J* = 8.5 Hz, 2H, H-2', H-6'), 6.90 (d, *J* = 8.5 Hz, 2H, H-3', H-5'), 6.01 (s, 1H, H-6), 5.96 (s, 1H, H-8), 5.01 (d, *J* = 12.0 Hz, 1H, H-2), 4.54 (d, *J* = 11.5 Hz, 1H, H-3)
^{13}C NMR (125 MHz, $CDCl_3$+ CD_3OD)	:	δ 196.5 (C-4), 167.6 (C-5), 163.8 (C-7), 163.3 (C-9), 157.9 (C-4'), 129.3 (2C, C-2',6'), 127.7 (C-1'), 115.7 (2C, C-3',5'), 100.7 (C-10), 96.9 (C-6), 96.0 (C-8), 83.6 (C-2), 72.5 (C-3)
HRMS (m/z)	:	287.0563 [$(M-H)^+$]; $C_{15}H_{12}O_6$, requires 288.0634

NMR spectral assignments were made on the basis of DEPT-135, 2D NMR analysis and on comparison with the literature reports.[27,31]

2.7.3.2.5. Isolation of compound 5

Twelfth fraction pool (Fr. 157-184) obtained by eluting the column with 40% ethyl acetate in hexane showed the presence of two UV active compounds with some impurities. This was further purified using silica gel column chromatography to yield compounds **5** and **6.** Compound **5** (280 mg) obtained as a viscous liquid was

characterized as the lignan lactone **matairesinol** based on the spectral data obtained, as given below.

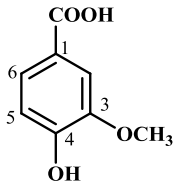


FT-IR (KBr, υ_{max}, cm^{-1})	:	3425, 2926, 2850, 1761, 1606, 1514, 1457, 1434, 1271, 1205, 1154, 1026
^1H NMR (500 MHz, $CDCl_3$)	:	δ 6.81 (m, 2H, H-5, H-5'), 6.61 (m, 2H, H-2, H-6), 6.51 (dd, *J* = 8.0, 1.5 Hz, 1H, H-2'), 6,42 (d, *J* = 1.5 Hz, 1H, H-6'), 5.61 (brs, 2H, -OH), 4.16 (dd, *J* = 9.0, 7.5 Hz, 1H, H-9a), 3.89 (dd, *J* = 8.8, 7.5 Hz, 1H, H-9b), 3.82 (s, 3H, $-OCH_3$), 3.82 (s, 3H, $-OCH_3$), 2.96 (dd, *J* = 14.3, 5.3 Hz, 1H, H-7a), 2.88 (dd, *J* = 14.0, 7.0 Hz, 1H, H-7b), 2.64-2.45 (m, 4H, H-7, H-8, H-8')
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 178.9 (C-9), 146.7 (C-3), 146.6 (C-3'), 144.5 (C-4), 144.4 (C-4'), 129.8 (C-1), 129.5 (C-1'), 122.0 (C-6), 121.3 (C-6'), 114.4 (C-5), 114.1 (C-5'), 111.5 (C-2), 110.9 (C-2'), 71.4 (C-9') , 55.8 ($-OCH_3$), 55.8 ($-OCH_3$), 46.5 (C-8), 41.0 (C-8'), 38.3 (C-7'), 34.6 (C-7)
HRMS (m/z)	:	381.1306 $[(M+Na)^+]$; $C_{20}H_{22}O_6$, requires 358. 1416
$[\alpha]_D^{26}$	:	-34.0° (MeOH; *c* 0.01), lit.[86] - 33.3° (MeOH; *c* 0.087).

NMR spectral assignments were made on the basis of DEPT-135, 2D NMR analysis and on comparison with the literature reports.[34-35]

2.7.3.2.6. Isolation of compound 6

15 mg of compound **6** obtained as a white amorphous powder on continued purification of the twelfth fraction pool (Fr. 157-184) was characterized as the phenolic acid, **vanillic acid** based on the spectral data obtained as given below, comparison with literature reports and by co-spotting TLC with authentic sample of **vanillic acid**.[43]



White powder; m.p. 210-212 °C, lit.[24b] 210-212 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 3481, 2921, 2852, 1684, 1600, 1522, 1432, 1296, 1209, 1111, 1028, 915

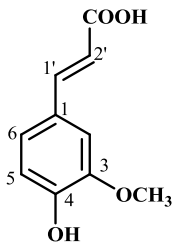
^{1}H NMR (500 MHz, $CDCl_3$) : δ 7.72 (dd, *J* = 8.3, 2.0 Hz, 1H, H-6), 7.59 (d, *J* = 2.0 Hz, 1H, H-2), 6.97 (d, *J* = 8.5 Hz, 1H, H-5), 6.08 (brs, 1H, -OH), 3.97 (s, 3H, $-OCH_3$)

^{13}C NMR (125 MHz,$CDCl_3$ + CD_3OD) : δ 169.0 (-COOH), 150.6 (C-4), 146.7 (C-3), 124.4 (C-6), 121.8 (C-1). 114.4 (C-2), 112.4 (C-5), 55.8 ($-OCH_3$)

HRMS (m/z) : 167.0343 $[(M-H)^+]$; $C_8H_8O_4$, requires 168.0423

2.7.3.2.7. Isolation of compound 7

Thirteenth fraction pool (Fr. 185-195) obtained by eluting the column with 45% ethyl acetate in hexane on crystallization using the same solvent yielded 12 mg of compound **7** as pale yellow crystals, which was characterized as **ferulic acid** on the basis of spectral data obtained as shown below and on comparison with the literature reports.[44]



Pale yellow crystals; m.p.: 168-170 °C, lit.[24b] 168-169 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 3435, 3009, 2923, 1686, 1621, 1595, 1514, 1461, 1377, 1271, 1205, 1179

^{1}H NMR (500 MHz, $CDCl_3$) : δ 7.71 (d, *J* = 15.5 Hz, 1H, H-1'), 7.11 (dd, *J* = 8.0, 2.0 Hz, 1H, H-6), 7.06 (d, *J* = 2.0 Hz, 1H, H-2), 6.94

(d, *J* = 8.5 Hz, 1H, H-5), 6.30 (d, *J* = 15.5 Hz, 1H, H-2'), 5.90 (brs, 1H, -OH), 3.97 (s, 3H, $-OCH_3$)

^{13}C NMR (125 MHz, $CDCl_3$ + CD_3OD) : δ 165.9 (-COOH), 144.5 (C-4), 143.4 (C-3), 141.9 (C-1'), 122.6 (C-1), 119.1 (C-2'), 111.1 (C-2), 111.0 (C-5), 105.9 (C-6), 51.8 ($-OCH_3$)

HRMS (m/z) : 193.0501 [$(M-H)^+$]; $C_{10}H_{10}O_4$, requires 194.0597

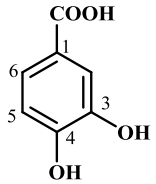
2.7.3.2.8. Isolation of compound 8

Fourteenth fraction pool (Fr. 196-220) obtained by eluting the column with 50% ethyl acetate in hexane yielded 10 mg of compound **8** as off white amorphous powder, which was characterized as **protocatechuic acid** on the basis of spectral data obtained as shown below and on comparison with the literature reports.[45]

Off white powder; m.p. 196-198 °C, lit.[87] 199 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 33291, 2921, 2852, 1672, 1603, 1526, 1423, 1304, 1251, 1096, 1057, 764

^{1}H NMR (500 MHz, $CDCl_3$) : δ 7.72 (dd, *J* = 8.3, 2.0 Hz, 1H, H-6), 7.59 (d, *J* = 2.0 Hz, 1H, H-2), 6.97 (d, *J* = 8.5 Hz, 1H, H-5)



^{13}C NMR (125 MHz,$CDCl_3$ + CD_3OD) : δ 169.3 (-COOH), 149.9 (C-4), 144.4 (C-3), 123.1 (C-1), 121.8 (C-6), 116.61 (C-2), 114.62 (C-5)

HRMS (m/z) : 153.0186 [$(M-H)^+$]; $C_7H_6O_4$, requires 154.0266

2.7.3.2.9. Isolation of compound 9

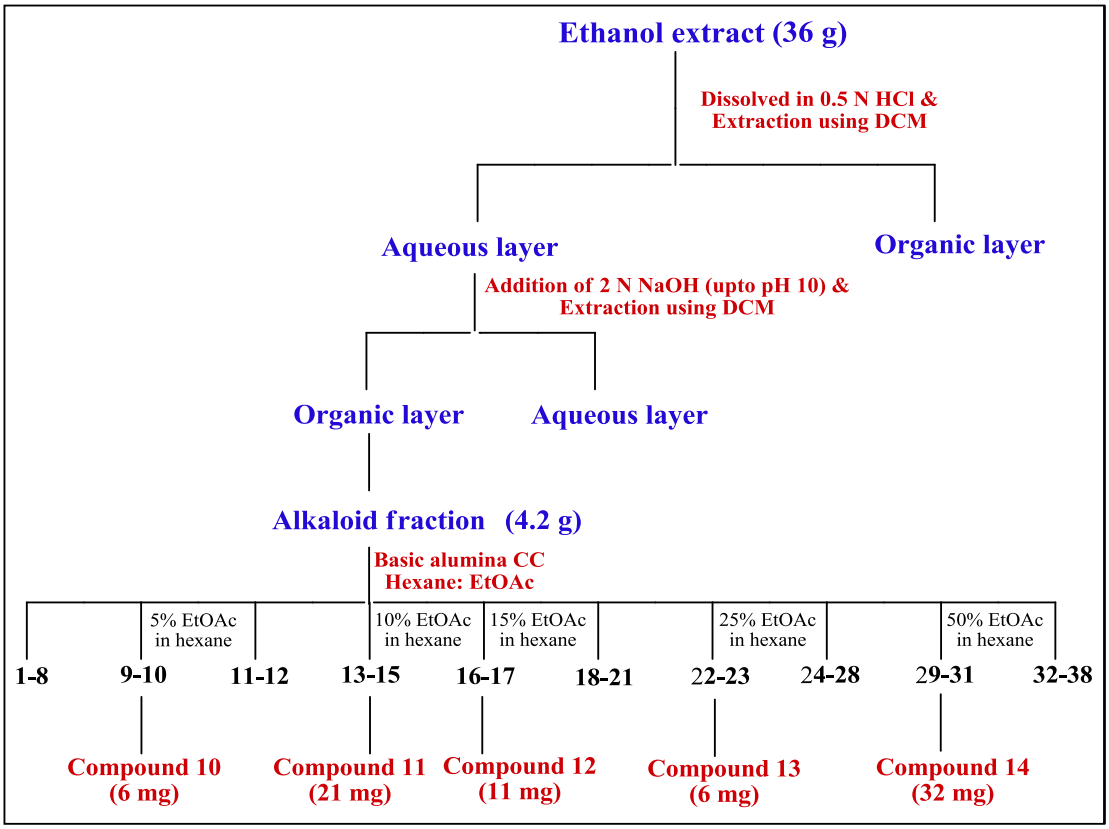
Nineteenth fraction pool (Fr. 275-301), obtained by eluting the column with 100% ethyl acetate followed by 5% methanol in ethyl acetate, gave 25 mg of compound **9** as a white amorphous powder. The compound was successfully characterized as

sitosterol-3-*O*-β-D-glucopyranoside based on the spectral data obtained and on comparison with the literature reports.[46]

2.7.3.3. Isolation of alkaloid constituents

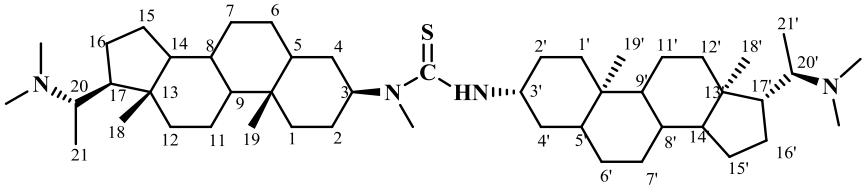
Alkaloids were isolated from the ethanol extract of *C. fragrans* roots (CfEE, 36 g). Alkaloid mixture was extracted from CfEE using standard acid-base extraction technique.[47] For this, the extract was dissolved in 0.5 N HCl and partitioned using dichloromethane. The aqueous layer obtained after the extraction with DCM was treated with 2 N sodium hydroxide solution until pH 10 was reached. The free alkaloids were then extracted from the aqueous layer using DCM. A total of 4.2 g of the alkaloid extract thus obtained was purified using column chromatography using basic alumina as the adsorbent and mixtures of hexane–ethyl acetate was used as the eluent. Column elution was started using 100% hexane and increase in polarity was carried out by increasing the amount of ethyl acetate. A total of 38 fractions of approximately 50 mL each were collected. Five alkaloids were thus isolated from various fractions and the pictorial representation of the isolation of alkaloid constituents from *C. fragrans* ethanol extract (CfEE) is given in chart 2.4.

Chart 2.4: Pictorial representation for the isolation of alkaloid constituents from CfEE



2.7.3.3.1. Isolation of compound 10

Fractions 9-10 obtained by eluting the column with 5% ethyl acetate in hexane yielded 6 mg of compound **10** as a white amorphous solid The compound was successfully characterized as the sulphur containing pregnane type steroidal alkaloid, **japindine** based on the spectral data obtained as given below.



White amorphous powder

FT-IR (KBr, υ_{max}, cm^{-1})	:	3377, 2926, 2914, 2851, 2836, 1528, 1455, 1378, 1361, 1350, 1170
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 5.07 (d, *J*= 7.5 Hz, 1H, -*N*H), 4.36 (m, 1H, H-3'), 2.89 (s, 3H, -*NC*H_3), 2.42 (m, 2H, H-20, H-20'), 2.17 (s, 12 H, 2 × -*N*$(CH_3)_2$), 0.87 (d, *J*= 7.5 Hz, 6H, H-21, H-21'), 0.80 (s, 6H, H-19, H-19'), 0.67 (s, 6H, H-18, H-18'), 2.45-0.70 (m, other aliphatic hydrogens)
^{13}C NMR (125 MHz,$CDCl_3$)	:	δ 179.9 (>C=S), 61.2 (2C, C-20, C-20'), 59.1 (C-3'), 56.5 (2C, C-14, C-14'), 56.8 (2C, C-17, C-17'), 54.2 (C-9), 54.1 (C-9'), 45.3 (C-5), 45.19, C-5'), 41.7 (4C, C-10, C-10', C-13, C-13'), 39.8 (4C, 2 × -*N*$(CH_3)_2$), 39.7 (2C, C-12, C-12'), 37.3 (2C, C-1, C-1'), 35.6 (2C), 35.4 (2C), 31.9 (2C), 31.6 (–*N*CH_3), 29.7, 28.7 (2C), 28.7, 28.6 , 27.6 (2C), 24.0 (2C, C-15, C-15'), 21.1 (2C, C-11, C-11'), 12.4 (2C, C-18, C-18'), 12.3 (2C, C-19, C-19'),

9.9 (2C, C-21, C-21')

HRMS (m/z) : 749.6507 [$(M+H)^+$]; $C_{48}H_{84}N_4S$, requires 748.6417

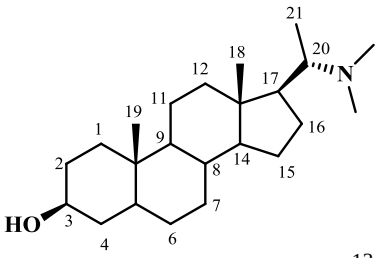
NMR spectral assignments were made on the basis of DEPT-135, 2D NMR (HOMO and HETERO COSY) analysis, comparing the spectral values of the compound with steroidal alkaloid monomers isolated from the plant (*N*-methylchonemorphine, *N*-formylchonemorphine) and on comparison with the literature reports.[17] *C. fragrans* is the sole source japindine and to the best of our knowledge ^{13}C NMR spectral details of the compound is being reported for the first time.

2.7.3.3.2. Isolation of compound 11

Fractions 13-15 obtained by eluting the column with 10% ethyl acetate in hexane yielded 21 mg of compound **11** as a colourless crystalline solid. The compound was successfully characterized as the pregnane type steroidal alkaloid, **sarcorucinine D (deaminooxochonemorphine)** based on the spectral data obtained as given below.

Colourless crystals; m.p. 148-150 °C, lit.[48] 149-150 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 3311, 2928, 2855, 1450, 1378, 1262, 1169, 1076, 1043



^1H NMR (500 MHz, $CDCl_3$) δ 3.59 (m, 1H, H-3), 2.51 (m, 1H, H-20), 2.22 (s, 6H, -*N*$(CH_3)_2$, 0.91 (d, *J* = 6.0 Hz, 3H, H-21), 0.80 (s, 3H, H-19), 0.65 (s, 3H, H-18), 0.63 (m, 1H, H-9), 1.99-0.88 (m, other aliphatic hydrogens)

^{13}C NMR (125 MHz,$CDCl_3$) : δ 71.3 (C-3), 61.5 (C-20), 56.6 (C-14), 54.6 (C-9), 54.3 (C-17), 44.8 (C-5), 41.9 (C-13), 39.8 (C-12), 39.8 (2C, -*N*$(CH_3)_2$), 38.2 (C-4), 37.0 (C-1), 35.5 (C-10), 35.4 (C-8), 32.0 (C-7), 31.5 (C-2), 28.7 (C-6), 27.6 (C-16), 24.1 (C-15), 21.2 (C-11), 12.3

(C-19), 12.3 (C-18), 10.1 (C-21)

HRMS (m/z) : 348.3271 [$(M+H)^+$] ; $C_{23}H_{41}NO$, requires 347.3188

NMR spectral assignments were made on the basis of DEPT-135, 2D NMR analysis and on comparison with the literature reports.[48,88] This is the first report on the isolation of sarcorucinine D from any plant belonging to Apocynaceae family.

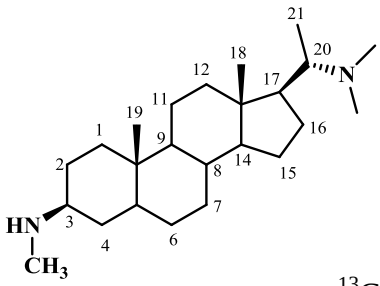
2.7.3.3.3. Isolation of compound 12

Fractions 16-17 obtained by eluting the column with 15% ethyl acetate in hexane yielded 11 mg of compound **12** as a white amorphous solid. The compound was successfully characterized as the pregnane type steroidal alkaloid, ***N*-methyl chonemorphine** or **dictyophlebine** based on the spectral data obtained as given below.

Colourless crystals; m.p. 170-172 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 3270, 2926, 2853, 1447, 1381, 1263, 1048, 1011

^{1}H NMR (500 MHz, $CDCl_3$) : δ 2.77 (m, 1H, H-3), 2.53 (s, 3H, -*N*H<u>C</u>H$_3$) 2.53 (m, 1H, H-20), 2.25 (s, 6H, -*N*$(CH_3)_2$, 0.93 (d, *J* = 6.5 Hz, 3H, H-21), 0.83 (s, 3H, H-19), 0.66 (s, 3H, H-18), 0.66 (m, 1H, H-9), 1.98-0.95 (m, other aliphatic hydrogens)



^{13}C NMR (125 MHz,$CDCl_3$) : δ 61.7 (C-20), 58.8 (C-3), 56.6 (C-14), 54.7 (C-9), 54.3 (C-17), 45.2 (C-5), 41.8 (C-13), 39.8 (2C, C-4, C-12), 39.8 (2C, -*N*$(CH_3)_2$), 37.1 (C-1), 35.8 (C-10), 35.4 (C-8), 32.0 (2C, C-7, C-2), 31.9 (-*N*HCH_3), 28.7 (C-6), 27.6 (C-16), 24.0 (C-15), 21.1 (C-11), 12.3 (C-19), 12.3 (C-18), 10.1 (C-21)

HRMS (m/z) : 361.3220 [$(M+H)^+$] ; $C_{24}H_{44}N_2$, requires 360.35104

NMR spectral assignments were made on the basis of DEPT-135, 2D NMR analysis and on comparison with the literature reports.

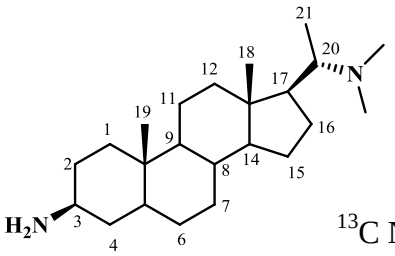
2.7.3.3.4. Isolation of compound 13

Fractions 22-23 obtained by eluting the column with 25% ethyl acetate in hexane yielded 6 mg of compound **13** as a white amorphous powder. The compound was successfully characterized as the pregnane type steroidal alkaloid, **chonemorphine** based on the spectral data obtained on comparison with the literature reports. The compound was reported earlier from *C. fragrans* root bark.[15]

White powder; m.p. 144-146 °C, lit.[15] 144-145 °C,

FT-IR (KBr, υ_{max}, cm^{-1}) : 3218, 2925, 2853, 2773, 1460, 1380, 1222, 1049, 1012

^{1}H NMR (500 MHz, $CDCl_3$) : δ 2.64 (m, 1H, H-3), 2.42 (m, 1H, H-20), 2.30 (s, 6H, -*N*$(CH_3)_2$, 0.86 (d, *J* = 6.5 Hz, 3H, H-21), 0.78 (s, 3H, H-19), 0.65 (s, 3H, H-18), 0.64 (m, 1H, H-9), 1.89-0.94 (m, other aliphatic hydrogens)



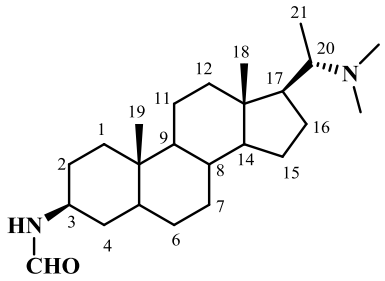
^{13}C NMR (125 MHz,$CDCl_3$) : δ 61.3 (C-20), 56.5, 54.5, 54.3, 51.0, 45.4, 42.1, 39.8, 39.6 (2C, -*N*$(CH_3)_2$), 38.5, 37.6, 35.5, 35.4, 32.0 (2C), 28.7, 27.4, 24.1, 21.1, 12.3 (2C C-19, C-18), 10.5 (C-21)

HRMS (m/z) : 347.3435 [$(M+H)^+$] ; $C_{23}H_{42}N_2$, requires 346.3348

2.7.3.3.5. Isolation of compound 14

Fractions 29-31 obtained by eluting the column with 50% ethyl acetate in hexane yielded 32 mg of compound **14** as pale yellow crystalline solid. The compound

was successfully characterized as the pregnane type steroidal alkaloid, ***N*-formyl chonemorphine** based on the spectral data obtained as given below.



Pale yellow crystals; m.p. 288-290 °C, lit.[17] 289-291 °C

FT-IR (KBr, υ_{max}, cm^{-1})	:	3282, 2932, 2855, 2774, 1661, 1539, 1451, 1381, 1265, 1161, 1045
^{1}H NMR (500 MHz, $CDCl_3$)		δ 8.09 (s, 1H, -*N*CHO), 5.40 (d, *J* = 7.5 Hz, 1H, -*N*HCHO), 3.86 (m, 1H, H-3), 2.45 (m, 1H, H-20), 2.19 (s, 6H, -*N*$(CH_3)_2$), 0.88 (d, *J* = 6.0 Hz, 3H, H-21), 0.79 (s, 3H, H-19), 0.65 (m, 1H, H-9), 0.64 (s, 3H, H-18), 2.05-0.89 (m, other aliphatic hydrogens)
^{13}C NMR (125 MHz,$CDCl_3$)	:	δ 160.4 (-*N*HCHO), 61.3 (C-20), 56.5 (C-14), 54.7 (C-17), 54.2 (C-9), 47.9 (C-3), 45.3 (C-5), 41.8 (2C, C-10, C-13), 39.8 (2C, -*N*$(CH_3)_2$), 39.7 (C-12), 37.3 (C-1), 35.4 (C-8), 35.3 (C-4), 31.9 (C-7), 28.8 (C-6), 28.5 (C-2), 27.6 (C-16), 24.0 (C-15), 21.0 (C-11), 12.3 (C-18), 12.2 (C-19), 9.9 (C-21)
HRMS (m/z)	:	375.3381 $[(M+H)^+]$; $C_{24}H_{42}N_2O$, requires 374.3297

NMR spectral assignments were made on the basis of DEPT-135, 2D NMR analysis and on comparison with the literature reports.[17] Some of the ^{13}C NMR values which were misinterpreted in the literature are corrected in this report.[58]

2.7.4. Antioxidant activity studies on *C. fragrans* root extracts and isolated phenolic compounds

2.7.4.1. Chemicals used

Spectroscopic grade methanol, butylated hydroxy anisole (BHA), gallic acid, quercetin, ascorbic acid, Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), sodium

nitrite ($NaNO_2$), aluminium chloride ($AlCl_3$) and sodium hydroxide (NaOH) were purchased from Merck, India. 1,1'-diphenyl-2-picrylhydrazyl radical ($DPPH^{\bullet}$) was procured from Sigma-Aldrich Co., USA and ammonium heptamolybdate from Nice chemicals, India. All chemicals used were analytical grade.

2.7.4.2. Total Phenolic Content (TPC)

The total phenolic content of *C. fragrans* extracts was determined using Folin – Ciocalteu colorimetric method as described by Slinkard *et al.* with slight modification.[67] Folin–Ciocalteu reagent (0.5 mL), previously diluted with distilled water (1:2) was added to appropriately diluted samples (0.5 mL) of different concentrations (200-1000 µg/mL) and mixed thoroughly. To this mixture, 1 mL saturated sodium carbonate was added and made up to 10 mL with distilled water. The mixture was allowed to stand at room temperature for 45 minutes. Contents were centrifuged if a precipitate was noticed. The absorbance was measured using colorimetric method against a reagent blank at 760 nm. Blank was prepared by adding all the reagents except the extract. Gallic acid was used as the standard. The TPC was assessed by plotting the gallic acid calibration curve (25 to 125 µg/mL) and expressed as milligrams of gallic acid equivalents (GAE) per 100 gram of dry weight of sample. Data are reported as mean ± SD for at least three replications.

2.7.4.3. Total Flavonoid Content (TFC)

Total flavonoid content was determined by aluminium chloride colorimetric method by employing quercetin as the standard.[69,89] To appropriately diluted samples (1 mL), 4 mL distilled water was added. To this 0.3 mL of 5% (w/v) $NaNO_2$ was added and kept for 5 minutes. After 5 minutes 0.3 mL of 10% (w/v) $AlCl_3$ was added to form a flavonoid – aluminium complex. After 6 minutes, 2 mL of 1 M NaOH was added and the total volume was made to 10 mL using distilled water. The solution was mixed well again and the absorbance was measured against a reagent blank, at 415 nm. Blank was prepared by adding all reagents except $AlCl_3$. Distilled water was added in place of $AlCl_3$ in the blank. Quercetin was used as the standard. The TFC was assessed by plotting the quercetin calibration curve (100 to 500 µg/mL) and expressed as milligrams of quercetin equivalents (mg QE/100 gram of dry weight of the sample). Data are reported as mean ± SD for at least three replications.

2.7.4.4. Total Antioxidant Capacity (TAC)

Total antioxidant capacity was determined by phosphomolybdenum method by using ascorbic acid as the standard.[71] 0.5 mL of the extract was combined with 5 mL of the reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate, 4 mM ammonium molybdate) and the reaction mixture was incubated at 95 °C for 90 minutes. After cooling to room temperature, absorbance of the resulting solution was measured at 695 nm against the blank. Blank was prepared by adding methanol in the place of extract. Ascorbic acid was used as the standard. The TAC was assessed by plotting the ascorbic acid calibration curve (100 to 500 µg/mL) and expressed as milligrams of ascorbic acid equivalents (mg AAE/100 gram of dry weight of the sample). Data are reported as mean ± SD for at least three replications.

2.7.4.5. Free radical scavenging activity

Radical scavenging capacities of *C. fragrans* extracts as well as the isolated phenolic compounds were determined by $DPPH^{\bullet}$ scavenging assay as explained by Brand Williams *et al*.,.[90] $DPPH^{\bullet}$ (0.1 mM) as well as the extracts (200–1000 µg/mL) and different concentrations of phenolic compounds were dissolved in methanol. To 0.5 mL of sample solutions taken in test tubes, about 5 mL of $DPPH^{\bullet}$ solution was added and shaken well. The test tubes were incubated at room temperature in darkness for 30 minutes. The control was prepared with 0.5 mL MeOH and 5 mL $DPPH^{\bullet}$ solution. After 30 minutes, absorbance was read at 517 nm. Percentage of radical scavenging was calculated using the formula:

$$\text{Percentage of radical scavenging activity} = [(A_0\text{-}A_t) / A_0] \times 100$$

Where, A_0 and A_t are the absorbance of control and sample respectively.

IC_{50} values were calculated from the graph plotted with percentage of radical scavenging capacity on Y axis and sample concentration on X axis. BHA was used as the standard for comparison and all determinations were done in triplicates.

2.7.5. Antimicrobial activity of alkaloids isolated from *C. fragrans*

2.7.5.1. Test microorganisms and antibiotic used

The following four Gram-positive bacteria viz., *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermis* and *Staphylococcus simulans* and five Gram-negative bacteria viz., *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Pseudomonas mirabilis* and *Salmonella typhi* were used in the present study. All the strains were cultured at 37 °C on nutrient agar (NA) medium and stored

at 4 °C. All the test microorganisms were purchased from Microbial Type Culture Collection Centre, IMTECH, Chandigarh, India.

The tetracycline antibiotic, minocycline (98% pure) was used in this study as a positive control. The compound was purchased from Sigma-Aldrich, USA. The structure of the compound is given in fig. 2.28.

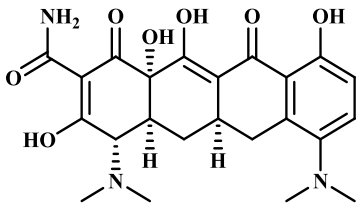


Figure 2.28: Structure of minocycline

2.7.5.2. Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs)

The test compounds and the antibiotic, minocycline (the positive control used in the study) were screened for antimicrobial activity using the macro broth dilution method in nutrient broth (NB).[91] To determine the MIC, the test compounds were dissolved in DMSO to afford a stock concentration of 4000 μg/mL; while the antibiotic was dissolved in sterile distilled water to afford stock concentrations of 1000 μg/mL. All stock concentrations of test compounds and antibiotics were filter sterilized using 0.22 μM syringe filter (Millipore). Two fold serial dilutions of the test compounds and antibiotic were prepared with NB to afford concentrations ranging from 1 to 1000 μg/mL. Five hundred microliter of 1×10^6 CFU/mL bacterial suspensions were added to the sterile test tubes affording an inoculum of 5×10^5 CFU/mL. Another 50 μL of antibiotics or test compound were pipetted into the tubes and incubated at 37 °C for 18 h. The tubes were read visually and spectrophotometrically at 600 nm. The lowest concentration of the test compounds that exhibited no turbidity was recorded as the MICs. The control tube did not have any antibiotics or test compounds, but contained the test bacteria and DMSO was used to dissolve the compounds. DMSO was found not to affect the growth of the bacteria during the experiments. MIC was defined as the lowest concentration of test compounds that inhibited the visual growth after incubating with the test bacteria for 18 h.

For MBC testing, an aliquot of inoculum was taken from MIC test tubes that did not show turbidity and serially diluted in saline and plated into nutrient agar plates for

each bacterial species. The agar plates were incubated for 24 h at 37 °C. The MBC value was read as the lowest concentration of the compounds at which 99.99% or more of the initial inoculum was killed.[92] The MIC and MBC experiments were carried out in triplicate.

2.7.5.3. Antibacterial activity determination using disc diffusion method

The antibacterial activity of the test compounds was determined by the disc diffusion method against the test bacteria on Muller–Hinton agar, according to the Clinical and Laboratory Standards Institute (CLSI). The media plates (MHA) were streaked with bacteria 2–3 times by rotating the plate at 60° angles for each streak to ensure the homogeneous distribution of the inoculums. After inoculation, discs (6 mm Hi-Media) loaded with 50 µg/mL of the test compounds were placed on the bacteria-seeded plates using sterile forceps. The plates were then incubated at 37 °C for 24 h. The inhibition zone around the discs was measured and recorded. Minocycline (Hi-Media) was used as the positive controls to compare the efficacy of the test samples. DMSO served as the negative control, and assays were carried out in triplicates.

2.7.5.4. Cytotoxicity of DOC against normal cell lines

2.7.5.4.1. Cell line maintenance

H9c2 cell lines (rat embryonic cardiomyoblasts) were obtained from ATCC (American Type Culture Collection, USA). For maintenance of cell lines, Dulbeccos Modified Eagle's Medium (DMEM) (Sigma) containing 10% fetal bovine serum (FBS) (Gibco), antibiotics (100 U/mL Penicillin and 100 µg/mL streptomycin) and amphotericin (0.25 µg/mL) (Hi-Media) was employed. The cells were maintained in cell culture flasks in CO_2 incubators at 37 °C with 5% CO_2 in air and 99% humidity. Passaging of cells when confluent was carried out using 0.25% trypsin and 0.02% EDTA (Hi-Media) in phosphate buffered saline (PBS).

2.7.5.4.2. MTT assay against normal human cell lines

Cell viability after incubating the cells with different concentrations of the test substance was determined by methyl thiazolyl tetrazolium (MTT) assay. It is a colorimetric assay based on the ability of live cells to reduce MTT (yellow) to a purple formazan product. The cells were spread in 96-well plates at 5×10^3 cells/well. After 36 h of seeding, they were incubated with different concentrations (10, 50, 100 and 200 µM) of the test compound for 24 h. Subsequently, the cells were exposed to MTT at a

concentration of 50 µg/well for 2.5 to 3 hrs at 37 °C in CO_2 incubator. The working solution of MTT was prepared in Hanks balanced salt solution (HBSS). After viewing formazan crystals under the microscope, the crystals were solubilized by treating the cells with DMSO: isopropanol at a ratio of 1:1 for 20 min at 37 °C. Plate was read at an absorbance of 570 nm. The relative cell viability in percent was calculated as: (Absorbance of treated/Absorbance of control) ×100. Control samples used were cells without any treatment. Cell viability of control cells were kept as 100%.

2.8. Conclusion

Nine non-alkaloid constituents viz., sitostenone, *β*-sitosterol, naringenin, aromadendrin, matairesinol, vanillic acid, ferulic acid, protocatechuic acid and sitosterol-3-*O*-*β*-D-glucopyranoside were isolated from the acetone extract of *C. fragrans* roots. Except *β*-sitosterol all other compounds are being reported for the first time from any part of *C. fragrans*. Five pregnane type steroidal alkaloids viz., japindine, deaminooxochonemorphine (sarcorucinine D), *N*-methylchonemorphine (dictyophlebine), chonemorphine and *N*-formylchonemorphine were isolated from the ethanol extract of *C. fragrans* roots. Among the alkaloid constituents isolated, deaminooxochonemorphine (sarcorucinine D) is being reported for the first time from *C. fragrans* as well as from Apocynaceae family. Preliminary antioxidant efficacy of acetone and ethanol extracts, radical scavenging activity of extracts as well as the isolated phenolic compounds were evaluated using *in vitro* methods. Antimicrobial potentials of the alkaloids isolated were examined against four different Gram-positive and five different Gram-negative pathogens. Deaminooxochonemorphine (DOC) was found to have the most promising activity against all the tested strains and can be considered as a potent lead for the development of new antimicrobial agent.

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Chapter 3A

Isolation and Characterization of Bioactive Compounds from *Piper cubeba* Seeds

3A.1. Introduction

Piperaceae or the pepper family is one of the earliest known families of flowering plants, consisting of more than 3,600 species, included under thirteen different genera. Plants belonging to this family usually grow as herbs, vines, shrubs, and rarely as trees. They are widely distributed throughout the tropical and subtropical regions of the world. Two important genera of the family are *Piper* and *Peperomia*.

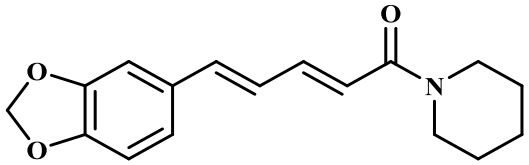
3A.1.1. *Piper*

Piper is an extremely large genus of shrubs, rarely herbs and trees containing more than 1,000 species distributed worldwide. Plants belonging to *Piper* species have attained worldwide attention due to their enormous medicinal, economical and commercial importance. The most popular member of the genus is *Piper nigrum*, the ripened fruit of which provides the important spice 'black pepper'.[1] Around 30 species of *Piper* are found in India. Among them, *P. nigrum* and *P. betle* are cultivated in India on a large scale,[2] whereas a few other members of the genus like *P. longum* are also cultivated in Kerala for medicinal purposes. Even though *P. cubeba* is not cultivated in Kerala, it is readily available in most of the Ayurvedic medicinal shops in the state. The above mentioned plants, belonging to *Piper* species, are widely used in the preparation of a large number of *Ayurvedic* formulations. Five important *Piper* plants available in Kerala which are commonly used in *Ayurveda* are given in table 3A.1.

Table 3A.1: Important *Piper* plants available in Kerala

Scientific name	Common name	Sanskrit name	Malayalam name
Piper betle	Betel	Nagavallari	Vettila
Piper brachystachyum	Wild pepper	Cavyah	Kattukurumulaku
Piper cubeba	Tailed pepper	Kankolam	Valmulaku
Piper longum	Long pepper	Pippali	Thippali
Piper nigrum	Black pepper	Maricam	Kurumulaku

Since plants belonging to this genus are extensively used in traditional systems of medicines like *Ayurveda*, *Sidha* etc., they have been studied in detail for their phytochemical constituents and several reviews are available on this topic.[1,3] Piperine (**A1**), the first amide alkaloid isolated from *Piper* species, is the most important compound present in almost all plants belonging to *Piper* species and is found in varying quantities. The compound possesses various bioactivities such as antioxidant,[4] antiarthritic, anti-inflammatory,[5] anticancer[6] activities and it can also act as a very good bioavailability enhancer.[7]



A1

Details of the major phytochemical constituents isolated from five important *Piper* species mentioned in table 3A.1 are discussed in detail in the following sections.

3A.1.1.1. *Piper nigrum*

Piper nigrum, commonly known as black pepper, is a perennial, climbing shrub found throughout India and is mostly cultivated as a mixed crop. It is one of the most popular spices in the world (known as 'King of spices') which possesses very high commercial and economic importance. The fruits of the plant possess medicinal properties and are used in traditional systems of medicine for the treatment of fever, cough, bronchitis, asthma, dysentery, flatulence, haemorrhoids etc.[8] Black pepper can be converted to white pepper after processing, which is preferred in European countries. For this, the pericarp of the fruit is removed and then dried.[9] The aroma of black pepper could be attributed to the essential oil, whereas the pungency is due to the presence alkaloid piperine.[10] Apart from piperine, other pungent alkaloids such as chavicine (an isomer of piperine), piperidine, piperittine are present in the seeds in small quantities.[11] The major constituents in the pepper oil were found to be α and β-pinene, limonene, piperonal, dihydrocarveol, epoxydihydrocaryophyllene, cryptone etc.[12] Phytochemical investigations of *P. nigrum* fruits led to the isolation of several bioactive amides and phenolic compounds. Apart from the seeds the roots of the plant are also a rich source of amidic alkaloids. Wei *et al.*, reported the presence of more than 50 alkaloids including nigramides A-S from the roots of *P. nigrum*.[13] Major alkaloids isolated from

the fruits of the plant are listed in table 3A.2 and their structures are given in chart 3A.1. Prefixes given to the compound numbers in table 3A.2 to 3A.5 are: **A**–alkaloid/amide, **F**–flavonoid, **L**–lignan, **N**–neolignan, **M**–miscellaneous and **P**–propenylphenols.

Table 3A.2: Alkaloids isolated from the fruits of *P. nigrum*

Plant	Name of the compound	No.	Reference
Piper nigrum	Brachyamide B	**A2**	14
	Dihydropipericide	**A3**	15,16
	3,4-dihydroxy-6-(*N*-ethylamino)benzamide	**A4**	17
	(2*E*, 4*E*)-N-dodecadienoyl pyrrolidine	**A5**	14
	N-trans-feruloyl tyramine	**A6**	18
	N-trans-feruloyl piperidine	**A7**	19
	N-formylpiperidine	**A8**	20
	Guineensine	**A9**	16
	N-5-(4-hydroxyphenyl)- 2*E*,4*E*-pentadienoyl piperidine	**A10**	18
	N-5-(4-hydroxy-3-methoxyphenyl)- 2*E*,4*E*-pentadienoyl piperidine	**A11**	19
	N-5-(4-hydroxy-3-methoxyphenyl)-2*E*-pentenoyl piperidine	**A12**	
	(2*E*, 4*E*)-*N*-isobutyldecadienamide	**A13**	21
	(2*E*, 4*E*)-*N*-isobutyleicosadienamide	**A14**	22
	(2*E*, 4*E*, 8*Z*)-*N*-isobutyleicosatrienamide	**A15**	23
	(2*E*, 4*E*)-*N*-isobutyloctadienamide	**A16**	
	(2*E*, 8*E*)-*N*-9-(3,4-methylenedioxyphenyl) nonadienoylpiperidine	**A17**	24
	(2*E*, 4*E*, 8*E*)-*N*-9-(3,4-methylenedioxy phenyl)nonatrienoylpyrollidine	**A18**	14
	(2*E*, 4*E*, 10*E*)-N-11-(3,4-methylenedioxy phenyl)undecatrienoylpiperidine	**A19**	25
	Pellitorine	**A20**	26
	Piperamide	**A21**	14

	Piperamine	**A22**	15
	Piperettine	**A22**	
	Piperine	**A1**	14
	Piperolein B	**A24**	14
	Pipilyasine	**A25**	27
	Pipnoohine	**A26**	26
	Pipyahyine	**A27**	
	Pipyaqubine	**A28**	27
	Pipzubedine	**A29**	
	Reteofractamide A	**A30**	14
	Sarmentine	**A31**	
	Sarmentosine	**A32**	
	Tricolein	**A33**	
	Trichostachine	**A34**	

Chart 3A.1: Structures of alkaloids isolated from the fruits of *P. nigrum*

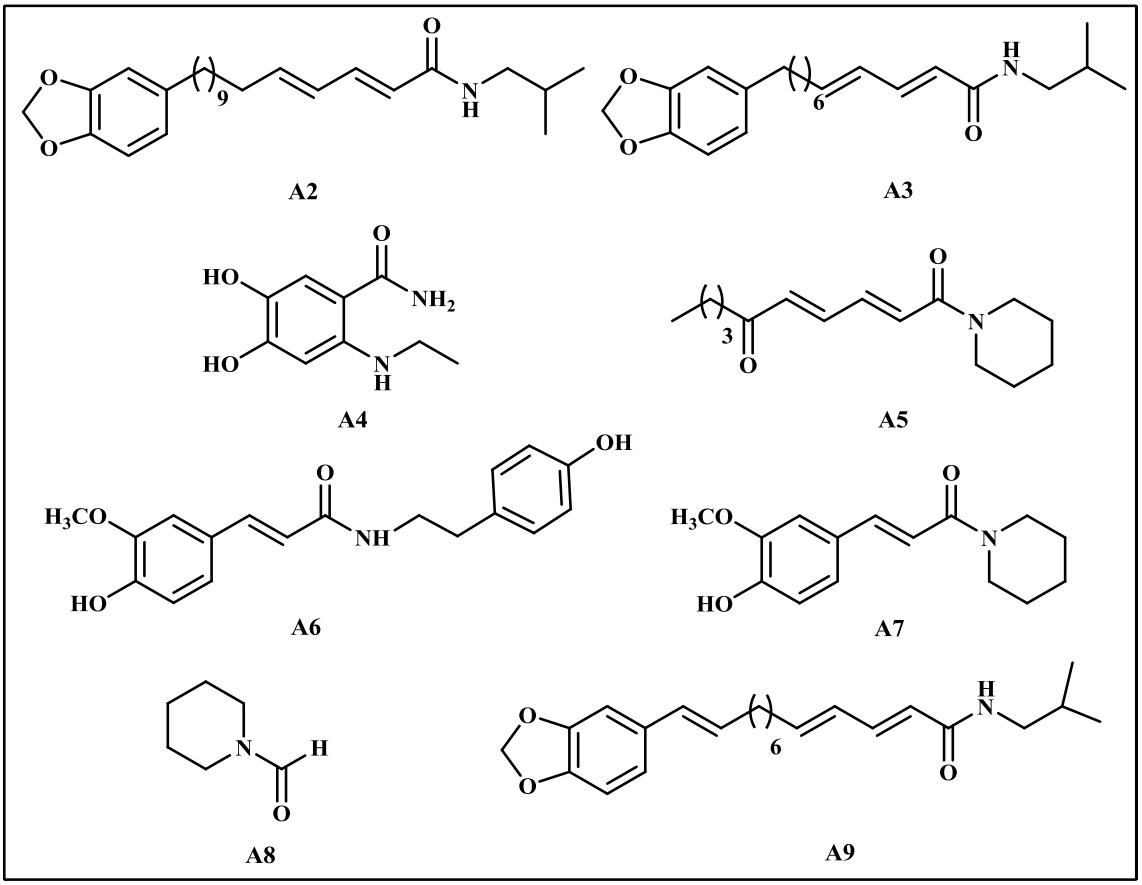
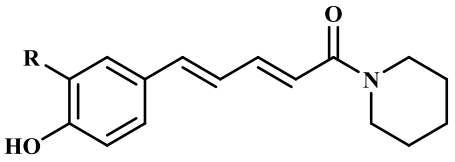
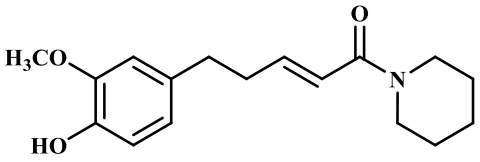


Chart 3A.1 continues.....



A10 R = H
A11 R = OH



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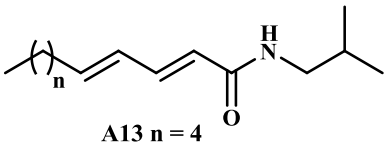

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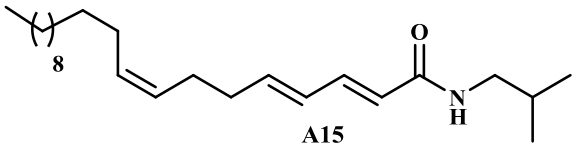
A14 n = 14
A16 n = 2



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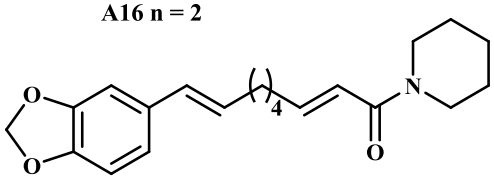

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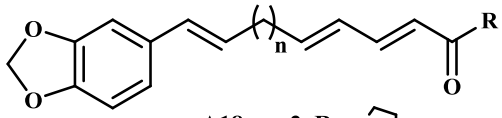
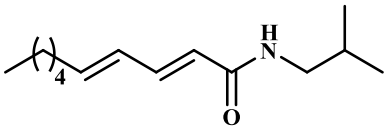


A18 n = 2, R=

A19 n = 4, R=



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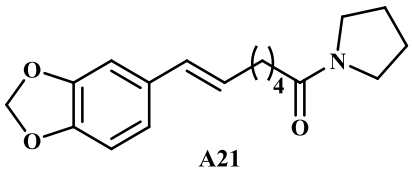

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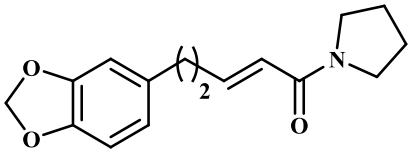

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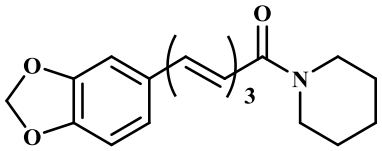

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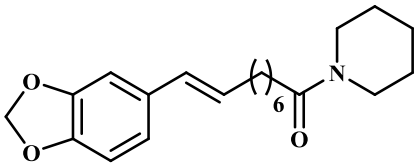

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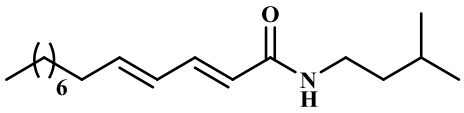

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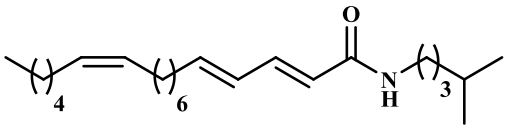

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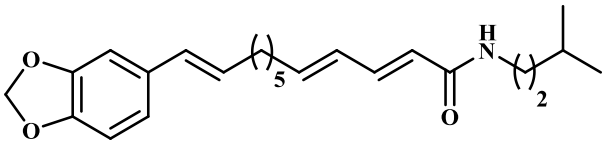

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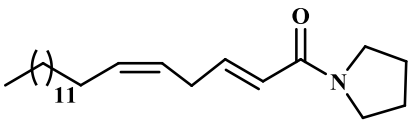

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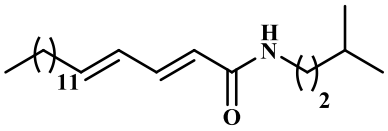

A29

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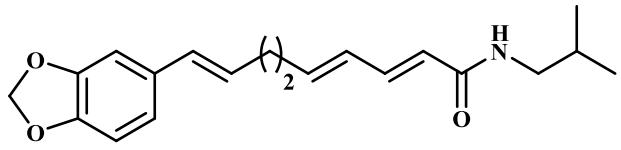

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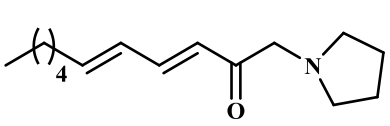

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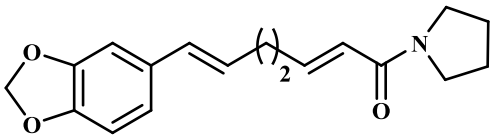

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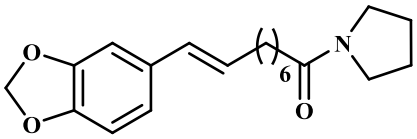

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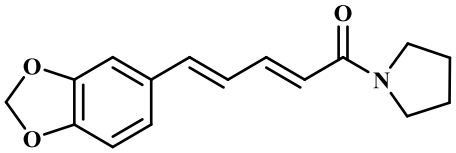


A34

3A.1.1.2. *Piper longum*

Piper longum is an aromatic climber grown throughout India. Fruits of the plant, which are sunk inside the solid fleshy spikes, are mainly used in *Ayurveda* for the treatment of problems related to respiratory system (cough, bronchitis and asthma) and digestive disorders. The matured, dried spikes of the plant are commercially sold as 'pippali'.[28] Usually, the dried spikes of other species such as *P. peepuloides* and *P. retrofractum* (syn. *P. Chaba)* are mistaken with that of *P. longum.* Apart from the seeds the dried roots of the plant (known as 'pipalmul') are also used as drug in *Ayurveda* and *Unani* for the treatment of gout, dyspepsia, stomachalgia, splenopathy etc.[29] Dried fruits of *P. longum* contain 0.7% of essential oil in it. The major constituent in the oil was found to be zingiberene followed by octadecane, heptadecane, eicosane, dihydrocarveol etc.[30] The main constituent present in the plant are alkaloids, including piperine, piperlongumine etc. Major compounds isolated from the fruits and roots of the plant are listed in table 3A.3 and their structures are shown in chart 3A.2.

Table 3A.3: Compounds isolated from *P. longum* (fruits and roots)

Plant	Name of the compound	No.	Reference
	Aristolactam A II	**A35**	31
	Brachystamide D	**A36**	32
	Cepharadione A	**A37**	31

Piper longum	Cepharadione B	**A38**	31
	Cepharanone B	**A39**	
	Desmethoxypiplartine dimer	**A40**	33
	5,6-dihydro-2(1H)-pyridinone	**A41**	34
	3β,4α-dihydroxy-2-piperidinone	**A42**	
	3β, 4α-dihydroxy-1-(3-phenylpropanoyl)-piperidine-2-one	**A43**	35
	Guineensine	**A9**	36
	(2*E*, 4*E*, 14*Z*)-6-hydroxyl-N-isobutyleicosa-2,4,14-trienamide	**A44**	35
	2-hydroxy-3-methoxy-4H-dibenzo-(d,e,g)quinoline-4,5-(6H)-dione	**A45**	31,37
	(2*E*, 4*E*)-*N*-isobutyldecadienamide	**A13**	38
	(2*E*, 4*E*)-*N*-isobutyleicosadienamide	**A14**	
	(2*E*, 4*E*, 8*Z*)-*N*-isobutyleicosatrienamide	**A15**	
	(2*E*, 4*E*)-*N*-isobutyloctadecadienamide	**A46**	
	(2*E*, 4*E*)-*N*-isobutyloctadienamide	**A16**	
	Isopiperlongumine	**A47**	32
	(2*E*, 8*E*)-*N*-9-(3,4-methylenedioxyphenyl) nonadienoyl piperidine	**A17**	34,36
	Norcepharadione	**A48**	31
	erythro-1-[1-oxo-9(3,4-methylenedioxy phenyl)-8,9-dihydroxy-2*E*-nonenyl]-piperidine	**A49**	34
	threo-1-[1-oxo-9(3,4-methylenedioxy phenyl)- 8,9-dihydroxy-2*E*-nonenyl]-piperidine	**A50**	
	1-[1-oxo-5(3-methoxyl-4-hydroxyphenyl)-2*E*-pentenyl]-piperidine	**A51**	
	Piperadione	**A52**	31
	Piperine	**A1**	25
	Piperlongumide	**A53**	39
	piperlongimin A	**A54**	40

	piperlongimin B	**A55**	
	Piperlongumine	**A56**	41
	Piperlonguminine	**A57**	25
	Pipernonaline	**A58**	42
	Piperoctadecalidine	**A59**	
	Piperolactam A	**A60**	31
	Piperolactam B	**A61**	
	Piperolactam C	**A62**	
	Sarmentine	**A63**	43
	Sylvetin	**A64**	44
	(+)-Asarinine	**L65**	36
	Bakuchiol	**M66**	45
	Bavachin	**F67**	
	Fargesin	**L68**	36
	Isobavachalcone	**M69**	45
	Pluviatilol	**L70**	36
	Sesamin	**L71**	

Chart 3A.2: Structures of the compounds isolated from *P. longum*

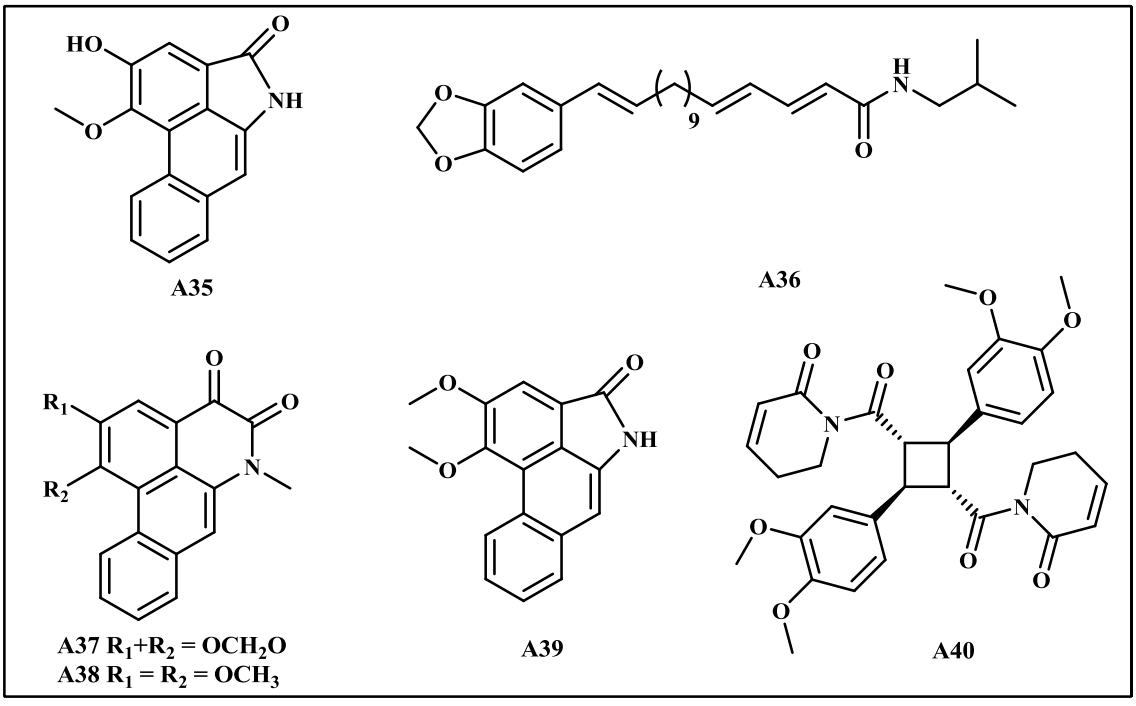
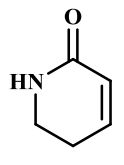


Chart 3A.2 continues.....



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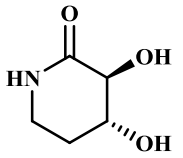

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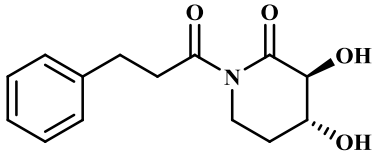

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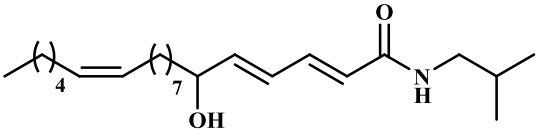

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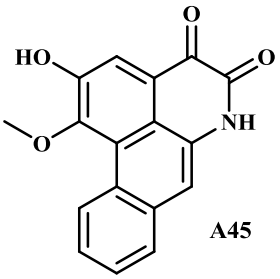

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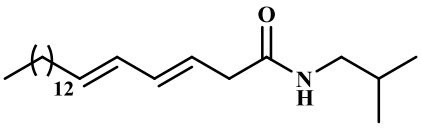

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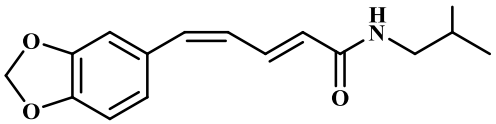

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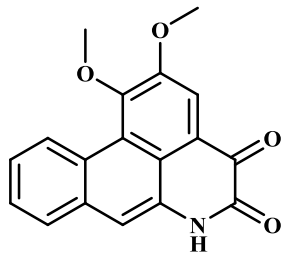

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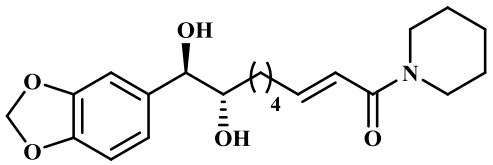

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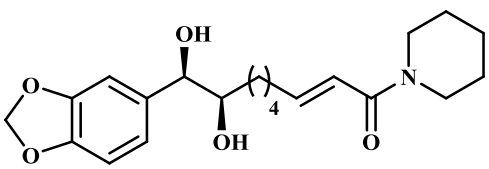

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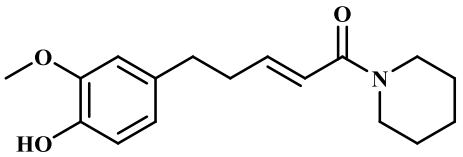

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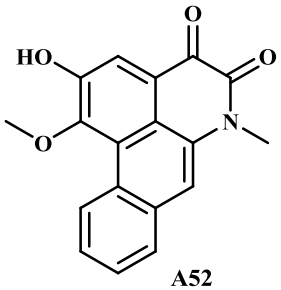

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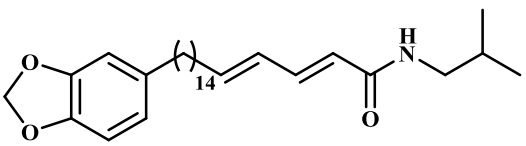

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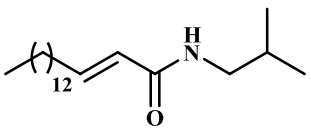

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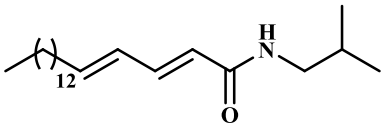

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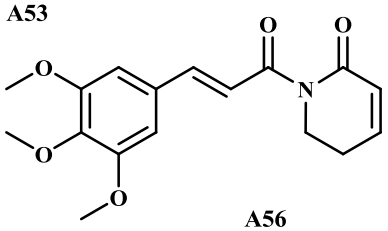

A56

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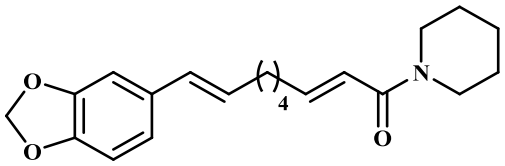

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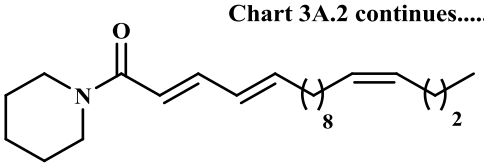

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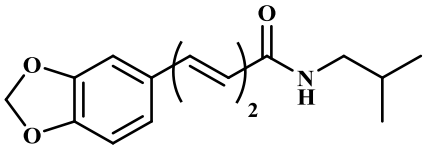
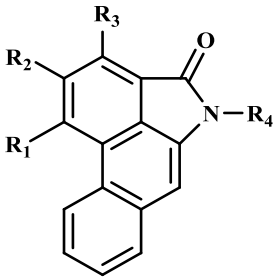
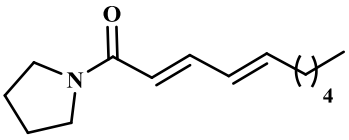


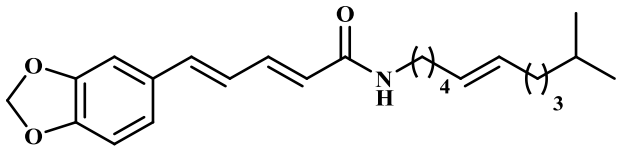
A59



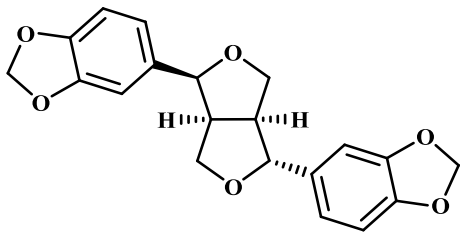
A60 R_1 = OH, R_2 = OCH_3, R_3 = R_4 = H
A61 R_1 = R_2 = OCH_3, R_3 = OH, R_4 = H
A62 R_1 = R_2 = R_3 = OCH_3, R_4 = H



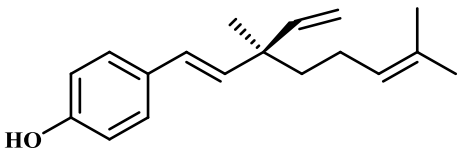
A63



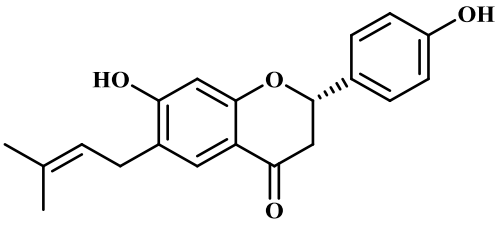
A64



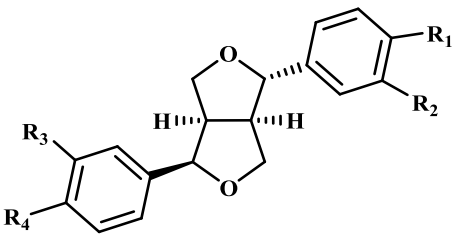
L65



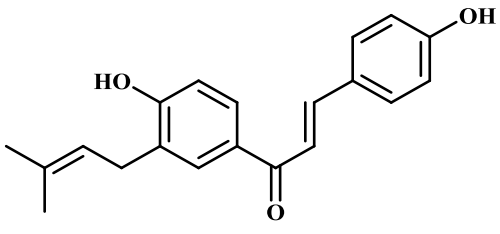
M66



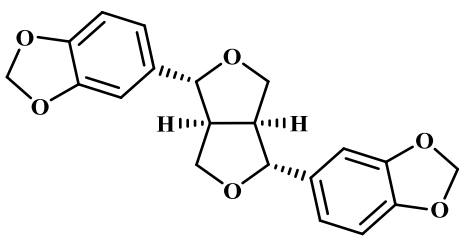
F67



L68 R_1+R_2 = OCH_2O R_3 = R_4 = OCH_3
L70 R_1+R_2 = OCH_2O R_3 = OCH_3 R_4 = OH



M69



L71

3A.1.1.3. *Piper brachystachyum (*syn. *Piper mullesua)*

P. brachystachyum is a branched woody climber, indigenous to India and is commonly known as 'wild pepper'. The roots of the plant possess medicinal value and is used for the treatment of asthma, bronchitis, dyspepsia, anorexia etc.[46,47] Oil of the inflorescence of the plant contains *β*-farnesene, myristicin and germacrene D as the major constituents.[48] Several amides and a few lignans were reported from this plant. Important compounds isolated from *P. brachystachyum* are given in table 3A.4 and structures of the compounds are given in chart 3A.3, along with that of *P. betle*.

3.1.1.4. *Piper betle*

P. betle is a perennial dioecious root climber with the semi-woody stem. The plant is native to Malaysia and is widely cultivated in India for its leaves. Betle chewing along with various adjuncts is an ancient traditional practice in India as well as other countries of East Asia.[49] Leaves have strong pungent aromatic flavour and they possess stimulant, carminative as well as antiseptic properties. In *Ayurveda,* the plant is used for the treatment of bronchitis, asthma, cough, leprosy, rheumatism, diarrhoea etc.[50] Essential oil of the leaves has been reported to contain eugenol as the major constituent, followed by eugenol acetate, germacrene-D, sabinene, *α* and *β*-selinene, *α*-farnesene etc.[51] Essential oil of the leaves possess antibacterial and antifungal activities. Leaves also possess antioxidant and antiseptic activity due to the presence of phenolic compounds in it.[52] Phytochemical investigation of the leaves and roots revealed the presence of amides and phenolic compounds in it. Major compounds isolated from *P. betle* are given in table 3A.4 and their structures are given in chart 3A.3.

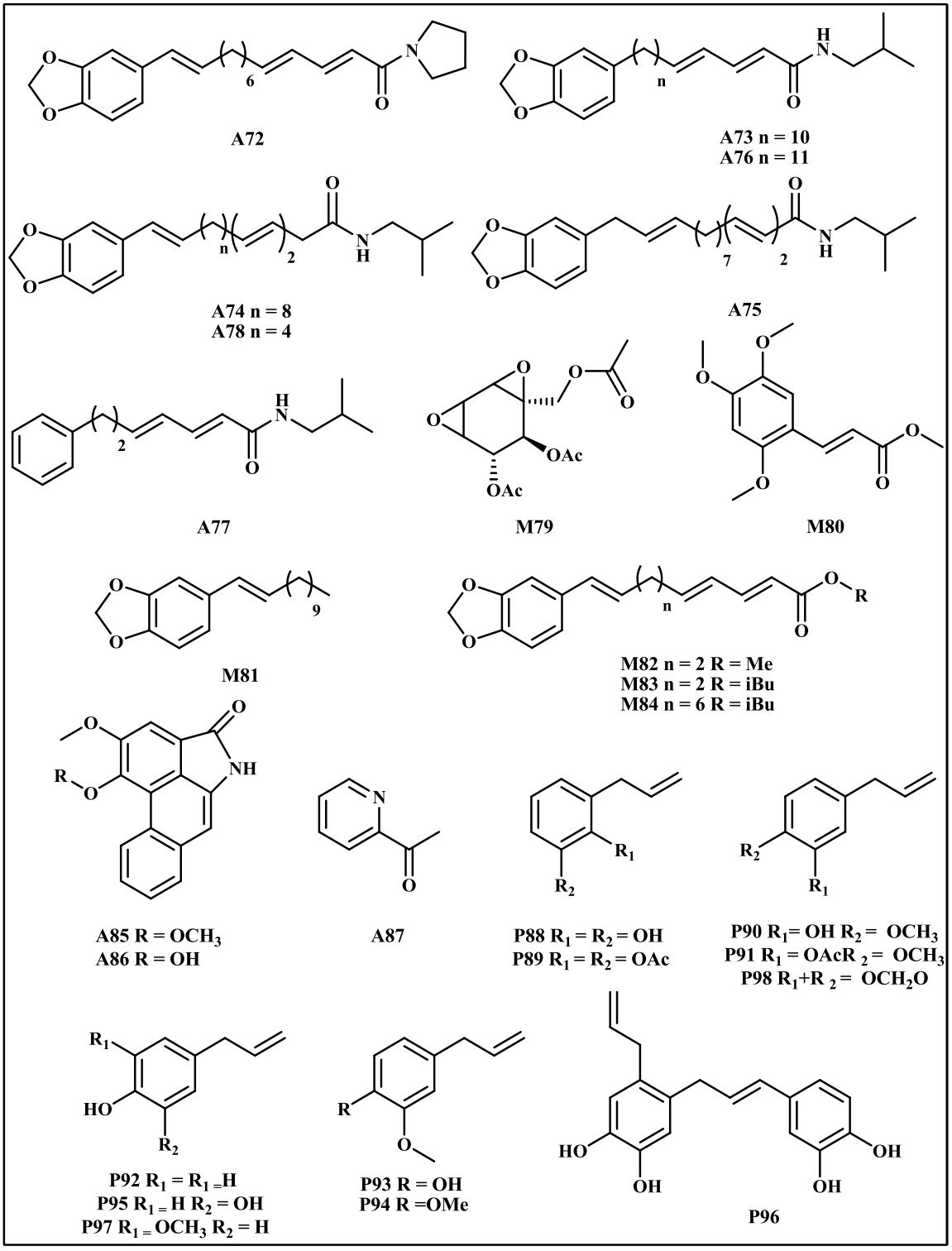
Table 3A.4: Compounds isolated from *P. brachystachyum* and *P. betle*

Plant	Name of the compound	No.	Reference
	Brachyamide A	**A72**	36
	Brachyamide B	**A2**	
	Brachystamide A	**A73**	53
	Brachystamide B	**A74**	
	Brachystamide C	**A75**	54
	Brachystamide D	**A36**	
	Brachystamide E	**A76**	
	Guineensine	**A9**	38

Piper brachystachyum	*N*-isobutyl-16-phenylhexadeca-2*E*,4*E*-dienamide	**A77**	55
	Pipericide	**A78**	36
	Retrofractamide A	**A30**	
	Sylvetin	**A64**	56
	(+)-Asarinine	**L65**	36,56
	Fargesin	**L68**	36
	Pluviatilol	**L70**	
	Sesamine	**L71**	
	Crotepoxide	**M79**	57
	Methyl-(*E*)-2,4,5-trimethoxycinnamate	**M80**	36
	Piptaline	**M81**	
	1,3-benzodioxole-5-(2,4,8-triene-methylnonaoate)	**M82**	58
	1,3-benzodioxole-5-(2,4,8-triene-isobutylnonaoate)	**M83**	
	iso-Bu 13-(1,3-benzdioxolo-5)trideca-2*E*,4*E*,12*E*-trienoate	**M84**	55
Piper betle	Aristololactam A II	**A35**	59
	Aristololactam B II	**A85**	
	Cepharadione A	**A37**	60
	Piperine	**A1**	
	Piperlonguminine	**A57**	
	Piperolactam A	**A86**	59
	1-(2 pyridinyl) ethanone	**A87**	61
	Allylpyrocatechol	**P88**	62
	Allylpyrocatecholdiacetate	**P89**	
	Chavibetol	**P90**	
	Chavibetol acetate	**P91**	
	Chavicol	**P92**	
	Eugenol	**P93**	63
	Eugenol methylether	**P94**	

	Hydroxychavicol	**P95**	63,64
	2-(γ′-hydroxychavicol)-hydroxychavicol	**P96**	59
	Isoeugenol	**P97**	63
	Safrole	**P98**	

Chart 3A.3: Structures of compounds isolated from *P. brachystachyum* and *P. betle*



3A.1.1.5. *Piper cubeba*

P. cubeba (fig. 3A.1), the plant used in the present study, is commonly known as 'Tailed pepper' in English, 'Kannkolam' in Sanskrit and 'Valmulaku' in Malayalam. It is a perennial woody climber, native to Indonesia and cultivated in the southern region of India. Fruits of the plant are globular with a slender stalk attached to its base and are hence known as 'tailed pepper'. The dried berries of the plant are used in traditional systems of medicine for the treatment of inflammation, helminthiasis, wound, ulcers, cough, rheumatism, hay fever etc.[65] Famous Indian *Ayurveda* physicians Charaka and Sushruta prescribed the paste of *P. cubeba* fruits as mouth wash. Dried fruits are also used for the treatment of dental diseases, fever, cough etc.[66] Nowadays, seeds of the plant are utilized in the preparation of many Ayurvedic formulations like 'dasamoolarishtam'.[67] In *Unani* system of medicine it is used for the treatment of renal diseases and recent studies have shown that the powdered berries show nephroprotective activity.[68]

Figure 3A.1: Berries of *Piper cubeba*

The essential oil obtained from *P. cubeba* seeds contains monoterpene hydrocarbons including sabinene, α-thujene, α-pinene *p*-cymene, etc., as well as sesquiterpenes like caryophyllene, copaene, α and β-cubebene, cubebol, δ-cadinene etc.[69] Essential oil of *P. cubeba* berries have been reported to have activity against the parasites *Schistosoma mansoni* (causative agent of schistosomiasis),[70] *Trypanosoma cruzi* (causative agent of Chagas disease) and *Leishmania amazonensis* (causative agent of leishmaniasis).[71] Essential oil and oleoresins of *P. cubeba* have been reported to show antimicrobial as well as antioxidant activities.[69b] It also showed activity against *Streptococcus faecalis, Bacillus pumilus* and *Pseudomonas solanacearum*.[72]

Ethanol extract of *P. cubeba* seeds have been reported to possess anti-inflammatory[73] and hepatoprotective[74] activities. Alcoholic extract showed cytotoxic activity against various breast cancer cell lines (MCF-7, MDA-MB-468, MDA-MB-

231)[75] and androgen-dependent prostate cancer growth.[76] *P. cubeba* extracts also showed activity against *Streptococcus salivarius, Streptococcus mitis, Enterococcus feacalis,*[77] promastigotes of *Leishmania donovani*[78] and hepatitis C virus.[79]

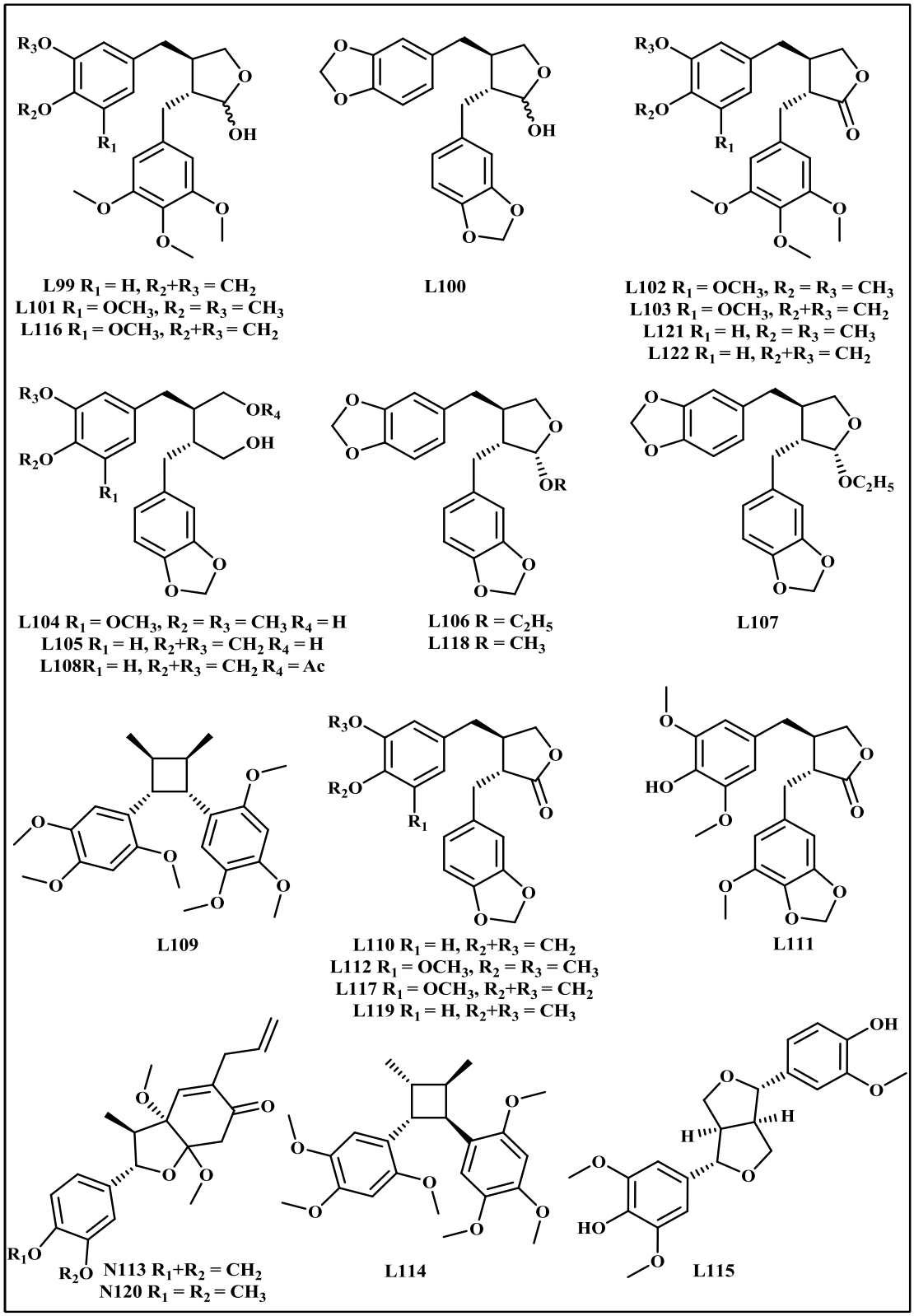
Phytochemical investigation of *P. cubeba* seeds have been carried out by several groups and it has been found that the berry of the plant is a rich source of bioactive lignans and neolignans.[80] Apart from lignans, two oxygenated cyclohexanes[81] and two sesquiterpenes[82] were also reported from the plant. Lignans isolated from the berries of the plant are listed in table 3A.5 and their structures are given in chart 3A.4.

Table 3A.5: Lignans isolated from the berries of *Piper cubeba*

Plant	Name of the compound	No	Reference
Piper cubeba	Clusin	**L99**	83
	Cubebin	**L100**	
	Cubebinin	**L101**	
	Cubebininolide	**L102**	84
	Cubebinone	**L103**	
	Dihydroclusin	**L104**	83
	Dihyrocubebin	**L105**	
	α-*O*-ethylcubebin	**L106**	85
	β-*O*-ethylcubebin	**L107**	
	Hemiariensin	**L108**	
	Heterotropan	**L109**	
	Hinokinin	**L110**	83
	4-hydroxycubebinone	**L111**	82
	Isoyatein	**L112**	84
	Kadsurin A	**N113**	81
	Magnosalin	**L114**	85
	Medioresinol	**L115**	82
	5-methoxyclusin	**L116**	
	5'-methoxyhinokinin	**L117**	85
	α-methylcubebin	**L118**	82
	2-(3",4"-methylenedioxy-benzyl)-3-(3',4'-dimethoxybenzyl) butyrolactone	**L119**	84

	Piperenone	**N120**	81
	Thujaplicatin Trimethylether	**L121**	84
	Yatein	**L122**	

Chart 3A.4: Structures of lignans isolated from *P. cubeba*



3A.2. Aim and Scope of the present study

From the introduction, it is evident that plants belonging to Piperaceae family are rich source of bioactive compounds. Even though many lignans were isolated from the *Piper cubeba* berries, there are only limited reports available on the isolation of other class of phytochemical constituents. Therefore, as part of this Ph.D. program, a detailed study of *Piper cubeba* berries was undertaken and semi-synthetic modifications on the major compound isolated from this plant was also carried out in an effort to obtain novel structures with wider bioactivity profiles.

3A.3. Isolation and characterization of compounds from *Piper cubeba* seeds

3A.3.1. Extraction

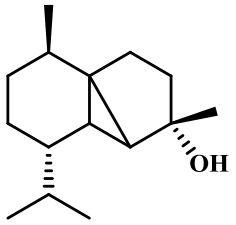
2.3 kg of the powdered *P. cubeba* seeds were subjected to repeated extraction with acetone (4 L × 4) at room temperature. The total extract was then concentrated under reduced pressure to yield approximately 412 g of crude acetone extract. For the ease of handling during the column chromatographic separation and to remove the oil content, the crude extract was then defatted using hexane. The defatted acetone extract weighed approximately 57 g.

3A.3.2. Isolation and characterization of phytochemicals

Defatted acetone extract was used for the detailed phytochemical investigation. After studying the TLC, 55 g of the defatted acetone extract was subjected to column chromatographic purification using silica gel (100-200 mesh). Column elution was started using 5% ethyl acetate in hexane and increase in polarity was carried out by increasing the amount of ethyl acetate. Final elution was carried out using 5% methanol in ethyl acetate. A total of 453 fractions of approximately 150 mL each were collected. According to the similarity in TLC, they were pooled into twenty major fraction pools.

Fraction pool three (Fr. 17-51), obtained by eluting the column with 5% ethyl acetate in hexane showed the presence of a UV active compound along with some impurities. It was again subjected to column chromatographic purification using silica gel to yield 310 mg of colourless crystals, which was labelled as compound **15.** IR spectrum of the compound showed a band at 3281 cm^{-1}, suggesting the presence of hydroxyl group in it. ^{1}H NMR spectrum (fig. 3A.2) of the compound showed the presence of 26 aliphatic hydrogens. Doublets, integrating for three protons each, at

δ 0.92, 0.94 and 0.97 along with a singlet at δ 1.28 suggested the presence of four methyl groups. ^{13}C NMR spectrum (fig. 3A.3) of the compound showed the presence of fifteen carbon atoms. DEPT-135 spectrum along with ^{13}C NMR spectrum suggested that there were four methyl, four methylene, five methine and two quaternary carbons in the molecule. Methyl carbons resonated at δ 18.7, 19.6, 20.1 and 22.6 and the peak at δ 80.3 could be attributed to the carbon bearing hydroxyl group. Mass spectrum of the compound showed molecular ion peak at m/z 245.2878, which is the $(M+Na)^+$ peak. From all these spectral data and on comparison with the literature reports,[86,87] compound **15** was confirmed as **cubebol,** which was earlier reported from the essential oil of *P. cubeba* seeds.[69b] The structure of the compound is shown below.



Compound **15 - Cubebol**

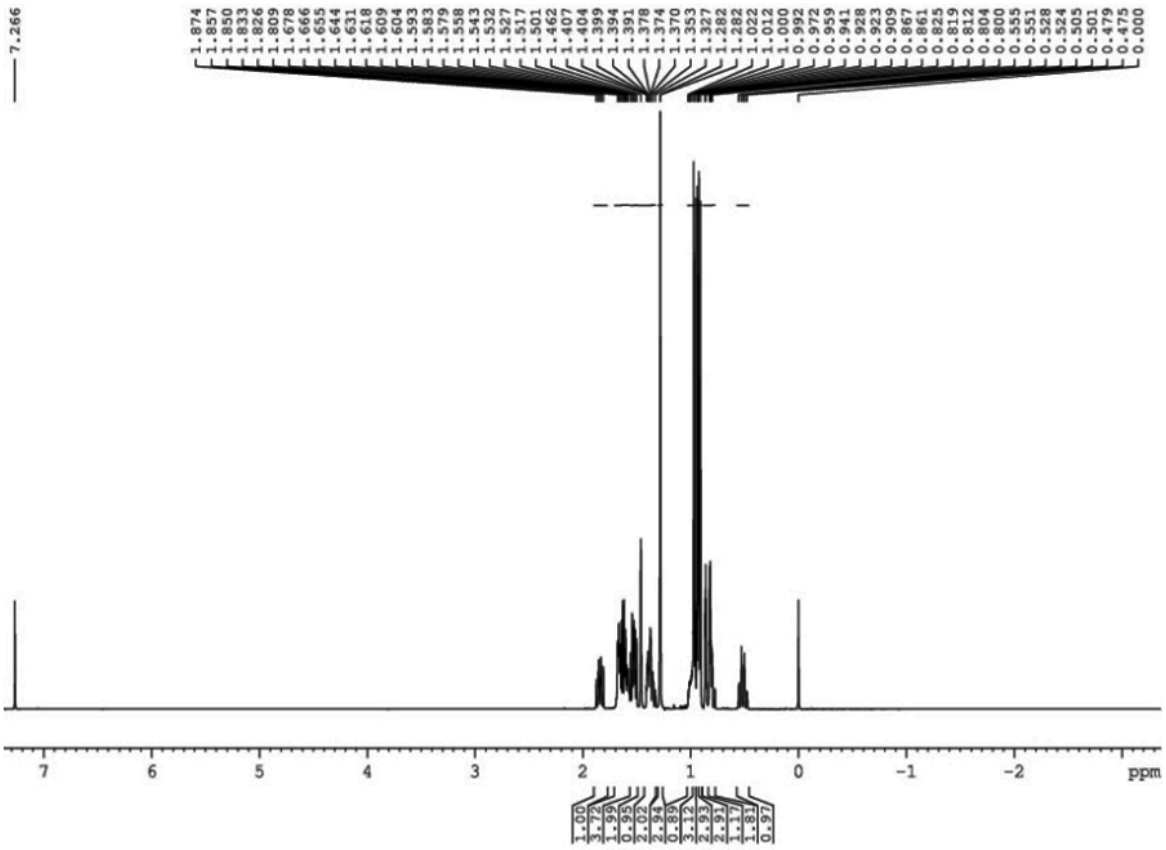


Figure 3A.2: ^{1}H NMR spectrum of cubebol

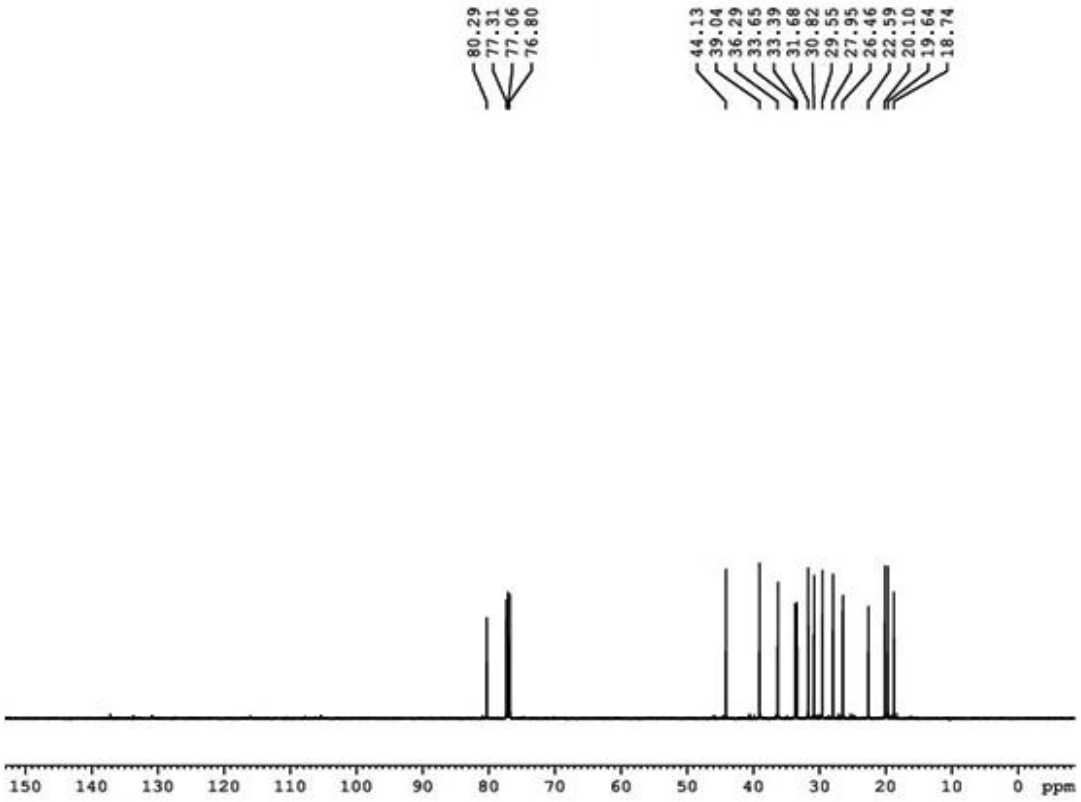
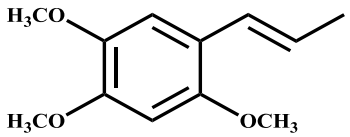


Fig. 3A.3: ^{13}C NMR spectrum of cubebol

Fraction pool four (Fr. 52-90) obtained by eluting the column with 10% ethyl acetate in hexane showed the presence of three UV active compounds in it. It was further purified using silica gel column chromatography. Subfractions 67-78 obtained by eluting the column with 4% ethyl acetate in hexane showed the presence of a single UV active compound, which on crystallization yielded 790 mg of colourless crystals of compound **16**. IR spectrum of the compound showed absorptions at 1607 and 1511 cm^{-1}, indicating the presence of an aromatic group in the molecule. In the ^{1}H NMR spectrum (fig. 3A.4) singlets at δ 6.94 and 6.48 integrating for one proton each, suggested the presence of two aromatic protons. Doublet centred at δ 6.64 (J = 15 Hz) and a multiplet at 6.08, integrating for one proton each, suggested the presence of a *trans* olefinic group attached to the aromatic system. Singlets at δ 3.86, 3.84 and 3.79, integrating for three protons each, indicated the presence of three methoxy groups. Doublet centred at δ 1.88, integrating for three protons could be ascribed to the methyl group attached to olefinic carbon in the molecule. The presence of three methoxy groups was again confirmed by the peaks at δ 56.6, 56.4 and 56.0 ppm in the ^{13}C NMR spectrum (fig. 3A.5). Mass spectrum of the compound showed molecular ion peak at m/z 231.0998, which is the $(M+Na)^{+}$ peak. From these spectral data and on comparison

with the literature reports,[88] compound **16** was confirmed as ***α*-asarone**, which was reported earlier from this plant. The structure of the compound is shown below.



Compound **16 - *α*-asarone**

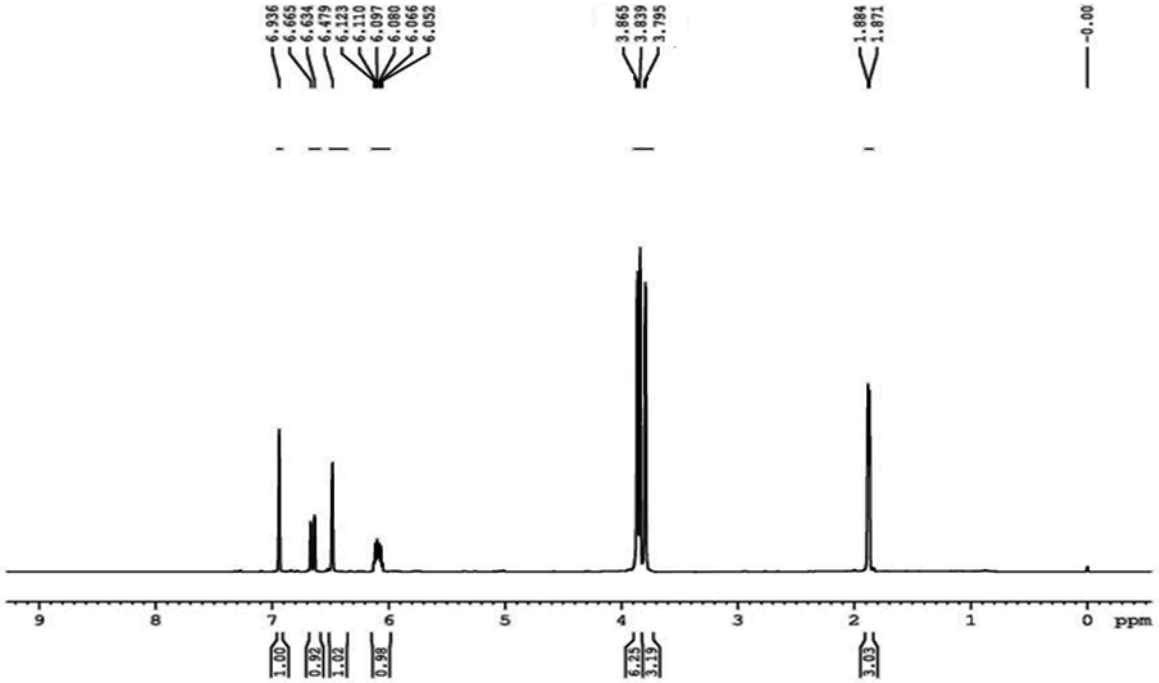


Figure 3A.4: ^{1}H NMR spectrum of *α*-asarone

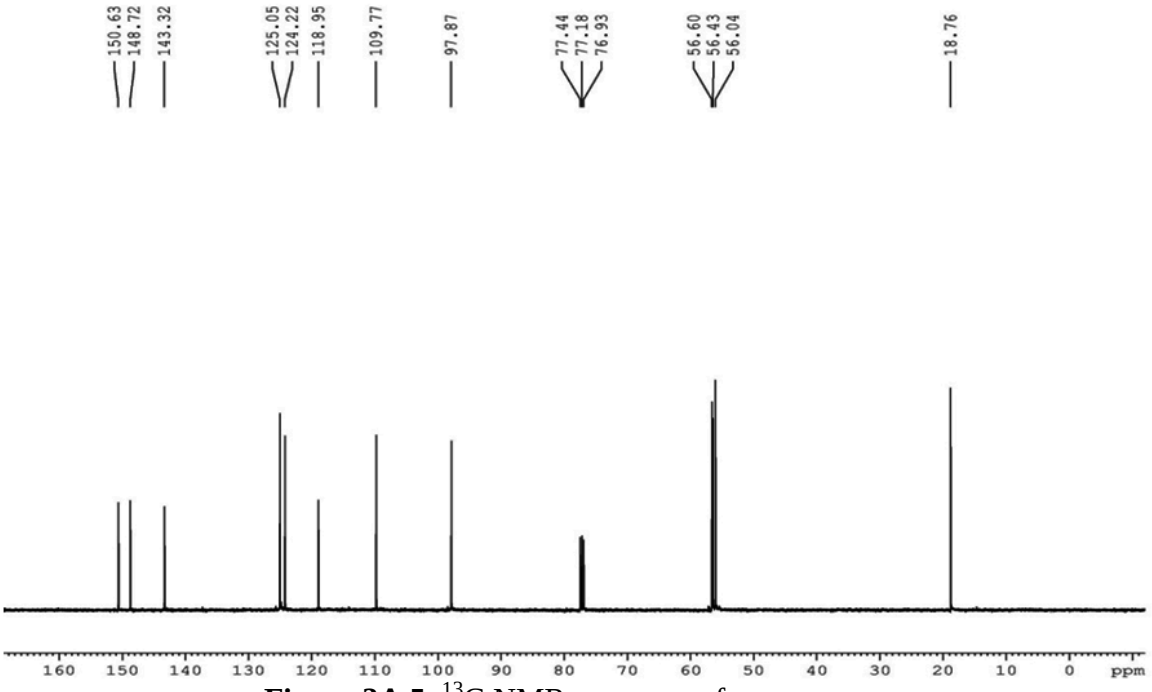
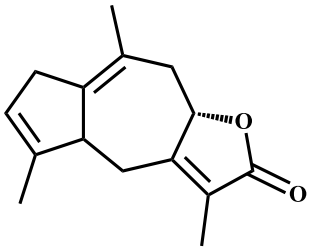


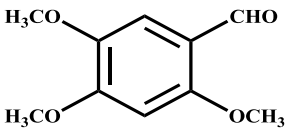
Figure 3A.5: ^{13}C NMR spectrum of *α*-asarone

Subfractions 84-88 obtained by eluting the column with 4% EtOAc in hexane on crystallization yielded 7 mg compound **17** as pale yellow crystals. IR spectrum of the compound showed absorption at 1753 cm^{-1} suggesting the presence of a five membered lactone carbonyl group. ^{1}H NMR spectrum (fig. 3A.6) of the compound showed doublets at δ 5.49 and 4.55, each integrating for one proton, suggested the presence of an olefinic hydrogen and the hydrogen adjacent to oxygen atom respectively. Singlets at δ 1.85, integrating for three protons, and δ 1.78, integrating for six protons, suggested the presence of three methyl groups attached to olefinic carbons. ^{13}C NMR spectrum (fig. 3A.7) of the compound showed a peak at δ 174.3 confirming the presence of carbonyl group in the lactone. Six peaks in between δ 163.5-121.3 ppm suggested the presence of six olefinic carbons in the molecule. Olefinic methyl groups resonated at δ 21.9, 14.9 and 8.3 ppm respectively. Mass spectrum of the compound showed molecular ion peak at m/z 253.1208, which is the $(M+Na)^{+}$ peak. From these spectral data and on comparison with the literature reports,[89] compound **17** was confirmed as the guaianolide type sesquiterpene lactone, **podoandin**. To the best of our knowledge, this is the first report of podoandin from *Piper* species. The compound has been previously isolated from the plant *Podocarpus andina* belonging to Podocarpaceae family.[89] The structure of compound **17** is shown below.



Compound **17 - Podoandin**

Subfractions 138-140 obtained by eluting the column with 50% ethyl acetate in hexane yielded 21 mg of compound **18** as a pale yellow solid. The compound as characterized as **2,4,5-trimethoxybenzaldehyde** (**asaronaldehyde**) from various spectral data obtained for the compound. Asaronaldehyde has been earlier isolated from *P. cubeba* by Badheka *et al*.[85] The structure of the compound is shown below.



Compound **18 - 2,4,5-trimethoxy benzaldehyde**

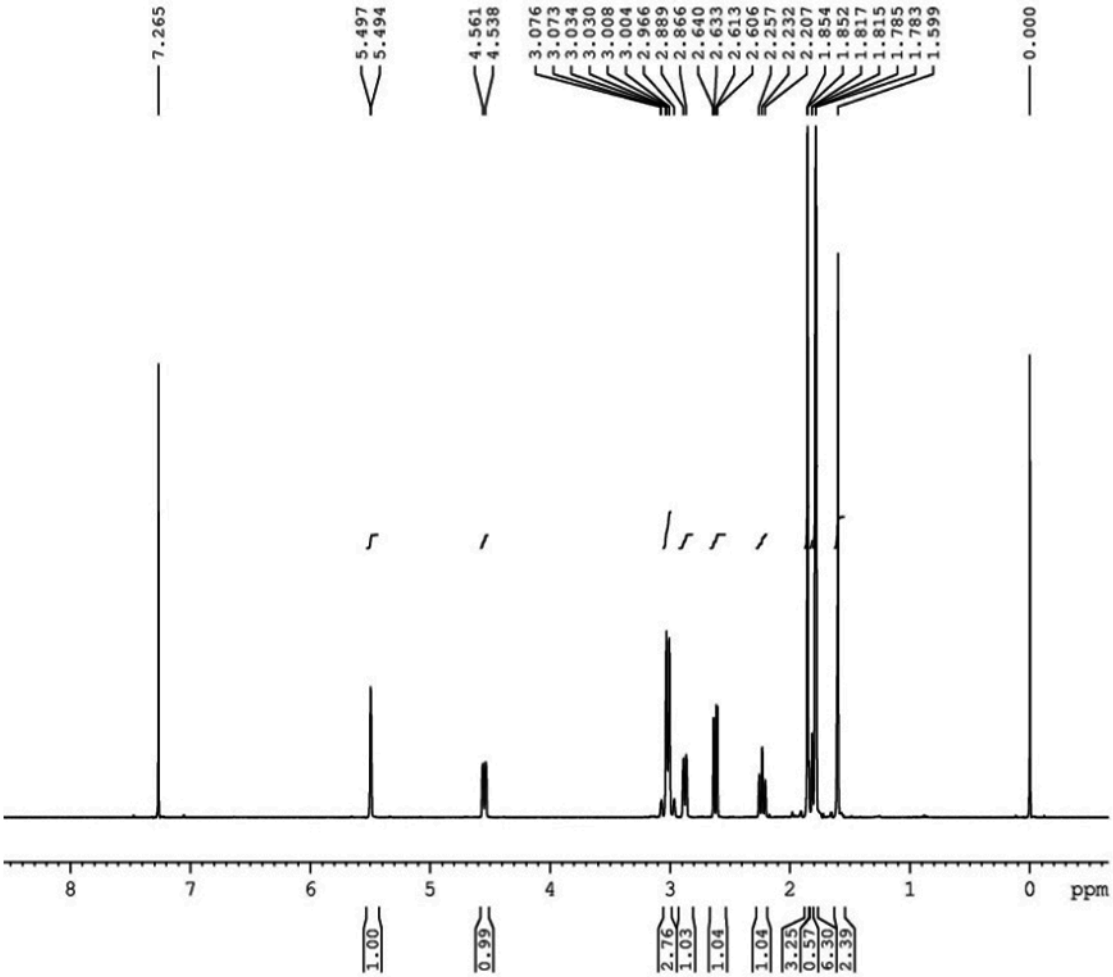


Figure 3A.6: ^{1}H NMR spectrum of podoandin

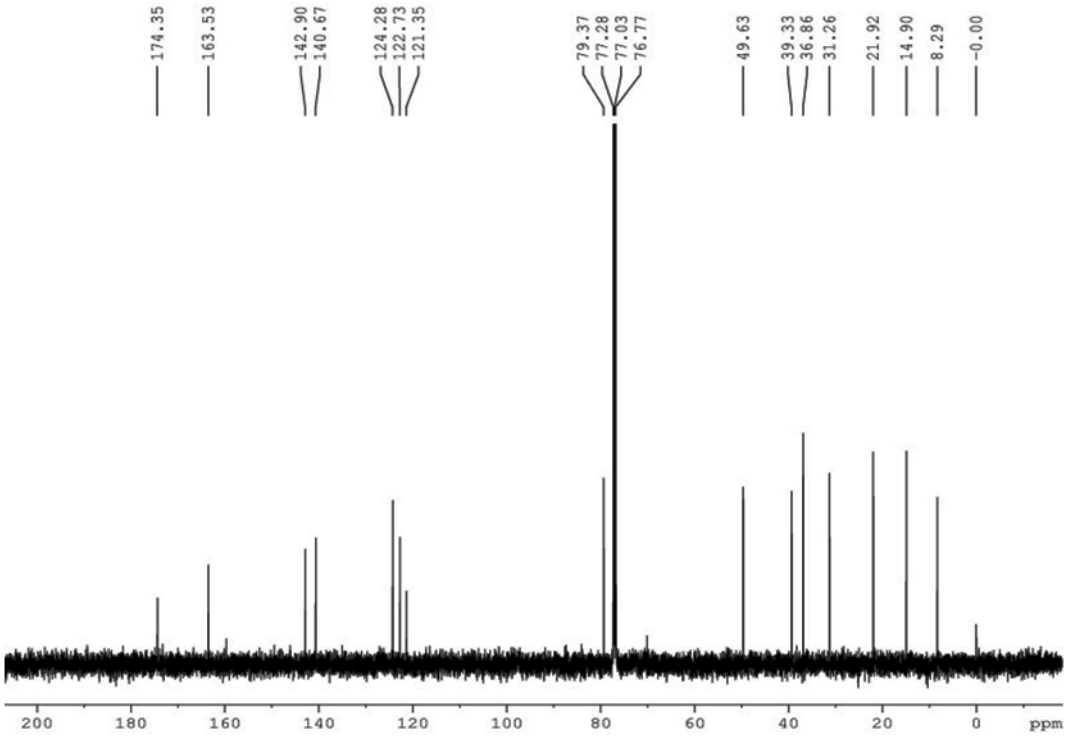
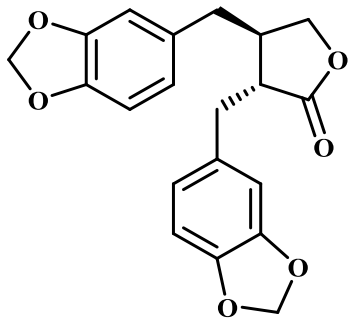


Figure 3A.7: ^{13}C NMR spectrum of podoandin

Major fraction pool six (Fr. 116-148) obtained by eluting the column with 20% ethyl acetate in hexane on further purification again on silica gel yielded 6.37 g of compound **19** as a gummy solid. IR spectrum of the compound showed absorption at 1769 cm^{-1}, indicating the presence of a five membered lactone ring in the molecule. Doublets centered at δ 6.73, 6.70, 6.64 and doublets of doublet centered δ 6.61, each integrating for one proton along with a multiplet centered at δ 6.47 integrating for two protons in the ^{1}H NMR spectrum (fig. 3A.8) suggested the presence of six aromatic protons. Singlet at δ 5.93 integrating for four protons suggested the presence of two methylenedioxy groups as seen in many natural products. Doublets of doublet at δ 4.13 and δ 3.86, each integrating for one proton could be attributed to diastereotopic protons in the lactone ring. The presence of lactone carbonyl was confirmed by the peak at δ 178.5 in the ^{13}C NMR spectrum (fig. 3A.9). ^{13}C NMR spectrum showed twelve peaks ranging from δ 147.8- 108.2, confirming the presence of twelve aromatic carbon atoms in the molecule. DEPT-135 spectrum of the compound suggested that there were five methylene carbons in it. The peak observed at δ 101.0 in the ^{13}C NMR, which appeared as a downward peak in the DEPT-135 spectrum could be attributed to the carbon atoms of two methylenedioxy groups. Mass spectrum of the compound showed molecular ion peak at m/z 377.0977, which is the $(M+Na)^{+}$ peak. From these spectral data and on comparison with the literature reports,[83] compound **19** was identified as the lignan lactone **hinokinin**, which was reported earlier from this plant. The structure of the compound is shown below.



Compound **19 - Hinokinin**

Hinokinin has been reported to have various bioactivities such as analgesic, anti-inflammatory,[90] antimutagenic[91] and trypanocidal activities.[92]

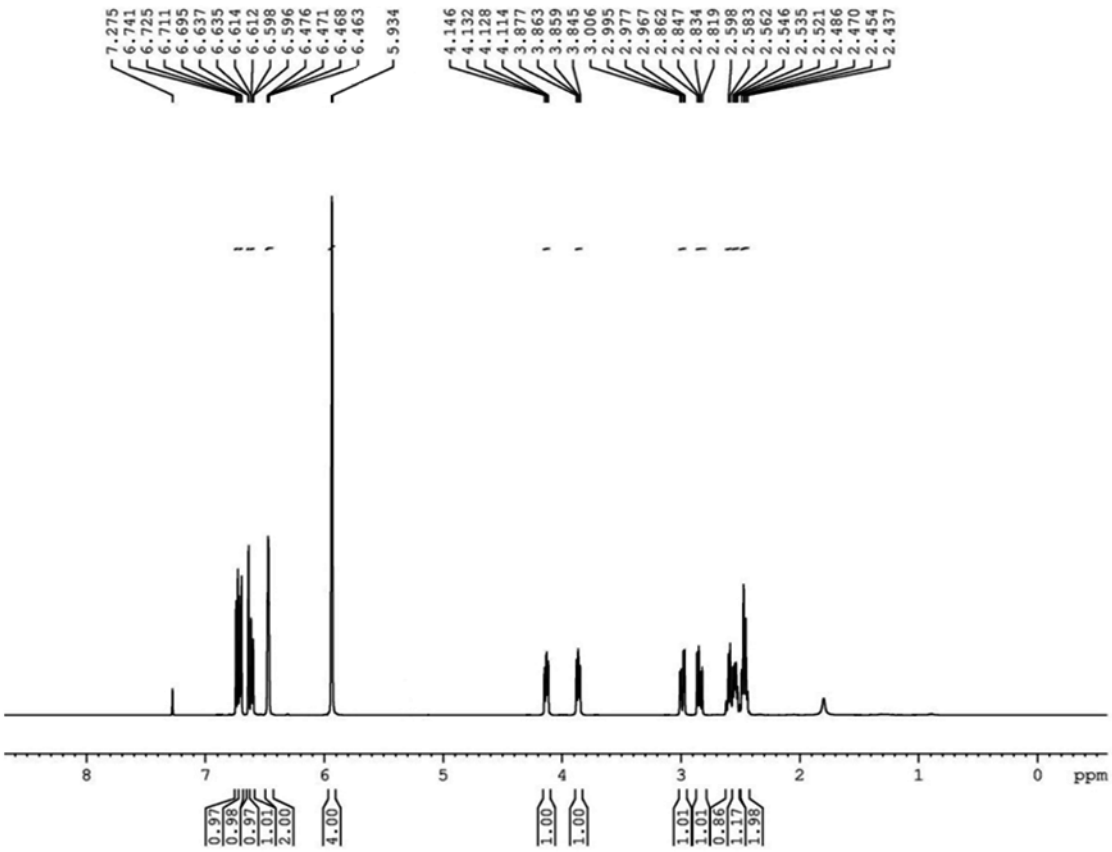


Figure 3A.8: ^{1}H NMR spectrum of hinokinin

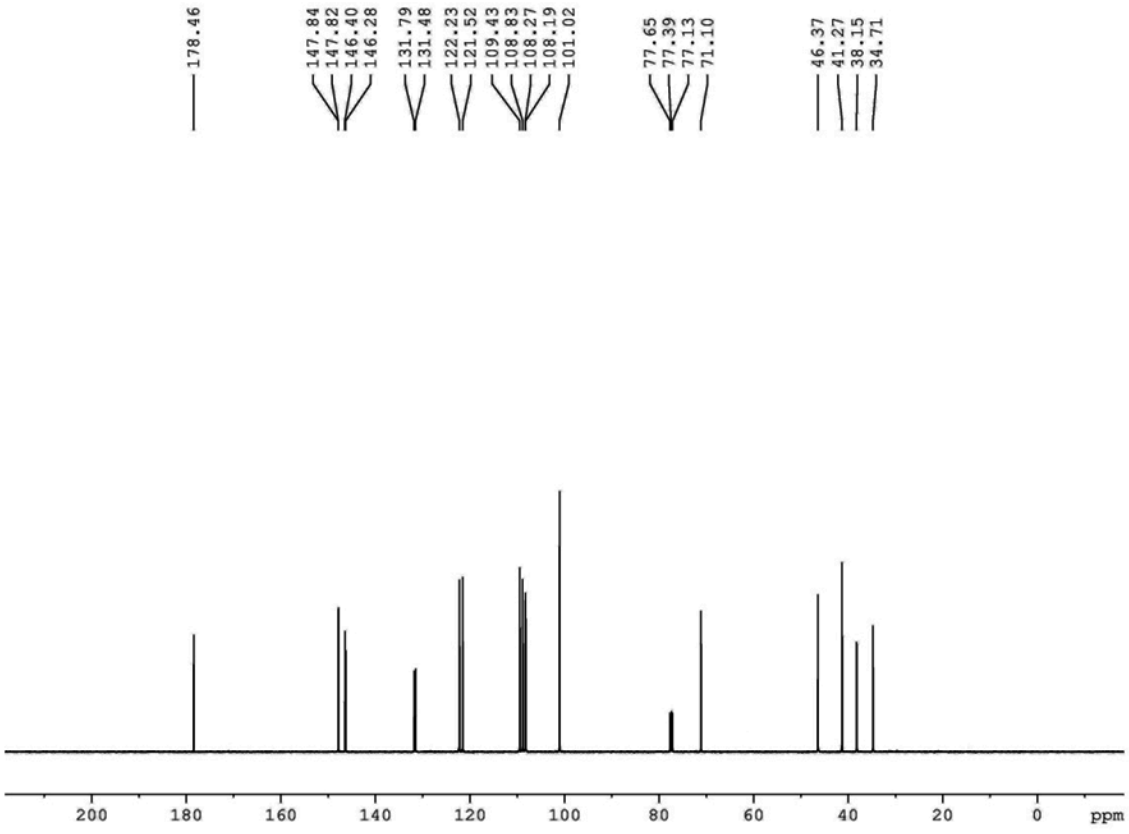
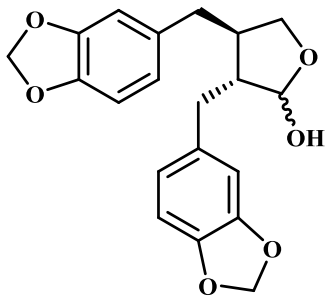


Figure 3A.9: ^{13}C NMR spectrum of hinokinin

The next major fraction pool viz., nine (Fr. 168-204) obtained by eluting the column with 25% ethyl acetate in hexane on crystallization using dichloromethane-hexane solvent system yielded 7.2 g of compound **20** as colourless crystals. IR spectrum of the compound showed a broad absorption at 3352 cm^{-1} suggesting the presence of a hydroxyl group. ^{1}H NMR spectrum (fig. 3A.10) of the compound showed multiplet in between δ 6.73-6.50, which could be attributed to the six aromatic hydrogens. Two sets of singlets at δ 5.93 and 5.92 suggested the presence of two methylenedioxy groups. The singlet at δ 5.22 could be attributed to the hydrogen on the carbon bearing the hydroxyl group (anomeric carbon) in the lactol ring. Diastereotopic protons of the lactol ring appeared as two pairs of triplets (at δ 4.01 & 3.57, δ 4.00 & 3.79), integrating for one proton each suggesting the presence of epimeric mixture. Benzylic protons and the two methine protons of the lactone ring appeared as multiplets in between δ 2.78-1.99. By analyzing the integration in the ^{1}H NMR spectrum, it has been found that the compound is a 3:2 mixture two epimers at C-9', i.e., *α*-cubebin and *β*-cubebin respectively, as reported in the literature. This was again confirmed from the ^{13}C NMR spectrum (fig. 3A.11) in which the hemiacetalic carbon gave peaks at δ 103.4 and 98.9, again confirming the presence of epimeric mixture. The peak at δ 100.9 and 100.8 in the ^{13}C NMR, which appeared as downward peaks in the DEPT-135 spectrum confirmed the presence of two methylenedioxy groups. Methylene carbon in the five membered lactol ring resonated at δ 72.6 and 72.2. Mass spectrum of the compound showed the molecular ion peak at m/z 379.1161, which is the $(M+Na)^{+}$ peak. From all these data and on comparison with the literature reports, compound **20** was confirmed as the 3:2 epimeric mixture of (-)-**cubebin**, which was reported earlier from this plant.[93] The structure of the compound is shown below.



Compound **20 - Cubebin**

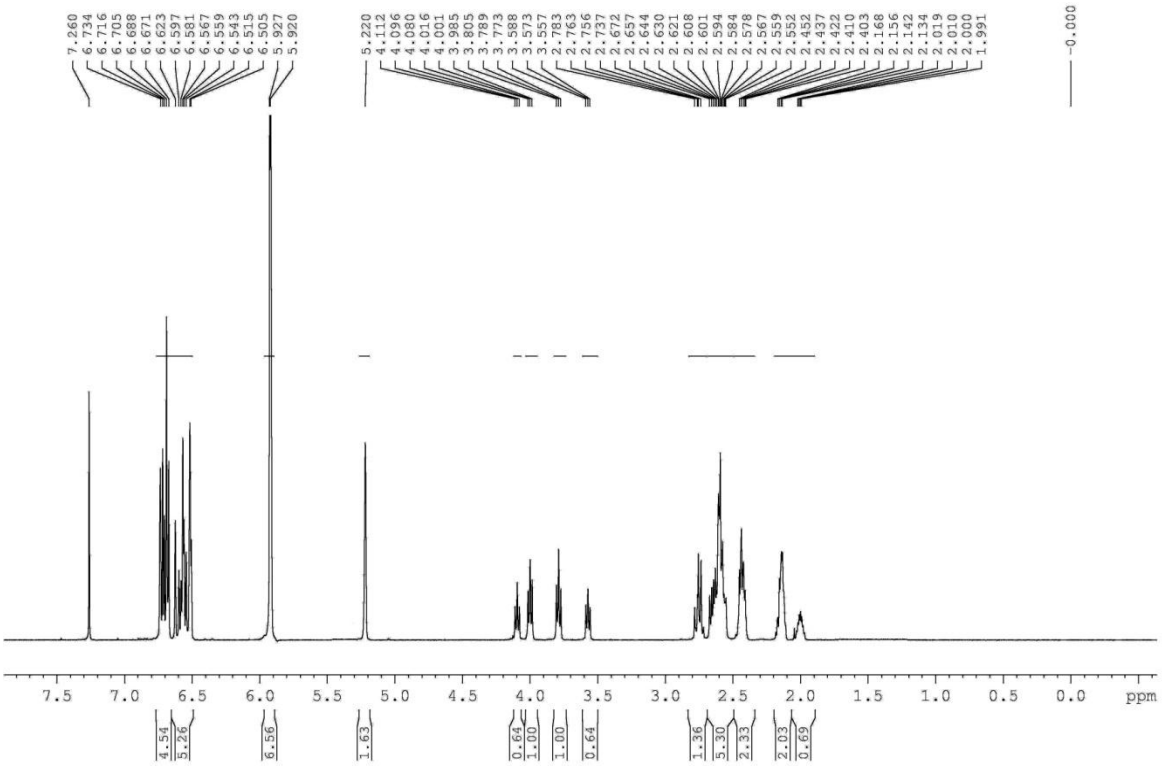


Figure 3A.10: ^{1}H NMR spectrum of cubebin

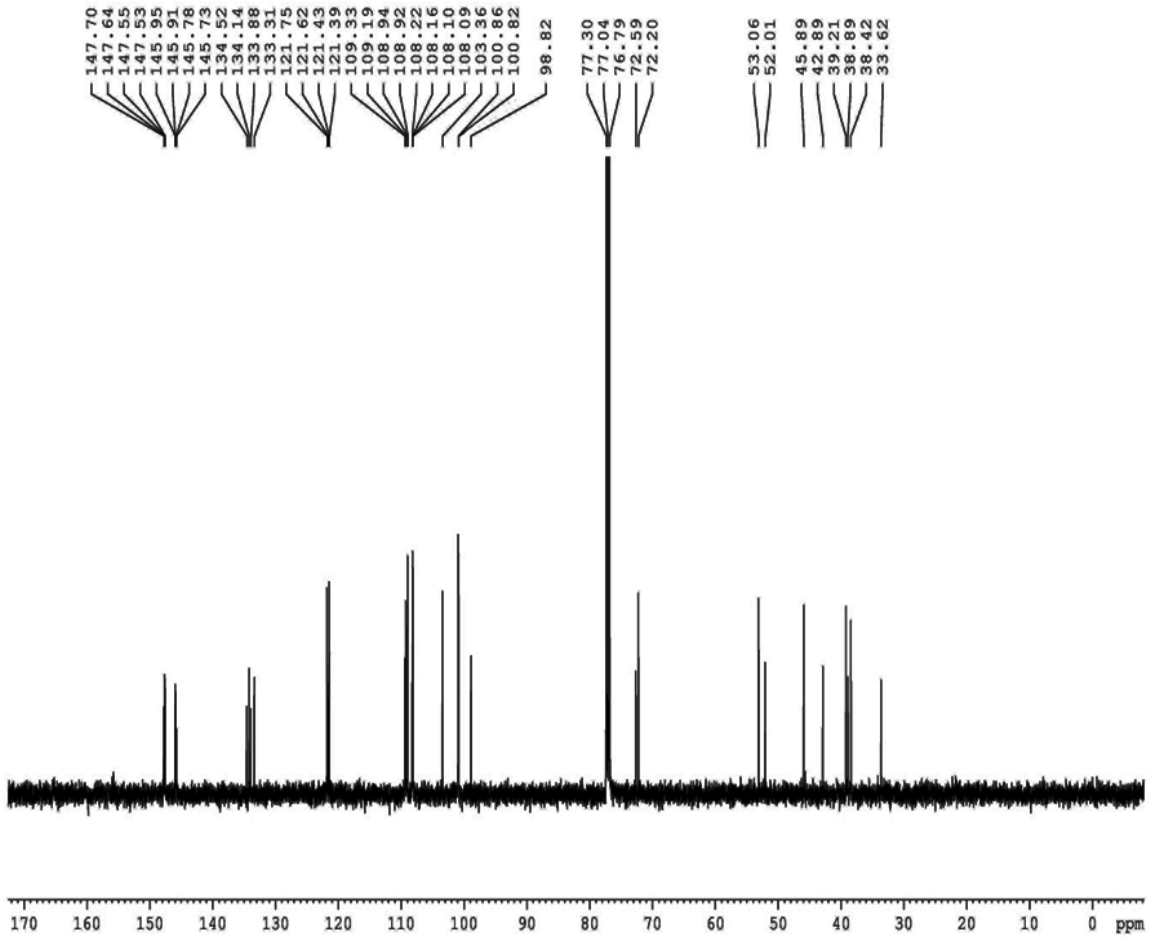
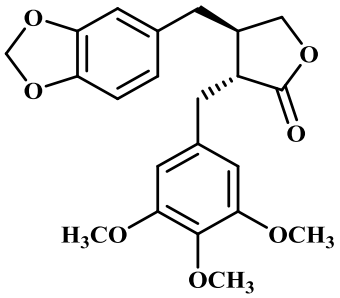


Figure 3A.11: ^{13}C NMR spectrum of cubebin

It has been found to that cubebin is the most abundant phytochemical constituent present in *P. cubeba* seed. It has been further known that irrespective of the source from which cubebin is obtained (whether natural source or synthesized), it occurs as a mixture of two 8,8'- *trans* diastereomers at the anomeric position.[94] Cubebin has been reported to have trypanocidal,[92b] anti-inflammatory,[95] analgesic,[96] vasorelaxant,[97] antimycobacterial and antiprotozoal activities.[98]

Mother liquor obtained after the crystallization of cubebin was subjected to silica gel column chromatographic purification using gradient mixtures of chloroform and ethyl acetate. From this, fractions 67-73 (20 mg) obtained as a gummy solid, by eluting the column with 8% ethyl acetate in chloroform showed the presence of a single UV active compound which was labelled as compound **21**. IR spectrum of the compound showed absorption at 1761 cm^{-1} suggesting the presence of a five membered lactone ring. ^{1}H NMR (fig. 3A.12) spectrum of the compound showed the presence of five aromatic protons [δ 6.71 (1H, d), 6.47 (2H, m), 6.36 (2H, s)]. Protons of methylenedioxy group were observed as doublets centred at δ 5.94 and 5.93. Methylene protons in the lactone ring appeared as two separate doublets of doublet centered at δ 4.18 and 3.88. A singlet at δ 3.83 integrating for nine protons suggested the presence of three methoxy groups. Multiplets centered at δ 2.91 integrating for two protons and δ 2.56 integrating for four protons could be attributed to the benzylic and the two methine protons of the lactone ring. The presence of lactone carbonyl was again confirmed by the peak at δ 178.6 in the ^{13}C NMR spectrum (fig. 3A.13) and it showed twelve peaks ranging from δ 153.3- 106.2, indicating the presence of twelve aromatic carbon atoms in the molecule. The peak observed at δ 101.1 could be attributed to the carbon atom of methylenedioxy group. 3,4,5-methoxy substitution of the molecule in the aromatic ring could be ascertained from the peaks at δ 60.9 and 56.1 (2C). It has been reported that signal of methoxy carbon in the centre will be shifted downfield by around 4.6 ppm in the case of three methoxy groups in a consecutive disposition, which is consistent with the result obtained. DEPT-135 spectrum of the compound suggested that there are four methylene carbons present in the molecule. Mass spectrum of the compound showed the molecular ion peak at m/z 423.1423, which is the $(M+Na)^{+}$ peak. From these spectral data and on comparison with the literature reports,[84,99] the compound was confirmed as the lignan lactone, **yatein** which has been reported earlier from *P. cubeba*. The structure of compound **21** is shown below.



Compound **21 - Yatein**

Yatein has been reported to have cytotoxic activity against various human cancer cell lines[100] and shows anti-platelet aggregation activity also.[101]

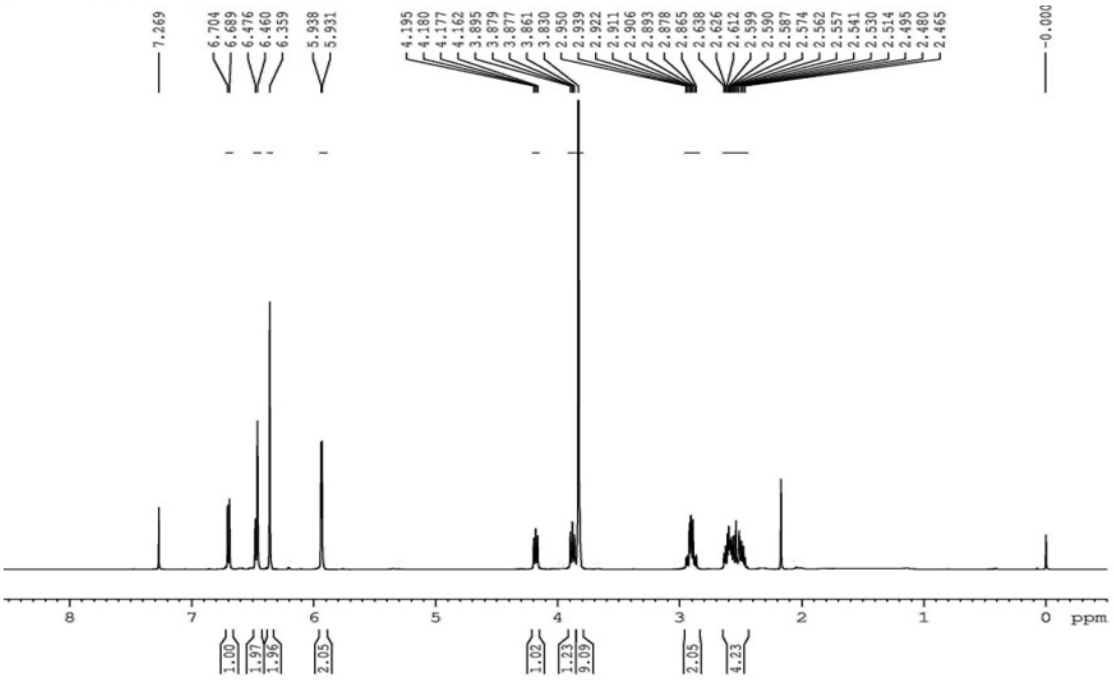


Figure 3A.12: ^{1}H NMR spectrum of yatein

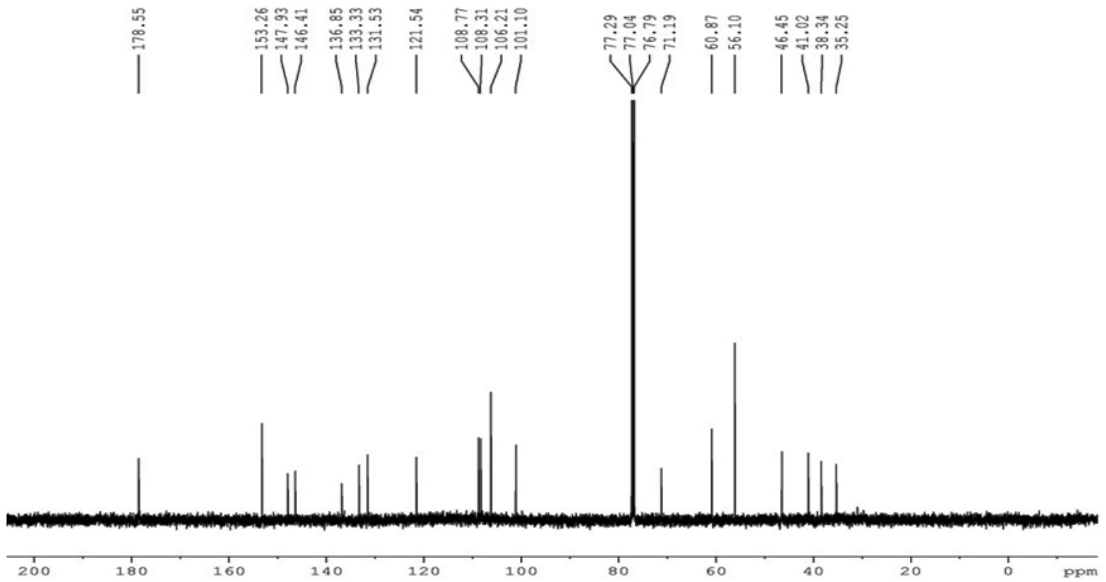
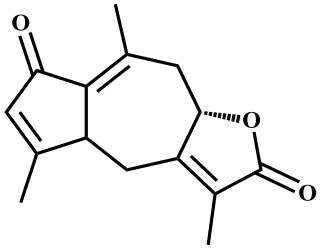


Figure 3A.13: ^{13}C NMR spectrum of yatein

Fraction pool twelve (Fr. 261-299) obtained by eluting the column with 35% ethyl acetate in hexane was further subjected to column chromatographic purification using silica gel to get 55 different fractions of approximately 100 mL each. Subfractions 22-26 was further purified by column chromatography over neutral alumina, using increasing polarity of hexane–ethyl acetate mixture which yielded 11 mg compound **22** as a pale yellow solid and 20 mg of compound **23** as a gummy solid.

IR spectrum of compound **22** showed absorptions at 1741 and 1680 cm^{-1} suggesting the presence of a five membered lactone ring and an α,β-unsaturated ketone in the molecule. ^{1}H NMR spectrum (fig. 3A.14) of the compound displayed singlet at δ 6.16, integrating for one proton, which could be ascribed to an olefinic methine proton. A doublet at δ 4.64 integrating for one proton, suggested the presence of a proton on the carbon also bearing oxygen atom. Three tertiary methyls were seen, which resonated at δ 2.46, 2.18 and 1.91. The ^{13}C NMR spectrum (fig. 3A.15) of the compound showed 15 carbon signals including that of a conjugated ketone (δ 195.6), a lactone carbonyl (δ 173.5), six olefinic carbons (in between δ 160.7–123.0) and an oxygen-substituted carbon (δ 77.9). Mass spectrum of the compound showed molecular ion peak at m/z 267.0987, which is the $[M+Na]^{+}$ peak. From these spectral data and on comparison with the literature reports compound **22** was identified as the guaianolide type sesquiterpene lactone, **(5α,8α)-2-oxo-1-(10),3,7(11)-guaiatrien-12,8-olide**, which was reported earlier from this plant.[82] The structure of compound **22** is as shown below.



Compound **22 - (5α,8α)-2-oxo-1-(10),3,7(11)-guaiatrien-12,8-olide**

IR spectrum of compound **23** showed absorption at 1768 cm^{-1} suggesting the presence of a five membered lactone ring. ^{1}H NMR spectrum (fig. 3A.16) of the compound showed two singlets at δ 6.40 and 6.22, each integrating for two protons suggested the presence of four aromatic hydrogens. Triplets centered at δ 4.19 and 3.90, integrating for one proton each could be attributed to the diastereotopic protons in the lactone ring. Singlets at δ 3.83 and 3.81, integrating for a total of eighteen protons suggested the presence of six methoxy groups in the compound. Multiplets in between

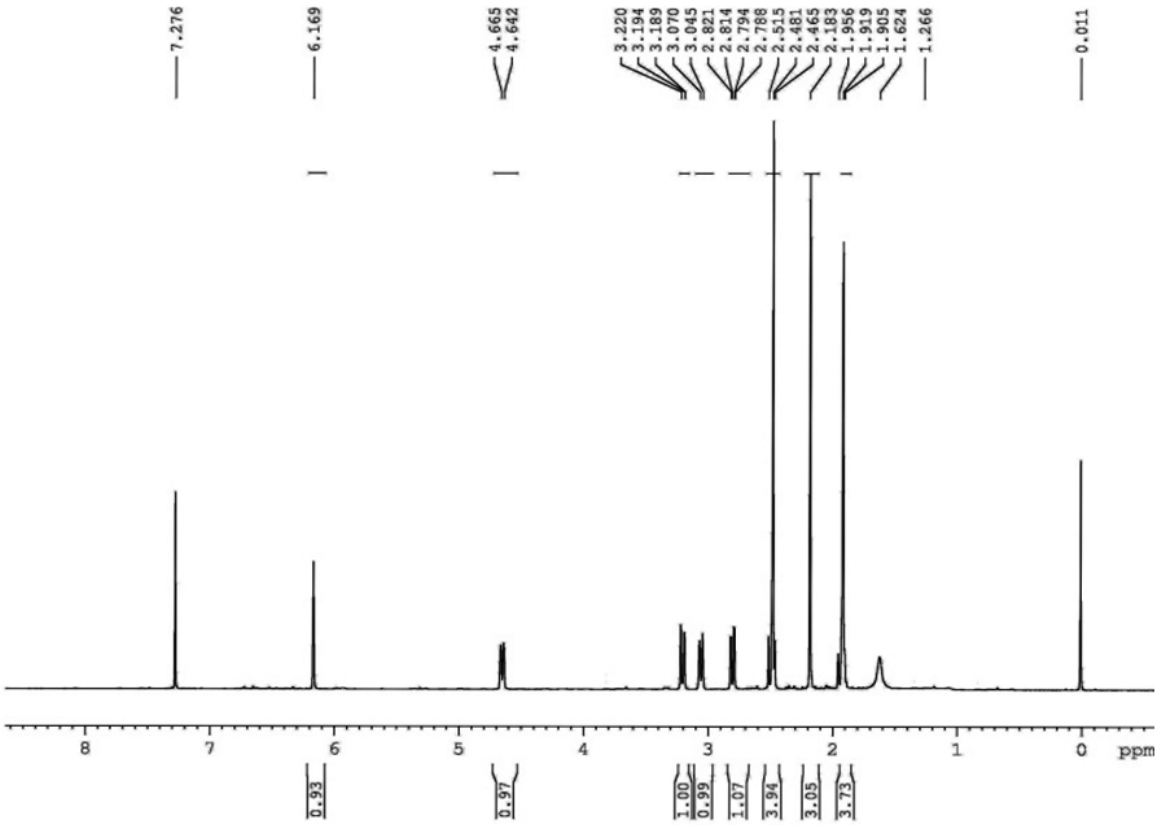


Figure 3A.14: ^{1}H NMR spectrum of (5*α*,8*α*)-2-oxo-1-(10),3,7(11)- guaiatrien-12,8-olide

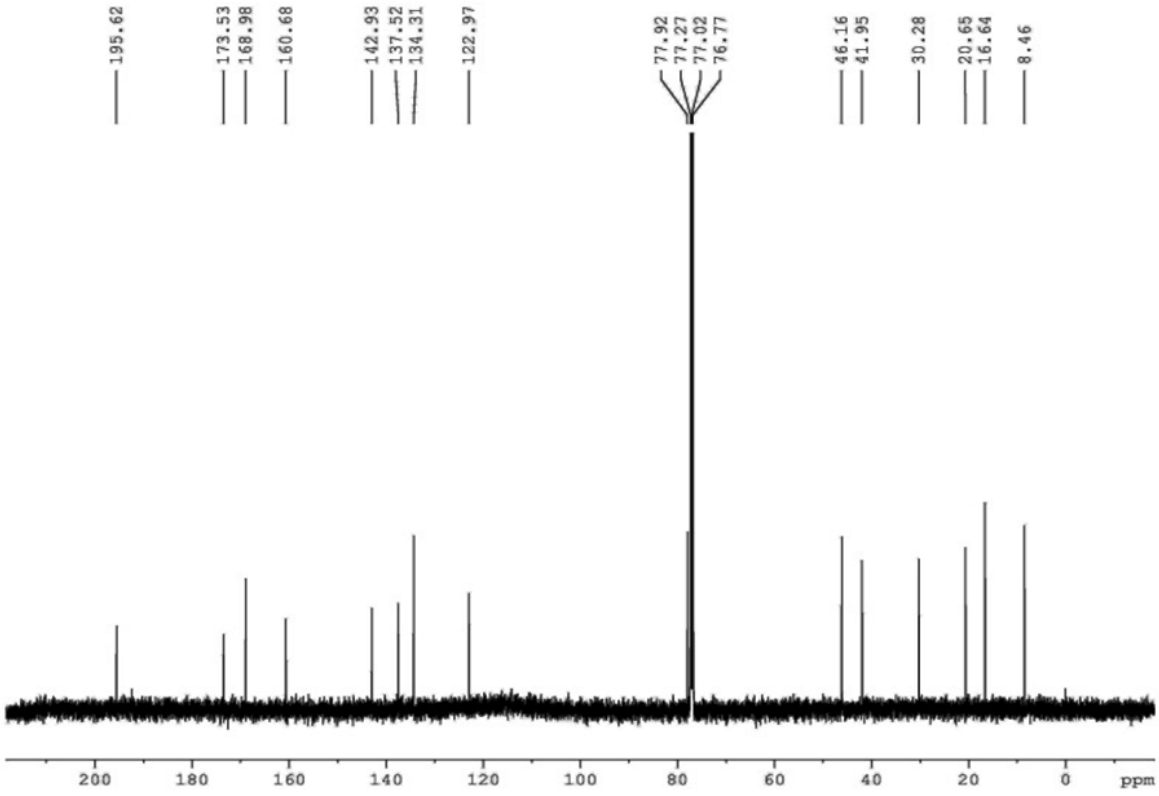
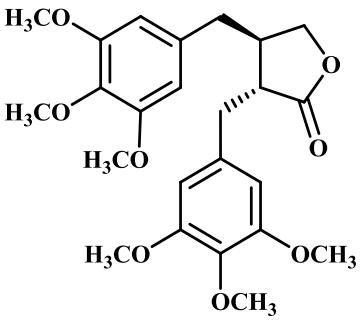


Figure 3A.15: ^{13}C NMR spectrum of (5*α*,8*α*)-2-oxo-1-(10),3,7(11)- guaiatrien-12,8-olide

δ 2.98 and 2.50 integrating for six protons could be attributed to the four benzylic protons and the methine protons of the lactone ring. ^{13}C NMR spectrum (fig. 3A.17) of the compound showed a peak at δ 178.5 confirming the presence of carbonyl group in the five membered lactone ring. Twelve aromatic carbon's resonance could be observed in between δ 153.4 and 105.5. Methoxy carbons resonated at δ 60.9, 60.8, 56.2 (2C) and 56.1 (2C). Mass spectrum of the compound gave the molecular ion peak at m/z 469.1841, which is the $(M+Na)^+$ peak. From these spectral data and on comparison with the literature reports, compound **23** was confirmed as the lignan lactone **cubebininolide,** which was reported earlier from this plant.[83] The structure of the compound is as shown below.



Compound **23 - Cubebininolide**

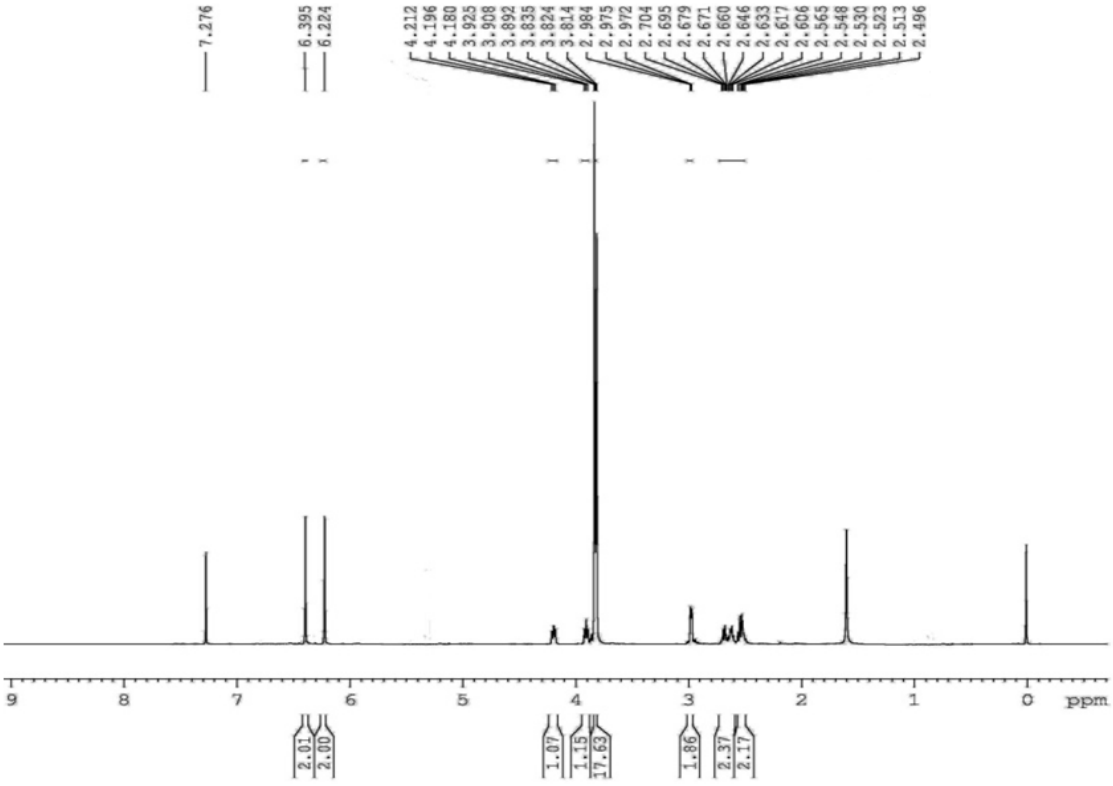


Figure 3A.16: ^{1}H NMR spectrum of cubebininolide

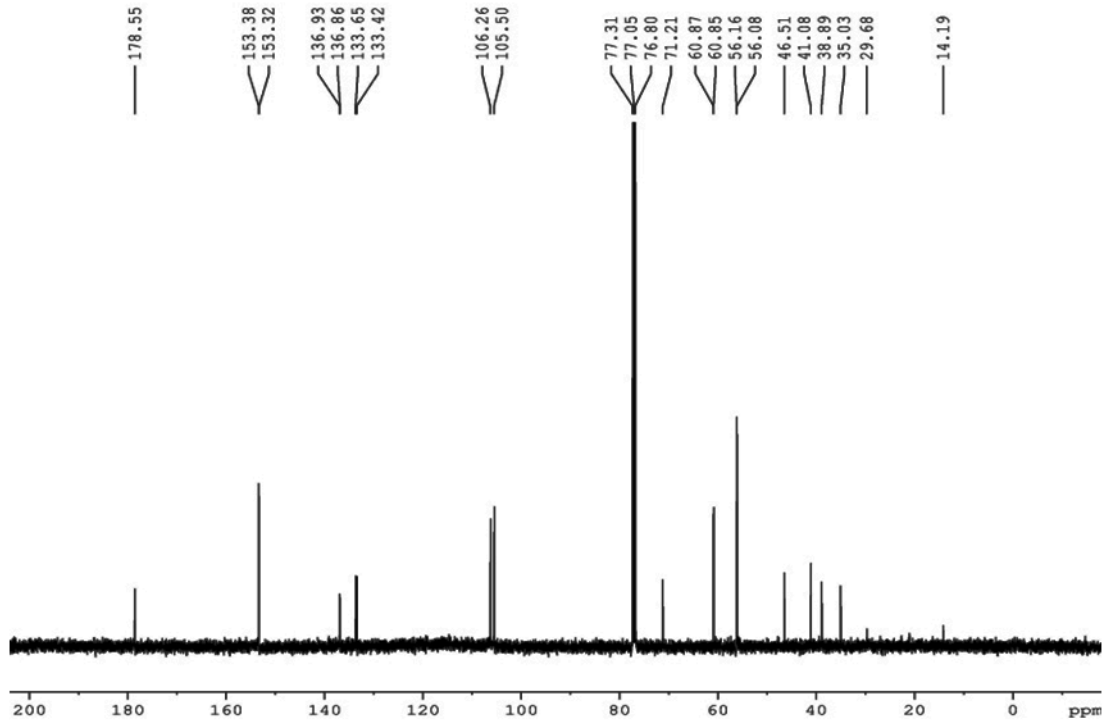
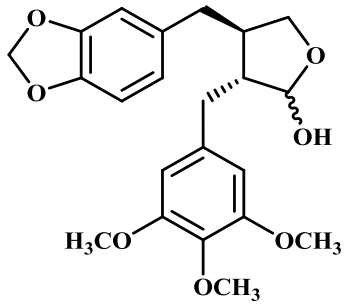


Figure 3A.17: ^{13}C NMR spectrum of cubebininolide

Subfractions 27-55 obtained during the purification of fraction pool twelve on further flash column chromatography over silica gel, using increasing polarities of chloroform-ethyl acetate mixture yielded 340 mg of compound **24** as a gummy solid and 158 mg of compound **25** as colourless crystals.

IR spectrum of compound **24** showed broad absorption at 3392 cm^{-1} suggesting the presence of hydroxyl group in the molecule. In the ^{1}H NMR spectrum (fig. 3A.18) peaks in between δ 6.72 and 6.32 indicated the presence of five aromatic hydrogens. Multiplet centred at δ 5.93 integrating for two protons may be assigned to the protons of methylenedioxy group. A singlet (δ 5.24) along with a doublet (δ 5.25), together integrating for one proton, could be attributed to the hemiacetalic proton. This observation suggested that the compound is a mixture of two epimers. Singlets at δ 3.84 and 3.81, integrating for three and six protons respectively, suggested the presence of three methoxy groups in the molecule. Diastereotopic protons of the lactone ring appeared as four separate multiplets, together integrating for two protons, in between δ 4.13 and 3.59. Multiplets in between δ 2.78 and 1.84 integrating for six protons could be attributed to the four benzylic and the methine protons of the lactone ring. By analyzing the integration in the ^{1}H NMR spectrum, it has been found that the compound is a 3:2 mixture two epimers at C-9', similar to that of cubebin. This was again

confirmed by analyzing the ^{13}C NMR spectrum (fig. 3A.19), which gave two sets of closely spaced signals. Peaks at δ 103.3 and 98.8 confirmed the presence of hemiacetalic carbons of the epimeric mixture. Two peaks at δ 100.9 and 100.8 in the ^{13}C NMR, which appeared as downward peaks in the DEPT-135 spectrum, confirmed the presence of a methylenedioxy group. Methylene carbon in the five membered lactol ring resonated at δ 72.5 and 72.1. Central methoxy carbon in the epimeric mixture resonated at δ 60.9 and 60.1, whereas the other two methoxy carbons resonated at δ 56.1 (2C) and 56.0 (2C). Mass spectrum of the compound gave the molecular ion peak at m/z 425.1575, which is the $(M+Na)^+$ peak. From these spectral data and on comparison with the literature reports, compound **24** was confirmed as **clusin** which was reported earlier from this plant[83] but first isolated from *Piper clusii*.[102] The structure of the compound is shown below.



Compound **24 -Clusin**

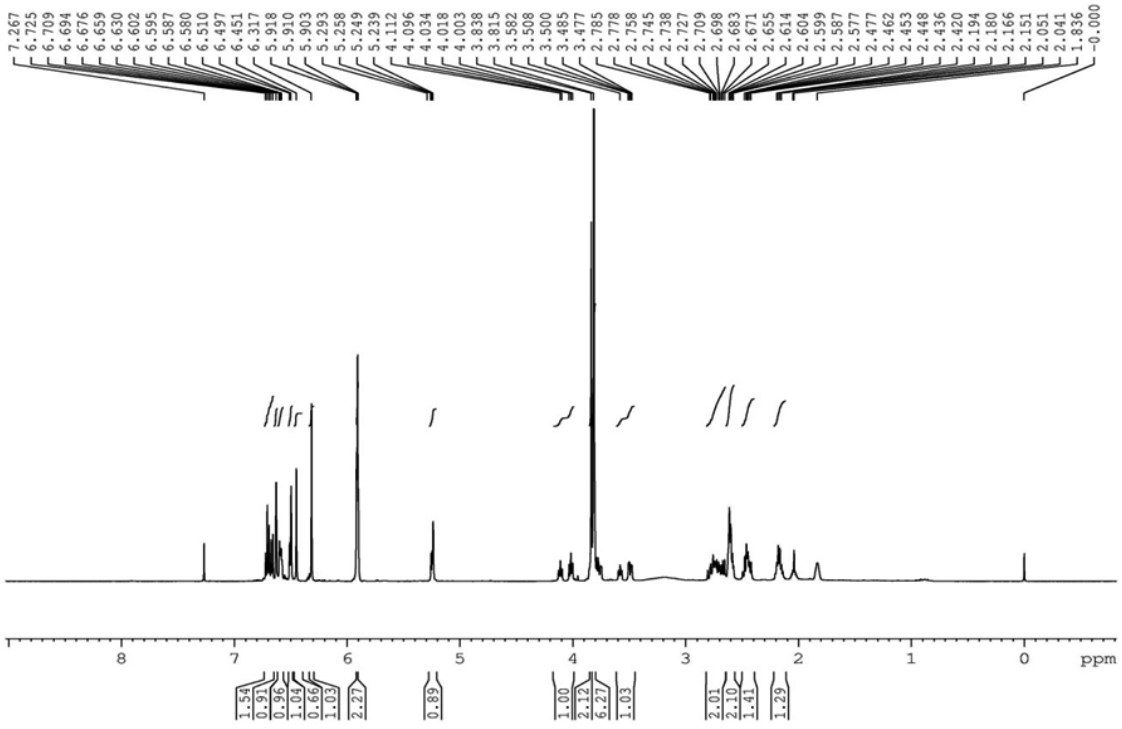


Figure 3A.18: ^1H NMR spectrum of clusin

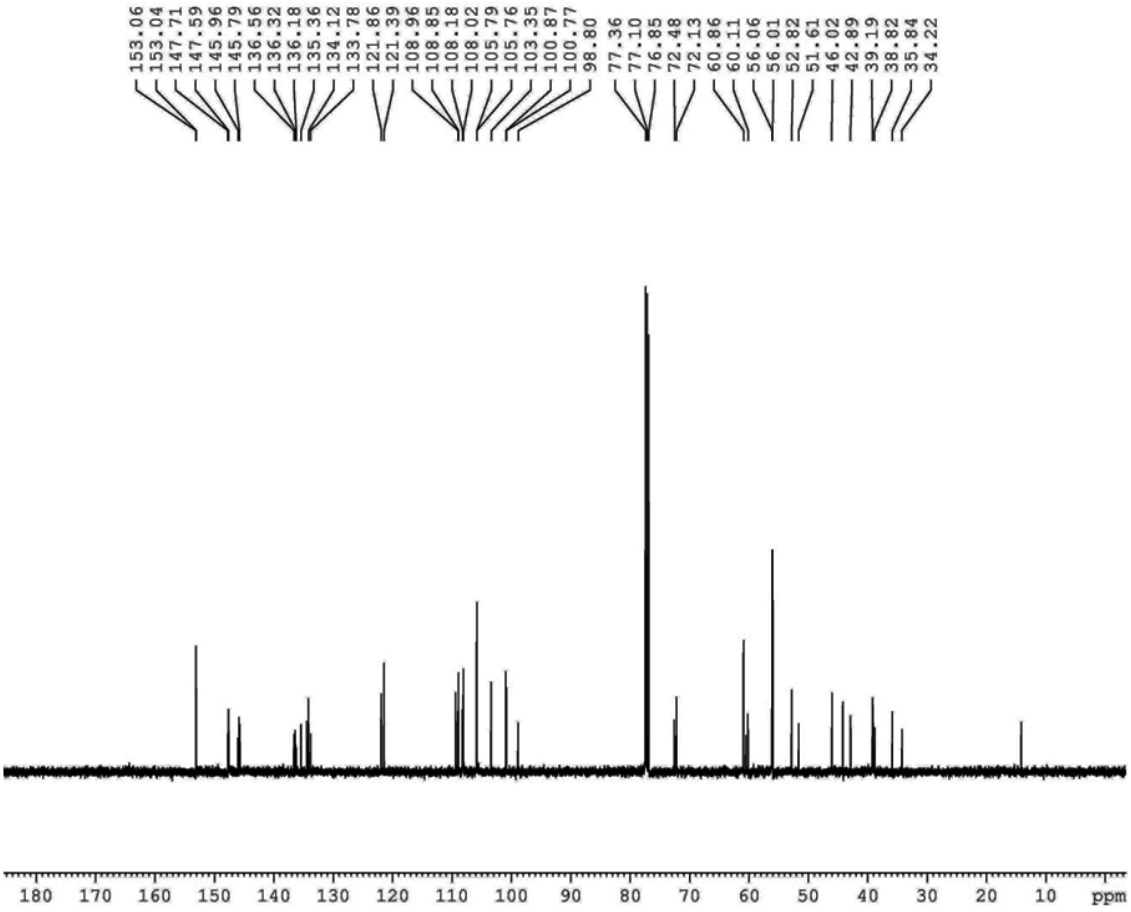
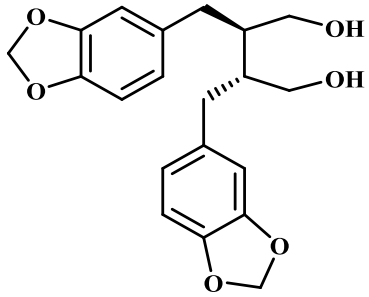


Figure 3A.19: ^{13}C NMR spectrum of clusin

IR spectrum of compound **25** showed absorption at 3425 cm^{-1} suggesting the presence of hydroxyl group in the molecule. Doublets centred at δ 6.71 and 6.61 along with a singlet at δ 6.64, each integrating for two protons in the ^{1}H NMR spectrum (fig. 3A.20) suggested the presence of six aromatic protons as in cubebin. Singlet at δ 5.92 integrating for four protons suggested the presence of two methylenedioxy groups again. Doublets of doublet centered at δ 3.77 and 3.49, integrating for two protons each could be attributed to the protons on the carbon bearing hydroxyl group. Doublets of doublet centered at δ 2.72 and 2.62 along with a multiplet centered at δ 1.84, each integrating for two protons, could be attributed to the four protons present at the benzylic position and the two methine protons of the lactone ring. Six signals resonating in between δ 147.6 and 108.1 in the ^{13}C NMR spectrum (fig. 3A.21) confirmed the presence of two sets of similarly trisubstituted aromatic ring reminiscent of cubebin structure. Methylenedioxy carbons were observed as a single signal at δ 100.8 ppm. The peak observed at δ 60.0 confirmed the presence of carbon bearing hydroxyl group. ^{13}C NMR spectrum of the compound, showing only ten peaks, clearly indicated the

symmetric nature of the molecule. Mass spectrum of the compound gave the molecular ion peak at m/z 381.1309, which is the $[M+Na]^+$ peak. From these spectral data and on comparison with the literature reports,[102] compound **25** was confirmed as **dihydrocubebin**, which was reported earlier from this plant. The structure of the compound is shown below.



Compound **25 - Dihydrocubebin**

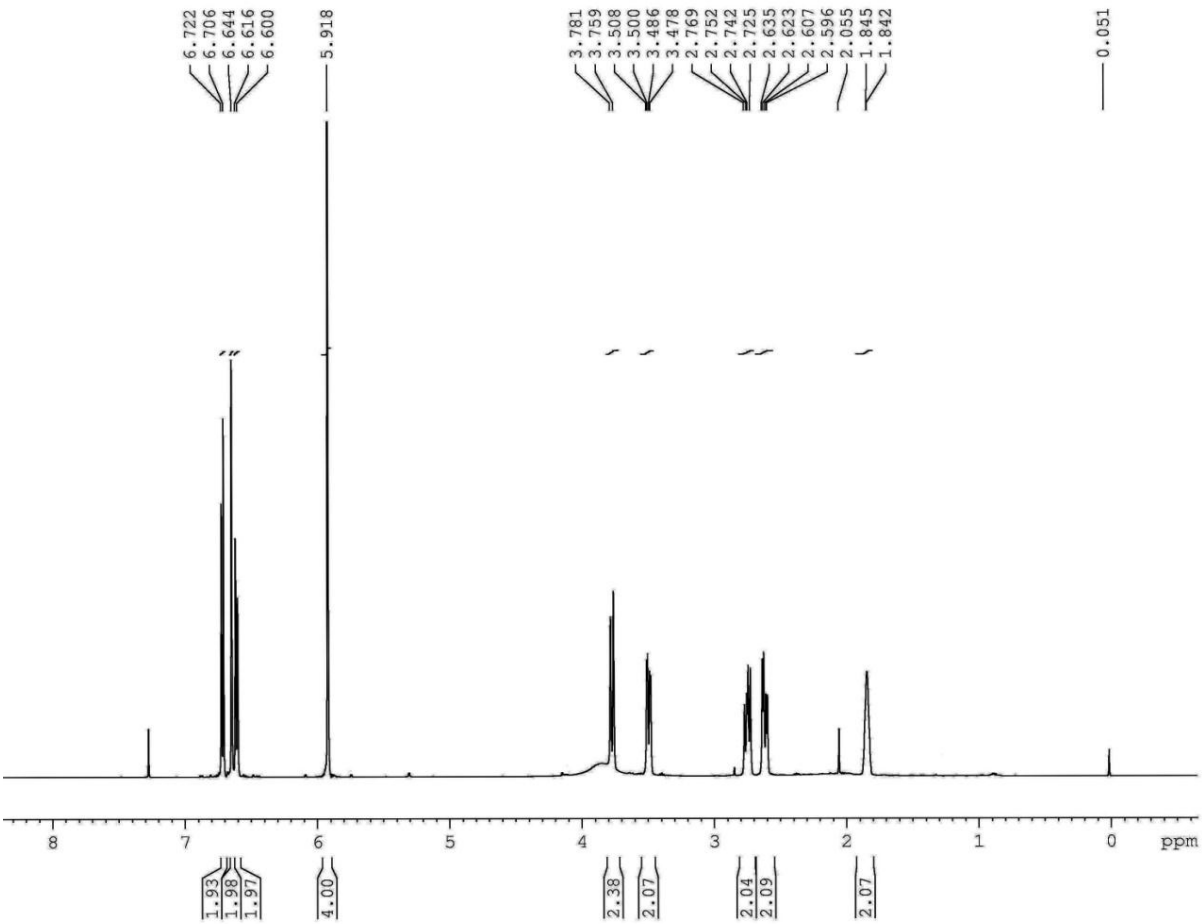


Figure 3A.20: ^{1}H NMR spectrum of dihydrocubebin

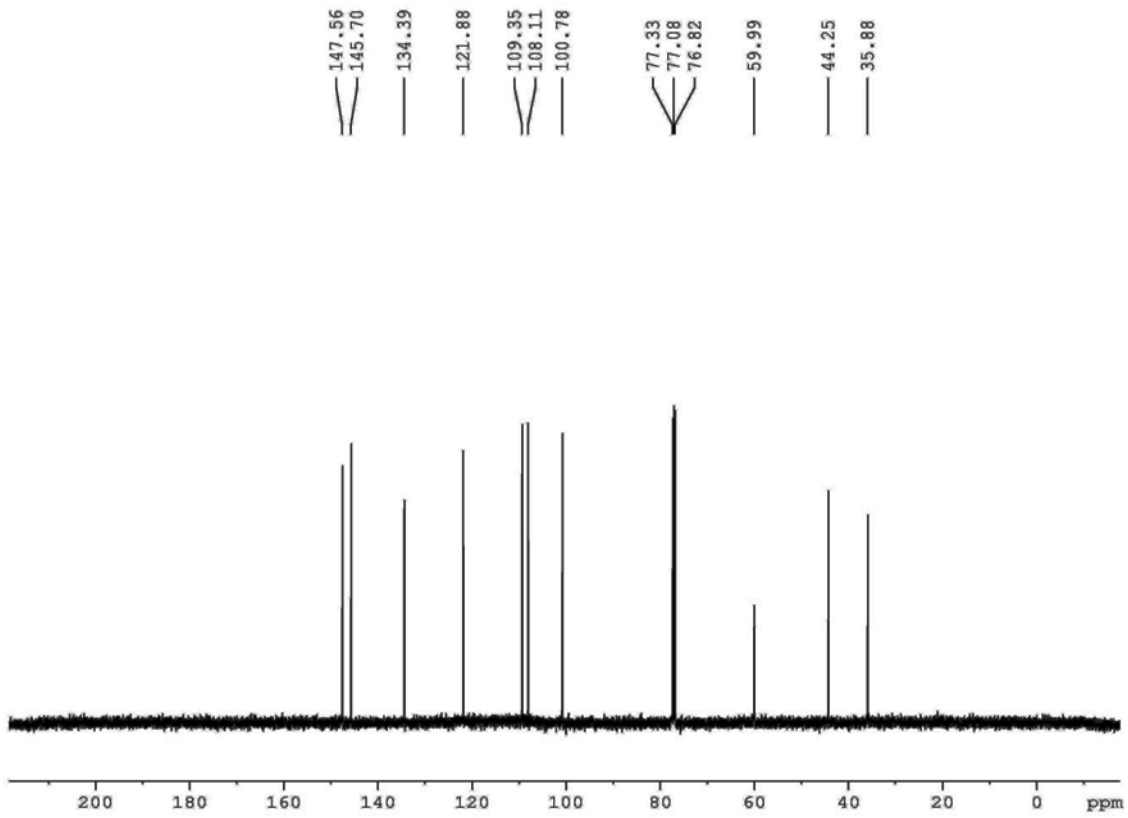
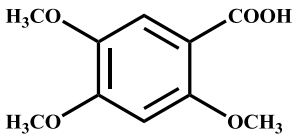


Figure 3A.21: ^{13}C NMR spectrum of dihydrocubebin

Fraction pool thirteen (Fr. 300-386) obtained by eluting the column with 50% ethyl acetate in hexane was further subjected to purification using silica gel column chromatography with gradient mixtures of chloroform and ethyl acetate. 25 different fractions of approximately 100 mL each were collected.

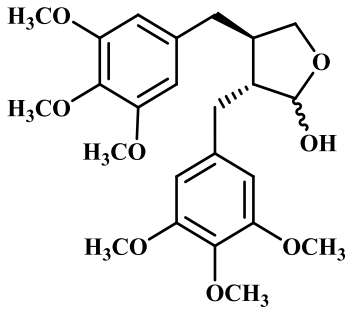
Subfractions 1-4 on further column chromatography over silica gel followed preparative TLC yielded 6 mg of compound **26** as a pale yellow solid. From the spectral data obtained, it was characterized as **2,4,5-trimethoxybenzoicacid (asaronic acid)**, which is being reported for the first time from *P. cubeba*. The structure of the compound is shown below.



Compound **26 - 2,4,5-trimethoxybenzoicacid**

Sub fraction 8 on repeated silica gel column chromatographic purification using gradient mixtures of dichloromethane–ethyl acetate solvent system yielded 76 mg of compound **27**, which was obtained as a gummy solid. IR spectrum of the compound

showed absorption at 3368 cm^{-1} suggesting the presence of hydroxyl group in the molecule. Nature of ^{1}H NMR spectrum (fig. 3A.22) of the compound suggested that it was a mixture of α and β epimers as in the case of cubebin and clusin. Four singlets at δ 6.47, 6.37, 6.36 and 6.29 suggested the presence four aromatic hydrogens. A doublet at δ 5.29 along with a singlet at δ 5.27, integrating for a total of one proton could be attributed to the hemiacetalic hydrogens of the epimeric mixture. Methylene protons of the lactol ring of the epimeric mixture appeared as different triplets at δ 4.14, 4.03, 3.80 and 3.63. Singlets at δ 3.85, 3.82 and 3.81 could be attributed to the six methoxy groups. The four protons present at the benzylic position and the two methine protons of the lactone ring appeared as multiplet in between δ 2.85 and 2.05 ppm. ^{13}C NMR spectrum (fig. 3A.23) also supported the presence of epimeric mixture. In the ^{13}C NMR spectrum, hemiacetalic carbon resonated at δ 103.3 and 98.8. Methylene carbon in the five membered lactol ring resonated at δ 72.7 and 72.3. Central methoxy carbons in the epimeric mixture resonated at δ 60.9 and 60.4, other four methoxy carbons resonated at δ 56.1 (2C) and 56.0 (2C). Mass spectrum of the compound gave the molecular ion peak at m/z 471.1997, which is the $[M+Na]^{+}$ peak. From these spectral data and on comparison with the literature reports, compound **27** was confirmed as 3:2 epimers of α and β–**cubebinin**, which was reported earlier from this plant.[83] The structure of the compound is as shown below.



Compound **27 - Cubebinin**

Subfractions 14-15 on repeated silica gel column chromatographic purification using gradient mixtures of dichloromethane–ethyl acetate solvent system yielded 67 mg of compound **28** as a gummy solid. IR spectrum of the compound showed absorption at 3425 cm^{-1} suggesting the presence of hydroxyl group in the molecule. ^{1}H NMR spectrum (fig. 3A.24) of the compound showed the presence of five aromatic protons resonating in between δ 6.57 and 6.21. A singlet observed at δ 5.77 integrating for two

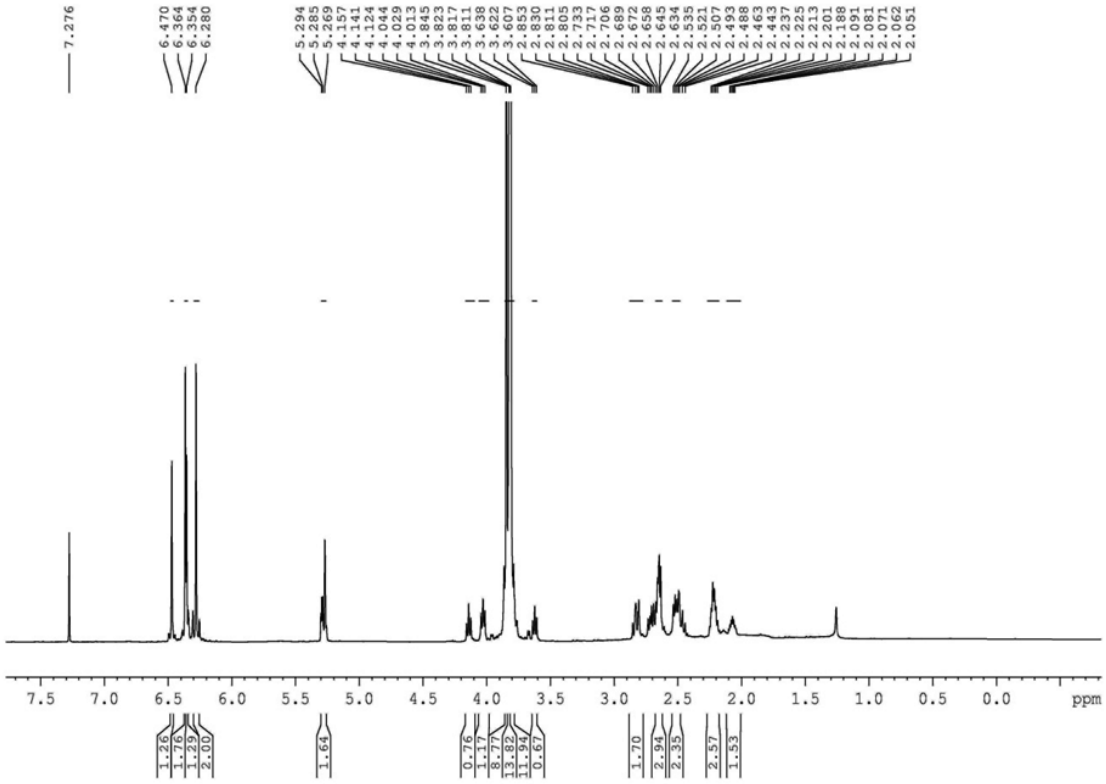


Figure 3A.22: ^{1}H NMR spectrum of cubebinin

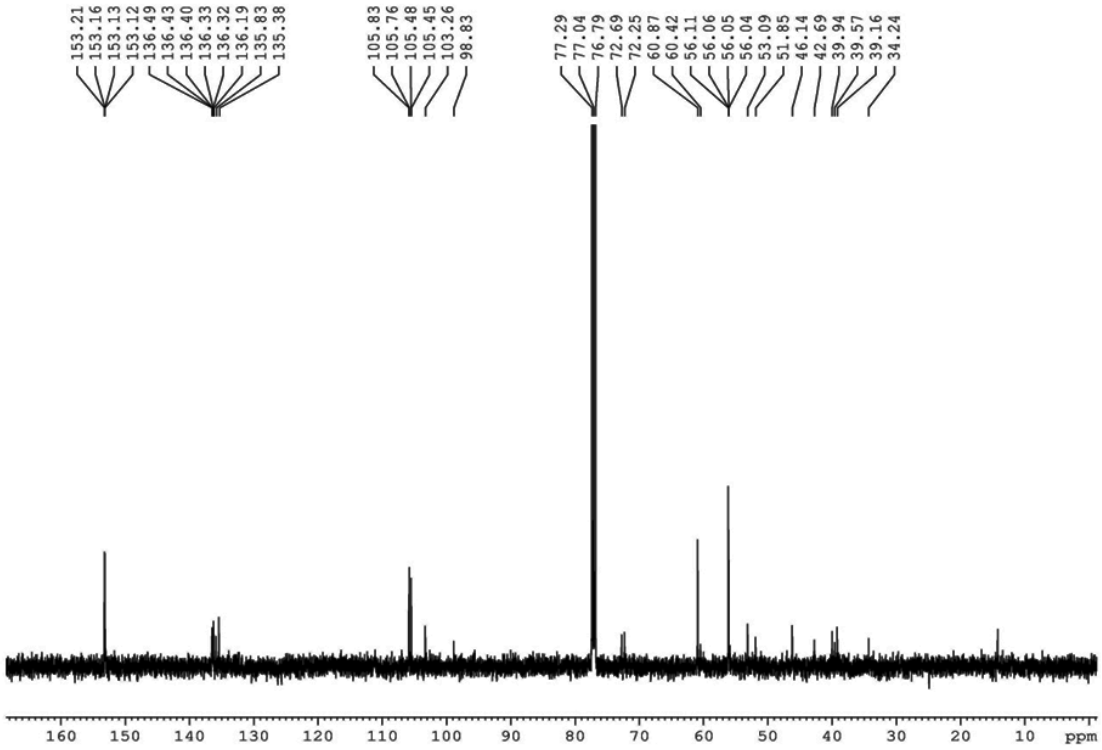
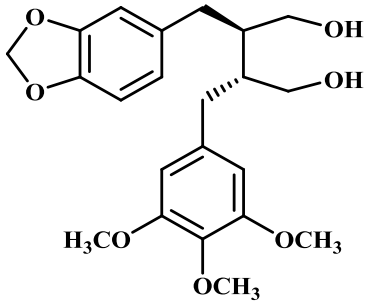


Figure 3A.23: ^{13}C NMR spectrum of cubebinin

protons could be attributed to the methylenedioxy group. Another singlet at δ 3.68 integrating for nine protons suggested the presence of three methoxy groups. Multiplets centred at δ 3.64 and 3.39, each integrating for two protons could be attributed to two

sets of methylene protons bearing hydroxy group. Multiplets in between δ 2.65 and 1.71, integrating for six protons in all, could be ascribed to the four protons at the benzylic position and the two methine protons. The presence of methylenedioxy carbon was confirmed by the peak at δ 100.8 in the ^{13}C NMR spectrum (fig. 3A.25) and the peak at δ 60.9 ppm could be attributed to the carbon bearing hydroxyl group. Methoxy carbons resonated at δ 60.5 and 56.1 (2C). Mass spectrum of the compound gave the molecular ion peak at m/z 427.1704, which is the $[M+Na]^+$ peak. From these spectral data and on comparison with the literature reports, compound **28** was confirmed as **dihydroclusin**, which was reported earlier from this plant.[83] The structure compound **28** is as shown below.



Compound **28 - Dihydroclusin**

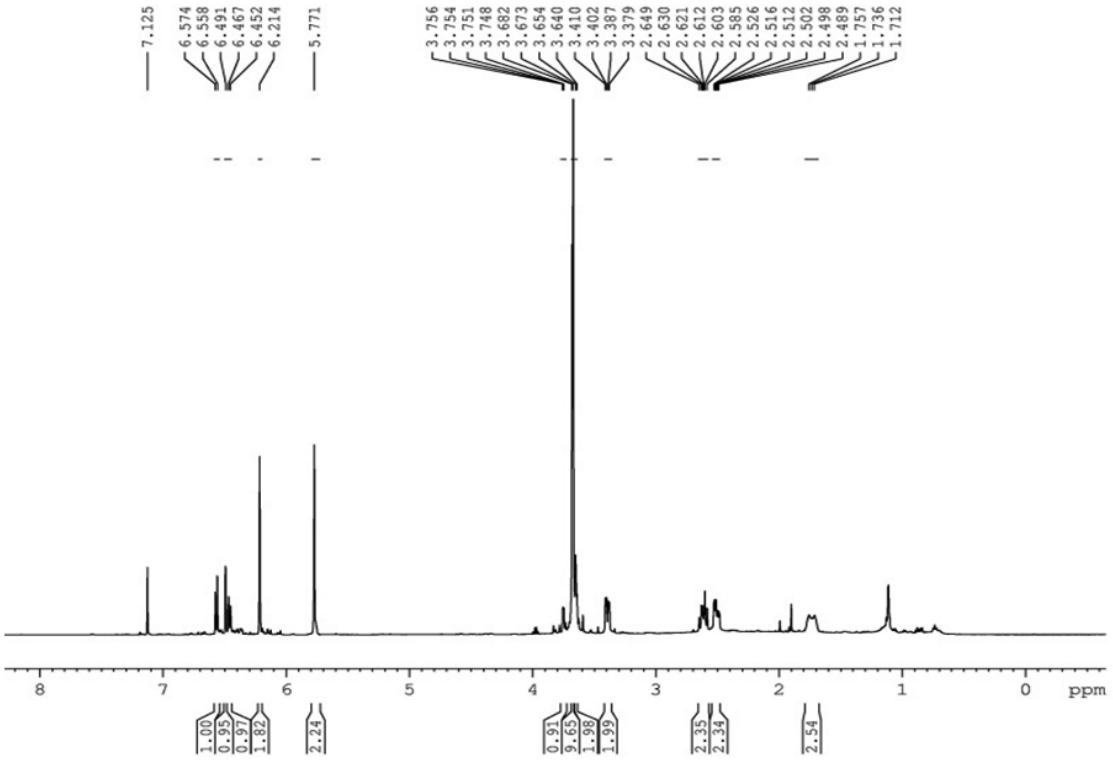


Figure 3A.24: ^{1}H NMR spectrum of dihydroclusin

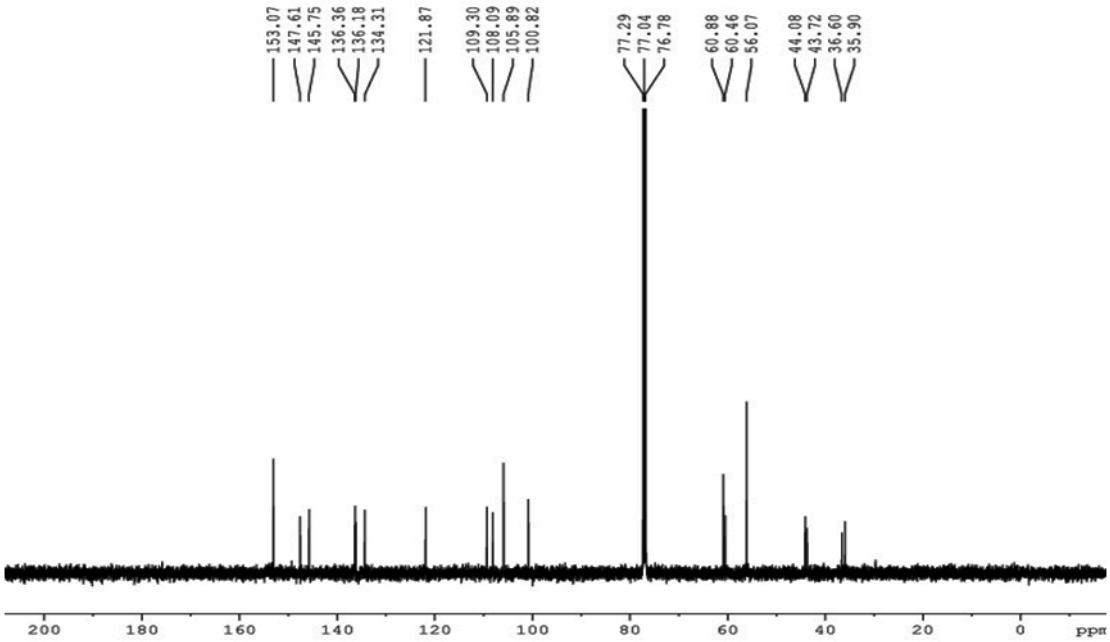


Figure 3A.25: ^{13}C NMR spectrum of dihydroclusin

3A.4. Experimental

General experimental procedures are described in chapter 2 of this thesis.

3A.4.1. Collection of plant material and extraction

The dried berries of *P. cubeba* were purchased from a registered medicinal plant vendor in Thiruvananthapuram. 2.3 kg of the powdered *P. cubeba* seeds were subjected to repeated extraction using acetone (4 L × 4) at room temperature. The total extract was then concentrated under reduced pressure using a rotary evaporator to yield approximately 412 g of crude acetone extract. For the ease of handling the oil content in the crude extract was removed by defattening using hexane. For this, the acetone extract was stirred with hexane for two hours and the hexane soluble portion decanted. The process was repeated four times. Defatted acetone extract weighed about 57 g.

3A.4.2. Isolation of compounds

Defatted acetone extract was used for the detailed phytochemical investigation. After studying the TLC, 55 g of the defatted acetone extract was subjected to purification using silica gel (500 g, 100-200 mesh) column chromatography. Column elution was started using 5% ethyl acetate in hexane and increase in polarity was carried out by increasing the amount of ethyl acetate. Final elution was carried out using 5% methanol in ethyl acetate. A total of 453 fractions of approximately 150 mL each were collected. According to the similarity in TLC, they were pooled into twenty

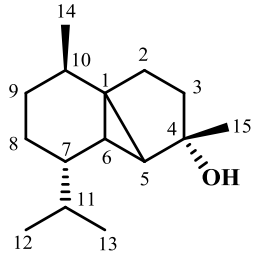
major fraction pools. Pictorial representation of the isolation of phytochemicals from *P. cubeba* seeds is given in chart 3A.5.

3A.4.2.1. Isolation of compound 15

Fraction pool three (Fr. 17-51), obtained by eluting the column with 5% ethyl acetate in hexane showed the presence of a UV active compound with some impurities. It was again subjected to column chromatographic purification using silica gel to yield 310 mg of colourless crystals. The compound was confirmed to be **cubebol** based on the spectral data obtained, as shown below.

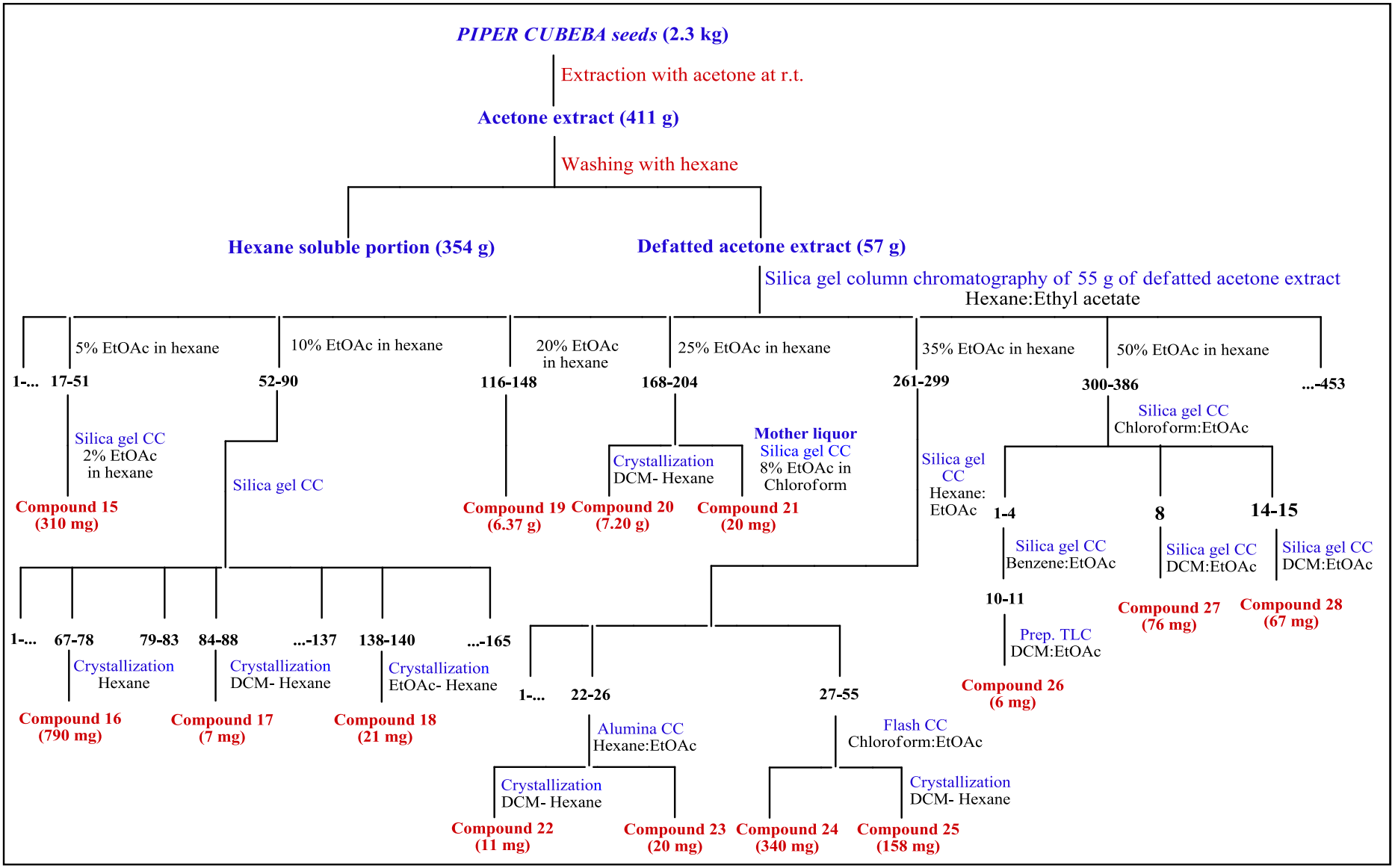
Colourless crystals; m.p. 57-59 °C, lit.[103] 61-62 °C

FT-IR (KBr, υ_{max}, cm^{-1})	:	3281, 2954, 2921, 2864, 1643, 1452, 1390, 1363, 1299, 1224, 1187, 1131
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 1.84 (m, 1H, H-2a), 1.62 (m, 3H, H-10, H-11, H-8a), 1.53 (m, 2H, H-2b, H-3a), 1.46 (s, 1H, -OH), 1.38 (m, 2H, H-9a, H-3b), 1.28 (s, 3H, H-15), 1.00 (m, 1H, H-7), 0.97 (d, *J* = 6.5 Hz, 3H, H-13), 0.94 (d, *J* = 6.5 Hz, 3H, H-14), 0.92 (d, *J* = 7.0 Hz, 3H, H-12), 0.86 (m, 1H, H-5), 0.81 (m, 2H, H-8b, H-6), 0.515 (m, 1H, H-9b)
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 80.3 (C-4), 44.1 (C-7), 39.0 (C-5), 36.3 (C-3), 33.6 (C-11), 33.4 (C-1), 31.7 (C-9), 30.8 (C-10), 29.5 (C-2), 27.9 (C-15), 26.5 (C-8), 22.6 (C-6), 20.1 (C-14), 19.6 (C-13), 18.7 (C-12)
HRMS (m/z)	:	245.2878 [$(M+Na)^+$]; $C_{15}H_{26}O$, requires 222.1984



NMR Spectral assignments were made on the basis of DEPT-135 spectrum and on comparison with literature reports.[86]

Chart 3A.5: Pictorial representation for the isolation of compounds from *Piper cubeba* seed

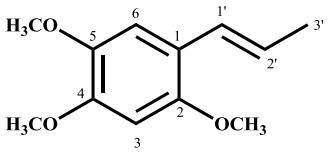


3A.4.2.2. Isolation of compound 16

Fraction pool four (Fr. 52-90) obtained by eluting the column with 10% ethyl acetate in hexane showed the presence of three UV active compounds in it. It was further purified using silica gel column chromatography. Subfractions 67-78 obtained by eluting the column with 4% ethyl acetate in hexane showed the presence of a single UV active compound, which on crystallization yielded 790 mg of colourless solid. The compound was confirmed to be ***α*-asarone,** based on the spectral data obtained, as shown below.

Colourless solid; m.p. 61-63 °C, lit.[104] 62-63 °C

FT-IR (KBr, υ_{max}, cm^{-1})	:	2955, 2920, 2861, 1607, 1511, 1461, 1400,1375, 1315, 1271, 1210, 1126, 1036
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 6.97 (s, 1H, H-6), 6.65 (d, *J* = 15.5 Hz, 1H, H-1'), 6.48 (s, 1H, H-3), 6.10 (m, 1H, H-2'), 3.86 (s, 3H, -OCH_3) 3.84 (s, 3H, -OCH_3), 3.79 (s, 3H, -OCH_3), 1.88 (d, *J* = 6.5 Hz, 3H, H-3')
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 150.6 (C-4), 148.7 (C-5), 143.3 (C-2), 125.0 (C-1'), 124.2 (C-2'), 118.9 (C-1), 109.8 (C-6), 97.9 (C-3), 56.6 (-OCH_3), 56.4 (-OCH_3), 56.0 (-OCH_3), 18.8 (C-3')
HRMS (m/z)	:	231.0998 [$(M+Na)^+$]; $C_{12}H_{16}O_3$, requires 208.1099



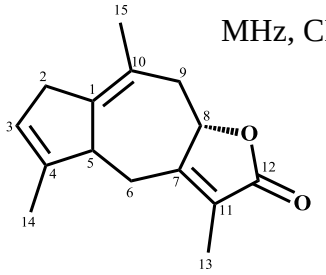
NMR Spectral assignments were made on the basis of DEPT-135 and 2D NMR analysis.

3A.4.2.3. Isolation of compound 17

Subfractions 84-88 obtained by eluting the column with 4% EtOAc in hexane on crystallization yielded 7 mg compound **17** as pale yellow crystals. The compound was confirmed to be **podoandin** based on the spectral data obtained, as shown below.

Pale yellow crystals; m.p.115-116 °C, lit.[89] 114-115 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 2920, 2859, 1753, 1440, 1380, 1314, 1214, 1096, 1017



^{1}H NMR (500 MHz, $CDCl_3$) : δ 5.49 (d, *J* = 11.5 Hz, 1H, H-3), 4.55 (d, *J* = 11.5 Hz, 1H, H-8), 3.03 (3H, m, H-2b, H-6), 2.88 (d, *J* = 11.5 Hz, 1H, H-5), 2.62 (dd, *J* = 13.5, 3.5 Hz, 1H, H-9b), 2.23 (t, *J* = 12.5 Hz, 1H, H-9a), 1.85 (s, 3H, H-13), 1.83 (m, 1H, H-2a), 1.78 (s, 6H, H-14, H-15)

^{13}C NMR (125 MHz, $CDCl_3$) : δ 174.3 (C-12), 163.5 (C-7), 142.9 (C-1), 140.7 (C-4), 124.3 (C-3), 122.7 (C-10), 121.3 (C-11), 79.4 (C-8), 49.6 (C-5), 39.3 (C-9), 36.9 (C-6), 31.3 (C-2), 21.9 (C-14), 14.9 (C-15), 8.3 (C-13)

HRMS (m/z) : 253.1208 $[(M+Na)^+]$; $C_{15}H_{18}O_2$, requires 230.1307

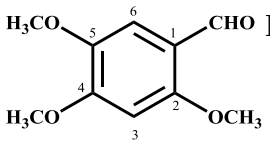
NMR Spectral assignments were made on the basis of literature reports.[89,105] The compound has been previously isolated from the plant *Podocarpus andina* belonging to Podocarpaceae family and to the best of our knowledge, this is the first report in the isolation of podoandin from *Piper* species.

3A.4.2.4. Isolation of compound 18

Subfractions 138-140 obtained by eluting the column with 50% ethyl acetate in hexane on crystallization yielded 21 mg of compound **18** as a pale yellow solid. The compound as characterized as **2,4,5-trimethoxybenzaldehyde** (**asaronaldehyde**) based on the spectral data obtained, as given below.

Pale yellow solid; m.p. 112-114 °C, lit.[106] 113-114 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 2932, 2853, 1755, 1669, 1608, 1510, 1465, 1441, 1407, 1279, 1215, 1124, 10269



^{1}H NMR (500 MHz, $CDCl_3$) : δ 10.32 (s, 1H, -CHO), 7.33 (s, 1H, H-6), 6.50 (s, 1H, H-3), 3.98 (s, 3H, $-OCH_3$), 3.93 (s, 3H, $-OCH_3$), 3.88 (s, 3H, $-OCH_3$)

^{13}C NMR (125 MHz, $CDCl_3$) : δ 188.1 (-CHO), 158.7 (C-4), 155.8 (C-5), 143.6 (C-2), 117.4 (C-1), 109.1 (C-6), 96.0 (C-3), 56.3 ($-OCH_3$), 56.2 ($-OCH_3$), 56.2 ($-OCH_3$)

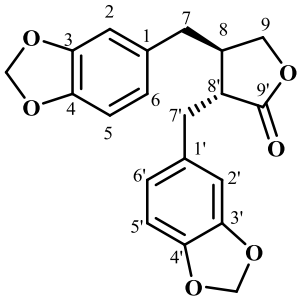
HRMS (m/z) : 197.0814 $[(M+H)^+]$; $C_{10}H_{12}O_4$, requires 196.0736

3A.4.2.5. Isolation of compound 19

Major fraction pool six (Fr. 116-148) obtained by eluting the column with 20% ethyl acetate in hexane on further purification yielded 6.37 g of gummy solid. The compound was confirmed to be (-)-**hinokinin** based on the spectral data obtained, as shown below.

Gummy solid

FT-IR (KBr, υ_{max}, cm^{-1}) : 2902, 2778, 1769, 1608, 1495, 1443, 1368, 1247, 1191, 1037, 929, 811



^{1}H NMR (500 MHz, $CDCl_3$) : δ 6.73 (d, *J* = 8.0 Hz, 1H, ArH), 6.70 (d, *J* = 8.0 Hz, 1H, ArH), 6.64 (d, *J* = 1.0 Hz, 1H, ArH), 6.61 (dd, *J* = 8.0, 1.0 Hz, 1H, ArH), 6.47 (m, 2H, ArH), 5.93 (s, 4H, $-OCH_2O-$), 4.13 (dd, *J* = 9.0, 7.0 Hz, 1H, H-9a), 3.86 (dd, *J* = 9.0, 7.0 Hz, 1H, H-9b), 2.99 (dd, *J* = 14.0, 5.3 Hz, 1H, H-7'a), 2.85 (dd, *J* = 14.0, 7.5 Hz, 1H, H-7a), 2.71 (m, 4H, H-7'b, H-7b, H-8, H-8')

^{13}C NMR (125 MHz, $CDCl_3$) : δ 178.5 (C-9'), 147.8 (C-4), 147.8 (C-4'), 146.4 (C-3), 146.3 (C-3'), 131.8 (C-1'), 131.5 (C-1), 122.2 (C-6'), 121.5 (C-6),

109.4 (C-2), 108.8 (C-2'), 108.3 (C-5), 108.2 (C-5'), 101.0 (2C, C-11, C-12), 71.1 (C-9), 46.4 (C-8'), 41.3 (C-8), 38.1 (C-7), 34.7 (C-7')

HRMS (m/z) : 377.0977 $[(M+Na)^+]$; $C_{20}H_{18}O_6$, requires 354.1103

$[\alpha]_D^{26}$: -30.0° ($CHCl_3$; *c* 0.51), lit.[94] -28.9° ($CHCl_3$; *c* 0.31).

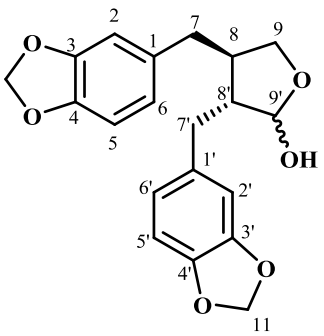
NMR Spectral assignments were made on the basis of DEPT-135 and 2D NMR analysis and on comparison with the literature reports.[83,107]

3A.4.2.6. Isolation of compound 20

Major fraction pool nine (Fr. 168-204) obtained by eluting the column with 25% ethyl acetate in hexane on crystallization using dichloromethane-hexane solvent system yielded 7.2 g of colourless crystals. The compound was confirmed to be the 3:2 epimeric mixture of (-)-**cubebin** based on the spectral data obtained, as shown below.

Colourless crystals; m.p. 133-135 °C, lit.[102] 131.5 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 3352, 2920, 2892, 1492, 1440, 1357, 1243, 1189, 1035, 1096 923, 807

^{1}H NMR (500 MHz, $CDCl_3$) : 

α-cubebin- δ 6.70 (m, 3H, ArH), 6.57 (m, 3H, ArH), 5.92 (s, 4H, -OCH_2O-), 5.22 (s, 1H, H-9'), 4.00 (t, *J* = 8.0 Hz, 1H, H-9a), 3.79 (t, *J* = 8.0 Hz, 1H, H-9b), 2.64 (m, 4H, H-7a, H-7', H-8), 2.42 (m, 1H, H-7b), 2.15 (m, 1H, H-8')

β-cubebin- δ 6.70 (m, ArH), 6.57 (m, ArH), 5.93 (s, -OCH_2O-), 5.22 (s, H-9'), 4.01 (t, *J* = 8.0 Hz, H-9a), 3.57 (t, *J* = 8.0 Hz, H-9b), 2.64 (m, H-7a, H-7', H-8), 2.42 (m,

H-7b), 2.01 (m, H-8')

^{13}C NMR (125 MHz, $CDCl_3$)	:	*α*-cubebin- δ 147.6 (C-3'), 147.5 (C-3), 145.9 (C-4), 145.7 (C-4'), 134.1 (C-1), 133.3 (C-1'), 121.7 (C-6), 121.4 (C-6'), 109.2 (C-2), 108.9 (C-2'), 108.1 (C-5), 108.1 (C-5'), 103.4 (C-9'), 100.9 (2C, -OCH_2O-), 72.2 (C-9), 53.1 (C-8'), 45.9 (C-8), 39.2 (C-7), 38.9 (C-7') *β*-cubebin- δ 147.7 (C-3'), 147.5 (C-3), 145.9 (C-4), 145.8 (C-4'), 134.5 (C-1), 133.9 (C-1'), 121.6 (C-6), 121.4 (C-6'), 109.3 (C-2), 108.9 (C-2'), 108.5 (C-5), 108.2 (C-5'), 100.8 (2C, -OCH_2O-), 99.8 (C-9'), 72.6 (C-9), 52.0 (C-8'), 42.9 (C-8), 38.9 (C-7), 33.6 (C-7')
HRMS (m/z)	:	379.1161 [$(M+Na)^+$]; $C_{20}H_{20}O_6$, requires 356.1260
$[\alpha]_D^{26}$	:	-45.6° ($CHCl_3$; *c* 0.54), lit.[108] -43.9° ($CHCl_3$; *c* 0.5).

NMR Spectral assignments were made on the basis of DEPT-135 and 2D NMR analysis and on comparison with the literature report.[94]

3A.4.2.7. Isolation of compound 21

Mother liquor obtained after the crystallization of cubebin was subjected to silica gel column chromatographic purification using gradient mixtures of chloroform and ethyl acetate. Fractions 67-73 (20 mg) obtained as a gummy solid; by eluting the column with 8% ethyl acetate in chloroform was found to be **yatein** based on the spectral data obtained for the compound.

Gummy solid

FT-IR (KBr, υ_{max}, cm^{-1})	:	2955, 2916, 2845, 1761, 1585, 1456, 1376, 1296, 1120, 1015
^1H NMR (500 MHz, $CDCl_3$)	:	δ 6.71 (d, *J* = 7.5 Hz, 1H, ArH), 6.47 (m, 2H, ArH), 6.36 (s, 2H, H-5', H-6'), 5.93 (d, *J* = 3.5 Hz 2H, $-OCH_2O-$), 4.18 (dd, *J* = 9.0, 7.5 Hz, 1H, H-9a), 3.89 (dd, *J* = 9.0, 8.0 Hz, 1H, H-9B), 3.83 (9H, s, $-OCH_3$), 2.91 (m, 2H, H-7a, H-7'a), 2.56 (m, 4H, H-7'b, H-7b, H-8, H-8')
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 178.5 (C-9'), 153.3 (2C, C-3', C-5'), 147.9 (C-3), 146.4 (C-4), 136.8 (C-4'), 133.3 (C-1'), 131.5 (C-1), 121.5 (C-6), 108.8 (C-2), 108.3 (C-5), 106.2 (2C, C-2', C-6'), 101.1 ($-OCH_2O-$), 71.2 (C-9), 60.9 ($-OCH_3$), 56.1 (2C, $-OCH_3$), 46.4 (C-8'), 41.0 (C-8), 38.3 (C-7'), 35.25 (C-7)
HRMS (m/z)	:	423.1423 $[(M+Na)^+]$; $C_{22}H_{24}O_7$, requires 400.1522

NMR Spectral assignments were made on the basis of DEPT-135 and 2D NMR analysis and on comparison with the literature reports.[99]

3A.4.2.8. Isolation of compound 22

Fraction pool twelve (Fr. 261-299) obtained by eluting the column with 35% ethyl acetate in hexane was further subjected to column chromatographic purification using silica gel to get 55 different fractions of approximately 100 mL each. Subfractions 22-26 on column chromatography over neutral alumina, using increasing polarity of hexane-ethyl acetate mixture yielded 11 mg of compound **22** as a pale yellow solid along with 20 mg of compound **23**. Compound **22** was characterized as **(5*α*,8*α*)-2-oxo-1-(10),3,7(11)-guaiatrien-12,8-olide** based on the spectral data obtained, as shown below.

Pale yellow solid; m.p. 231-233 °C

FT-IR (KBr, υ_{max}, cm^{-1})	:	2922, 2853, 1741, 1680, 1433, 1378, 1305, 1252, 1196, 1098, 1023
^1H NMR (500 MHz, $CDCl_3$)	:	δ 6.16 (s, 1H, H-3), 4.64 (d, *J* = 11.5 Hz, 1H, H-8), 3.20 (dd, *J* = 14.0, 2.0 Hz, 1H, H-6a) 3.05 (d, *J* = 12.0 Hz, 1H, H-5), 2.80 (dd, *J* = 13.5, 3.5 Hz, 1H, H-9a) 2.48 (m, 1H, H-9b), 2.46 (s, 3H, H-14), 2.18 (s, 3H, H-15), 1.92 (m, 1H, H-6b), 1.91 (s, 3H, H-13)
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 195.6 (C-2), 173.5 (C-12), 169.0 (C-4), 160.7 (C-7), 142.9 (C-10), 137.5 (C-1), 134.3 (C-1), 123.0 (C-11), 77.9 (C-8), 46.2 (C-5), 41.9 (C-9), 30.3 (C-6), 20.6 (C-14), 16.6 (C-15), 8.5 (C-13)
HRMS (m/z)	:	267.0987 $[(M+Na)^+]$; $C_{15}H_{16}O_3$, requires 244.1099

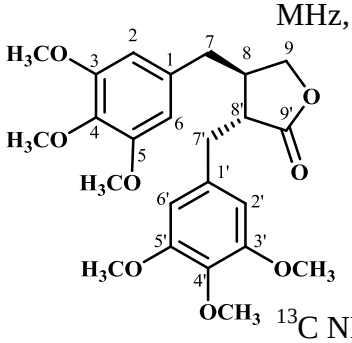
NMR Spectral assignments were made on the basis of DEPT-135 and 2D NMR analysis and on comparison with the literature reports.[82]

3A.4.2.9. Isolation of compound 23

Fraction pool twelve (Fr. 261-299) obtained by eluting the column with 35% ethyl acetate in hexane was further subjected to purification using silica gel to get 55 different fractions of approximately 100 mL each. Subfractions 22-26 on column chromatography over neutral alumina, using increasing polarity of hexane-ethyl acetate mixture gave 20 mg of compound **23** as a gummy solid. The compound was characterized as **cubebininolide** based on the spectral data obtained, as shown below.

Gummy solid

FT-IR (KBr, υ_{max}, cm^{-1})	:	2931, 2845, 1768, 1590, 1506, 1459, 1424, 1332, 1240, 1126, 101



^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 6.39 (s, 2H, ArH), 6.22 (s, 2H, ArH), 4.19 (t, *J* = 8.0 Hz, 1H, H-9a), 3.90 (t, *J* = 8.0 Hz, 1H, H-9b), 3.83 (s, 6H, -OCH_3), 3.82 (s, 6H, -OCH_3), 3.81 (s, 6H, -OCH_3), 2.97 (m, 2H, H-7a, H-7'a), 2.60 (m, 4H, H-7'b, H-7b, H-8, H-8')
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 178.5 (C-9'), 153.4 (2C, C-3', C-5'), 153.3 (2C, C-3, C-5), 136.9 (C-4'), 136.9 (C-4), 133.6 (C-1'), 133.4 (C-1), 106.3 (2C, C-2', C-6'), 105.5 (2C, C-2, C-6), 71.2 (C-9), 60.9 (-OCH_3), 60.8 (-OCH_3), 56.2 (2C, -OCH_3), 56.1 (2C, -OCH_3), 46.5 (C-8'), 41.1 (C-8), 38.9 (C-7), 35.0 (C-7')
HRMS (m/z)	:	469.1841 [$(M+Na)^+$]; $C_{24}H_{30}O_8$, requires 446.1941

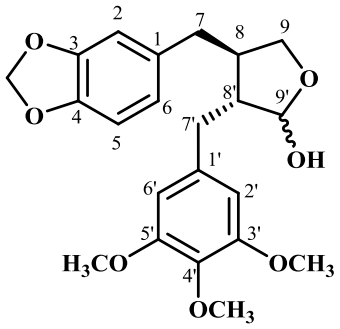
NMR Spectral assignments were made on the basis of DEPT-135 and 2D NMR analysis and on comparison with the literature reports.[83]

3A.4.2.10. Isolation of compound 24

Subfractions 27-55 obtained during the purification major fraction pool twelve on flash column chromatography over silica gel, using increasing polarity of chloroform-ethyl acetate mixture yielded 340 mg of gummy solid. The compound was characterized as 2:3 epimeric mixture of **clusin** based on the spectral data obtained, as shown below.

Gummy solid

FT-IR (KBr, υ_{max}, cm^{-1})	:	3392, 2937, 2887, 1590, 1503, 1488, 1441, 1213, 1035
^{1}H NMR (500 MHz, $CDCl_3$)	:	α-clusin- δ 6.72-6.32 (m, 5H, ArH), 5.91 (m, 2H, -OCH_2O-), 5.25 (d, *J* = 4.5 Hz) & 5.24 (s) – 1H, H-9'a, 4.03 (t, *J* = 8.0 Hz,



1H, H-9a), 3.80 (m, 1H, H-9b), 3.84 (s, 3H, -OCH_3), 3.81 (s, 6H, -OCH_3), 2.78-1.84 (m, 6H, H-8, H-8', H-7, H-7')

β-clusin- δ 6.72-6.32 (m, ArH), 5.91 (m, -OCH_2O-), 5.25 (d, *J* = 4.5 Hz) & 5.24 (s), - H-9'a, 4.14 (t, *J* = 8.0 Hz, H-9a), 3.62 (t, *J* = 8.0 Hz, H-9b), 3.84 (s, -OCH_3), 3.81 (s, -OCH_3), 2.78-1.84 (m, H-8, H-8', H-7, H-7')

^{13}C NMR (125 MHz, $CDCl_3$) : *α*-clusin- δ 153.0 (2C, C-3', C-5'), 147.6 (C-3), 145.8 (C-4), 136.3 (C-4'), 135.4 (C-1'), 134.1 (C-1), 121.4 (C-6), 108.8 (C-2), 108.0 (C-5), 105.8 (2C, C-2', C-6'), 103.3 (C-9'), 100.9 (-OCH_2-), 72.5 (C-9), 60.1 (-OCH_3), 56.0 (2C, -OCH_3), 52.9 (C-8'), 46.0 (C-8), 39.2 (C-7), 35.9 (C-7')

β-clusin- δ 153.1 (2C, C-3', C-5'), 147.7 (C-3), 146.0 (C-4), 136.5 (C-4'), 136.2 (C-1'), 133.8 (C-1), 121.9 (C-6), 109.0 (C-2), 108.2 (C-5), 105.8 (2C, C-2', C-6'), 100.9 (-OCH_2-), 98.8 (C-9'), 72.1 (C-9), 60.9 (-OCH_3), 56.1 (2C, -OCH_3), 51.6 (C-8'), 42.9 (C-8), 38.8 (C-7), 34.2 (C-7')

HRMS (m/z) : 425.1575 [$(M+Na)^+$]; $C_{22}H_{26}O_7$, requires 402.1679

$[\alpha]_D^{26}$: -36.2° ($CHCl_3$; *c* 0.14), lit.[102] -34.5° ($CHCl_3$; *c* 1.0),

NMR Spectral assignments were made on the basis of DEPT-135 and 2D NMR analysis and on comparison with the literature reports.[102]

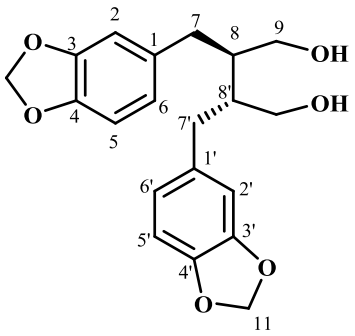
3A.4.2.11. Isolation of compound 25

Subfractions 27-55 obtained during the purification major fraction pool twelve on flash column chromatography over silica gel, using increasing polarity of chloroform-ethyl acetate mixture yielded 158 mg of as colourless crystals. The compound was characterized as **dihydrocubebin** based on the spectral data obtained, as shown below.

Colorless crysals; m.p.108-110 ^{o}C, lit.[109] 100-102 ^{o}C

FT-IR (KBr, υ_{max}, cm^{-1}) : 3425, 2931, 1589, 1497, 1457, 1243, 1125, 1038, 1007, 929

^{1}H NMR (500 MHz, $CDCl_3$) : δ 6.71 (d, *J* = 8.0 Hz, 2H, H-6, H-6'), 6.64 (s, 2H, H-2, H-2'), 6.61 (d, *J* = 8.0 Hz, 2H, H-5, H-6), 5.92 (s, 4H, -OCH_2-), 3.77 (d, *J* = 11.0 Hz, 2H, H-9b, H-9b'), 3.49 (dd, *J* = 11.0, 4.0 Hz, 2H, H-9a, H-9a'), 2.75 (dd, *J* = 13.5, 8.5 Hz, 2H, H-7b, H-7b'), 2.62 (dd, *J* = 13.8, 6.0 Hz, 2H, H-7a, H-7a') 1.84 (m, 2H, H-8, H-8')



^{13}C NMR (125 MHz, $CDCl_3$) : δ 147.6 (2C, C-4, C-4'), 145.7 (2C, C-3, C-3'), 134.4 (2C, C-1, C-1'), 121.9 (2C, C-5, C-5'), 109.3 (2C, C-2, C-2'), 108.1 (2C, C-6, C-6'), 100.8 (2C, -OCH_2O-), 60.0 (2C, C-9, C-9'), 44.2 (2C, C-8, C-8'), 35.9 (2C, C-7, C-7')

HRMS (m/z) : 381.1309 [$(M+Na)^+$]; $C_{20}H_{22}O_6$, requires 358.1416

$[\alpha]_D^{26}$: -34.1° ($CHCl_3$; *c* 0.12), lit.[109] -36.8° ($CHCl_3$; *c* 2.2).

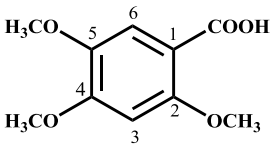
NMR Spectral assignments were made on the basis of DEPT-135 and 2D NMR analysis and on comparison with the literature reports.[102]

3A.4.2.12. Isolation of compound 26

Fraction pool thirteen (Fr. 300-386) obtained by eluting the column with 50% ethyl acetate in hexane was further subjected to purification using silica gel column chromatography with gradient mixtures of chloroform and ethyl acetate. 25 different fractions of approximately 100 mL each were collected. Subfractions 1-4 on further column chromatography over silica gel followed preparative TLC yielded 6 mg of a pale yellow solid. The compound was characterized as **2,4,5-trimethoxybenzoicacid (asaronic acid)** based on the spectral data obtained, as shown below.

Pale yellow solid; m.p. 142-144 ^{o}C, lit.[110] 143-144 ^{o}C

FT-IR (KBr, υ_{max}, cm^{-1}) : 3244, 2936, 2854, 1720, 1609, 1612, 1521, 1451, 1362, 1407, 1194, 1080



^{1}H NMR (500 MHz, $CDCl_3$) : δ 7.65 (s, 1H, H-6), 6.59 (s, 1H, H-3), 4.09 (s, 3H, -OCH_3), 3.99 (s, 3H, -OCH_3), 3.92 (s, 3H, -OCH_3)

^{13}C NMR (125 MHz, $CDCl_3$) : δ 165.3 (-COOH), 154.3 (C-4), 153.6 (C-5), 144.1 (C-2), 114.4 (C-1), 109.0 (C-6), 96.2 (C-3), 57.3 (-OCH_3), 56.4 (-OCH_3), 56.3 (-OCH_3)

HRMS (m/z) : 235.9577 [$(M+Na)^{+}$]; $C_{10}H_{12}O_{5,}$ requires 212.0685

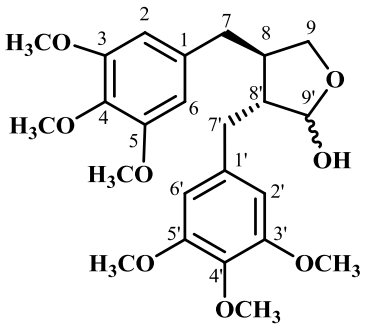
3A.4.2.13. Isolation of compound 27

Subfraction 8, obtained during the purification the fraction pool thirteen, on repeated silica gel column chromatographic purification using gradient mixtures of dichloromethane–ethyl acetate solvent system yielded 76 mg of a gummy solid. The compound was characterized as 2:3 epimeric mixture of **cubebinin** based on the spectral data obtained, as shown below.

Gummy solid

FT-IR (KBr, υ_{max}, cm^{-1}) : 3425, 2931, 2779, 1589, 1541, 1423, 1125, 1038, 1007

^{1}H NMR (500 MHz, $CDCl_3$) : α-cubebinin- δ 6.37 (s, 2H, ArH), 6.29 (s, 2H, ArH), 5.29 (d, *J* = 4.5 Hz) & 5.27 (s) – 1H, H-9'a, 4.03 (t, *J* = 8.0 Hz, 1H, H-9a), 3.80 (m, 1H, H-9b), 3.84 (s, 6H, $-OCH_3$), 3.82 (s, 6H, $-OCH_3$), 3.81 (s, 6H, $-OCH_3$), 2.85-2.05 (m, 6H, H-8, H-8', H-7, H-7')



β-cubebinin- δ 6.47 (s, ArH), 6.36 (s, ArH), 5.29 (d, *J* = 4.5 Hz) & 5.27 (s) – H-9'a, 4.14 (t, *J* = 8.0 Hz, H-9a), 3.62 (t, *J* = 8.0 Hz, H-9b), 3.84 (s, $-OCH_3$), 3.82 (s, $-OCH_3$), 3.81 (s, $-OCH_3$), 2.85-2.05 (m, H-8, H-8', H-7, H-7')

^{13}C NMR (125 MHz, $CDCl_3$) : α-cubebinin- δ 153.2 (2C, C-3', C-5'), 153.1 (2C, C-3, C-5), 136.4 (C-4'), 136.3 (C-4), 136.2 (C-1'), 135.4 (C-1), 105.8 (2C, C-2', C-6'), 105.5 (2C, C-2, C-6), 103.3 (C-9'), 72.2 (C-9), 60.9 (2C, $-OCH_3$), 56.1 (2C, $-OCH_3$), 56.0 (2C, $-OCH_3$), 53.1 (C-8'), 46.1 (C-8), 39.9 (C-7), 39.2 (C-7')

β-cubebinin- δ 153.2 (2C, C-5', C-3'), 153.1 (2C, C-5, C-3), 136.5 (C-4'), 136.4 (C-4), 136.3 (C-1'), 135.8 (C-1), 105.8 (2C, C-2', C-6'), 105.4 (2C, C-2, C-6), 98.8 (C-9'), 72.7 (C-9), 60.8 (2C,$-OCH_3$), 56.1 (2C, $-OCH_3$), 56.0 (2C, $-OCH_3$), 51.8 (C-8'), 42.7 (C-8), 39.6 (C-7), 34.2 (C-7')

HRMS (m/z) : 471.1997 $[(M+Na)^+]$; $C_{24}H_{32}O_{8,}$ requires 448.2097

NMR Spectral assignments were made on the basis of DEPT-135 and 2D NMR analysis, on comparison with the spectral data obtained for cubebin and cubebininolide

as well as on comparison with the literature reports.[83]

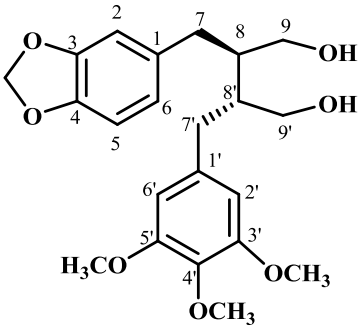
3A.4.2.14. Isolation of compound 28

Subfractions 14-15, obtained during the purification major fraction pool thirteen, on repeated silica gel column chromatographic purification using gradient mixtures of dichloromethane–ethyl acetate solvent system yielded 67 mg of a gummy solid. The compound was characterized as **dihydroclusin** based on the spectral data obtained, as shown below.

Gummy solid

FT-IR (KBr, υ_{max}, cm^{-1}) : 3245, 2932, 2890, 1590, 1514, 1492, 1438, 1242, 1128, 1038, 1006

^{1}H NMR (500 MHz, $CDCl_3$) : δ 6.56 (d, *J* = 8.0 Hz, 1H, ArH), 6.49 (s, 1H, ArH), 6.46 (d, *J* = 7.5 Hz, 1H, ArH), 6.21 (s, 2H, ArH), 5.77 (s, 2H, -OCH_2O-), 3.75 (m, 1H, H-9a), 3.68 (s, 9H, -OCH_3), 3.64 (m, 1H, H-9'a,), 3.39 (dd, *J* = 11.0, 4.0 Hz, 2H, H-9b, H-9'b), 2.62 (m, 2H, H-7a, H-7'a), 2.51 (m, 2H, H-7b, H-7'b), 1.74 (m, 2H, H-8, H-8')



^{13}C NMR (125 MHz, $CDCl_3$) : δ 153.1 (2C, C-3', C-5'), 147.6 (C-3), 145.8 (C-4), 136.3 (C-4'), 136.2 (C-1'), 134.3 (C-1), 121.9 (C-6), 109.3 (C-2), 108.1 C-5), 105.9 (2C, C-2', C-6'), 100.82 (-OCH_2O-), 60.9 (2C, C-9, C-9'), 60.5 (-OCH_3), 56.1 (2C, -OCH_3), 44.08 (C-8'), 43.73 (C-8), 36.60 (C-7'), 35.90 (C-7)

HRMS (m/z) : 427.1704 [$(M+Na)^+$]; $C_{22}H_{28}O_{7,}$ requires 404.1835

NMR Spectral assignments were made on the basis of DEPT-135 and 2D NMR analysis and on comparison with the literature reports.[83]

3A.5. Conclusion

Fourteen compounds viz., cubebol, *α*-asarone, 2,4,5-trimethoxybenzaldehyde, podoandin, hinokinin, cubebin, yatein, (5*α*,8*α*)-2-oxo-1-(10),3,7,(11)-guaiatrien-12,8-olide, cubebininolide, clusin, dihydrocubebin, 2,4,5-trimethoxybenzoic acid, cubebinin and dihydroclusin were isolated from the dried berries of *P. cubeba.* Among these, the sesquiterpene lactone, podoandin, is being reported for the first time from *Piper* species. The lignan lactol (-)-cubebin was found to be the major compound present in the berries, followed by the lignan lactone (-)-hinokinin.

3A.6. References

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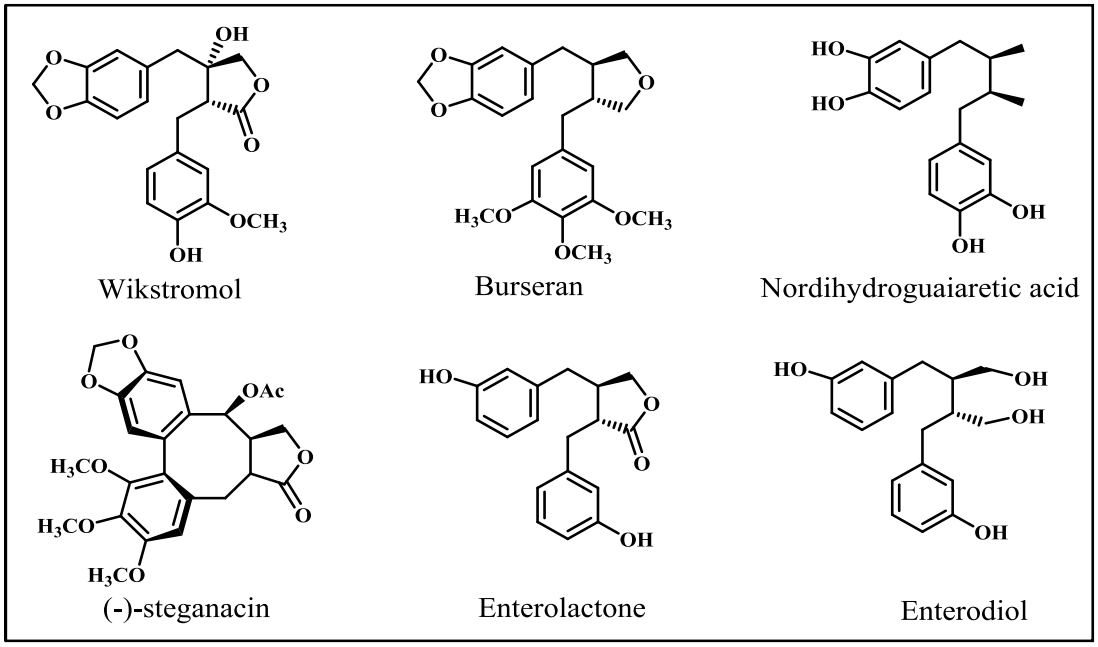
Chapter 3B

Synthesis of Cubebin Analogues and their Anticancer Activity Studies

3B.1. Introduction

As explained in the introduction chapter, cancer is one of the fatal diseases claiming millions of lives across the globe. Despite the fast growth of modern medicine, plant-derived natural products and their synthetic analogues contribute their fair share in cancer chemotherapy.[1] Lignans, an important class of plant secondary metabolites have served as lead compounds for the development of novel anticancer agents. Success stories include that of the anticancer lignans such as etoposide and teniposide, which are the semi-synthetic derivatives of the lignan lactone podophyllotoxin[2,3] (details given in chapter 1). Apart from podophyllotoxin, lignans such as wikstromol, burseran, nordihydroguaiaretic acid, steganacin, enterodiol, enterolactone etc., (chart. 3B.1) are also known to have very good *in vitro* anticancer activities.[4]

Chart 3B.1: Structures of some important anticancer lignans



P. cubeba seed extract has been reported to be effective against breast cancer[5] and prostate cancer growth.[6] Phytochemical investigation of *P. cubeba* seed, as described in chapter 3A, as well as the literature reports clearly indicate that *P. cubeba*

is a rich source of bioactive lignans and the major compound present in *P. cubeba* seeds is the butyrolactol lignan, (-)-cubebin (**20**).[7,8] (-)-Cubebin has been reported to have trypanocidal,[9] anti-inflammatory,[10] analgesic,[11] vasorelaxant,[12] antimycobacterial and antiprotozoal[13] activities. (-)-Hinokinin (**19**), a butyrolactone lignan, isolated from the same plant has been reported to have very high trypanocidal,[9a,14] analgesic, anti-inflammatory,[15] antimutagenic,[16,17] chemopreventive,[18] modulatory effects on human monoamine and GABA transporter activities[19] as well as anticancer activities.[20,21] The compound was also found to be effective against oral pathogens including *Streptococcus mutans*.[22]

Even though (-)-cubebin is the most abundant compound in *P. cubeba*, the only studies reported on its synthetic derivatives and their bioactivity was that of Silva *et al.*[23] who reported the anti-inflammatory and analgesic activities. The same derivatives were also evaluated for their activity against *Trypanosoma cruzi*,[24] the causative agent of Chaga's disease.

3B.2. Aim and scope of the present study

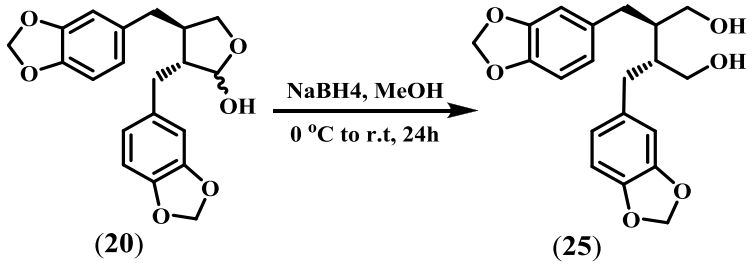
As mentioned in the introduction, even though (-)-cubebin is the most abundant compound in *P. cubeba* seeds, only a few derivatives of the compound have been synthesized. Most importantly, apart from these semi-synthetic derivatives, there have been no further reports in the area of chemical modification of (-)-cubebin and neither cubebin nor its derivatives have been explored in detail for their anticancer activities. Therefore in the present study, we have embarked upon studying the anticancer activities of (-)-cubebin, its chemical diversification and structure-activity effect of different functionalities in providing the anticancer activity.

3B.3. Synthetic transformation of cubebin

(-)-Cubebin (**20**), isolated as described in chapter 3A, was converted into five different functionalities in order to check whether structural modification on the lactol ring can enhance the anticancer activity. The two naturally occurring lignans hinokinin (**19**) and dihydrocubebin (**25**) (earlier isolated from *P. cubeba* seeds, as explained in chapter 3A) were synthesized from cubebin via the oxidation and reduction reactions respectively. Cubebin was also converted into its oxolane, amide as well as imide derivatives by modifying the lactol ring.

3B.3.1. Conversion of (-)-cubebin to (-)-dihydrocubebin

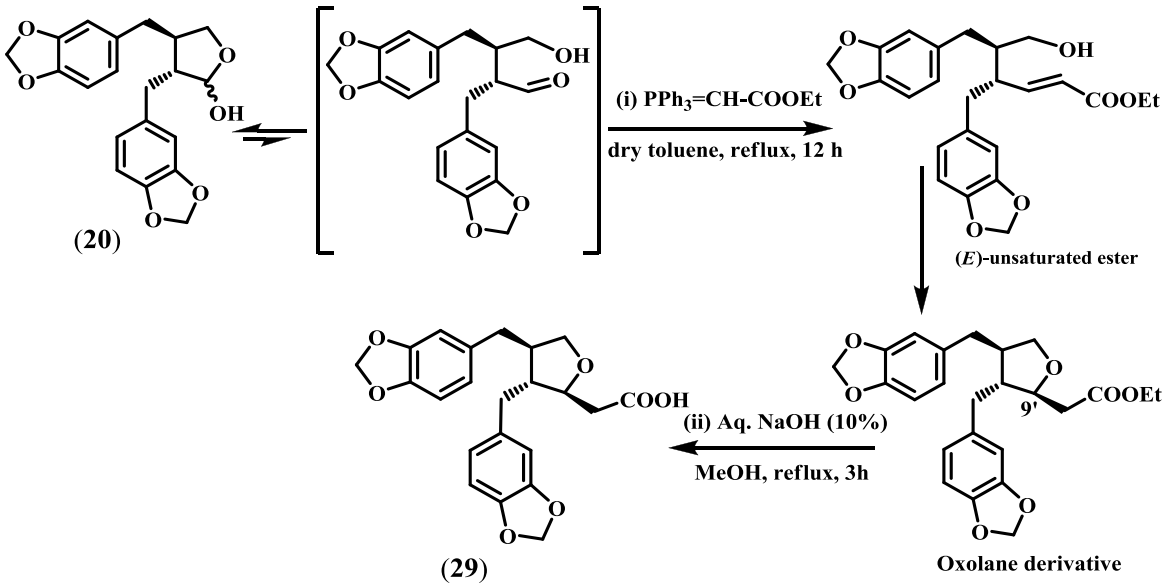
(-)-Dihydrocubebin (**25**) was obtained by the reduction of cubebin using sodium borohydride (scheme 3B.1). The structure of the compound was confirmed by various spectroscopic techniques as explained in chapter 3A.



Scheme 3B.1

3B.3.2. Conversion of (-)-cubebin to oxolane derivative

Cubebin was readily converted into its oxolane derivative for the first time under Wittig reaction condition using ethyl (triphenylphosphoranylidene). The cyclic hemiacetal moiety of cubebin reacted with Wittig reagent to form the oxolane derivative via (*E*)-unsaturated ester intermediate, which readily underwent *in situ* cycloaddition leading to the oxolane formation.[25] The ester group of 9'-substituted oxolane derivative was then hydrolysed to the corresponding carboxylic acid by alkaline hydrolysis using 10% aqueous sodium hydroxide to give compound **29** (scheme 3B.2).



Scheme 3B.2

IR spectrum of compound **29** showed absorptions at 3180 (-OH) and 1712 (-C=O) cm^{-1} suggesting the presence of a carboxylic acid group. ^{1}H NMR spectrum (fig. 3B.1) of the compound showed the presence of six aromatic protons [δ 6.52 (s, 2H), 6.56 (s, 2H), 6.70 (m, 2H)]. A multiplet observed at δ 5.93 integrating for four protons could be attributed to the two methylenedioxy groups. Presence of carboxylic acid group was confirmed by the peak at δ 175.9 in the ^{13}C NMR spectrum (fig. 3B.2). Aromatic carbons appeared in between δ 147.8 and 108.1. Methylenedioxy carbon atoms resonated at δ 100.9. Peaks at δ 81.0 and 72.2 could be attributed to the methine and methylene carbons attached to oxygen in the oxolane ring respectively. The mass spectrum of the compound showed molecular ion peak at m/z 421.1264 which is the $(M+Na)^{+}$ peak. Connecting all these spectral data, structure of compound **29** was confirmed as shown in scheme 3B.2.

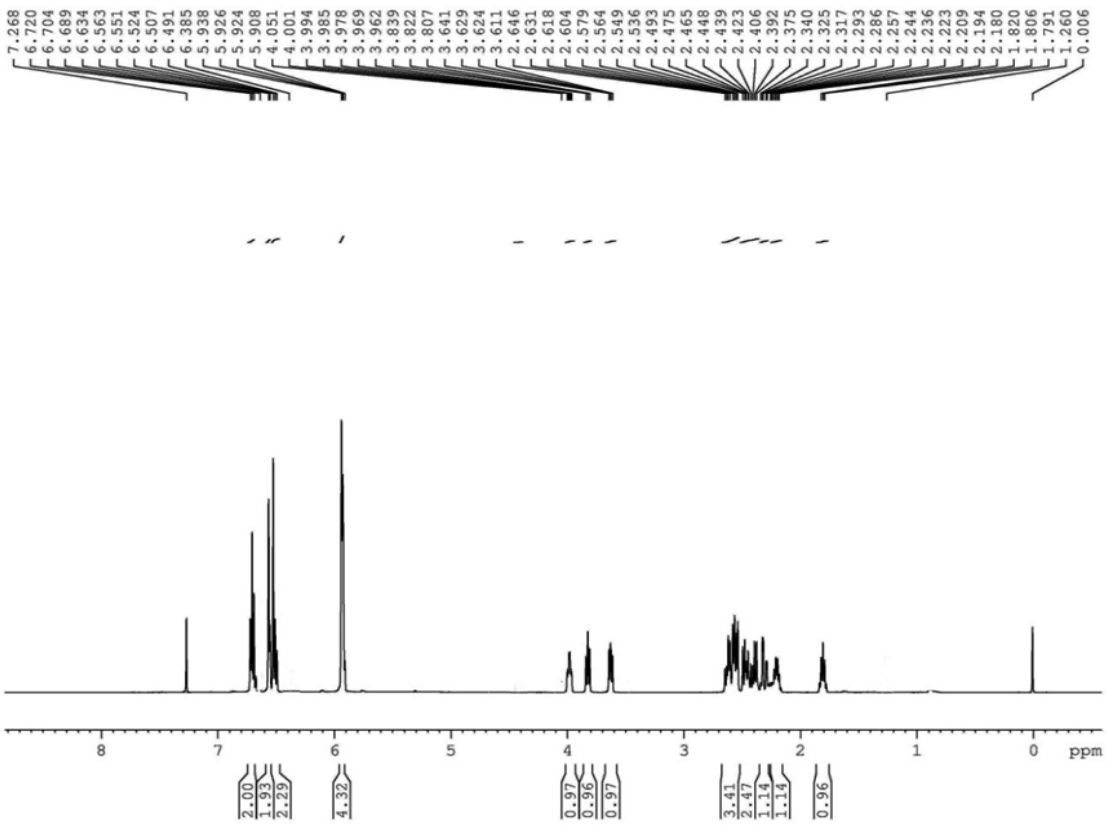


Figure 3B.1: ^{1}H NMR spectrum of compound **29**

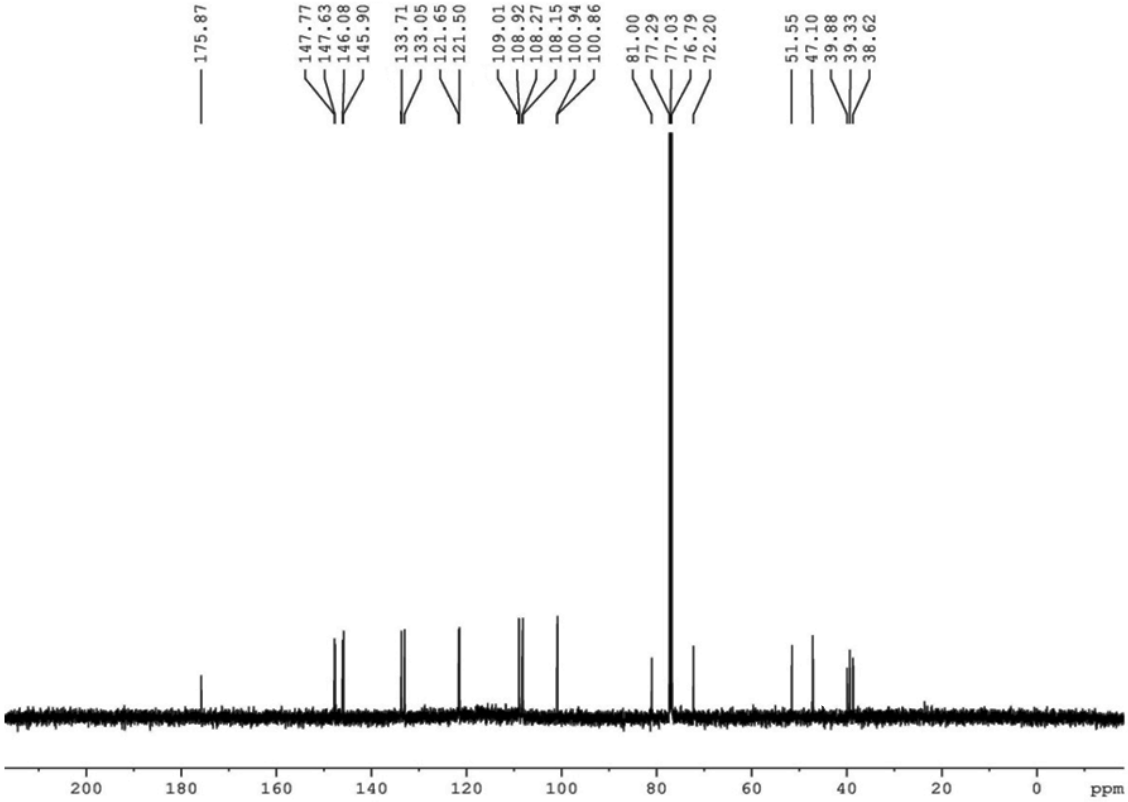
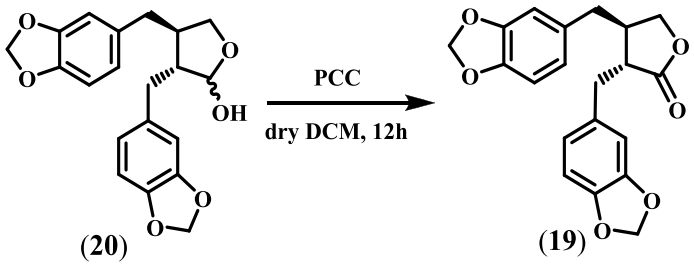


Figure 3B.2: ^{13}C NMR spectrum of compound **29**

3B.3.3. Conversion of (-)-cubebin to (-)-hinokinin

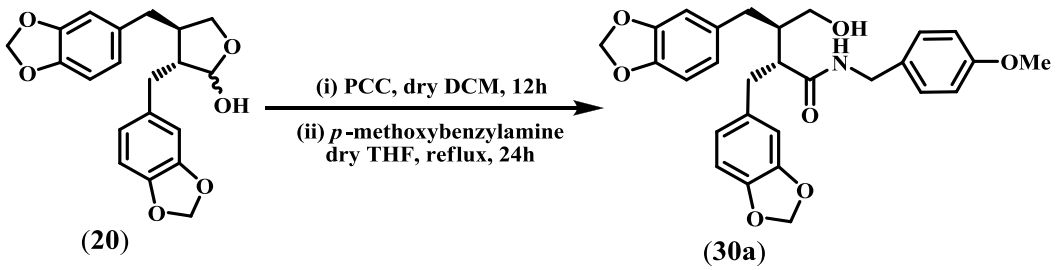
Cubebin was also converted into the natural lignan lactone (-)-hinokinin (**19**) by PCC oxidation (scheme 3B.3). The structure of the compound was confirmed by various spectroscopic techniques as explained chapter 3A.



Scheme 3B.3

3B.3.4. Conversion of (-)-cubebin to amide and imide derivatives

Cubebin was first converted into (-)-hinokinin (**19**) and the lactone ring of hinokinin was then opened using a primary amine (*p*-methoxy benzylamine) to give the respective amide **30a** (scheme 3B.4). The structure of the compound **30a** was confirmed by employing various spectroscopic techniques.



Scheme 3B.4

IR spectrum of the compound **30a** showed absorptions at 3400 (-OH), 3301 (-NH), 1639 (-C=O) cm^{-1} suggesting the presence of hydroxyl and amide groups. ^{1}H NMR spectrum (fig. 3B.3) of the compound gave two doublets centred at δ 6.95 and 6.80, each integrating for two protons, revealing the presence of *para* disubstituted benzene ring. Other six aromatic protons resonated in between δ 6.68-6.58. A multiplet at δ 5.90 integrating for four protons could be attributed to the methylenedioxy groups. Methoxy protons resonated as a singlet at δ 3.79. –NH and –OH protons resonated at δ 5.65 and 3.50 respectively. Presence of amide group was again confirmed by the peak at δ 174.7 in the ^{13}C NMR spectrum (fig. 3B.4). Carbon bearing hydroxyl group and the methoxy carbon resonated at δ 60.8 and δ 55.3 respectively. Mass spectrum of the compound showed molecular ion peak at m/z 514.1848 which is the $(M+Na)^{+}$ peak. These spectral details fully supported the proposed structure of compound **30a** as depicted in scheme 3B.4.

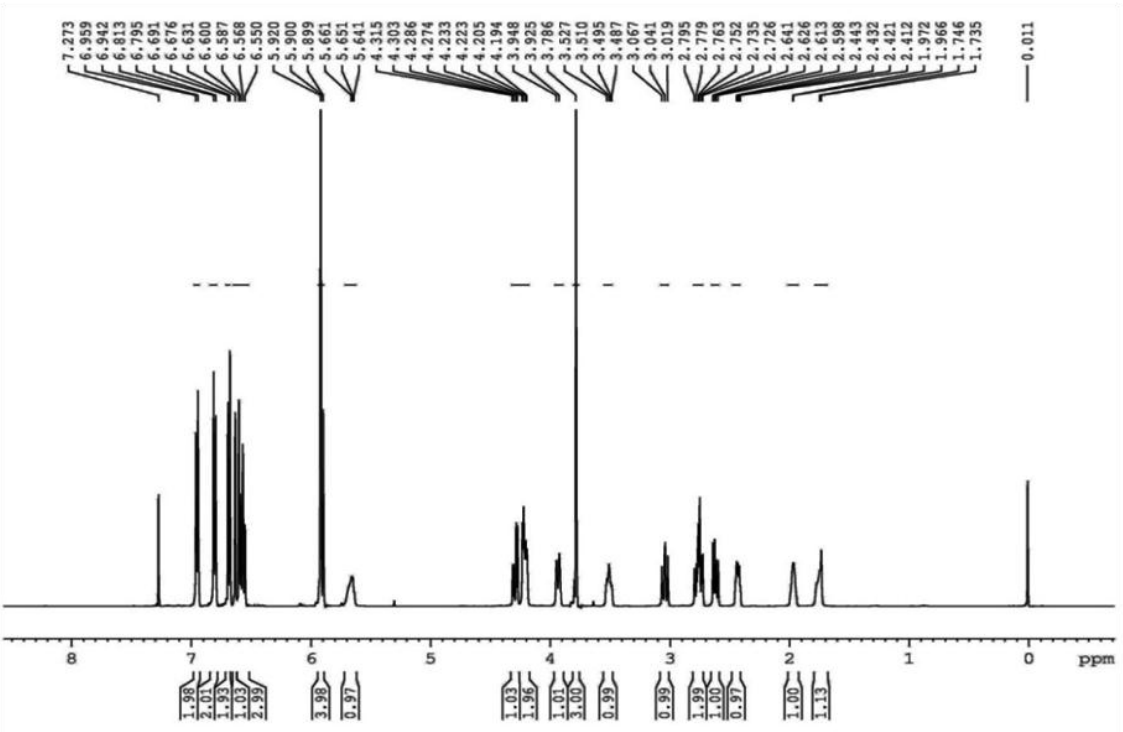


Figure 3B.3: ^{1}H NMR spectrum of amide **30a**

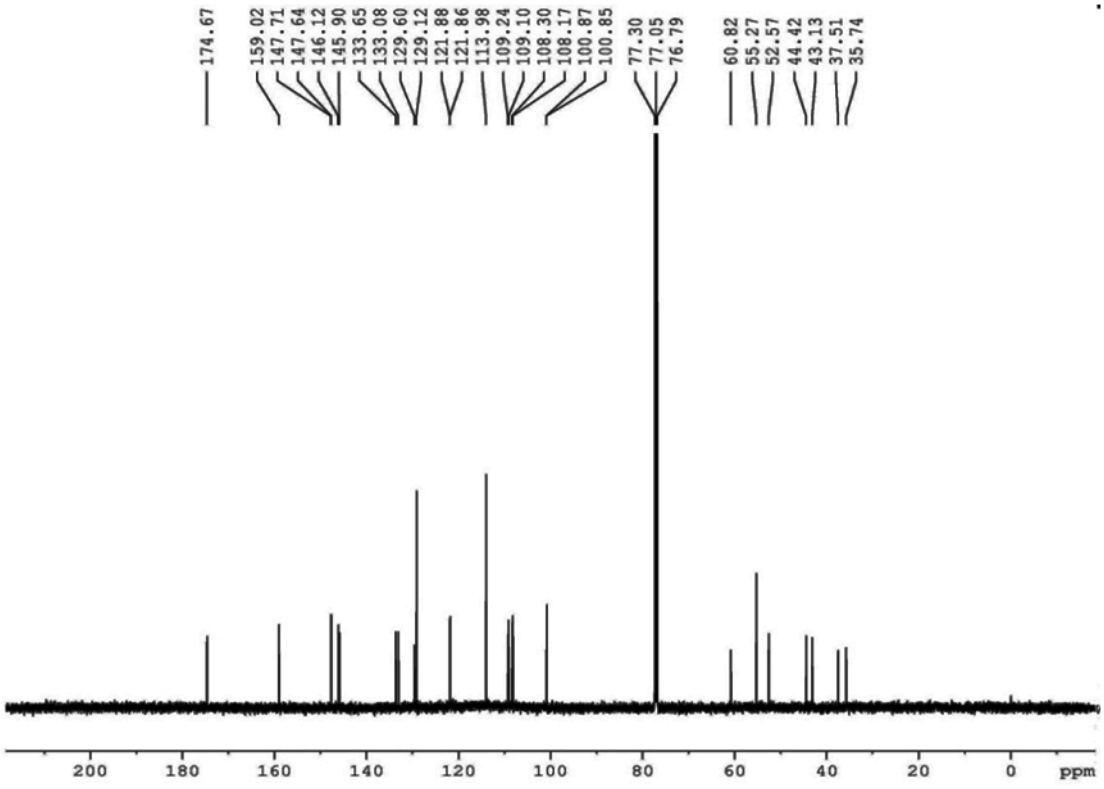


Figure 3B.4: ^{13}C NMR spectrum of amide **30a**

Preliminary investigation on the *in vitro* anticancer activity of these five compounds (**20, 25, 29, 19** and **30a**) bearing various functionalities revealed that amide derivative (**30a**) showed higher activity and in some of the tested cell lines, better than the natural products, cubebin (**20**) and hinokinin (**19**). Compounds containing dihydro (**25**) and oxolane (**29**) functionalities were found to be less effective (table 3B.1).

Table 3B.1: IC_{50} value of cubebin and its derivatives

Compound	[a]IC_{50} (μM)					
	A549	**K562**	**SiHa**	**KB**	**HCT116**	**HT29**
20	8.30±0.16	8.66±0.43	>100	8.16±0.41	45.06±3.7	45.2±0.87
25	75.55±1.1	30.17±5.65	>100	**7**.82±0.38	85.32±6.4	>100
29	52.86±6.9	7.94±0.45	>100	73.88±1.3	>100	>100
19	7.86±0.54	9.07±0.41	68.4±4.0	7.68±0.53	72.58±6.2	35.7±1.23
30a	6.61±0.42	8.37±0.19	91.50±0.31	9.17±0.26	46.06±1.6	51.1±0.90

[a] IC_{50}- Data represented as mean ± S.D from results obtained in triplicate

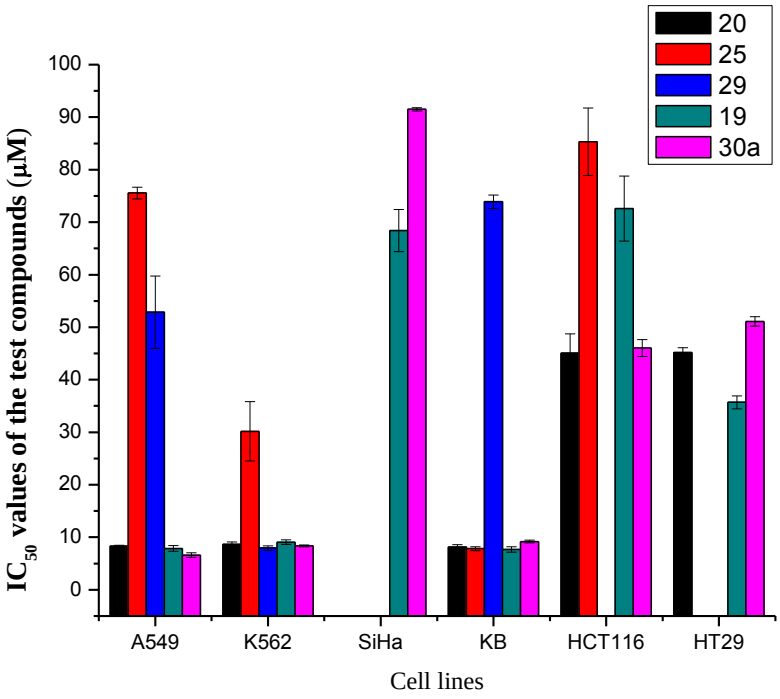
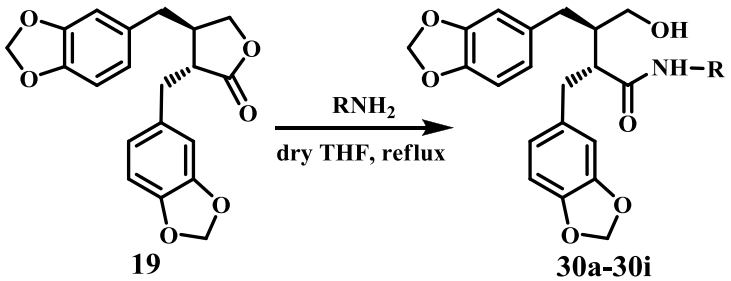


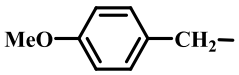
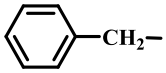
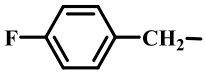
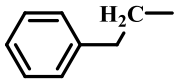
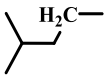
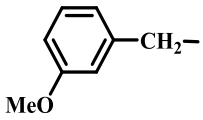
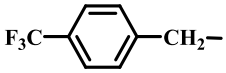
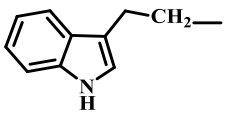
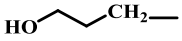
Figure 3B.5: Graphical representation of the IC_{50} values of cubebin and its derivatives (Compounds which showed IC_{50} values >100 μM are not shown)

Since the amide derivative **30a** showed significant anticancer activity as compared to cubebin and hinokinin in some of the tested cell lines, a series of novel amide derivatives as shown in table 3B.2 were synthesized in order to study the structure activity relationship. The amide derivatives (**30a-30i**) were synthesized in good yield by treating (-)-hinokinin (**19**) with respective primary amines under reflux condition, without the use of any catalyst. The absence of any metal/non-metal catalyst makes the process an environmentally benign one. Structure and stereochemistry of all the amide derivatives were unambiguously confirmed from their spectral data. In addition, single crystal X-ray diffraction was used to confirm the structure of **30d** (fig 3B.6). All the benzyl amines gave fairly good yield, but in the case of tryptamine and hydroxy substituted alkyl amine reduction in yield was observed. Secondary amines did not give the expected product under the above reaction conditions.

Table 3B.2: Synthesis of amide derivatives **30a-30i**



19 **30a-30i**

Entry	Compound	R	Time (h)	%Yield[a]
1	**30a**		36	73
2	**30b**		36	70
3	**30c**		36	78
4	**30d**		36	84
5	**30e**		36	71
6	**30f**		48	71
7	**30g**		48	74
8	**30h**		48	40
9	**30i**		48	54

%Yield[a] - Percentage yield of isolated compound

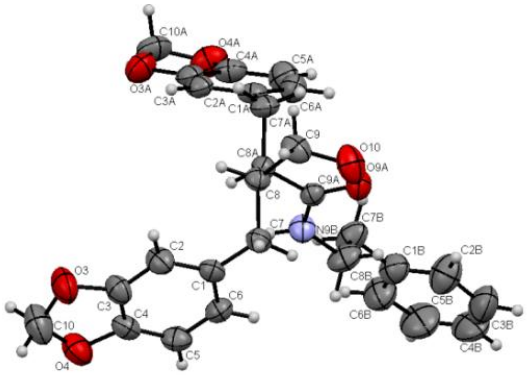
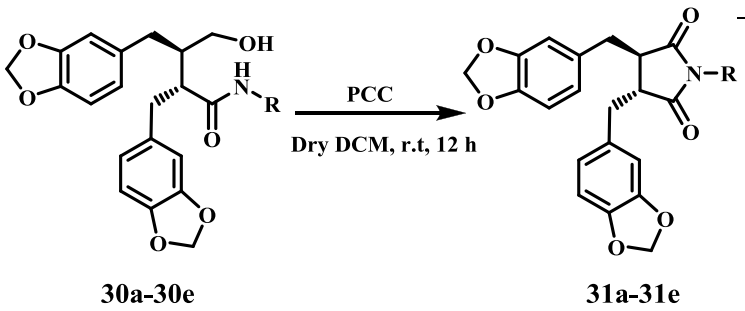


Figure 3B.6: ORTEP diagram of **30d**

In order to expand the scope of the study, amides **30a-30e** were then oxidized into corresponding succinimide derivatives (**31a-31e,** scheme 3B.5) using PCC as the oxidant.



Compound	R
31a	MeO–CH_2–
31b	CH_2–
31c	F–CH_2–
31d	H_2C–
31e	H_2C–

Scheme 3B.5: Synthesis of **31a-31e**

The compounds isolated were successfully characterized using various spectral techniques. The salient spectral features of compound **31a** (obtained as a semi solid) are as follows. IR spectrum of the compound showed absorption at 1697 cm^{-1} suggesting the presence of an imide group. ^{1}H NMR spectrum (fig. 3B.7) of the compound showed the presence of ten aromatic protons. Protons of the methylenedioxy groups appeared as two separate singlets at δ 5.94 and 5.91. Methoxy protons resonated as a singlet at δ 3.79. A multiplet seen at δ 4.55 could be ascribed to the benzylic protons attached to nitrogen atom of the imide ring. Other four benzylic protons and two protons of the imide ring appeared together as multiplets centered at δ 2.74. Presence of imide group was further confirmed by the peak at δ 178.2 in the ^{13}C NMR spectrum (fig. 3B.8). The peak at δ 55.2 could be ascribed to the methoxy carbon. The mass spectrum of the compound showed molecular ion peak at m/z 510.1531 which is the $(M+Na)^{+}$ peak. These spectral details confirmed the proposed structure of **31a** as given in scheme 3B.5.

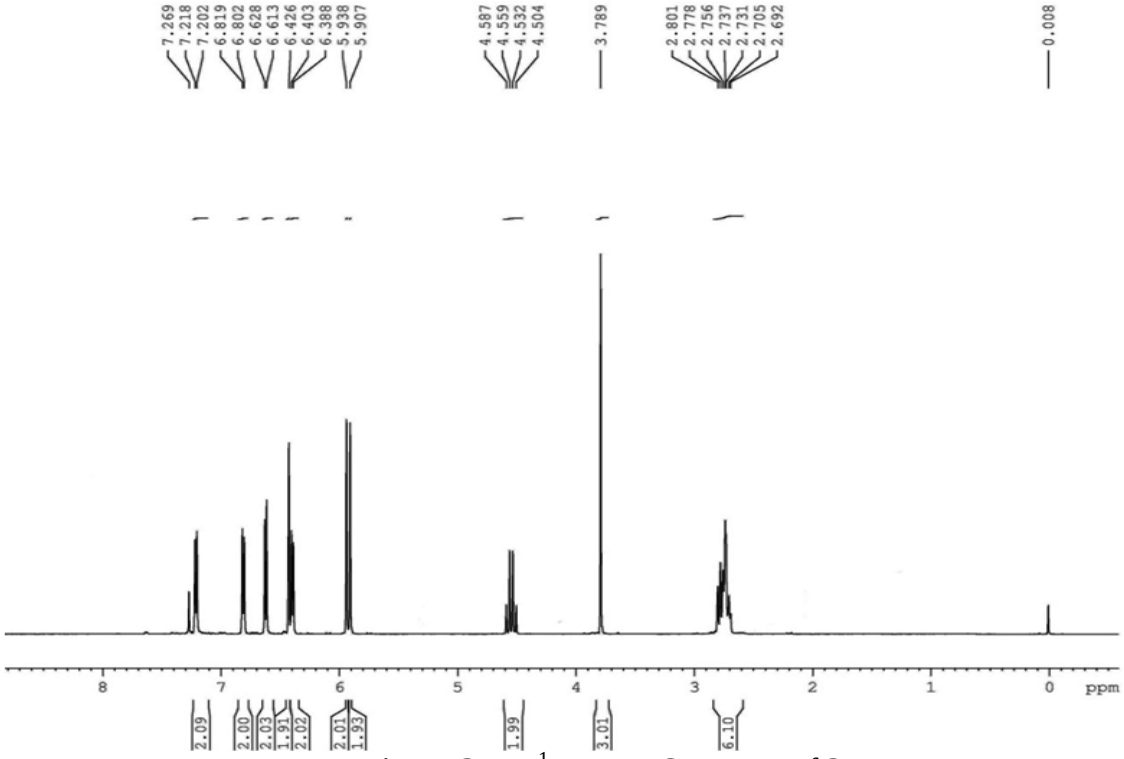


Figure 3B.7: ^{1}H NMR Spectrum of **31a**

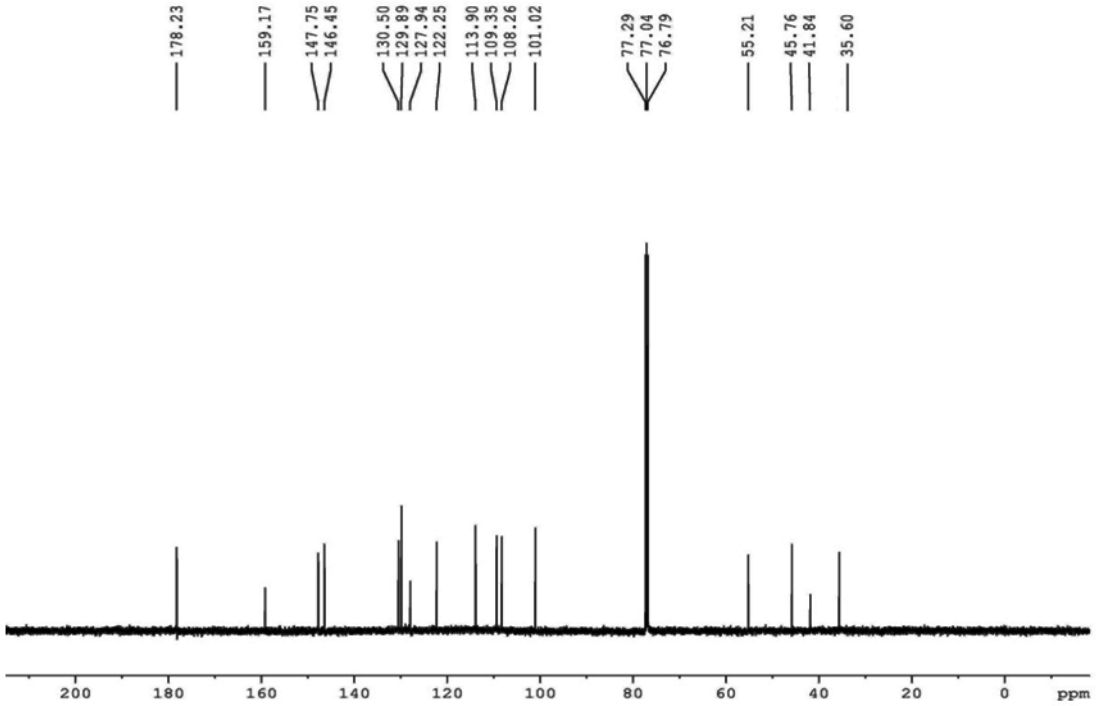


Figure 3B.8: ^{13}C NMR Spectrum of **31a**

Cytotoxicities of these compounds against different cancer cell lines were also checked. To the best of our knowledge, this is the first report on the synthesis of amide and succinimide derivatives of (-)-cubebin.

3B.4. Biological evaluation

3B.4.1. *In vitro* anticancer activity and structure activity relationship

As the first study, cubebin (**20**) and its derivatives **19, 25, 29** and **30a** were tested for their *in vitro* cytotoxicity against six human cancer cell lines viz., A549 (human lung adenocarcinoma), KB (human nasopharyngeal carcinoma), K562 (human chronic myeloid leukemia), SiHa (human cervical carcinoma), HCT116 and HT29 (human colon carcinoma) using MTT assay and the results are reported in terms of IC_{50} values (the concentrations of test compounds required to inhibit 50% of cell growth). From the IC_{50} values (table 3B.1) it was clear that the natural lignans, (-)-cubebin (**20**) and (-)-hinokinin (**19**) have very good activity against A549, K562 and KB cell lines but was found to be less effective against other cell lines. Hinokinin showed better activity compared to cubebin in most of the tested cell lines, suggesting that compound with lactone ring is more active. Even though cubebin and hinokinin showed good anticancer activity, the natural lignan (-)-dihydrocubebin (**25**) devoid of the lactone ring was found to be less effective against all the tested cell lines. Similarly, the oxolane derivative **29** was also found to be less effective. The amide derivative **30a** showed better results than the parent compounds cubebin/hinokinin in most of the tested cell lines. From the results obtained, it became clear that compounds possessing lactone ring (**19**) and free amide group (**30a**) showed slightly higher activity as compared to the lactol compound, viz., the naturally occurring major constituent of *P. cubeba* seeds, cubebin (**20**). The dihydro and oxolane derivatives **25** and **29** were not found to be effective.

Promising results obtained in the case of amide derivative **30a**, prompted us to invest our time in synthesizing a library of amide derivatives (**30a-30i)** and to evaluate their cytotoxic activity. The results obtained are summarized in table 3B.3. On comparing the IC_{50} values obtained for lactone and amide derivatives as seen from table 3B.3, it has been found that some of the amide derivatives have significantly higher activity than the parent compounds cubebin and hinokinin. In the case of A549 cell lines, the amide derivatives **30a, 30d** and **30f** were more active than the natural lignans cubebin (**20**) and hinokinin (**19**). Against K562 cell lines, the amide **30c** was found to be most active one while all the other benzyl amide derivatives showed lower but comparable activities. Very interestingly, in the case of KB cell lines **30h** was found to be the most potent one, followed by **30d**. The compound **30h,** which is the amide derived from tryptamine, showed significant activity only against KB cell lines. All the

tested compounds were found to be less effective against SiHa, HCT116 and HT29 cell lines.

Table 3B.3: IC_{50} value of compounds **30a-31e**

Compound	[a]IC_{50} (μM)					
	A549	**K562**	**SiHa**	**KB**	**HCT116**	**HT29**
30a	6.61±0.42	8.37±0.19	91.50±0.31	9.17±0.26	46.06±1.6	51.1±0.90
30b	10.85±2.1	8.60±1.35	>100	9.16±1.1	93.51±3.1	68±1.56
30c	8.94±0.96	7.76±0.24	80.0±3.34	33.8±0.41	34.10±6.2	24.6± 1.11
30d	7.28±0.35	7.91±0.24	57.46±9.8	7.09±0.61	7.83±1.2	58.9±0.87
30e	8.75±0.71	48.84±4.19	95.82±7.6	8.94±0.42	66.83±6.4	23.8± 0.97
30f	7.28±0.39	7.98±0.56	25.32±0.87	80.5±6.5	>100	25.5±0.78
30g	>100	8.86±0.16	73.70±8.9	19.2±0.51	> 100	64.91±0.10
30h	>100	>100	>100	6.38±0.35	> 100	52.1±1.90
30i	>100	87.38±11.3	85.18±2.7	7.96±0.45	45.19±4.2	51.1±2.1
31a	45.49±2.9	8.69±0.18	33.35±2.6	9.13±0.13	>100	72.57±0.46
31b	58.61±7.5	8.88±0.18	>100	7.36±0.38	65.43±3.3	71.8±0.13
31c	>100	>100	>100	8.95±1.0	>100	72.1±0.99
31d	>100	9.15±0.45	96.26±3.9	62.57±3.4	64.04±5.3	66.55±0.89
31e	>100	> 100	89.29±3.6	7.71±0.78	67.70±0.97	66.21±0.08
CP	-	-	-	19.2±0.19	-	-
PL	0.01±0.01	-	-	-	-	-
PT	13.62[26]	-	-	-	0.01[27]	2.29±0.97[28]
ET	14.8[29]	4.39 ± 1.2[30]	30.7[29]	3.88± 0.12[31]	-	1.47±0.06[32]

[a] IC_{50} - Data represented as mean ± S.D from results obtained in triplicate;

CP- Cisplatin, PL- Paclitaxel, PT- Podophyllotoxin, EP- Etoposide

From the results obtained, it can be concluded that the ethyl phenyl substituted (**30d**) and benzyl substituted amides were found to be more active and among the various benzyl substituted amides, the one with *para* substituent is found to be more effective than unsubstituted or *meta* substituted ones. The amide formed from tryptamine was found to be effective only against KB cell lines and amide containing hydroxyl group showed only a very little effect against all the tested cell lines.

Succinimide derivatives (**31a-31e**) obtained by the oxidation of corresponding amides were also tested for their *in vitro* anticancer activity. These were found to be less effective than the parent amide. They showed only poor anticancer activity against all the tested cell lines except in the case of K562 and KB cell lines. Comparison of the IC_{50} values of the test compounds with literature values obtained for podophyllotoxin and etoposide (currently used as chemotherapeutic agent) indicates that cubebin as well as its lactone and amide derivatives possess promising *in vitro* anticancer activity.

3B.4.2. Morphological analysis

Induction of apoptosis (the natural and programmed cell death through a non-inflammatory pathway) is one of the sensitive and constructive strategies in anticancer chemotherapy.[33,34] Apoptosis is characterized by the morphological changes such as membrane blebbing, cell shrinkage, chromatin condensation etc.[35] To check, whether in the present study, the cell death occurred through necrosis or apoptosis, morphological analysis were carried out. For this, two cell lines A549 and KB were selected since the tested compounds showed very good activity against these two cell lines. Cells were treated with (-)-hinokinin (**19**) and the respective amide which showed maximum activity i.e. **30a** against A549 and **30h** against KB.

3B.4.2.1. By light microscopy

After 48 h of incubation, cell shrinkage was observed and there is loss of normal nuclear architecture. As shown in fig 3B.9, cells were found detached and floated in the medium when analyzed using inverted microscope.

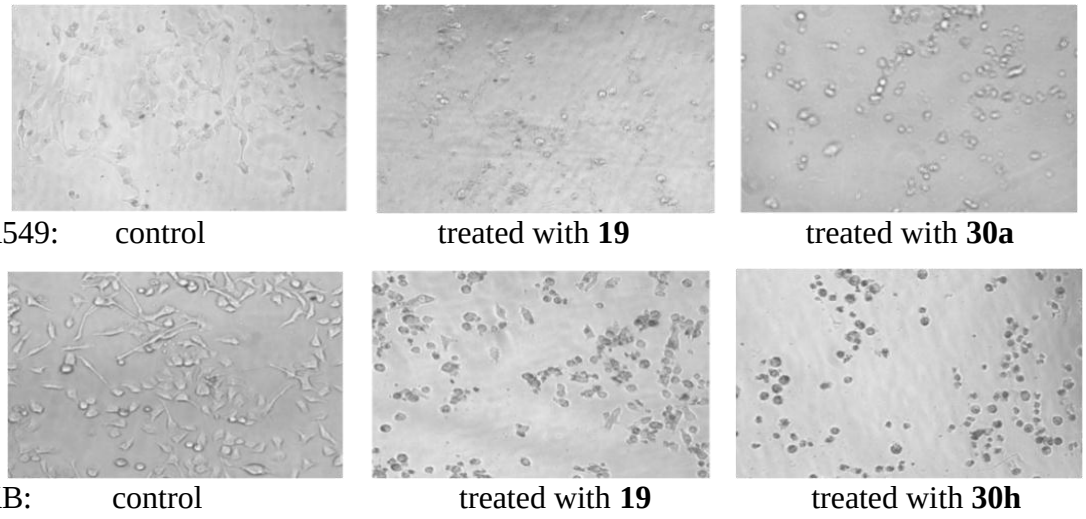


Figure 3B.9: Morphological changes observed by phase-contrast microscopy

3B.4.2.2. Acridine orange/ethidium bromide staining

In Acridine orange/ethidium bromide staining assay, normal cells appeared green and the apoptotic cells showed red coloured condensed nuclei when viewed under fluorescence microscope (fig 3B.10). Cell shrinkage, membrane blebbing, and chromosomal condensation were observed in cells treated with the compounds indicating that cell death occurred through apoptosis mediated path way.

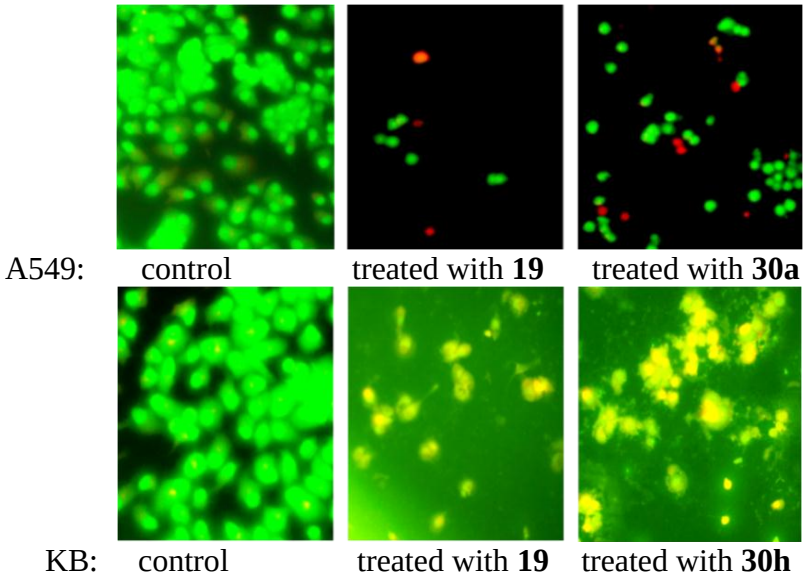


Figure 3B.10: Apoptotic morphology using Acridine orange/ethidium bromide staining

3B.4.2.3. Hoechst 33342 staining

In Hoechst 33342 staining assay, blue fluorescence was observed for normal cells which contained regular and round shaped nuclei in them. Apoptotic cells were characterized by the condensation and the fragmentation of nuclei with increased

brightness (fig. 3B.11). An increased number of cells with condensed and fragmented nuclei were observed after treatment with the compounds, which is the salient morphological feature of apoptosis. Morphological analysis using the most active, novel amide derivatives of (-)-cubebin against A549 and KB cell lines are being reported for the first time.

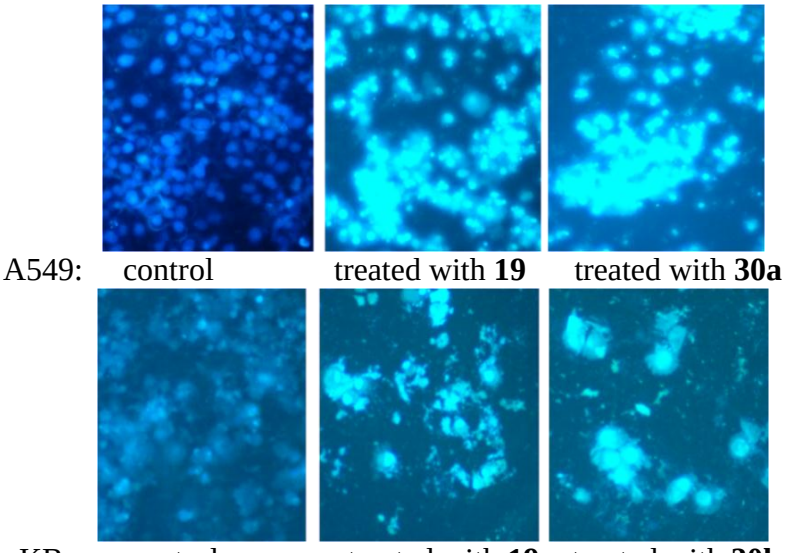


Figure 3B.11: Apoptotic morphology using Hoechst 33342 staining

3B.5. Experimental

General experimental procedures are given in chapter 2. Amines used were purchased from Sigma Aldrich. Dry solvents used for the synthesis were purchased from Merck. Isolation and spectral details of cubebin are given in chapter 3A.

3B.5.1. Synthesis of cubebin derivatives

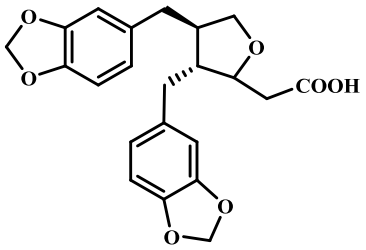
3B.5.1.1. Synthesis of (-)-dihydrocubebin (25)

To an ice cold solution of cubebin (**20**) (100 mg, 0.281 mmol) in dry methanol (10 mL), sodium borohydride (140 mg, 3.70 mmol) was added over 5 minutes time and the reaction mixture was stirred at 0 °C for an hour and then allowed attain room temperature and again stirred for 24 h. After that, the reaction mixture was quenched with water; methanol was removed under *vacuo* and the product was extracted with ethyl acetate. Pure compound was obtained in 88% yield after column chromatography (silica gel, 100-200 mesh, eluent: 30% hexane-EtOAc mixture). Spectral details of the compound matched well with those reported values for natural dihydrocubebin (**25**),[36] isolated earlier from *P. cubeba.* Spectral details are the same as given in chapter 3A.

3B.5.1.2. Synthesis of oxolane derivative 29

Ethyl (triphenylphosphoranylidene) acetate (196 mg, 0.563 mmol) was added to a solution of cubebin **20** (100 mg, 0.281 mmol) dissolved in dry toluene (10 mL), by stirring it for five minutes at 90 °C. The reaction mixture was stirred under reflux condition for about 12 h. Product obtained in 92% yield after column chromatography (silica gel, 100-200 mesh, eluent: 20% hexane-EtOAc mixture) was subjected to alkaline hydrolysis using 10% aqueous sodium hydroxide in methanol. Compound **29** was isolated in 90% yield after acid work up followed by column chromatography (silica gel, 100-200 mesh, eluent: 40% hexane-EtOAc mixture). Spectral details of the compound **29** are given below.

2-((3*R*,4*R*)-3,4-bis(benzo[d][1,3]dioxol-5-ylmethyl)tetrahydrofuran-2-yl)aceticacid (**29**)



Colourless crystals; m.p. 118-120 ^{o}C

FT-IR (KBr, υ_{max}, cm^{-1})	:	3180, 2917, 1712, 1394, 1494, 1442, 1246, 1192, 929
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 1.81 (m, 1H), 2.20 (m, 1H), 2.30 (dd, *J* = 15.8, 3.8 Hz, 1H), 2.44 (m, 2H), 2.59 (m, 3H), 3.63 (dd, *J* = 8.8, 6.5 Hz, 1H), 3.82 (t, *J* = 8.0 Hz, 1H), 3.98 (m, 1H), 5.93 (m, 4H), 6.52 (s, 2H), 6.56 (s, 2H), 6.70 (m, 2H)
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 175.9, 147.8, 147.6, 146.1, 145.9, 133.7, 133.0, 121.6, 121.5, 109.0, 108.9, 108.3, 108.1, 100.9, 100.9, 81.0, 72.2, 51.5, 47.1, 39.9, 39.3, 38.6
HRMS (m/z)	:	421.1264 [$(M+Na)^{+}$]; $C_{22}H_{22}O_{7,}$ requires 398.1366

3B.5.1.3. Synthesis of (-)-hinokinin (19)

A solution of cubebin (**20**) (150 mg, 0.421 mmol) dissolved in 5 mL dry DCM was added to a stirred mixture of PCC (181.50 mg, 0.842 mmol) and molecular sieves (91 mg) in 20 mL of dry DCM. Reaction mixture was stirred for 12 h at room

temperature and then the byproducts were removed by filter column. Product was obtained in 98% yield after column chromatographic purification (silica gel, 100-200 mesh, eluent: 20% hexane-EtOAc mixture). The structure of the compound was confirmed by analyzing various spectral data obtained for the compound which was consistent with that of natural hinokinin (**19**), isolated from *P. cubeba* and with the reported values.[37] Spectral details are the same as that given in chapter 3A.

3B.5.1.4. Synthesis of amide derivatives (30a-30i)

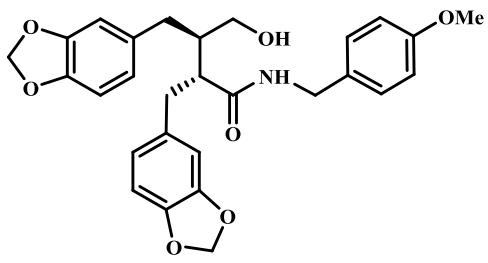
In a typical experiment, amine (4 equivalents) was added to a solution of compound **20** (1 equivalent) dissolved in dry THF and refluxed for 36-48 h. Completion of the reaction was monitored using thin layer chromatography. Product was obtained by removing the solvent under *vacuo* followed by column chromatography (silica gel, 100-200 mesh, eluent: 40% to 80% hexane-EtOAc mixtures). Structures of the compounds were confirmed using various spectroscopic techniques. Spectral details of the compounds **30a**-**30i** are given below.

(2*R*,3*R*)-4-(benzo[d][1,3]dioxol-5-yl)-2-(benzo[d][1,3]dioxol-5-ylmethyl)-3-(hydroxy methyl)-N-(4-methoxybenzyl)butanamide (**30a**)

Colourless crystals; m.p. 119-121 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 3400, 3301, 2894, 1639, 1494, 1443, 1247, 1185, 1038, 930, 811

^{1}H NMR (500 MHz, $CDCl_3$) : δ 1.97 (m, 1H), 2.43 (m, 1H), 2.62 (dd, *J* = 10.5, 7.5 Hz, 1H), 2.76 (m, 2H), 3.04 (m, 1H), 3.51 (m, 1H), 3.79 (s, 3H), 3.94 (d, *J* = 11.5 Hz, 1H), 4.21 (m, 2H), 4.30 (dd, *J* = 14.5, 6.0 Hz, 1H), 5.65 (t, *J* = 5.0 Hz, 1H), 5.91 (m, 4H), 6.58 (m, 3H), 6.63 (s, 1H), 6.68 (d, *J* = 7.5 Hz, 2H), 6.80 (d, *J* = 9.0 Hz, 2H), 6.95 (d, *J* = 8.5 Hz, 2H)



^{13}C NMR (125 MHz, $CDCl_3$) : δ 174.7, 159.0, 147.7, 147.6, 146.1, 145.9, 133.6, 133.1, 129.6, 129.1 (2C), 121.9, 121.9, 114.0 (2C), 109.2, 109.1, 108.2,

108.2, 100.9, 100.8, 60.8, 55.3, 52.6, 44.4, 43.1, 37.5, 35.7

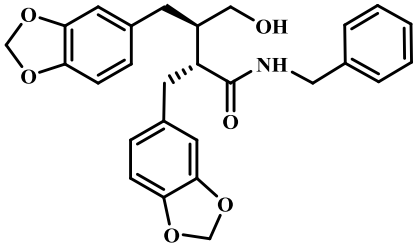
HRMS (m/z) : 514.1848 [$(M+Na)^+$]; $C_{28}H_{29}NO_7$, requires 491.1944

(2*R*,3*R*)-4-(benzo[d][1,3]dioxol-5-yl)-2-(benzo[d][1,3]dioxol-5-ylmethyl)-N-benzyl-3-(hydroxymethyl)butanamide (**30b**)

Colourless crystals; m.p. 143-145 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 3403, 3299, 2889, 1640, 1494, 1444, 1246, 1191, 1039, 930, 809

^{1}H NMR (500 MHz, $CDCl_3$) : 1.98 (m, 1H), 2.47 (m, 1H), 2.63 (dd, *J* = 14.0, 7.5 Hz, 1H), 2.77 (m, 2H), 3.05 (dd, *J* = 13.0, 11.0 Hz, 1H), 3.51 (m, 1H), 3.92 (d, *J* = 11.5 Hz, 1H), 4.17 (d, *J* = 7.5 Hz, 1H), 4.27 (dd, *J* = 14.8, 10.5 Hz, 1H), 4.38 (dd, *J* = 14.8, 6.3 Hz, 1H), 5.77 (t, *J* = 10.0 Hz, 1H), 5.91 (m, 4H), 6.63 (m, 2H), 6.62 (d, *J* = 5.0 Hz, 2H), 6.68 (dd, *J* = 7.8, 2.3 Hz, 2H), 7.01 (m, 2H), 7.28 (m, 3H)



^{13}C NMR (125 MHz, $CDCl_3$) : δ 174.8, 147.7, 147.7, 146.1, 145.9, 137.5, 133.6, 133.1, 128.6 (2C), 127.7 (2C), 127.6, 121.9, 121.8, 109.2, 109.1, 108.3, 108.2, 100.9, 100.9, 60.8, 52.6, 44.4, 43.7, 37.5, 35.7

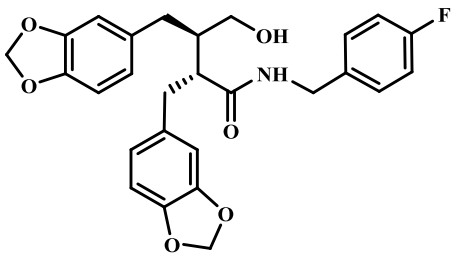
HRMS (m/z) : 484.1741 [$(M+Na)^+$]; $C_{27}H_{27}NO_6$, requires 461.1838

(2*R*,3*R*)-4-(benzo[d][1,3]dioxol-5-yl)-2-(benzo[d][1,3]dioxol-5-ylmethyl)-N-(4-fluoro benzyl)-3-(hydroxymethyl)butanamide (**30c**)

Colourless crystals; m.p. 124-126 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 3400, 3299, 2891, 1640, 1495, 1442, 1395, 1247, 1192, 1039, 930, 812

^{1}H NMR (500 MHz, $CDCl_3$) : δ 1.98 (m, 1H), 2.45 (m, 1H), 2.63 (dd, *J* = 14.0, 7.5 Hz, 1H), 2.78 (m, 2H), 3.03 (t, *J* = 12.3 Hz, 1H), 3.51 (d, *J* = 11.0 Hz, 1H), 3.92 (d, *J* = 12.0 Hz, 1H), 4.02 (brs, 1H), 4.19 (dd, *J* = 14.8, 5.3 Hz, 1H), 4.37 (dd, *J* = 14.8, 6.5 Hz, 1H), 5.69 (t, *J* = 5.0 Hz, 1H), 5.91 (m, 4H), 6.57 (m, 3H), 6.63 (m, 1H), 6.68 (m, 2H), 6.95 (m, 4H)



^{13}C NMR (125 MHz, $CDCl_3$) : δ 174.8, 162.2 ($^1J_{CF}$ = 245 Hz), 147.8, 147.7, 146.2, 146.0, 133.5, 133.3, 133.0, 129.4 ($^3J_{CF}$ = 8.75 Hz, 2C), 121.9, 121.8, 115.4 ($^2J_{CF}$ = 21.25 Hz, 2C), 109.2, 109.1, 108.3, 108.2, 100.9 (2C), 60.9, 52.6, 44.5, 42.9, 37.5, 35.7

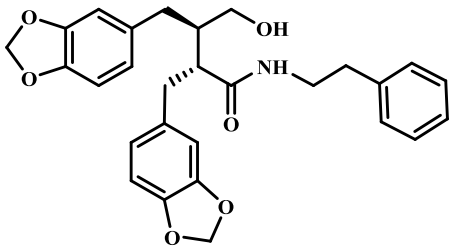
HRMS (m/z) : 502.1646 [$(M+Na)^+$]; $C_{27}H_{26}NO_6F$, requires 479.1744

(2*R*,3*R*)-4-(benzo[d][1,3]dioxol-5-yl)-2-(benzo[d][1,3]dioxol-5-ylmethyl)-3-(hydroxy methyl)-N-phenethylbutanamide (**30d**)

Colourless crystals; m.p. 134-135 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 3409, 3300, 2888, 1639, 1494, 1443, 1246, 1038, 930, 809

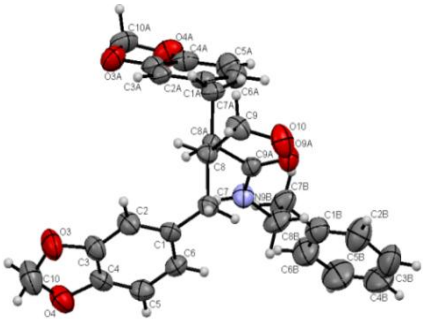
^{1}H NMR (500 MHz, $CDCl_3$) : δ 1.91 (m, 1H), 2.32 (m, 1H), 2.52 (dd, *J* = 14.0, 7.5 Hz, 1H), 2.60 (dd, *J* = 14.0,



7.0 Hz, 1H), 2.73 (m, 3H), 3.02 (dd, *J* = 13.3, 10.8 Hz, 1H), 3.45 (m, 3H), 3.88 (m, 1H), 4.08 (dd, *J* = 8.8, 2.3 Hz, 1H), 5.42 (t, *J* = 5.5 Hz, 1H), 5.92 (m, 4H), 6.52 (dd, *J* = 7.5, 1.5 Hz, 1H), 6.58 (dd, *J* = 8.0, 1.5 Hz, 1H), 6.60 (s, 2H), 6.68 (d, *J* = 8.0 Hz, 1H), 6.71 (d, *J* = 7.5 Hz, 1H), 7.05 (d, *J* = 7.0 Hz, 2H), 7.22 (m, 1H), 7.28 (m, 2H)

^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 175.0, 147.7, 147.6, 146.1, 145.9, 138.5, 133.7, 133.2, 128.6 (2C), 128.6 (2C), 126.6, 121.9 (2C), 109.2, 109.1, 108.3, 108.1, 100.9, 100.8, 60.7, 52.7, 44.3, 40.5, 37.6, 35.7, 35.4
HRMS (m/z)	:	498.1893 $[(M+Na)^+]$; $C_{28}H_{29}NO_6$, requires 475.1995

Crystal Parameters of 30d



CCDC Number	:	1014420
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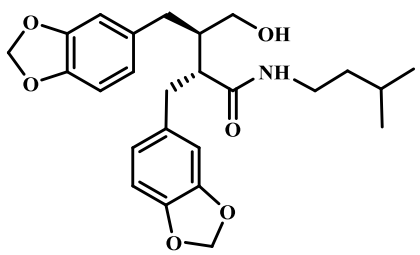
Chemical formula sum	:	$C_{28}H_{29}NO_6$
Chemical formula weight	:	475.52
Symmetry cell setting	:	Monoclinic
Symmetry space group name	:	P21
Cell length a	:	6.850(9)
Cell length b	:	20.466(4)
Cell length c	:	8.826(2)
Cell angle alpha	:	90.00
Cell angle beta	:	92.31(4)
Cell angle gamma	:	90.00
Cell volume	:	1236.3(17)
Cell formula units Z	:	2
Cell measurement temperature	:	301(2)

Cell measurement reflns used	:	2292
Cell measurement theta min	:	3.1
Cell measurement theta max	:	27.5

(2*R*,3*R*)-4-(benzo[d][1,3]dioxol-5-yl)-2-(benzo[d][1,3]dioxol-5-ylmethyl)-3-(hydroxy methyl)-N-isopentylbutanamide (**30e**)

Colourless crystals; m.p. 115-117 °C

FT-IR (KBr, υ_{max}, cm^{-1})	:	3404, 3299, 2954, 1635, 1547, 1494, 1443, 1246, 1040, 931, 809
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 0.84 (d, *J* = 6.5 Hz, 3H) 0.86 (d, *J* = 6.0 Hz, 3H), 1.19 (m, 2H), 1.39 (m, 1H), 1.95 (m, 1H), 2.36 (m, 1H), 2.60 (dd, *J* = 14.0, 7.5 Hz, 1H), 2.71 (dd, *J* = 13.5, 4.5 Hz, 1H), 2.78 (dd, *J* = 14.0, 8.0 Hz, 1H), 3.02 (dd, *J* = 13.0, 11.0 Hz, 1H), 3.15 (m, 2H), 3.51 (m, 1H), 3.95 (d, *J* = 12.0 Hz, 1H), 4.34 (d, *J* = 7.5 Hz, 1H), 5.25 (brs, 1H), 5.91 (s, 2H), 5.94 (s, 2H), 6.60 (m, 3H), 6.70 (m, 3H)



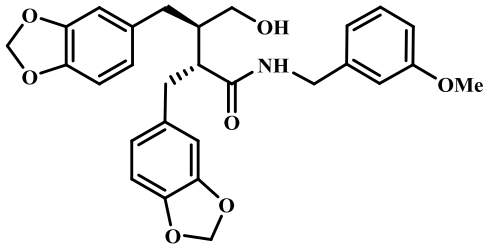
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 174.8, 147.7, 147.6, 146.1, 145.9, 133.8, 133.2, 121.8, 121.8, 109.3, 109.1, 108.2, 108.2, 100.9 (2C), 60.8, 52.9, 44.4, 38.2, 37.9, 37.7, 35.9, 25.6, 22.3 (2C)
HRMS (m/z)	:	464.2039 [$(M+Na)^+$]; $C_{25}H_{31}NO_6$, requires 441.2151

(2*R*,3*R*)-4-(benzo[d][1,3]dioxol-5-yl)-2-(benzo[d][1,3]dioxol-5-ylmethyl)-3-(hydroxy methyl)-N-(4-methoxybenzyl)butanamide (**30f**)

Colourless crystals; m.p. 98-100 °C

FT-IR (KBr, υ_{max}, cm^{-1})	:	3405, 3303, 2894, 1641, 1494, 1443, 1247, 1209, 1159, 1040, 930, 810

^{1}H NMR (500 MHz, $CDCl_3$) : δ 1.98 (m, 1H), 2.44 (m, 1H), 2.63 (dd, *J* = 14.0, 7.5 Hz, 1H), 2.77 (m, 2H), 3.06 (t, *J* = 6.0 Hz, 1H), 3.53 (m, 1H), 3.78 (s, 3H), 3.95 (d, *J* = 11.5 Hz, 1H), 4.12 (d, *J* = 8.0 Hz, 1H), 4.30 (m, 2H), 5.66 (brs, 1H), 5.91 (m, 4H), 6.59 (m, 5H), 6.67 (m, 3H), 6.80 (d, *J* = 8.5 Hz, 1H), 7.20 (t, *J* = 8.0 Hz, 1H)



^{13}C NMR (125 MHz, $CDCl_3$) : δ 174.8, 159.8, 147.7, 147.6, 146.1, 145.9, 139.0, 133.6, 133.0, 129.7, 121.9, 121.8, 120.0, 113.5, 113.0, 109.2, 109.0, 108.3, 108.2, 100.9, 100.8, 60.8, 55.2, 52.5, 44.4, 43.7, 37.5, 35.7

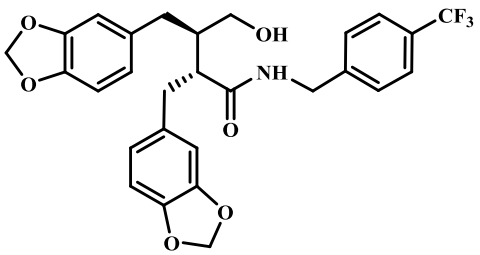
HRMS (m/z) : 514.1843 $[(M+Na)^+]$; $C_{28}H_{29}NO_7$, requires 491.1944

(2*R*,3*R*)-4-(benzo[d][1,3]dioxol-5-yl)-2-(benzo[d][1,3]dioxol-5-ylmethyl)-3-(hydroxy methyl)-N-(4-(trifluoro methyl)benzyl)butanamide (**30g**)

Colourless crystals; m.p. 135-137 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 3410, 3294, 2924, 1630, 1545, 1493, 1441, 1247, 1247, 1119, 1038, 930, 810

^{1}H NMR (500 MHz, $CDCl_3$) : δ 2.02 (m, 1H), 2.51 (m, 1H), 2.67 (dd, *J* = 14.0, 7.5 Hz, 1H), 2.78 (m, 2H), 3.05 (t, *J* = 12.0 Hz, 1H), 3.54 (m, 1H), 3.81 (d, *J* = 7.0 Hz, 1H), 3.92 (d, *J* = 11.5 Hz, 1H), 4.23 (dd, *J* = 15.5, 5.0 Hz, 1H), 4.54 (dd, *J* = 15.0, 6.5 Hz, 1H), 5.77 (t, *J* = 5.5 Hz, 1H), 5.92 (m, 4H), 6.32 (m, 3H), 6.70 (m, 3H), 7.07 (d, *J* = 7.5 Hz, 2H), 7.51 (d, *J* = 8.0 Hz, 2H)

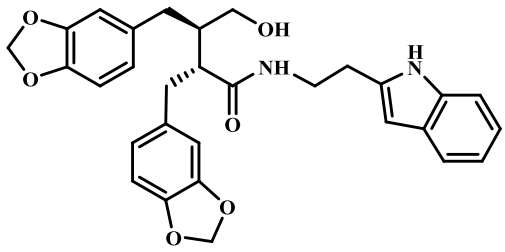


^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 175.1, 147.8, 147.7, 146.2, 146.0, 141.7, 133.5, 133.0, 129.7 ($^2J_{CF}$ = 32.5 Hz), 127.8 (2C), 125.5 ($^3J_{CF}$ = 3.75 Hz, 2C), 124.1 ($^1J_{CF}$ = 270 Hz), 121.9, 121.8, 109.2, 109.2, 108.3, 108.2, 100.9, 100.9, 60.8, 52.5, 44.5, 43.0, 37.3, 35.6
HRMS (m/z)	:	552.1611 [$(M+Na)^+$]; $C_{28}H_{26}NO_6F_3$, requires 529.1712

(2*R*,3*R*)-N-(2-(1H-indol-2-yl)ethyl)-4-(benzo[d][1,3]dioxol-5-yl)-2-(benzo[d][1,3] dioxol-5-ylmethyl)-3-(hydroxymethyl)butanamide (**30h**)

Colourless solid; m.p. 100-102 °C

FT-IR (KBr, υ_{max}, cm^{-1})	:	3410, 3295, 2894, 1640, 1493, 1443, 1246, 1038, 929, 742
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 1.89 (m, 1H), 2.29 (m, 1H), 2.48 (dd, *J* = 14.0, 7.5 Hz, 1H), 2.72 (m, 3H), 2.91 (m, 1H), 3.02 (dd, *J* = 13.5, 5.5 Hz, 1H), 3.47 (dd, *J* = 11.8, 3.8 Hz, 1H), 3.54 (q, *J* = 6.5 Hz, 2H), 3.90 (dd, *J* = 11.8, 2.3 Hz, 1H), 4.28 (m, 1H), 5.47 (t, *J* = 5.5 Hz, 1H), 5.91 (m, 4H), 6.44 (dd, *J* = 7.5 , 1.5 Hz, 1H), 6.58 (m, 4H), 6.69 (d, *J* = 8.0 Hz, 1H), 6.83 (d, *J* = 2.0 Hz, 1H), 7.12 (m, 1H), 7.21 (m, 1H), 7.37 (d, *J* = 8.5 Hz, 1H), 7.51 (d, *J* = 7.5 Hz, 1H), 8.18 (brs, 1H)



^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 175.0, 147.6, 147.6, 146.0, 145.8, 136.4, 133.7, 133.2, 127.1, 122.3, 122.0, 121.9 (2C), 119.5, 118.5, 112.5, 111.3, 109.2 (2C), 108.3, 108.1, 100.9, 100.8, 60.7,

52.7, 44.3, 39.5, 37.6, 35.7, 25.0

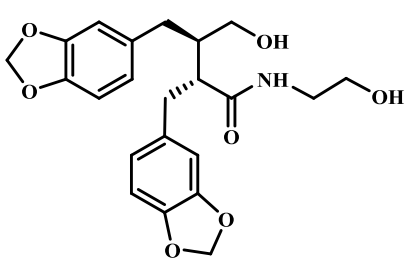
HRMS (m/z) : 537.2005 [$(M+Na)^+$]; $C_{30}H_{30}N_2O_6$, requires 514.2104

(2*R*,3*R*)-4-(benzo[d][1,3]dioxol-5-yl)-2-(benzo[d][1,3]dioxol-5-ylmethyl)-3-(hydroxy methyl)-N-(3-hydroxypropyl)butanamide (**30i**)

Colourless solid; m.p. 97-99 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 3400, 3314, 2927, 3887, 1638, 1493, 1442, 1246, 1038, 930, 810

^{1}H NMR (500 MHz, $CDCl_3$) : δ 1.55 (t, *J* = 6.25 Hz, 2H), 1.95 (m, 1H), 2.44 (m, 1H), 2.63 (dd, *J* = 14.0, 7.5 Hz, 1H), 2.76 (m, 2H), 3.00 (dd, *J* = 13.3, 11.3 Hz, 1H), 3.25 (m, 1H), 3.42 (m, 1H), 3.48 (m, 3H), 3.85 (dd, *J* = 12.0, 2.5 Hz, 1H), 4.04 (brs, 1H), 5.88 (m, 4H), 6.60 (m, 3H), 6.74 (m, 1H), 6.70 (m, 2H)



^{13}C NMR (125 MHz, $CDCl_3$) : δ 175.7, 147.7, 147.6, 146.1, 145.9, 133.7, 133.1, 121.9 (2C), 109.3, 109.1, 108.2 (2C), 100.9 (2C), 60.9, 59.8, 52.5, 44.4, 37.5, 36.9, 35.7, 31.7

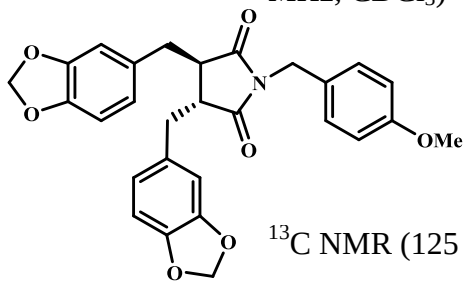
HRMS (m/z) : 452.1684 [$(M+Na)^+$]; $C_{23}H_{27}NO_7$, requires 429.1788

3B.5.1.5. Synthesis of imide derivatives (31a-31e)

In a typical experiment, a solution of amide (50 mg, 1 equivalent) dissolved in 5 mL dry DCM was added to a suspension of PCC (2 equivalents) and molecular sieves (half the amount of PCC) in 20 mL of dry DCM. Reaction mixture was stirred for 12 h at room temperature and after that the byproducts were removed by filter column. The product was obtained in good yield after column chromatographic purification (silica gel, 100-200 mesh, eluent: 20% hexane-EtOAc mixture). Structures of the compounds

were confirmed using various spectroscopic techniques. Spectral details of the compounds **31a-31e** are given below

(3*R*,4*R*)-3,4-bis(benzo[d][1,3]dioxol-5-ylmethyl)-1-(4-methoxybenzyl) pyrrolidine-2,5-dione (**31a**)



Gummy solid

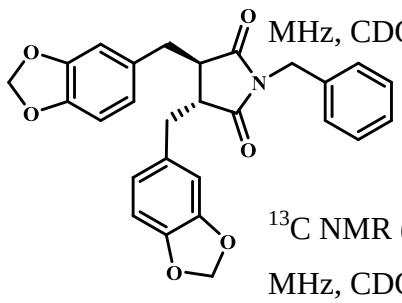
FT-IR (KBr, υ_{max}, cm^{-1}) : 2916, 2848, 1697, 1493, 1438, 1299, 771

^{1}H NMR (500 MHz, $CDCl_3$) : δ 2.74 (m, 6H), 3.79 (s, 3H), 4.55 (m, 2H), 5.91 (s, 2H), 5.94 (s, 2H), 6.40 (d, *J* = 7.5 Hz, 2H), 6.43 (s, 2H), 6.62 (d, *J* = 7.5 Hz, 2H), 6.81 (d, *J* = 8.5 Hz, 2H), 7.21 (d, *J* = 8.0 Hz, 2H)

^{13}C NMR (125 MHz, $CDCl_3$) : δ 178.2 (2C), 159.2, 147.7 (2C), 146.4 (2C), 130.5 (2C), 129.9 (2C), 127.9, 122.2 (2C), 113.9 (2C), 109.3 (2C), 108.3 (2C), 101.0 (2C), 55.2, 45.7 (2C), 41.84, 35.60 (2C)

HRMS (m/z) : 510.1531 [$(M+Na)^+$]; $C_{28}H_{25}NO_7$, requires 487.1631

(3*R*,4*R*)-3,4-bis(benzo[d][1,3]dioxol-5-ylmethyl)-1-benzylpyrrolidine-2,5-dione (**31b**)



Colourless crystals; m.p. 105-107 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 2920, 1700, 1493, 1440, 1396, 1246, 1172, 1037, 927, 738

^{1}H NMR (500 MHz, $CDCl_3$) : δ 2.77 (m, 6H), 4.62 (m, 2H), 5.92 (s, 2H), 5.95 (s, 2H), 6.41 (d, *J* = 8.0 Hz, 2H), 6.45 (s, 2H), 6.63 (d, *J* = 8.0 Hz, 2H), 7.26 (m, 5H)

^{13}C NMR (125 MHz, $CDCl_3$) : δ 178.2 (2C), 147.8 (2C), 146.5 (2C), 135.6, 130.5 (2C), 128.6 (2C), 128.3 (2C),

127.8, 122.3 (2C), 109.4 (2C), 108.3 (2C), 101.0 (2C), 45.8 (2C), 42.4, 35.6 (2C)

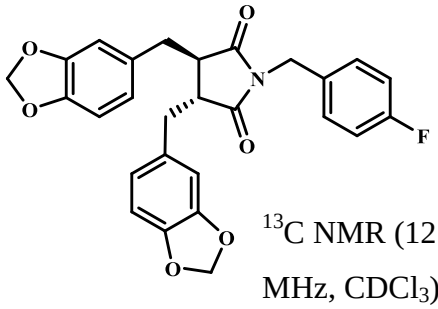
HRMS (m/z) : 480.1420 [$(M+Na)^+$]; $C_{27}H_{23}NO_6$, requires 457.1525

(3*R*,4*R*)-3,4-bis(benzo[d][1,3]dioxol-5-ylmethyl)-1-(4-fluorobenzyl)pyrrolidine-2,5-dione (**31c**)

Colourless crystals; m.p. 72-75 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 2898, 1703, 1498, 1441, 1397, 1343, 1248, 1040, 929, 806

^{1}H NMR (500 MHz, $CDCl_3$) : δ 2.77 (m, 6H), 4.57 (m, *J* = 2H), 5.92 (s, 2H), 5.95 (s, 2H), 6.41 (d, *J* = 8.0 Hz, 2H), 6.43 (s, 2H), 6.64 (d, *J* = 7.5 Hz, 2H), 6.97 (t, *J* = 8.75 Hz, 2H), 7.24 (dd, *J* = 8.5, 5.5 Hz, 2H)



^{13}C NMR (125 MHz, $CDCl_3$) : δ 178.1 (2C), 162.6 ($^1J_{CF}$ = 245 Hz), 147.8 (2C), 146.5 (2C), 133.1, 131.5 (2C), 130.3 ($^3J_{CF}$ = 8.75 Hz, 2C), 122.3 (2C), 115.4 ($^2J_{CF}$ = 21.25 Hz, 2C) 109.3 (2C), 108.3 (2C), 101.0 (2C), 45.7 (2C), 41.6, 35.5 (2C)

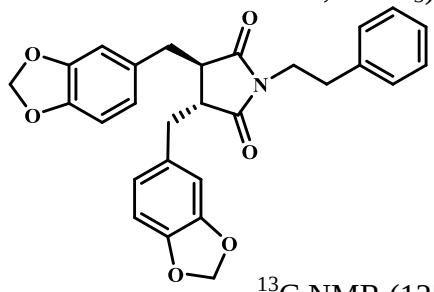
HRMS (m/z) : 498.1324 [$(M+Na)^+$]; $C_{27}H_{22}NO_6F$, requires 475.1431

(3*R*,4*R*)-3,4-bis(benzo[d][1,3]dioxol-5-ylmethyl)-1-phenethylpyrrolidine-2,5-dione (**31d**)

Gummy solid

FT-IR (KBr, υ_{max}, cm^{-1}) : 3025, 2923, 1697, 1493, 1442, 1399, 1357, 1245, 1039, 865, 699

^{1}H NMR (500 : δ 2.54 (dd, *J* = 13.5, 7.5 Hz, 2H), 2.66 (m,



MHz, $CDCl_3$) 2H), 2.78 (dd, *J* = 13.5, 7.5 Hz, 2H), 2.86 (m, 2H), 3.72 (m, 2H), 5.92 (s, 2H), 5.95 (s, 2H), 6.40 (d, *J* = 8.5 Hz, 2H), 6.43 (s, 2H), 6.66 (d, *J* = 7.5 Hz, 2H), 7.16 (d, *J* = 7.0 Hz, 2H), 7.23 (m, 1H), 7.29 (m, 2H)

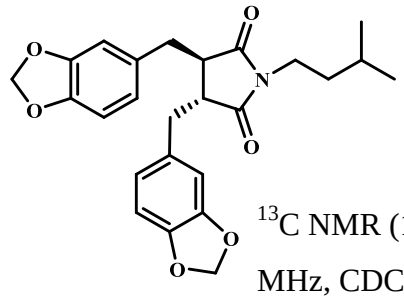
^{13}C NMR (125 MHz, $CDCl_3$) : δ 178.3 (2C), 147.7 (2C), 146.4 (2C), 137.7, 130.6 (2C), 128.9 (2C), 128.5 (2C), 126.7, 122.2 (2C), 109.3 (2C), 108.2 (2C), 101.1 (2C), 45.8 (2C), 39.7, 35.9 (2C), 33.4

HRMS (m/z) : 494.1582 [$(M+Na)^+$]; $C_{28}H_{25}NO_6$, requires 471.1682

(3*R*,4*R*)-3,4-bis(benzo[d][1,3]dioxol-5-ylmethyl)-1-isopentylpyrrolidine-2,5-dione (**31e**)

Colourless crystals; m.p. 76-78 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 2922, 2854, 1698, 1494, 1442, 1246, 1039, 930



^{1}H NMR (500 MHz, $CDCl_3$) : δ 0.91 (d, *J* = 4.0 Hz, 3H), 0.92 (d, *J* = 4.0 Hz, 3H), 1.40 (m, 3H), 2.73 (m, 4H), 2.82 (m, 2H), 3.47 (m, 2H), 5.93 (s, 2H), 5.96 (s, 2H), 6.46 (d, *J* = 8.0 Hz, 2H), 6.48 (s, 2H), 6.68 (d, *J* = 8.0 Hz, 2H);

^{13}C NMR (125 MHz, $CDCl_3$) : δ 178.5 (2C), 147.8 (2C), 146.5 (2C), 130.6 (2C), 122.3 (2C), 109.3 (2C), 108.3 (2C), 101.0 (2C), 45.8 (2C), 37.3, 36.6, 35.8 (2C), 25.8, 22.3, 22.2

HRMS (m/z) : 460.1721 [$(M+Na)^+$]; $C_{25}H_{27}NO_6$, requires, 437.1838

3B.5.2. Biology

3B.5.2.1. Cell viability assay

Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, US) supplemented with 10% heat inactivated fetal bovine serum (Pan Biotech, US), and were incubated in a humidified atmosphere of 5% CO_2 at 37 °C. Cell viability was measured by the colorimetric MTT assay, based on the ability of mitochondrial reductase enzyme present in living cells to cleave yellow coloured soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into an insoluble purple formazan crystals, which can be dispersed using DMSO.[38] Briefly, cells (100 µL/well) were seeded at seeding densities of 5×10^3 cells into 96 well microtitre plates and allowed to adhere by overnight incubation. The cells were treated with varying concentrations of test compounds (10-200 µM) and incubated for 48 h. DMEM with 0.1% DMSO was considered as negative control. Cell viability was assessed by adding 20 µL MTT (5 mg/ml in PBS) and incubated for 2 h in dark. At the end of incubation, the formazan crystals were dissolved in DMSO (100 µL) by incubating at 37 °C for 30 min. The absorbance at 570 nm was measured with a plate reader (Power wave XS, BioTek). The percentage of growth inhibition was calculated as follows,

% of Growth inhibition = 100 – [(Absorbance of treated cells/Absorbance of control) × 100]

A graph was plotted using Origin Pro8 software to obtain the IC_{50} values (expressed in µM), which is defined as concentration required for inhibiting 50% of cell growth.

3B.5.2.2. Morphological analysis

3B.5.2.2.1. Morphological analysis under light microscope

A549 and KB cells (2×10^4 cells/well) were seeded into a 96-well plate and incubated overnight at 37 °C and 5% CO_2. The cells were treated with IC_{50} concentration of each compound (**19** and **30a** in A549 cells; **19** and **30h** in KB cells) for 48 h and then observed and photographed under an inverted microscope (Model IX51; Olympus, Japan).

3B.5.2.2.2. Acridine orange –Ethidium bromide (AO/EB) dual staining

AO/EB staining was performed to detect morphological changes due to apoptosis as previously described.[39] Viable cells stain uniformly green only with AO

whereas apoptotic cells showed red nuclear staining with condensed chromatin. In brief, A549 and KB cells were seeded into a 96-well plate (2×10^4 cells/well) and incubated overnight at 37 °C and 5% CO_2. The cells were treated with IC_{50} concentration of each compound (**19** and **30a** in A549 cells; **19** and **30h** in KB cells) for 48 h. The treated cells were stained with 10 µL of dye mixture containing AO (1 mg/L) and EB (1 mg/L) in PBS. The cells were observed by fluorescence microscope and photographed.

3B.5.2.2.3. Hoechst 33342 staining

Characteristic apoptotic morphological changes such as condensation of chromatin and fragmentation of DNA were assessed by fluorescent microscopy using Hoechst 33342 staining as reported earlier.[40] The dye has higher affinity to condensed chromatin in apoptotic cells and therefore showed increased brightness compared to normal cells. In brief, A549 and KB cells (2×10^4 cells/well) were seeded into a 96-well plate and incubated overnight at 37 °C and 5% CO_2. The cells were treated with IC_{50} concentration of each compound (**19** and **30a** in A549 cells; **19** and **30h** in KB cells) for 48 h. The treated cells were stained with 10 µL of Hoechst 33342 (10 mg/mL) for 30 min at room temperature. The cells were examined using a fluorescence microscope (Olympus 1X51, Japan), to determine DNA fragmentation and chromatin condensation.

3B.6. Conclusion

Development of novel chemotherapeutic agents with fewer side effects, for the treatment of cancer still remains a challenging problem to the scientific community. Plant-derived anticancer compounds and their semi-synthetic analogues with structural diversity play a crucial role in solving this problem to some extent. In the present study we have carried out the chemical diversification of (-)-cubebin, the butyrolignan lactol isolated from the seeds of *P. cubeba*, under simple reaction conditions to yield five different types of derivatives. All the compounds were screened for their anticancer activity against six human cancer cell lines (A549, K562, SiHa, KB, HCT116 and HT29) using MTT assay. From the results obtained, it has been found that the compounds containing amide group are the most active ones, followed by lactone and lactol compounds. Compounds containing dihydro, oxolane and imide functionalities were found to be less effective. Among the various amides, amide derived from ethyl phenyl amine and *para* substituted benzyl amides were the most active ones. From the morphological analysis it has been confirmed that these compounds act through

apoptosis mediated pathway and further detailed studies are required for establishing the actual mechanism for apoptosis induction (*Bioorg. Med. Chem. Lett.*, **2016**, *26* (7), 1767-1771).

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Chapter 4

Isolation of Nimbolide from *Azadirachta indica* Leaves and Evaluation of its Anticancer and Antibacterial Activities

4.1. Introduction

Azadirachta indica (fig. 4.1), belonging to Meliaceae family has been accepted as a wonder tree, all over the world, because of its diverse utility and is considered as one of the most promising plants of the 21st century.[1] The plant is indigenous to South Asia and distributed all over Indian subcontinent. *A. indica* is now naturalized in African regions also.[2] It is popularly known as Indian neem (Margosa tree) or Indian lilac. In Sanskrit, it is called 'Nimba' and in Malayalam, it is called 'Aryaveppu'. Since the tree is used for the treatment of many diseases in different traditional systems of medicine practiced in the Indian subcontinent, it is called '*Sarvaroganivarini*', which means reliever of all diseases. Virtually, all parts of the tree (leaves, seeds, twig, bark, flowers etc.) find application in traditional and folklore medicines for the treatment of various ailments[3] and hence it is also known as "living pharmacy".[4] Besides its medicinal applications, the tree's various parts also find extensive applications in the field of pesticides, insecticides and agrochemicals.

Figure 4.1: Neem Neem leaves

Therapeutic efficacy of neem has been understood from time immemorial. Indigenous people in India used neem leaves for the treatment of gastrointestinal disorders.[5,6,7] Aqueous/ethanolic extracts of neem leaves have been used in the Ayurvedic system of medicine for the treatment of malaria.[8] The medicinal uses of neem leaves are wide ranging and include immunomodulatory, anti-inflammatory, antioxidant, antimutagenic and anticarcinogenic effects. It has been reported that the aqueous extract of *A. indica* leaves significantly reduces blood sugar levels and

prevents adrenaline and glucose-induced hyperglycemia[9] as well as decreases hyperglycemia in streptozotocin-induced diabetes.[10] The aqueous extract of the leaves shows antiulcer and antioxidant activities. Methanolic extract of neem leaves shows chemopreventive,[11] antibacterial and antisecretory activity (against *Vibrio cholera*).[12]

The leaves of *A. indica* are a rich source of bioactive limonoids (which are modified triterpenoids) as well as glycosides, phenolic and steroidal compounds. Till to date, more than eighty compounds have been isolated from various extracts of *A. indica* leaves. Major compounds isolated from neem leaves are listed in table 4.1 (compounds are listed in the order of their year of isolation) and the structures of important limonoids and other triterpenes isolated from *A. indica* leaves are shown in chart 4.1 (compounds are arranged according to the similarity in their structure).

Table 4.1: List of compounds isolated from *A. indica* leaves

Name of the compound	**Reference**
Nimbolide	13,14,15
Vilasinin	16
Quercetin-3-glu (6 ⟶1) rhamnoside	17,18
Nimbandiol	19
1,3-diacetylvilasinin	20,21
Nimbaflavone	22
Azadirachtanin	23
2',3'-dehydrosalannol	24
Isoazadirolide	25
Isonimbocinolide	
Scopoletin	
Nimbocenone	26
β-sitosterol	
Stigmasterol	
Isonimocinolide	27
Nimocinolide	
6-desacetylnimbinolide	28
6-desacetylisonimbonolide	
Nimbin	29

Quercetin-3-*O*-β-D-glucoside	10
Myricetin-3-*O*-rutinoside	
Quercetin-3-*O*-rutinoside	
Kaempferol-3-*O*-rutinoside	
Kaempferol-3-*O*-β-D-glucoside	
6-desacetylnimbin	30
6-desacetylnimbinal	
Azadirachtolide	31
Deoxyazadirachtolide	
Nimonol	32,33
6-desacetylnimbinolactone	32
28-deoxonimbolide	14,15
Nimbothalin	34
n-tridecyl benzene	
23-*O*-methylnimocinolide	35
7-*O*-desacetal-23-*O*-methyl-7-*O*-senecioylnimocinolide	
Odoratone	36
6α-*O*-acetyl-7-deacetylnimocinol	37
Meliacinol	
Melianol	38
Desfurano-desacetylnimbin-17-one	
Meliatetraone	
Azadirachtol	39
(+)-dehydro-vomifoliol	40,18
22,23-dihydronimocinol	41
Desfurano-6α-hydroxyazadiradione	
Meliatetraolenone	42
Azadirone	43
Zeeshanol	44
6-*O*-acetylnimbandiol	11a
Dehydronimonol	33
4α-hydroperoxy-6-*O*-acetylnimbandiol	45

24,25-epoxy-3β-hydroxy-20-oxo-7-tirucallene	45
22,23;24,25-diepoxy-3β-hydroxy-7-tirucallene	
24,25-epoxy-3β,23-dihydroxy-7-tirucallene	
6β-hydroxykulactone	
24-methylenecycloartane-3,28-diol	
2α,3β-dihydroxy-5-pregnen-16-one	46
3β-hydroxy-5α,6α-epoxy-7-megastigmen-9-one	47,18
3β,4β,20α-trihydroxy-5-pregnen	48,18
17-(5-methoxy-2-oxofuran-3-yl)-28-deoxonimbolide	18
2α,4α-dihydroxy-pregn-5-en-16-one-3α-*O*-D-glucopyranoside	
24,25,26,27-tetranor-apotirucall-6α-hydroxy-7α-acetoxy-1,14-dien-3-one-21,24-anhydride	49
24,25,26,27-tetranor-apotirucall-6α-hydroxy-7α-acetoxy-14-en-3-one-21,24-anhydride	
24,25,26,27-tetranor-apotirucall-6α,22-dihydroxy-7α-acetoxy-1,14,20(21)-trien-3-one-21,23-olide	
24,25,26,27-tetranorapotirucall-6α-hydroxy-7α-acetoxy-14-en-3-one-21,23-olide.	
24,25,26,27-tetranorapotirucall-6α,22-dihydroxy-7α-acetoxy-14,20(21)-dien-3-one-21,23-olide	
1-tigloylazadirachtol	
17-desfuran-17-(22-hydroxy-20(21)-ene-21,23-γ-lactone) nimbandiol	
17-desfuran-17-(21-hydroxy-20(22)-ene-21,23-γ-lactone) nimbandiol	
Isobutyric acid 2-*O*-(6-*O*-*trans*sinapoyl)-β-D-glucopyranoside.	
17-defurano-17-(2,5-dihydro-2-oxofuran-3-yl)-28-deoxonimbolide	50
17-defurano-17-(2ξ-2,5-dihydro-2-hydroxy-5-oxofuran-3-yl)-28-deoxonimbolide	
17-defurano-17-(5 ξ -2,5-dihydro-5-hydroxy-2-oxofuran-3-yl)-2',3'-dehydrosalannol	
6-homodesacetylnimbin	
6-desacetylnimbinene	
Munronolide	
Nimbinene	

Nimonolactone	50
Nimbolide B	51
Nimbicacid B	

Chart 4.1: Structures of important compounds isolated from *A. indica* leaves

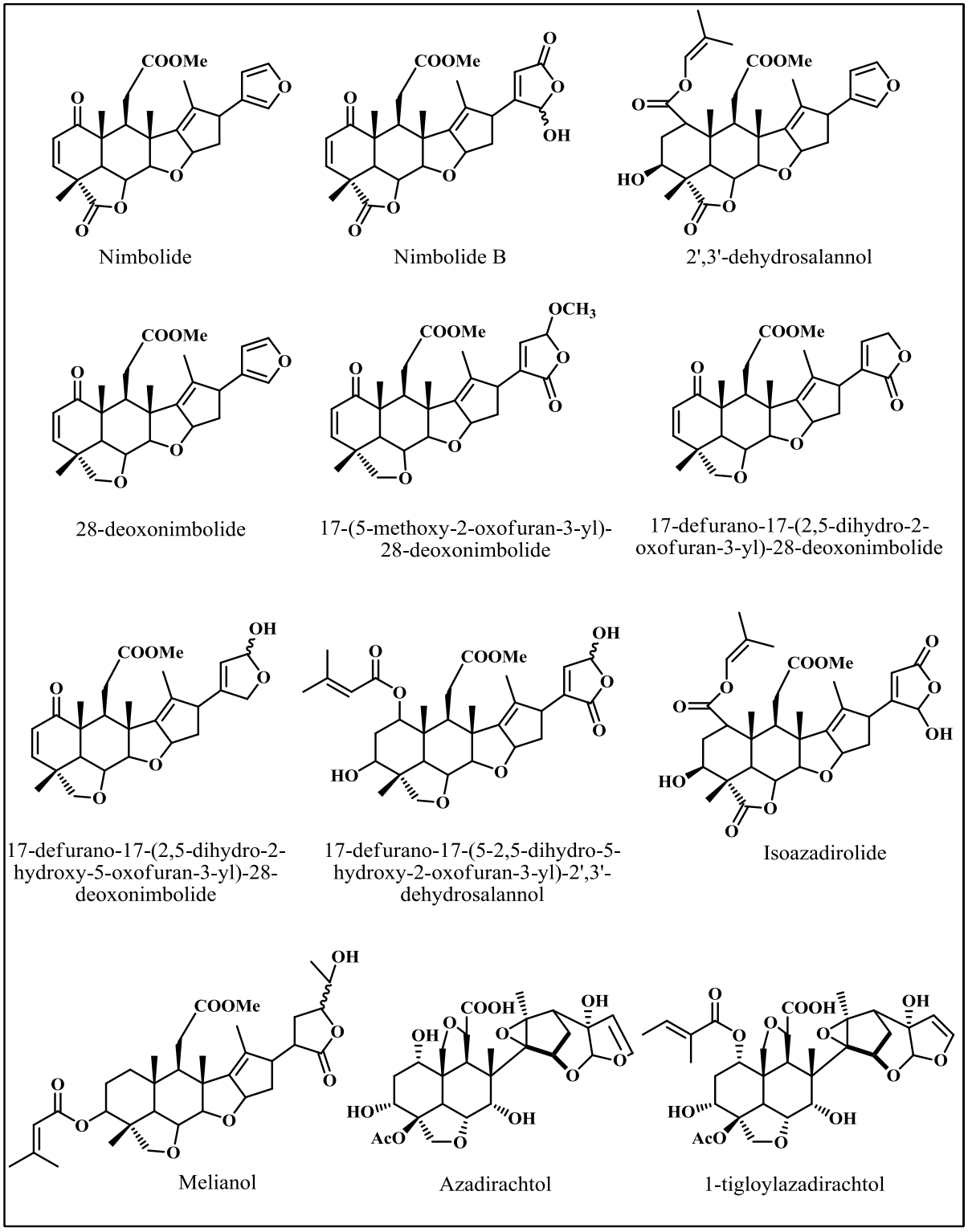
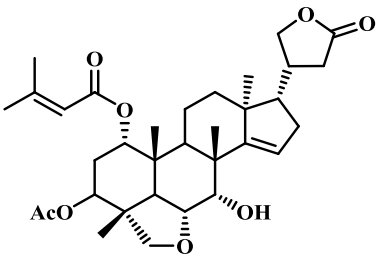
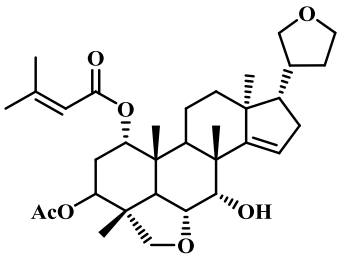


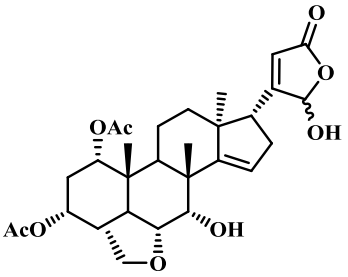
Chart **4.1** continues.....



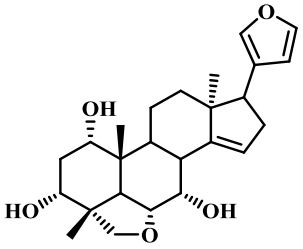
Azadirachtolide



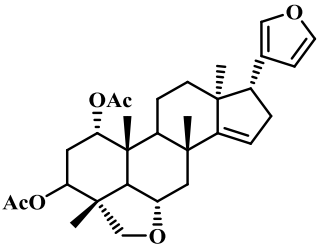
Deoxyazadirachtolide



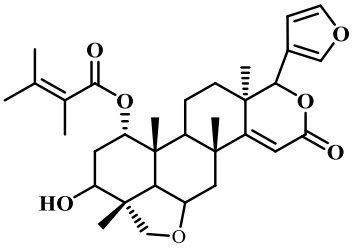
Muranoilide



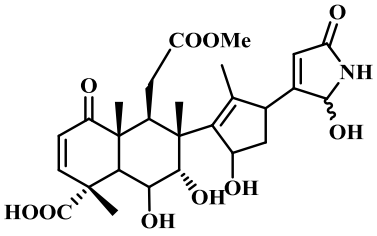
Vilasinin



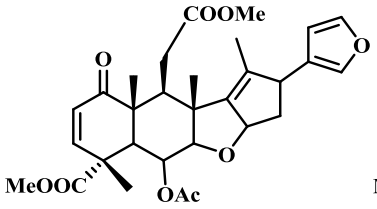
1,3-diacetylvilasinin



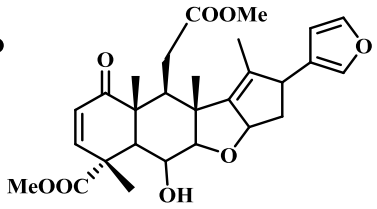
Meliacinol



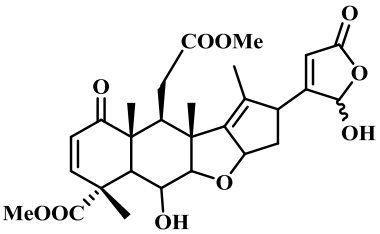
Nimbicacid B



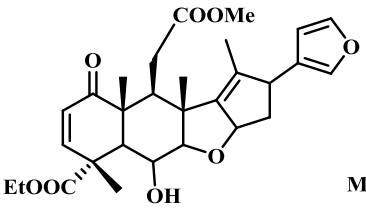
Nimbin



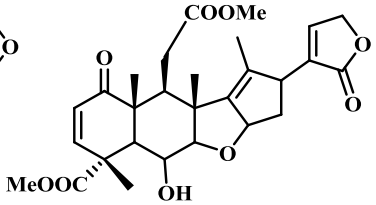
6-desacetylnimbin



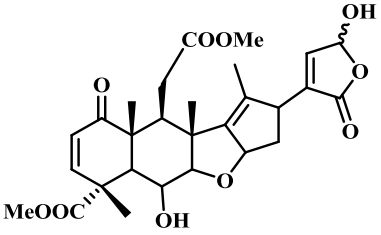
6-desacetylisonimbonolide



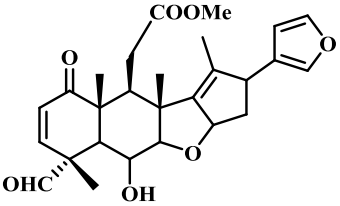
6-homodesacetylnimbin



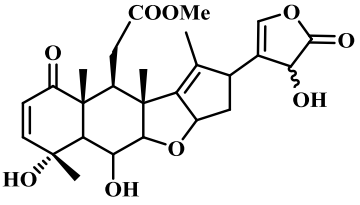
6-desacetylnimbinolactone



6-desacetylnimbinolide

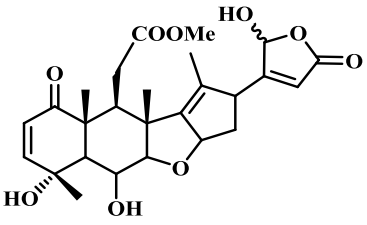


6-desacetylnimbinal

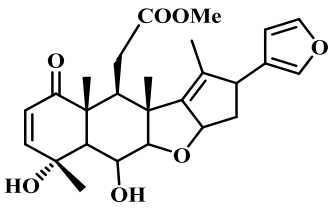


17-desfuran-17-(22-hydroxy-20(21)-ene-21,23-γ-lactone)-nimbandiol

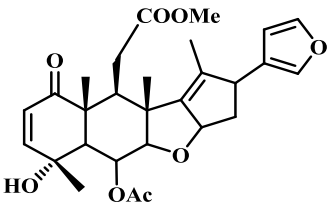
Chart **4.1** continues.....



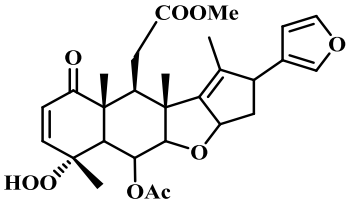
17-desfuran-17-(21-hydroxy-20(22)-ene-21,23-γ-lactone)-nimbandiol



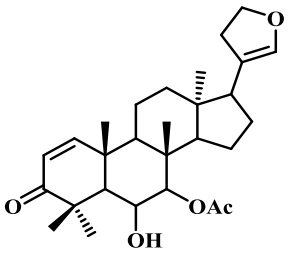
Nimbandiol



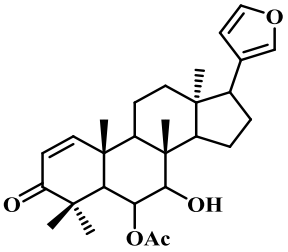
6-*O*-acetylnimbandiol



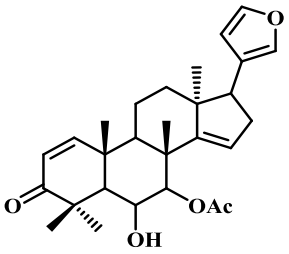
4α-hydroperoxy-6-*O*-acetylnimbandiol



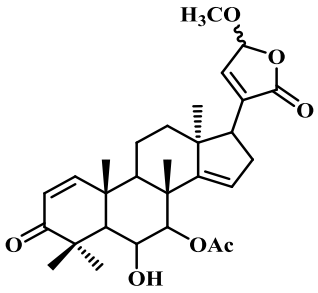
22,23-dihydronimocinol



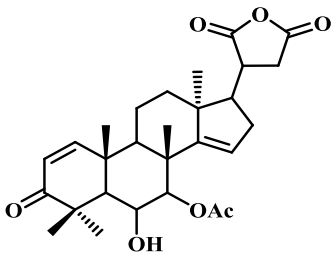
6 -*O*-acetyl-7-deacetylnimocinol



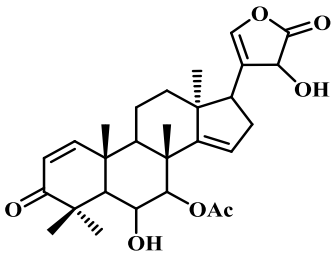
Nimonol



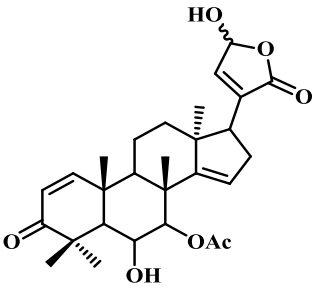
23-*O*-methylnimocinolide



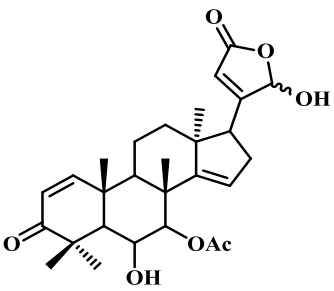
24,25,26,27-tetranor-apotirucall-6α-hydroxy-7α-acetoxy-1,14-dien-3-one-21,24-anhydride



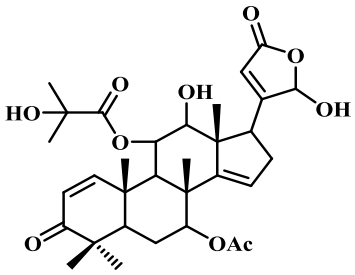
24,25,26,27-tetranor-apotirucall-6α,22-hydroxy-7α-acetoxy-1,14,20(21)-trien-3-one-21,23-olide



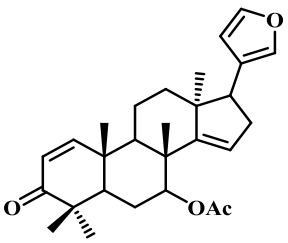
Nimocinolide



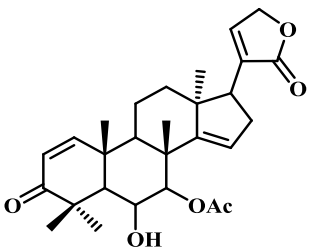
Isonimocinolide



Isonimbocinolide

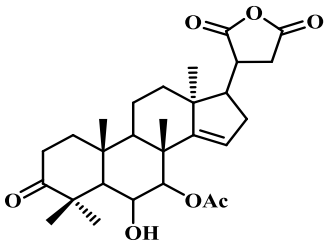


Azadirone

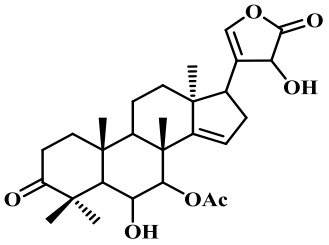


Nimonolactone

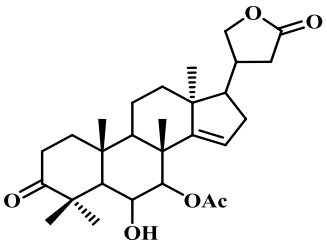
Chart **4.1** continues.....



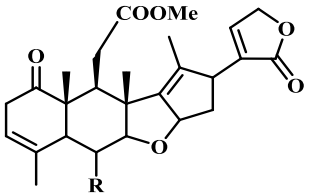
24,25,26,27-tetranor-apotirucall-6α-hydroxy-7α-acetoxy-14-en-3-one-21,24-anhydride



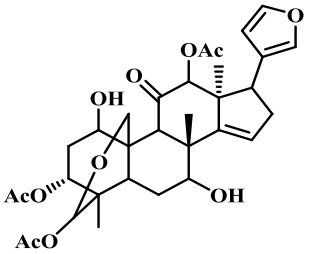
24,25,26,27-tetranor-apotirucall-6α,22-dihydroxy-7α-acetoxy-14,20(21)-en-3-one-21,23-olide



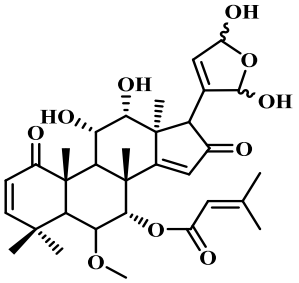
24,25,26,27-tetranor-apotirucall-6α-hydroxy-7α-acetoxy-14-en-3-one-21,23-olide



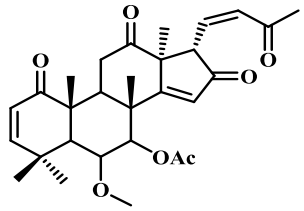
R = OAc; Nimbinene
R = H; 6-desacetylnimbinene



Azadirachtanin



Meliatetraolenone



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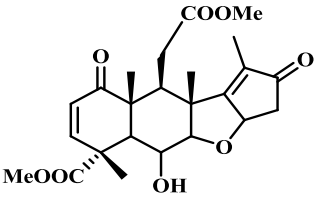
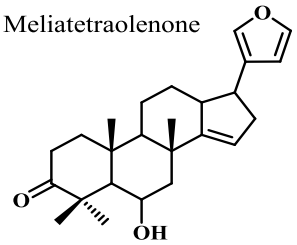
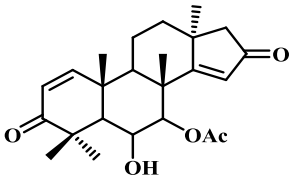


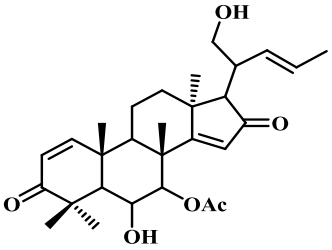
Desfurano-desacetylnimbin-17-one



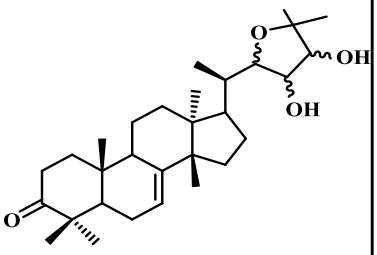
Dehydronimonol



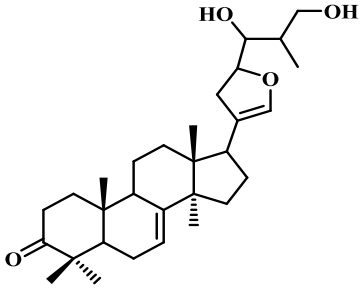
Desfurano-6 -hydroxyazadiradione



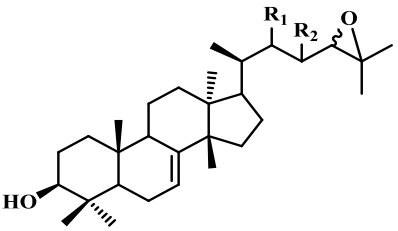
Zeeshanol



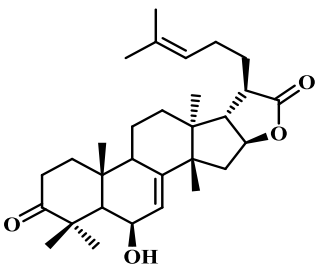
Odoratone



Nimbocinone



R1+R2 = O; 22,23;24,25-diepoxy-3β-hydroxy-7-tirucallene
R_1 = H, R_2 = O; 24,25-epoxy-3β-hydroxy-20-oxo-7-tirucallene
R_1 = H, R_2 = OH; 24,25-epoxy-3β,23-dihydroxy-7-tirucallene



6β-hydroxykulactone

4.2. Biological importance of nimbolide

Among the various limonoids isolated from *A. indica* leaves, the one which drew the maximum attention by the scientific community is nimbolide. It was first isolated from fresh leaves by Ekong *et al.*[13] in 1967. Nair M. S. and coworkers reported the optimized isolation procedure for nimbolide from dry leaves in the year 1997.[15] Chemically; nimbolide has a classical limonoid skeleton with α,β-unsaturated ketone system and δ-lactonic ring. The compound has been reported to have various bioactivities such as antimalarial,[52] antibacterial,[53] antifeedant,[54] anti-HIV and anticancer activities.[55] Studies revealed that nimbolide possesses promising *in vitro* anticancer activity and is emerging as a new lead molecule for the fight against cancer.

4.2.1. Anticancer activity of nimbolide

It has been reported that nimbolide has immense potential in cancer prevention and therapy based on its antiproliferative and apoptosis-inducing effects.[55] Nimbolide has been found to have antiproliferative effect on various human cancer cell lines such as lymphoma (BC-1), fibro sarcoma (HT-1080), nasopharyngeal (KB),[14] chorio carcinoma (BeWo),[56] neuroblastoma (NIE-115), osteo sarcoma (143B-TK),[57] prostate carcinoma (PC3), ovarian carcinoma (OVCAR-5),[58] colon carcinoma (COL-2, HT29, SW-480, SW-620, WiDr),[14,59] cervical carcinoma (HeLa),[60] hepato carcinoma (HepG2, Hepa 1c1c7, SMMC-7721),[45,61] leukemia (HL-60, P-388, THP-1), melanoma (B16, SK-MEL-2),[14,57,62] lung carcinoma (A549, HOP-62, DK-LU-1),[14,58,62] breast carcinoma (MDA-MB-231, MCF-7),[63] giloblastoma[64] etc.

Earlier studies from this laboratory have shown that nimbolide possesses promising activity against human colon cancer cell lines and it sensitizes tumor cells to chemotherapeutic agents through interaction with IKK, leading to inhibition of NF-κB-regulated proteins.[65] The anticancer activity of nimbolide may arise due to the modulation of numerous cell signalling molecules such as proliferating cell nuclear antigen (PCNA), p21, cyclin D1, glutathione S-transferase pi (GST-P), p53, Fas, Bcl-2, Bax, Bid, Apaf-1, cytochrome C, survivin, and caspases.[56] It was further noticed that nimbolide effectively inhibited proliferation of WiDr colon cancer cells through inhibition of cyclin A leading to S-phase arrest. It also caused activation of caspase-mediated apoptosis through the inhibition of ERK1/2 and activation of p38 and JNK1/2. Furthermore, nimbolide effectively retarded tumor cell migration and invasion through inhibition of metalloproteinase-2/9 (MMP-2/9) expression, both at the mRNA

and protein level and also strongly inhibited the VEGF expression, promoter activity, and *in vitro* angiogenesis. Nimbolide also suppressed the nuclear translocation of p65/p50 and DNA binding of NF-κB, which is an important transcription factor for controlling MMP-2/9 and VEGF gene expression.[59]

The α,β- unsaturated ketone group present in nimbolide plays a major role in exhibiting its anticancer activity.[66] In 2006 Sastry *et al.* reported the synthesis of amide derivatives of nimbolide, without affecting the unsaturated ketone and prepared several derivatives by opening the lactone ring. It has been found that some of the amide derivatives possessed better cytotoxic activities as compared to nimbolide.[58] From the above discussions, it is evident that nimbolide and its derivatives possess excellent antiproliferative effect against various human cancer cell lines and the limonoid, nimbolide has the potential for development as a promising drug candidate/drug lead for the treatment of cancer.

4.3. Aim and scope of the present study

From the literature reports, it is clear that nimbolide is one of the promising molecules which shows various biological activities, especially potential *in vitro* anticancer activity against colon cancer cell lines. Despite the significant advances in detection, surgery, and chemotherapy, colorectal cancer is one of the leading causes of cancer deaths. Although targeted therapies are currently used for colorectal cancer, many cases develop resistance to such treatments. Thus, safe and effective agents are required to replace or compliment current therapies. Therefore in the present work, we aimed at isolation of nimbolide in larger quantities in order to carry out detailed *in vitro* as well as *in vivo* studies against colorectal cancer. *In vivo* studies were performed to ascertain the real efficacy of nimbolide on the living systems.

Secondly, even though nimbolide and its derivatives have been well explored for their anticancer activity; they have not been studied in detail for their antimicrobial activities. Antibacterial activities of *A. indica* leaf extract against wound associated pathogens have been reported earlier, but there were only limited reports available on the antibacterial activity of nimbolide. Therefore, studies on the antibacterial activity of nimbolide and its derivatives against major wound associated bacterial pathogens have also been undertaken as part of the present study.

4.4. Isolation of nimbolide from *Azadirachta indica* leaves

4.4.1. Collection of plant material and extraction

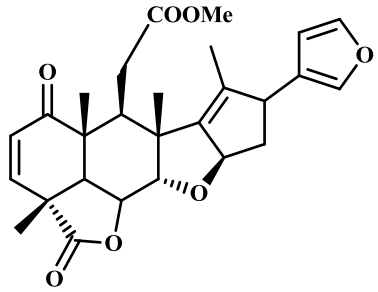
Fresh leaves of *A. indica* were collected from Thiruvananthapuram district, dried (40-45 °C, using RRL NC drier) and powdered. 2.5 kg of the powdered leaves were extracted using acetone at room temperature (2.5 L × 3). The solvent was removed under reduced pressure using a rotary evaporator to yield 160 g of the crude extract. The total extract was subjected to column chromatographic purification for obtaining nimbolide in a larger quantity. For ease of handling and for improving the yield, the separation was done in five different lots.

4.4.2. Isolation and characterization of nimbolide and desacetylnimbin

Acetone extract (30 g) was subjected to careful column chromatography using silica gel (100-200 mesh). Column elution was started using 5% ethyl acetate in hexane and further elution was done using the solvent of increasing polarity by increasing the amount of ethyl acetate in hexane. Different fractions of approximately 100 mL each were collected in conical flasks. A total of 214 fractions were thus collected. TLC of each fraction was checked and the fractions whose TLC profile was alike were pooled together to get 35 different fraction pools. Nimbolide containing fraction pool (TLC comparison with standard sample), obtained by eluting the column with 25% ethyl acetate in hexane was subjected to crystallization using petroleum ether–dichloromethane solvent system to yield 450 mg of pale yellow crystals (fig. 4.2) of nimbolide (compound **32**). The compound was analyzed using various spectroscopic techniques.

IR spectrum of the compound showed absorptions at 1778, 1730 and 1672 cm^{-1} which suggested the presence of a lactone carbonyl, ester carbonyl and an *α,β*-unsaturated ketone respectively. The ^{1}H NMR spectrum (fig. 4.3) confirmed the presence of four methyl groups at δ 1.22, 1.37, 1.47 and 1.70. The –OMe protons were confirmed by the presence of sharp singlet integrating for three protons at δ 3.54. Protons of the furan ring resonated at δ 7.32 (t), 7.22 (s) and 6.25 (m). Protons of *α, β*-unsaturated ketone were observed as two separate doublets integrating for one proton each at δ 7.28 and 5.93. The presence of three different carbonyl moieties was again confirmed by the peaks at δ 200.8, 175.0 and 173.0 in the ^{13}C NMR spectrum (fig. 4.4). The peak at δ 51.8 could be attributed to the methoxy carbon. The mass spectrum of the compound showed molecular ion peak at m/z 467.2079, which is the $(M+H)^{+}$ peak.

Spectral details of compound **32** matched well with the reported data for **nimbolide** and the structure is shown below.[14] As explained in the introductory section, the compound possesses a wide range of bioactivities.



Compound **32** - **Nimbolide**

Isolation procedure for nimbolide was repeated for four more times to get sufficient quantity of the compound inorder to carry out the animal studies.

Figure 4.2: Pale yellow Crystals of nimbolide

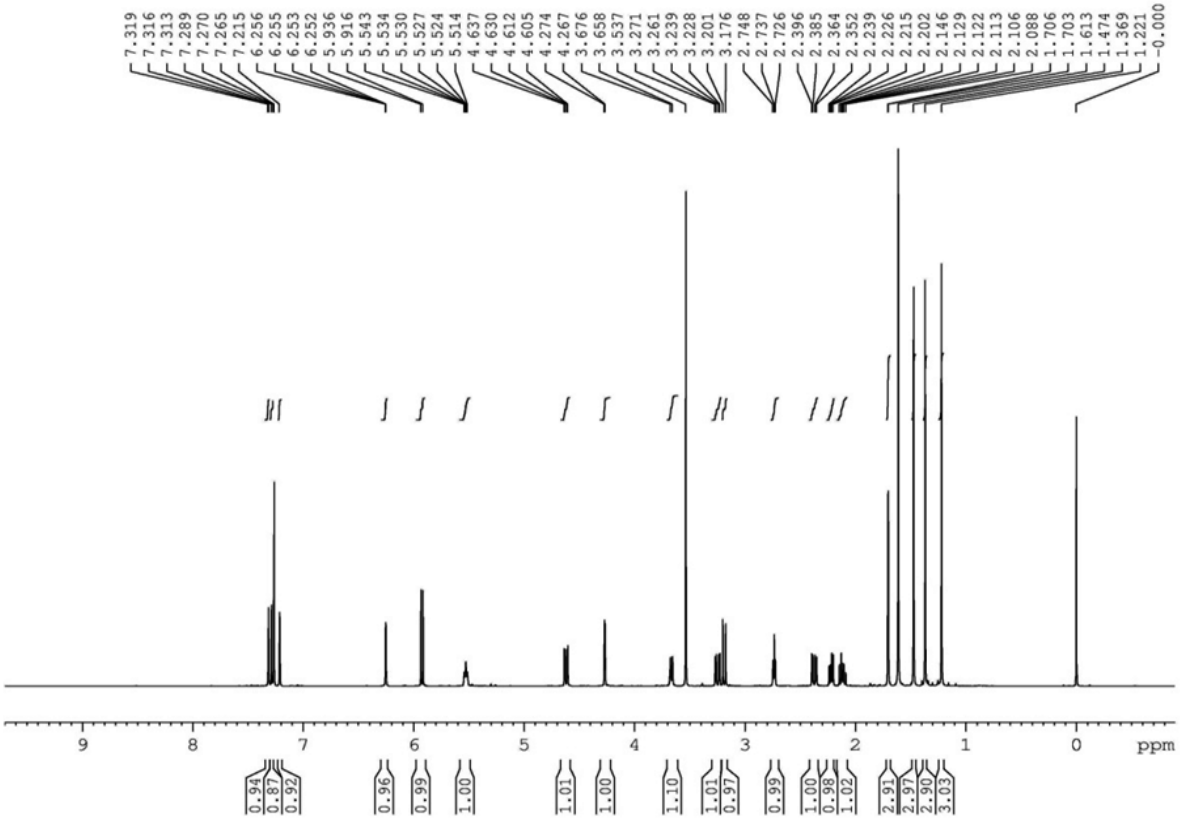


Figure 4.3: ^{1}H NMR spectrum of nimbolide

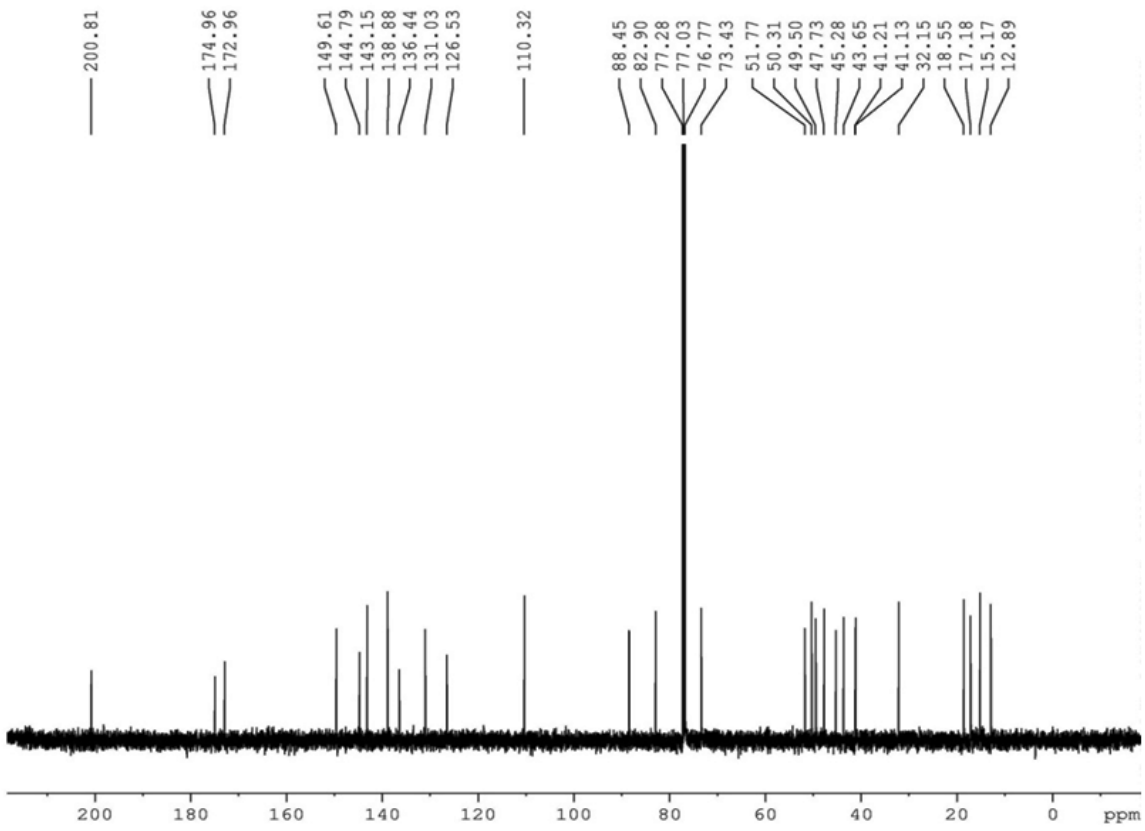
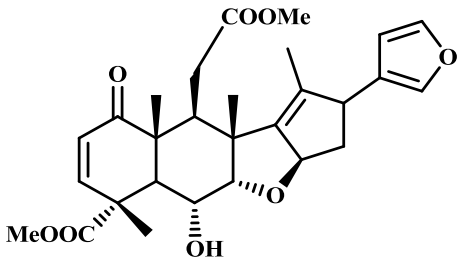


Figure 4.4: ^{13}C NMR spectrum of nimbolide

The supernatant liquid left after the crystallization of nimbolide on further purification by flash column chromatography using hexane-ethyl acetate as eluent, provided a pure compound which on crystallization using hexane-dichloromethane solvent system yielded 20 mg of colourless crystals of compound **33.** The compound was characterized as **desacetylnimbin** using various spectroscopic techniques and on comparison with the literature reports.[28]

IR spectrum of the compound showed broad absorption at 3574 cm^{-1}, which suggested the presence of hydroxyl group in the molecule. Absorptions at 1771 and 1731 cm^{-1} suggested the presence of two ester carbonyl groups and the peak at 1681 cm^{-1} could be attributed to the carbonyl group of an α,β-unsaturated ketone. ^{1}H NMR spectrum (fig. 4.5) showed the presence of four methyl groups which resonated at δ 1.22, 1.29, 1.59 and 1.69. Presence of two methoxy groups was indicated by the sharp singlets integrating for three protons at δ 3.71 and 3.66 respectively. Protons of the furan ring resonated at δ 7.33 (t), 7.24 (s) and 6.33 (d). Protons of α,β-unsaturated ketone were observed as two separate doublets integrating for one proton each at δ 6.41 and 5.86. The presence of three different carbonyl moieties was again confirmed by the peaks at δ 201.6, 175.6 and 173.7 in the ^{13}C NMR spectrum (fig. 4.6). The peak at

δ 66.21 confirmed the presence of a carbon bearing hydroxyl group. The peaks at δ 53.0 and 51.7 could be attributed to the two methoxy carbons. The mass spectrum of the compound showed molecular ion peak at 499.2343 which is the $(M+H)^+$ peak. From all these spectral data and on comparison with the literature, compound **33** was identified as **desacetylnimbin**. The structure of the compound is shown below.



Compound **33** - **Desacetylnimbin**

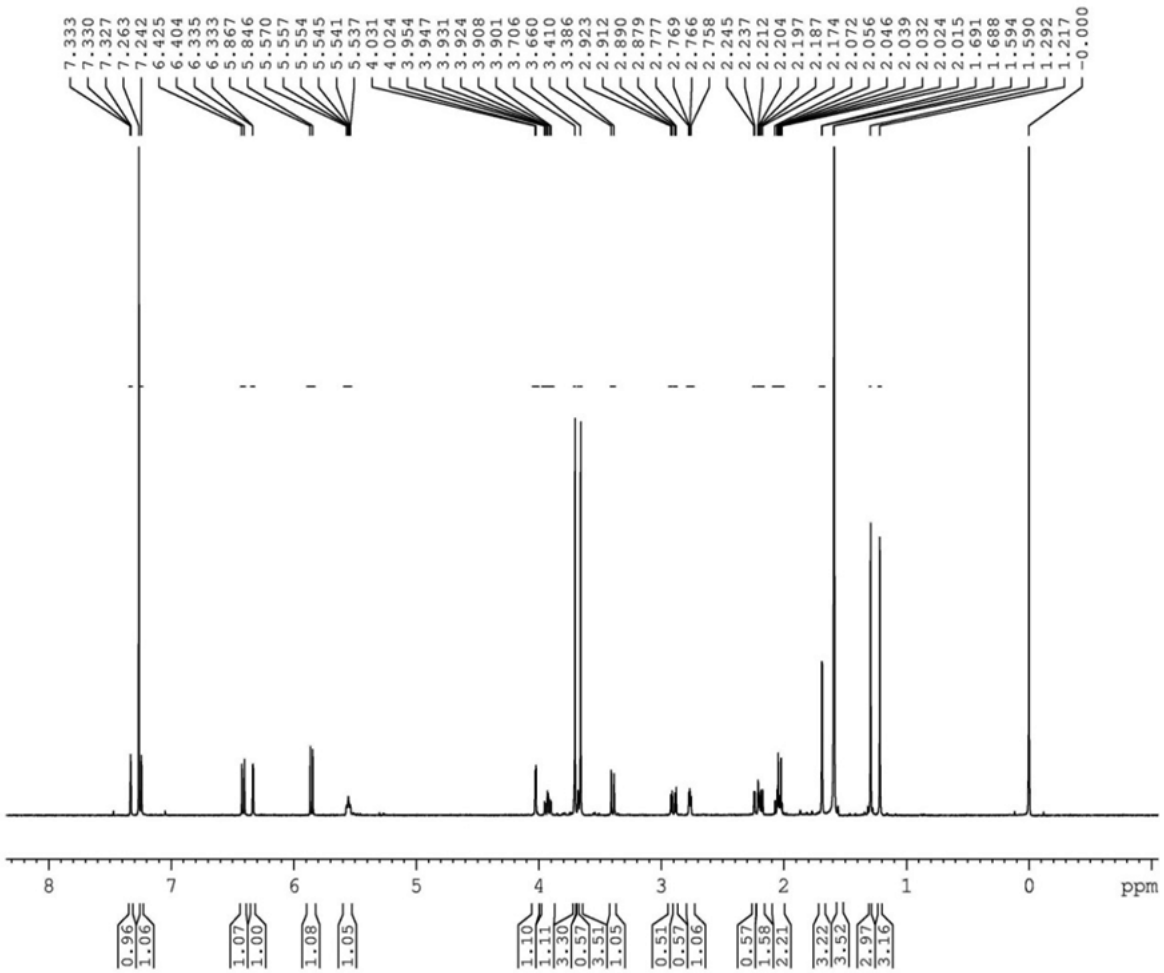


Figure 4.5: ^{1}H NMR spectrum of desacetylnimbin

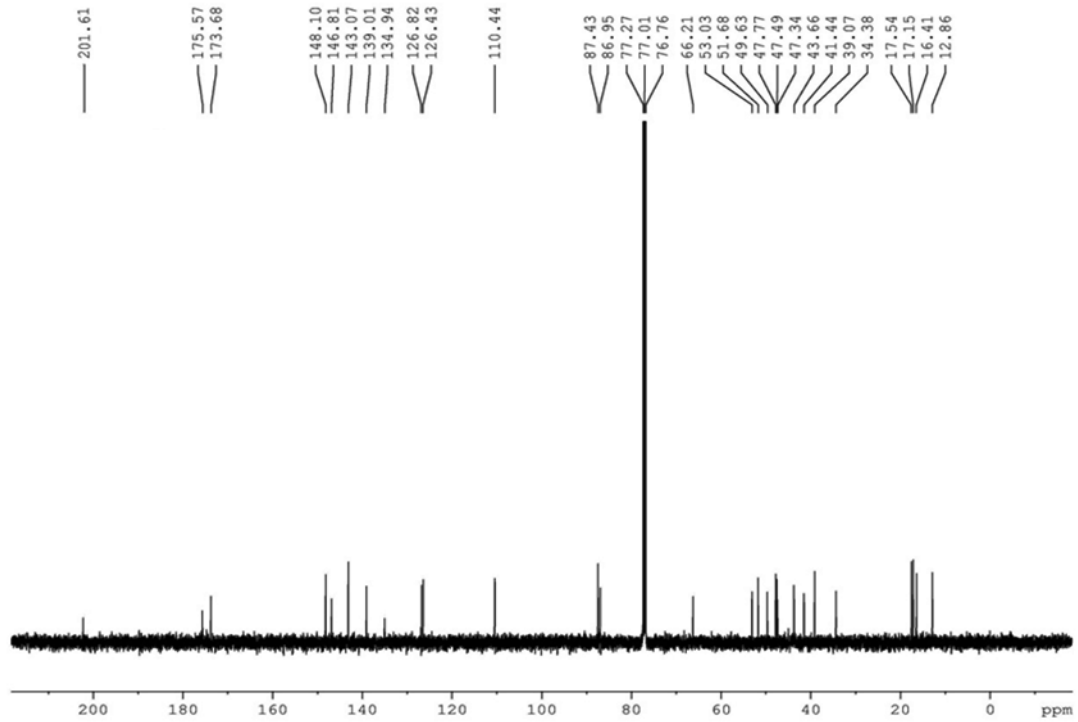


Figure 4.6: ^{13}C NMR spectrum of desacetylnimbin

4.5. Anticancer activity of nimbolide on human colon cancer cells

Cancer is one of the fatal diseases of the twenty-first century and colorectal cancer is one of the most common cancers found in both men and women. More than one million new cases of colorectal cancer (CRC) are diagnosed worldwide each year.[67] Despite major advances in detection, surgery and chemotherapy, CRC is the second leading cause of cancer-related deaths in the United States, with about 143,460 new cases and 51,690 deaths being claimed in 2012.[68] In India, the annual incidence rate for CRC among men and women are approximately 4.3 and 3.4 per 100,000 respectively.[69] Most of the anticancer activities assigned to nimbolide have been based on *in vitro* studies. Only a limited number of animal studies revealing the anticancer activities of nimbolide have been conducted. In order to establish the real efficacy of the compound in the living system, it is necessary to conduct the *in vivo* studies in detail. Extensive research over the past decade has revealed that the proinflammatory microenvironment plays a critical role in the development of colorectal cancer (CRC). Therefore in the present study, we have investigated the inhibitory activity of nimbolide against the growth of CRC, using both *in vitro* and *in vivo* method. *Studies were done in collaboration with Dr. Bharat B. Aggarwal, University of Texas, MD Anderson Cancer Center, Houston, Texas, USA.*

4.5.1. Effect of nimbolide on colorectal cancer cells

The effect of nimbolide on the proliferation of human colorectal cancer cells was determined by measuring mitochondrial dehydrogenase activity, using MTT as the substrate. Results indicated that, nimbolide suppressed the proliferation of HCT-116, HT-29, and Caco-2 cell lines in a dose and time dependent manner. At concentrations as low as 2 µM, nimbolide inhibited the proliferation of cancer cells after 3 days of treatment (fig. 4.7). At the highest concentration, significant inhibition of cell growth was observed after 1 day. These results indicate that nimbolide exhibits potent antiproliferative effects against CRC cells. To confirm the results obtained by the MTT assay, the CRC cells were treated with various concentrations of nimbolide for 24 hours and then performed live/dead assay. As indicated in fig. 4.8, cytotoxicity was induced in 52.5%, 38.4%, and 43.1% in the case of HCT-116, HT-29, and Caco-2 cells, respectively at 10 µM concentration of nimbolide. These results suggest that HCT-116 cells are relatively more sensitive to nimbolide.

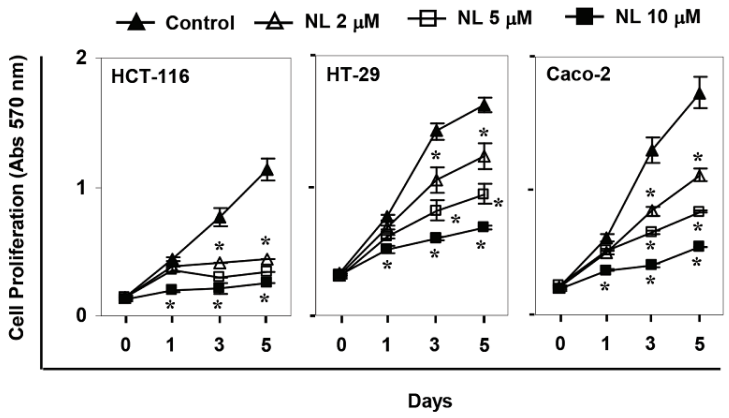


Figure 4.7: Analysis of cell growth using MTT assay

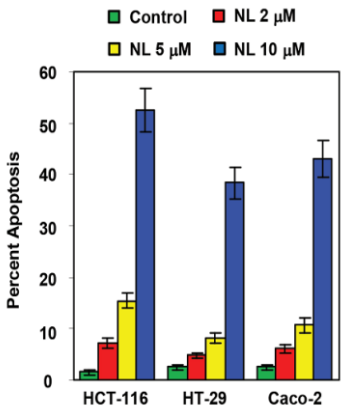


Figure 4.8: Cytotoxicity measured by live/dead assay

Since HCT-116 cells were found to be more sensitive to nimbolide, the ability of the limonoid to suppress HCT-116 colon cancer cells to form colonies was assessed. Cells were treated with 2 to 10 mmol/L nimbolide for 12 hours, washed and then allowed to form colonies for 10 days in fresh medium. After 10 days, cells were stained with crystal violet and the number of cells was counted. At the highest concentration of nimbolide, the total number of colonies was reduced from 907 to 152 (fig. 4.9).

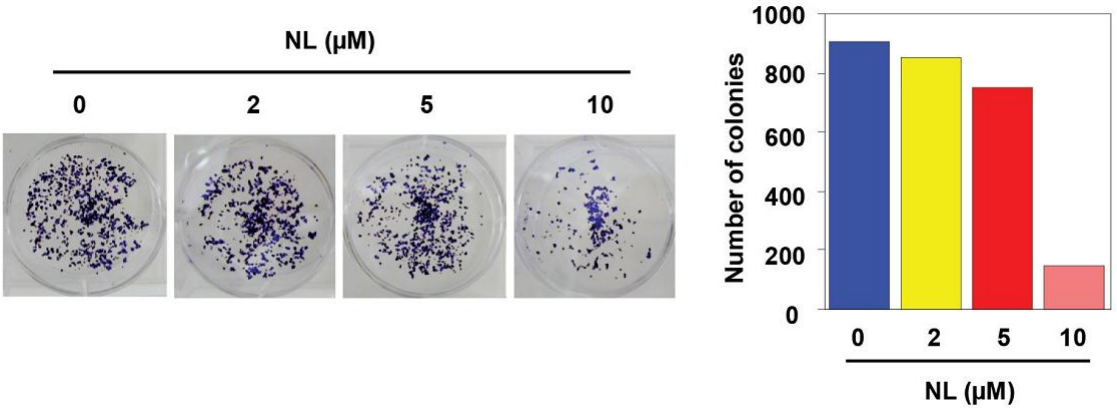


Figure 4.9: Clonogenic assay

Induction of apoptosis by nimbolide in colon cancer cells was examined by Western blot analysis. HCT-116 and HT-29 cells were treated with different concentrations of nimbolide for 12 h and then caspase activation as well as PARP cleavage were examined by Western blot analysis. The limonoid induced caspase activation and PARP cleavage at the highest concentration (10 μM).

Since nimbolide inhibited the survival of CRC cells, we investigated whether this limonoid can inhibit the expression of gene products involved in tumor cell survival. It has been observed that nimbolide inhibited the expression of antiapoptotic proteins such as Bcl-2, Bcl-xL, c-IAP-1 and survivin. The compound also inhibited the expression of cyclin D1 and c-Myc, both of which are known to contribute to cell proliferation. Furthermore, nimbolide inhibited the expression of proteins involved in tumor cell invasion, metastasis, and angiogenesis (MMP-9, ICAM-1, CXCR4, VEGF) in a concentration-dependent manner. The effect of nimbolide on the expression of gene products was greatest at a concentration of 10 μM. Nimbolide was also found to inhibit the expression of tumorigenic proteins at the transcriptional level and it suppressed the mRNA expressions of Bcl-2, c-IAP-1, survivin, c-Myc, ICAM-1 and MMP-9 in HCT-116 cells. A more prominent effect was observed at the mRNA levels of Bcl-2, c-IAP-1, and survivin.

Inflammatory response modulated by the proinflammatory transcription factor NF-kB plays a crucial role in the development of colorectal cancer and since Bcl-2, Bcl-xL, c-IAP-1, survivin, cyclin D1, c-Myc, MMP-9, ICAM-1, CXCR4 and VEGF are all regulated by NF-κB, we investigated the efficiency of nimbolide to suppress the expression of these gene products by inhibiting NF-κB activation. It has been found that HCT-116 cells exhibited constitutive NF-κB and treatment with nimbolide

inhibited NF-κB activation in a time-dependent manner. It has also been revealed that at 10 μM, nimbolide almost completely suppressed constitutive NF-κB after 12 hours. The inhibition of NF-kB activation by nimbolide was found to be specific since it neither inhibited STAT3 phosphorylation nor inhibited the expression of STAT3 (other proinflammatory transcription factor).

4.5.2. Inhibition of tumor growth by nimbolide in xenograft nude mice model

The *in vivo* efficacy of nimbolide to inhibit tumor growth in a xenograft-implanted nude mice model was also examined. Luciferase-transfected HCT-116 cells were xenograft-implanted into the leg of mice. One week later, mice were randomized into three different groups. Group I (vehicle) was given DMSO (50 μL, intraperitoneally daily) for 10 days. Groups II and III were given nimbolide at 5 mg/kg and 20 mg/kg of body weight (intraperitoneally daily), respectively, for 10 days (fig. 4.10).

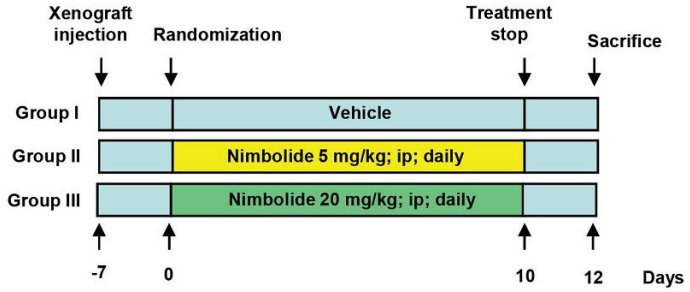


Figure 4.10: Grouping of mice

Noninvasive bioluminescence imaging after 10 days revealed that nimbolide significantly suppressed tumor growth in nude mice at a dose as low as 5 mg/kg. More specifically, administration of nimbolide at 20 mg/kg of body weight reduced tumor growth by almost 90% by day 10 (fig. 4.11). When the tumor volume was measured with Vernier calipers every other day, it was found that tumor growth had increased rapidly in the vehicle treated group (fig. 4.11). A comparison of tumor volumes between days 0 and 10 showed a 4.2-fold increase in tumor volume in the vehicle-treated group. However, in the nimbolide treated groups, an insignificant increase in tumor volume was observed. From these observations, it can be concluded that the tumor growth was significantly suppressed by the nimbolide treatment. Furthermore, it has also been established that the limonoid did not affect the body weight of mice (fig. 4.12 right).

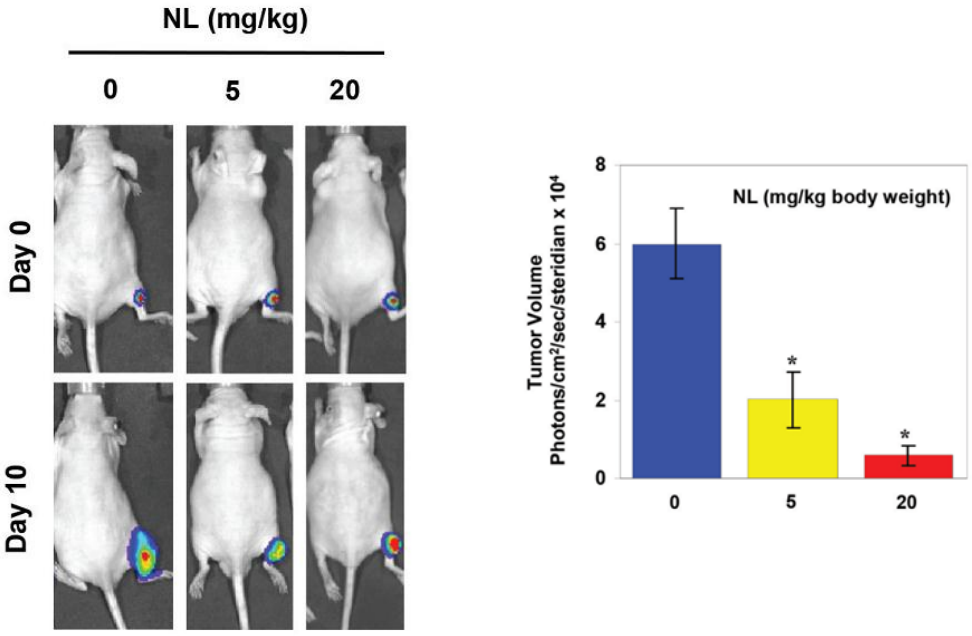


Figure 4.11: Suppression of tumor growth in nude mice model

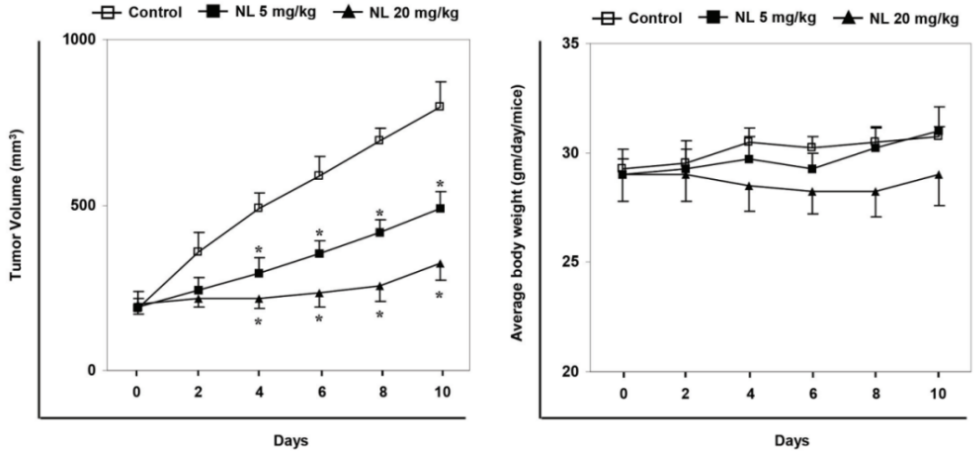


Figure 4.12: Tumor volume and average body weight per day

The availability of nimbolide in the blood plasma and tumors of the treated mice were also examined using HPLC method and it has been found that nimbolide was bio available in the plasma obtained from treated mice. More specifically, nimbolide levels of 222 ng/mL and 409 ng/mL of plasma were detected in the mice treated with nimbolide at 5 mg/kg and 20 mg/kg of body weight, respectively. Similarly, nimbolide levels of 345 ng/g and 868 ng/g of tumor tissue were obtained from the mice treated with nimbolide at 5 mg/kg and 20 mg/kg of body weight, respectively. Overall, these results reflect the bioavailability of nimbolide in blood plasma and tumor tissues.

Further studies revealed that nimbolide can mediate antitumor activity *in vivo* by modulating the expression of numerous tumorigenesis- related proteins. Nimbolide downregulated the expression of Bcl-2, Bcl-xL, c-IAP-1, surviving and Mcl-1 (all

known to promote tumor survival). The compound also downregulated the expression of c-Myc and cyclin D1, which are known to be overexpressed in colorectal cancer and to promote tumor growth[70] as well as downregulated the expression of proteins involved in tumor invasion, metastasis, and angiogenesis such as MMP-9, ICAM-1, CXCR4, and VEGF. Constitutively active NF-kB, known to regulate the expression of all of these proteins, was also inhibited by the nimbolide. Finally, expression of Ki67, a marker of proliferation, was also decreased by the limonoid treatment. The inhibition of CXCR4 expression by nimbolide may have clinical relevance, as increased expression of CXCR4 has been associated with increased risk of recurrence and poor survival in colorectal cancer.[71] This study provides strong evidence for evaluating the safety and efficacy of nimbolide by clinical studies.

4.6. Antibacterial activity studies of nimbolide and its derivatives

Chronic wounds are an increasingly urgent health problem and bacterial infection plays a significant role in the inability of these wounds to heal.[72] Treatment of such infections often involves combinations of antibiotics in an effort to increase efficacy and stem antibiotic resistance. Synergism of natural compounds and antimicrobial agents is a thrust area of phytomedicinal research, developing novel viewpoint of phytopharmaceuticals. The synergism of plant-derived compounds and antimicrobial drugs has been evaluated previously against many human pathogenic microorganisms. The approach is not exclusive for extract combinations, since effective combinations between single natural products, essential oils or extracts with chemosynthetics or antibiotics have been described in literature.[73] Even then, there are only limited reports available on the synergistic effect of individual phytochemicals with antibiotics (examples include that of lupulone, xanthohumol,[74] eugenol,[75] baicalien[76] etc.).

Neem leaf extracts have been reported to have promising antibacterial activity,[77] even then, nimbolide-the chief constituent of neem leaves, and its derivatives have not been studied in detail for their antimicrobial activity. Previous studies have shown that nimbolide possesses antimalarial activity[52] and shows antibacterial activity against *Staphylococcus aureus* and *S. coagulase*.[53] In 2006 Sastry *et al.* reported the synthesis of amide derivatives of nimbolide and studied their *in vitro* anticancer activities. It has been found that some of the amide derivatives possessed better activity than nimbolide.[58] Albeit, nimbolide and its amide derivatives were explored for their

anticancer activity, they have not been studied in detail for their antimicrobial activity. Therefore, in the present study we were interested in the evaluation of *in vitro* efficacy of nimbolide, desacetylnimbin isolated from *A. indica* and the amide derivatives of nimbolide against major wound associated bacterial pathogens.

Till date, no studies have evaluated the use of neem limonoids in combination with antibiotics. Hence it is not known if these compounds might have synergistic or antagonistic effects when co-administered with antibiotics. Therefore in the present study we have also investigated the synergistic activity of nimbolide as well as desacetylnimbin and the amide derivatives of nimbolide along with first generation cephalosporin antibiotics (cefalexin and cefazolin) against bacteria associated with wound infection. *Antibacterial studies were done in collaboration with Dr. Nishanth Kumar and Dr. Dileep Kumar B. S., Agroprocessing and Natural Products Division, CSIR-NIIST.*

4.6.1. Synthesis of amide derivatives of nimbolide

Three different amide derivatives (**34a-34c**) of nimbolide were synthesized by reacting nimbolide with respective primary amines under reflux condition using THF as the solvent. Structures of all the compounds were characterized using various spectroscopic techniques. The structures of the compounds are shown in fig. 4.13.

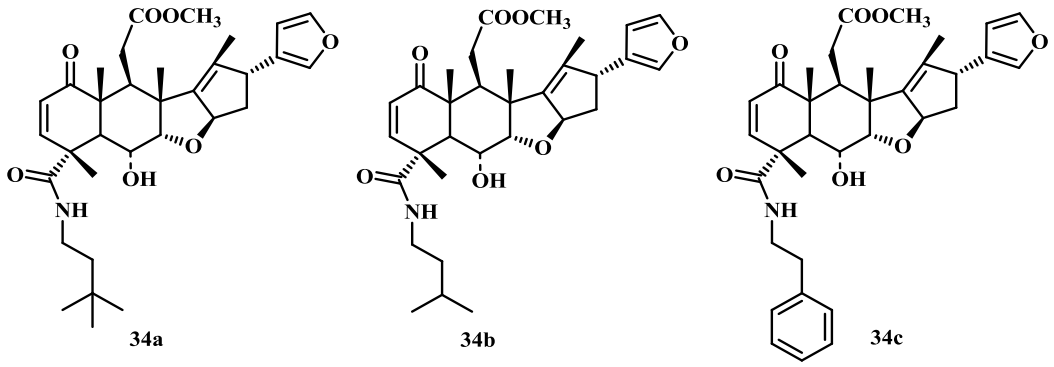


Figure 4.13: Structures of amide derivatives of nimbolide

IR spectrum of compound **34a** showed absorptions at 3558 cm^{-1} and 3380 cm^{-1}, suggesting the presence of –OH and –NH groups respectively. Absorptions at 1739, 1680 and 1640 cm^{-1} could be attributed to the ester carbonyl group, *α,β*-unsaturated ketone and amide carbonyl groups respectively. A singlet at δ 0.92 integrating for nine protons in the ^{1}H NMR spectrum (fig. 4.14) suggested the presence of a tertiary butyl group. Other four methyl protons resonated at δ 1.24, 1.29, 1.59 and 1.68. The methylene group attached to tertiary butyl group appeared as a triplet at

δ 1.39 and the methylene group attached to the amide functionality appeared as a multiplet at δ 3.25. The –OMe protons were confirmed by the presence of sharp singlet integrating for three protons at δ 3.54. The α and β protons of the α,β-unsaturated ketone were observed as doublets at δ 5.87 and 6.36 respectively. Peaks at δ 7.33, 7.32 and 6.32 integrating for one proton each confirmed the presence of three protons in the furan ring. The presence of three different carbonyl groups were again confirmed by the peaks at δ 202.4, 175.0 and 173.6 in the ^{13}C NMR spectrum (fig. 4.15). Carbon bearing the hydroxyl group resonated at δ 66.4. The peak at δ 51.7 could be attributed to the methoxy carbon where as the methyl carbons of tertiary butyl group resonated at δ 29.4 ppm. The mass spectrum of the compound showed molecular ion peak at m/z 568.3244, which is the $(M+H)^+$ peak. From all the spectral details, the structure of the compound was confirmed to be amide derivative of nimbolide (**34a**), obtained by opening the lactone ring using 3,3-dimethylbutamine. Structures of other two derivatives were also confirmed using various spectral techniques.

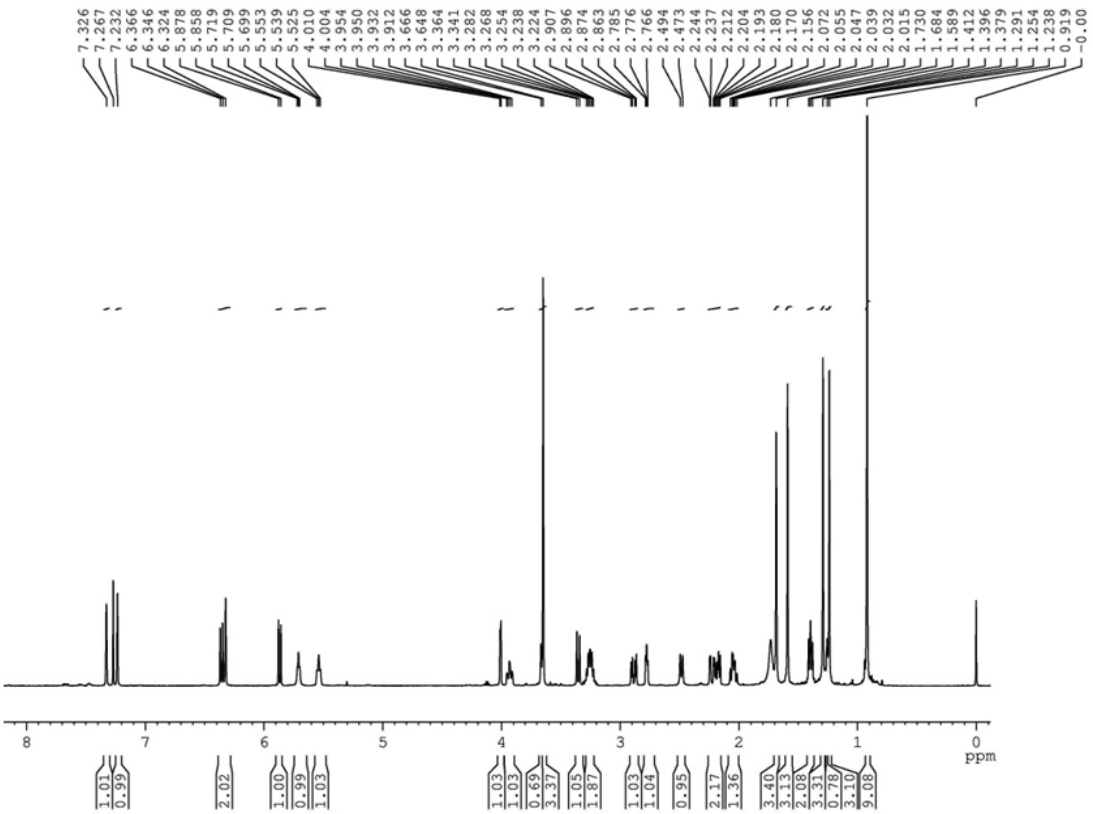


Figure 4.14: ^{1}H NMR spectrum of compound **34a**

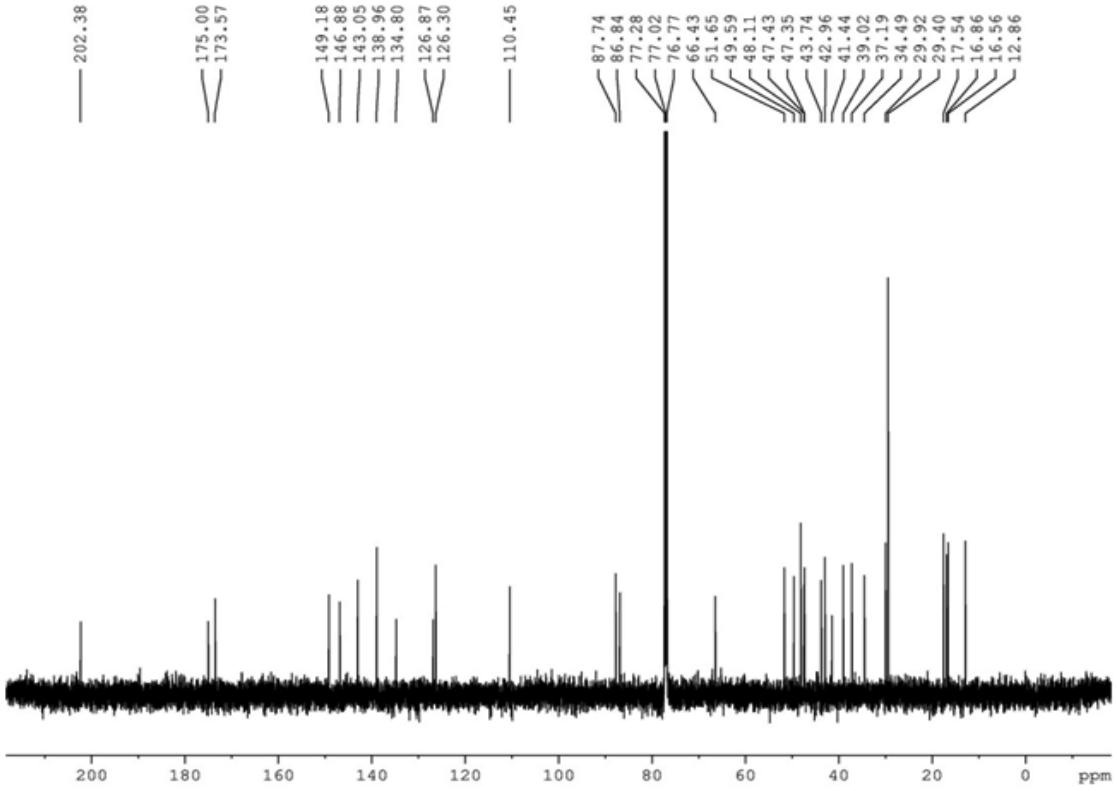


Figure 4.15: ^{13}C NMR spectrum of compound **34a**

4.6.2. Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs)

MIC (the lowest concentration of test compounds that inhibited the visual growth after incubating the test bacteria for 18 h) and MBC (the lowest concentration of the compounds at which 99.99% or more of the initial inoculum was killed) ranges of antibiotics, nimbolide, desacetylnimbin and the amide derivatives of nimbolide used alone for wound pathogens are shown in table 4.2. The ranges of MIC/MBC for the test pathogens were 64 to 1000 µg/mL for *S. aureus*, 125 to 2000 µg/mL for *S. epidermis*, 32 to 500 µg/mL for *K. pneumoniae*, 125 to 2000 µg/mL for *P. aeruginosa* and 250 to 2000 µg/mL for MR-*S. aureus*. Nimbolide recorded highest activity followed by desacetylnimbin, but the amide derivatives showed less activity when compared to the natural limonoids.

Table 4.2: Antibacterial activity of the test compounds and antibiotics against bacteria

	MIC/MBC (µg/mL)				
Test compounds	***S. aureus***	***S. epidermis***	***K. pneumoniae***	***P. aeruginosa***	**MR-*S. aureus***
Nimbolide	64/125	125/125	32/64	250/500	250/250
Desacetylnimbin	125/250	250/250	125/125	250/500	500/1000
Compound **34a**	125/250	125/250	250/250	500/500	1000/2000
Compound **34b**	250/500	500/1000	500/500	125/250	-
Compound **34c**	500/1000	1000/2000	500/500	2000/2000	-
Cefalexin	4/4	2/4	2/2	2/4	16/32
Cefazolin	2/4	4/4	1/2	2/4	64/64

(-) Recorded no MIC up to 2000 µg/mL, Values represent mean of three replications

4.6.3. Antibacterial activity by disc diffusion method

Quantification of antibacterial activity was carried out using agar disc diffusion method and the results are shown in table 4.3. Nimbolide recorded significant zone of inhibition against test bacteria, followed by desacetylnimbin. Best zone of inhibition was recorded by nimbolide against *K. pneumoniae* (29 mm). Photographs of zone of inhibition by nimbolide against the test bacteria are given in fig. 4.16. The natural limonoid, nimbolide was found to be effective against the important drug resistant strain MR-*S. aureus* also.

Table 4.3: Zone of inhibition of the test compounds and antibiotics against bacteria

	Diameter of zone of inhibition (in mm using 100 µg/mL of test compound)				
Test compounds	***S. aureus***	***S. epidermis***	***K. pneumoniae***	***P. aeruginosa***	**MR-*S. aureus***
Nimbolide	25±0.52	21±1.77	29±2.52	18±1.12	17±1.52
Desacetylnimbin	19±0.77	15±0.52	18±2.1	13±0.77	10±0.52
Compound **34a**	17±1.52	19±0.77	14±1.52	7.0±0.52	6.0±1.0
Compound **34b**	8.0±1.52	-	6.0±0.52	18±1.12	-
Compound **34c**	5.0±1.0	-	6.0±0	-	-
Cefalexin	31±0.52	28±1.0	32±1.12	30±1.72	21±0
Cefazolin	29±0	27±0	31±0	29±1	20±0

(-) no zone of inhibition, Values represent mean of three replications

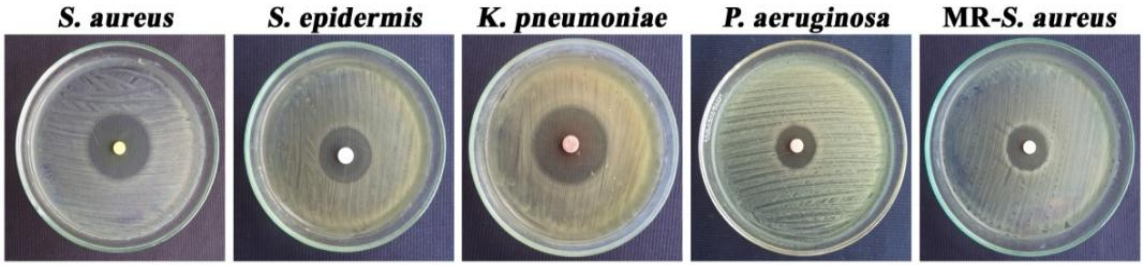


Figure 4.16: Photographs of zone of inhibition by nimbolide

4.6.4. Checkerboard assay

Checkerboard testing is one of the most widely used standard methods to determine the synergistic activity of test compound and antibiotic combinations. It is based on microdilution susceptibility testing of antibiotic combinations. The combined activities of the natural limonoids viz., nimbolide and desacetylnimbin as well as the amide derivatives of nimbolide with two first generation cephalosporin antibiotics from the *in vitro* checkerboard interactions against the medically important wound bacteria are summarized in table 4.4 and 4.5. FIC, FBC, FIC index, FBC index and interpretations for the activities of test compounds and antibiotics against the wound pathogens predominantly showed a synergistic interaction. But some combinations of amide derivatives with antibiotics recorded indifference. Antagonism was not recorded for any of the combinations.

During this synergistic combination, the concentration of the test compounds and antibiotics required to inhibit the pathogens has been reduced by many folds when compared with the concentration of compounds/antibiotics when given individually. When nimbolide was combined with cefazolin for the inhibition of *S. aureus*, a significant synergistic effect (FIC=0.09) was observed and the MIC values of nimbolide and cefazolin were reduced to 6 and 8 times below their individual MIC values, respectively (table 4.4). Another prominent synergism was observed (FIC=0.18) for desacetylnimbin and cefalexin against *K. pneumoniae* and the concentration of the desacetylnimbin was reduced to 4 times below its individual MIC value (table 4.5).

Interestingly, the combination of nimbolide and desacetylnimbin with antibiotics recorded significant synergistic effect against drug resistant *S. aureus* (MRSA) also, whereas the combination of amide derivatives of nimbolide with antibiotics did not record any synergistic interaction.

Table 4.4: Synergistic effects of the test compounds with cefazolin against bacteria

Test bacteria	Agent	MIC/MBC (µg/mL)		FIC/FBC	FICI[2]/FBCI[3]	Outcome
		Alone	Combination[1]			
S. aureus	Nimbolide	64/125	2/4	0.03/0.03	**0.09/0.09**	Synergistic/synergistic
	Cefazolin	2/4	0.12/0.25	0.06/0.06		
	Desacetylnimbin	125/250	32/64	0.26/0.26	0.39/0.39	Synergistic/synergistic
	Cefazolin	2/4	0.25/0.5	0.13/0.13		
	Compound 34a	125/250	64/64	0.51/0.26	0.101/0.51	Indifference/additive
	Cefazolin	2/4	1/1	0.50/0.25		
	Compound 34b	250/500	32/32	0.13/0.06	0.38/0.31	Synergistic/synergistic
	Cefazolin	2/4	0.5/1	0.25/0.25		
	Compound 34c	500/1000	125/250	0.25/0.25	0.75/0.50	Additive /synergistic
	Cefazolin	2/4	1/1	0.50/0.25		
S. epidermis	Nimbolide	125/125	16/32	0.13/0.26	0.26/0.39	Synergistic/synergistic
	Cefazolin	4/4	0.5/0.5	0.13/0.13		
	Desacetylnimbin	250/250	32/32	0.13/0.13	0.38/0.38	Synergistic/synergistic
	Cefazolin	4/4	1/1	0.25/0.25		
	Compound 34a	125/250	32/64	0.26/0.26	0.51/0.51	Additive/additive
	Cefazolin	4/4	1/1	0.25/0.25		
	Compound 34b	500/1000	125/125	0.25/0.13	0.38/0.38	Synergistic/synergistic
	Cefazolin	4/4	0.5/1	0.13/0.25		
	Compound 34c	1000/2000	250/250	0.25/0.13	0.75/0.63	Additive/additive
	Cefazolin	4/4	2/2	0.50/0.50		
K. pneumoniae	Nimbolide	32/64	4/8	0.13/0.13	0.25/0.26	Synergistic/synergistic
	Cefazolin	1/2	0.12/0.25	0.12/0.13		
	Desacetylnimbin	125/125	16/16	0.13/0.13	0.25/0.19	Synergistic/synergistic
	Cefazolin	1/2	0.12/0.12	0.12/0.06		
	Compound 34a	250/250	32/64	0.13/0.26	0.38/0.51	Synergistic/additive
	Cefazolin	1/2	0.25/0.5	0.25/0.25		
	Compound 34b	500/500	64/125	0.13/0.25	0.38/0.50	Synergistic/synergistic
	Cefazolin	1/2	0.25/0.5	0.25/0.25		
	Compound 34c	500/500	64/125	0.13/0.25	0.25/0.38	Synergistic/synergistic
	Cefazolin	1/2	0.12/0.25	0.12/0.13		
P. aeruginosa	Nimbolide	250/500	64/64	0.26/0.13	0.32/0.19	Synergistic/synergistic
	Cefazolin	2/4	0.12/0.25	0.06/0.06		
	Desacetylnimbin	250/500	32/64	0.13/0.13	0.38/0.38	Synergistic/synergistic
	Cefazolin	2/4	0.5/1	0.25/0.25		
	Compound 34a	500/500	32/32	0.06/0.06	0.31/0.19	Synergistic/synergistic
	Cefazolin	2/4	0.5/0.5	0.25/0.13		
	Compound 34b	125/250	16/32	0.13/0.13	0.63/0.63	Additive/additive
	Cefazolin	2/4	1/2	0.50/0.50		
	Compound 34c	2000/2000	250/250	0.13/0.13	0.38/0.38	Synergistic/synergistic
	Cefazolin	2/4	0.5/1	0.25/0.25		
MR-*S. aureus*	Nimbolide	125/250	16/16	0.13/0.06	0.38/0.31	Synergistic/synergistic
	Cefazolin	16/32	4/8	0.25/0.25		
	Desacetylnimbin	500/1000	32/64	0.06/0.06	0.31/0.31	Synergistic/synergistic
	Cefazolin	16/32	4/8	0.25/0.25		
	Compound 34a	1000/2000	500/500	0.50/0.25	1.0/0.75	Indifference/additive
	Cefazolin	16/32	8/16	0.50/0.50		
	Compound 34b	4000/4000	1000/2000	0.25/0.50	1.25/1.0	Indifference/additive
	Cefazolin	16/32	16/16	1.0/0.50		
	Compound 34c	4000/8000	2000/4000	0.50/0.50	1.0/0.75	Indifference/additive
	Cefazolin	16/32	8/8	0.50/0.25		

[1]The MIC and MBC of the test compounds with cefazolin

[2]The fractional inhibitory concentration index (FIC index)

[3]The fractional bactericidal concentration index (FBC index)

Significant FICI/FBCI values are shown in bold

Table 4.5: Synergistic effects of the test compounds with cefalexin against bacteria

Test bacteria	Agent	MIC/MBC (µg/mL)		FIC/FBC	FICI[2]/FBCI[3]	Outcome
		Alone	Combination[1]			
S. aureus	Nimbolide	64/125	16/32	0.25/0.26	**0.31/0.32**	Synergistic/synergistic
	Cefalexin	4/4	0.25/0.25	0.06/0.06		
	Desacetylnimbin	125/250	16/16	0.13/0.06	0.38/0.31	Synergistic/synergistic
	Cefalexin	4/4	1/1	0.25/0.25		
	Compound 34a	125/250	8/8	0.06/0.03	0.19/0.28	Synergistic/synergistic
	Cefalexin	4/4	0.5/1	0.13/0.25		
	Compound 34b	250/500	64/125	0.26/0.25	0.51/0.50	Additive/synergistic
	Cefalexin	4/4	1/1	0.25/0.25		
	Compound 34c	500/1000	64/64	0.13/0.06	0.38/0.56	Synergistic/indifference
	Cefalexin	4/4	1/2	0.25/0.50		
S. epidermis	Nimbolide	125/125	8/16	0.06/0.13	0.38/0.26	Synergistic/synergistic
	Cefalexin	2/4	0.5/1	0.25/0.25		
	Desacetylnimbin	250/250	16/32	0.06/0.13	0.31/0.26	Synergistic/synergistic
	Cefalexin	2/4	0.5/0.5	0.25/0.13		
	Compound 34a	125/250	16/32	0.13/0.13	0.63/0.38	Indifference/synergistic
	Cefalexin	2/4	1/1	0.50/0.25		
	Compound 34b	500/1000	32/64	0.06/0.06	0.31/0.19	Synergistic/synergistic
	Cefalexin	2/4	0.5/0.5	0.25/0.13		
	Compound 34c	1000/2000	250/500	0.25/0.25	0.75/0.75	Indifference/indifference
	Cefalexin	2/4	1/2	0.50/0.50		
K. pneumoniae	Nimbolide	32/64	4/8	0.13/0.13	0.26/0.26	Synergistic/synergistic
	Cefalexin	2/2	0.25/0.25	0.13/0.13		
	Desacetylnimbin	125/125	16/16	0.13/0.13	**0.19/0.26**	Synergistic/synergistic
	Cefalexin	2/2	0.12/0.25	0.06/0.13		
	Compound 34a	250/250	32/32	0.13/0.13	0.38/0.38	Synergistic/synergistic
	Cefalexin	2/2	0.5/0.5	0.25/0.25		
	Compound 34b	500/500	32/64	0.06/0.13	0.56/0.63	Indifference/indifference
	Cefalexin	2/2	1/1	0.50/0.50		
	Compound 34c	500/500	64/64	0.13/0.13	0.63/0.63	Indifference/indifference
	Cefalexin	2/2	1/1	0.50/0.50		
P. aeruginosa	Nimbolide	250/500	64/64	0.26/0.13	0.39/0.19	Synergistic/synergistic
	Cefalexin	2/4	0.25/0.25	0.13/0.06		
	Desacetylnimbin	250/500	32/64	0.13/0.13	0.38/0.26	Synergistic/synergistic
	Cefalexin	2/4	0.5/0.5	0.25/0.13		
	Compound 34a	500/500	64/64	0.13/0.13	0.28/0.28	Synergistic/synergistic
	Cefalexin	2/4	0.5/1	0.25/0.25		
	Compound 34b	125/250	16/32	0.13/0.13	0.28/0.28	Synergistic/synergistic
	Cefalexin	2/4	0.5/1	0.25/0.25		
	Compound 34c	2000/2000	125/250	0.06/0.13	0.56/0.63	Indifference/indifference
	Cefalexin	2/4	1/2	0.50/0.50		
MR-*S. aureus*	Nimbolide	125/250	16/32	0.13/0.13	0.26/0.26	Synergistic/synergistic
	Cefalexin	64/64	8/8	0.13/0.13		
	Desacetylnimbin	500/1000	64/125	0.13/0.13	0.26/0.38	Synergistic/synergistic
	Cefalexin	64/64	8/16	0.13/0.25		
	Compound 34a	1000/2000	500/500	0.50/0.25	0.75/0.75	Additive/additive
	Cefalexin	64/64	16/32	0.25/0.50		
	Compound 34b	4000/4000	1000/2000	0.25/0.50	0.75/1.0	Additive/indifference
	Cefalexin	64/64	32/32	0.50/0.50		
	Compound 34c	4000/8000	2000/4000	0.50/0.50	1.0/1.5	Indifferent/indifference
	Cefalexin	64/64	32/64	0.50/1.0		

[1]The MIC and MBC of compounds with cefalexin

[2]The fractional inhibitory concentration index (FIC index)

[3]The fractional bactericidal concentration index (FBC index)

Significant FICI/FBCI values are shown in bold

4.6.5. Time-kill study

The antibacterial effect of nimbolide, desacetylnimbin and the amide derivatives of nimbolide with antibiotics against test bacteria were confirmed by time-kill curve experiments. The time-kill assay was conducted to determine the rates of killing of test bacteria when exposed to test compounds with cefalexin or cefazolin.

When the test compounds with cefalexin or cefazolin was tested against *S. aureus* (fig. 4.17), maximum reduction in the colony count was observed between 6 and 12 h when compared to the control. Between 24 and 48 h, almost complete bactericidal effect was observed with treatments of combination of nimbolide and cefazolin. In the case of *S. epidermis* maximum reduction was observed between 2 and 6 h. Here also significant effect was noted for the combination of nimbolide and cefazolin. Similar pattern of results were observed for *K. pneumoniae.* Regrowth was observed for the compounds and antibiotics alone after 24 h, whereas it was not observed for the combination even at 48 h. In the case of *P. aeruginosa*, maximum reduction was observed between 4 and 6 h. For this organism, complete reduction in the colony count was observed after 24 h. In the case of MR-*S. aureus* maximum reduction was observed between 12 and 24 h. Interestingly, amide derivatives of nimbolide in combination with antibiotics recorded less activity when compared with that of nimbolide and desacetylnimbin. Moreover, regrowth was observed for the amide derivatives of nimbolide in combination with antibiotics after 24 h.

4.6.6. Cytotoxicity test against normal human cell lines

The cytotoxic activity of the compounds was tested against H9c2 (rat embryonic cardiomyoblasts) cell line by MTT assay. Fig. 4.18 represents cytotoxicity of compounds expressed in bar graph (control, 10, 25, 50, 100 and 200 µM) and corresponding confocal image of H9c2 cells (control, 100 and 200 µM). The results showed that there is no significant cytotoxicity up to 200 µM except in the case of compound **34c** (fig. 4.18). Compound **34c** recorded slight cytotoxicity at 200 µM concentration and approximately 16% of cells were dead at this concentration, which indicated that this compound may have slight toxicity towards normal cells. Cells were found to be viable in presence of all other tested compounds. These results of the present study clearly indicated that the compounds may be safe for the normal human cells. *These studies were done in collaboration with Dr. Raghu K G and Dr. Vandana Sankar, Agroprocessing and Natural Products Division, CSIR-NIIST.*

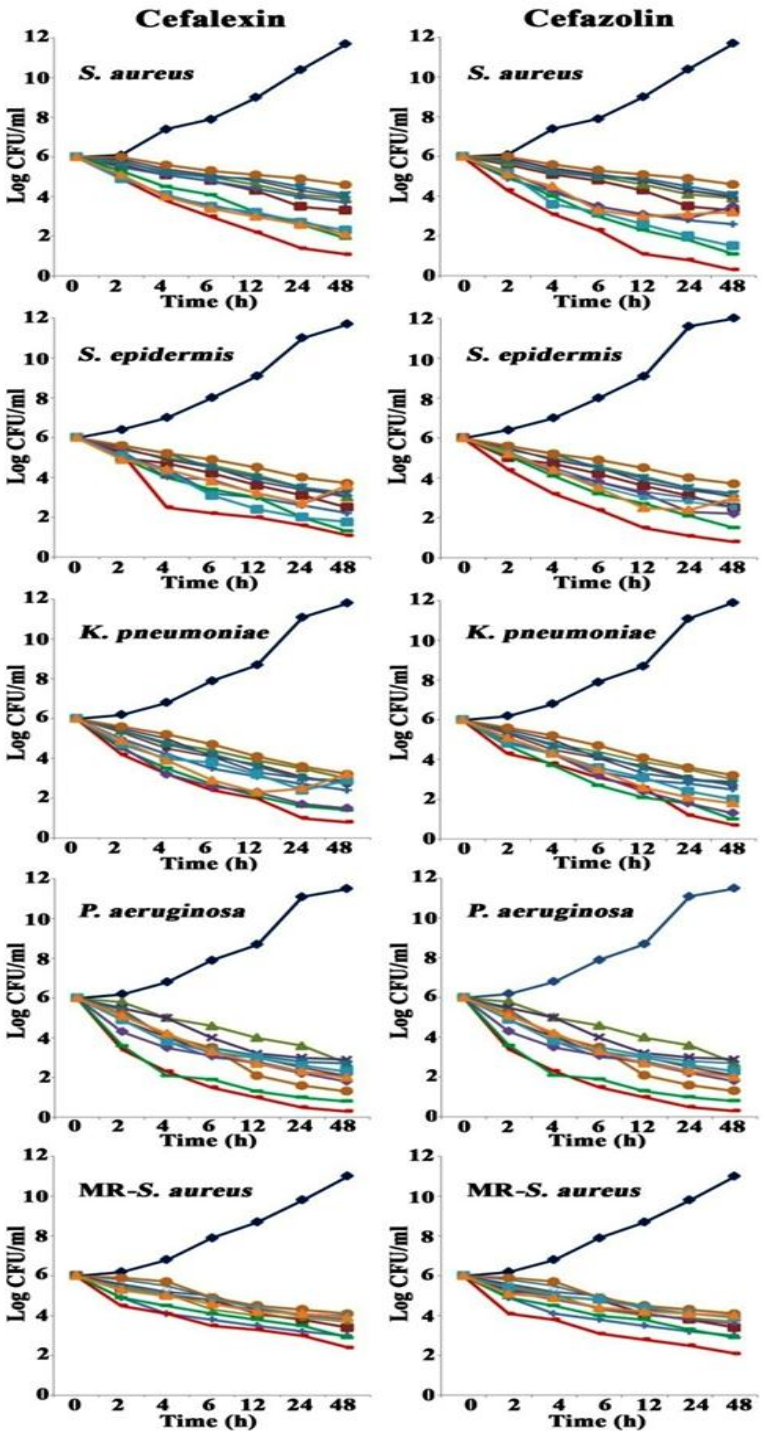


Figure 4.17: Time-kill curve of the compounds and antibiotics alone and in combination against bacterial species. control, **32**, **33**, **34a**, **34b**, **34c**, corresponding antibiotics, **32** plus corresponding antibiotics, **33** plus corresponding antibiotics, **34a** plus corresponding antibiotics, **34b** plus corresponding antibiotics, and **34c** plus corresponding antibiotics. Data are expressed as mean ± standard deviation. The X-axis represents time, and the Y-axis represents logarithmic bacterial survival.

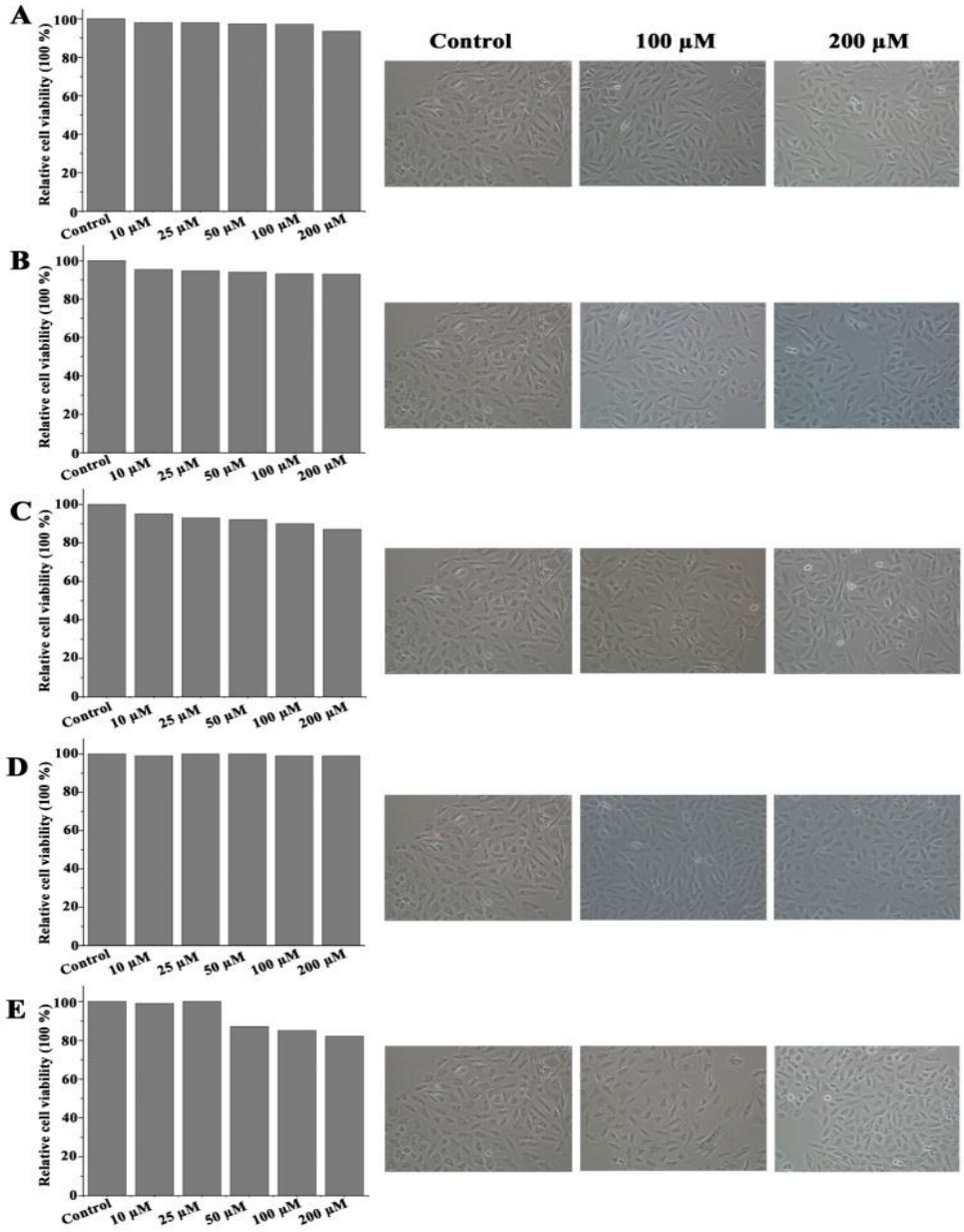


Figure 4.18: Cytotoxicity of the compounds on H9c2 cell line conducted by MTT assay; A- **32**, B- **33,** C- **34a,** D- **34b,** E- **34c**

4.7. Experimental

General experimental procedures are given in chapter 2 of this thesis. Determination of MICs and MBCs values of test compound against different wound infecting pathogens, antibacterial activity using disc diffusion method and determination of cytotoxicity against normal cell lines were performed as explained in chapter 2 of this thesis (sections **2.7.5.2**, **2.7.5.3** and **2.7.5.4** respectively).

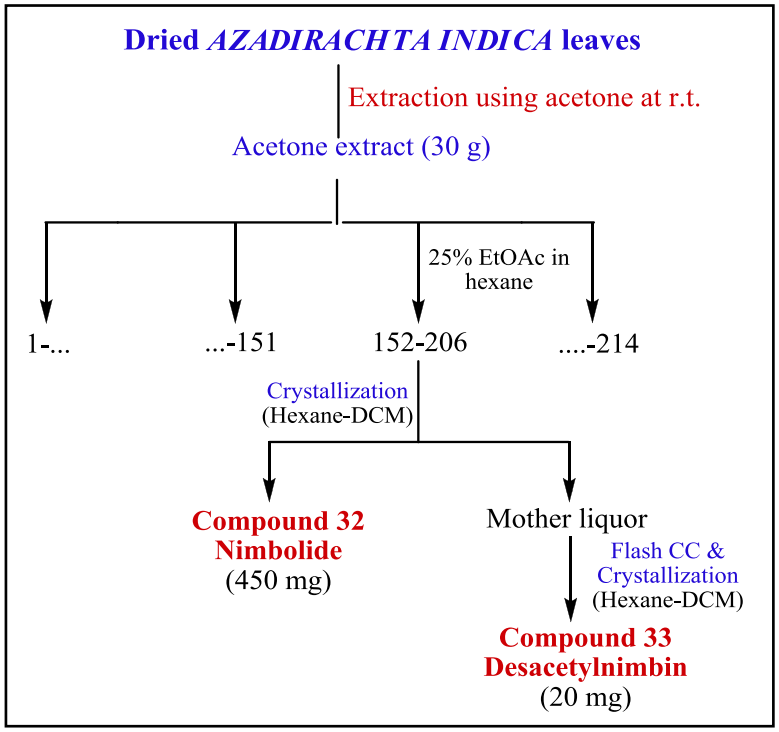
4.7.1. Extraction

Fresh leaves of *A. indica* were collected from Thiruvananthapuram district. It was then dried in an air oven maintained at 40-45 °C for five days and coarsely powdered. 2.5 kg of the powdered leaves were extracted using acetone at room temperature (2.5 L × 3). The total extract was concentrated under reduced pressure, in a rotary evaporator, to yield 160 g of the crude extract. For the ease of handling, the separation was done in five different lots.

4.7.2. Isolation of compounds

After studying the TLC of acetone extract, 30 g of the total extract was subjected to purification using silica gel (500 g, 100-200 mesh) column chromatography. Column elution was started using 5% ethyl acetate in petroleum ether and increase in polarity was carried out by increasing the amount of ethyl acetate in hexane. Final elution was carried out with 100% ethyl acetate. Pictorial representation of the isolation of compounds is shown in chart 4.2.

Chart 4.2: Pictorial representation for the isolation of nimbolide & desacetylnimbin from *A. indica* leaves



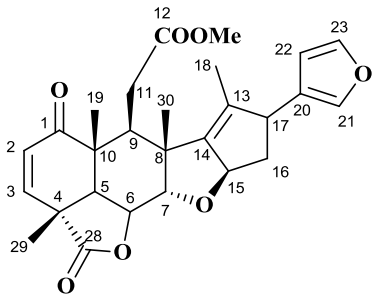
4.7.2.1. Isolation of compound 32 (nimbolide)

Different fractions of approximately 100 mL each were collected in conical flasks. A total of 214 fractions were thus collected. TLC of each fraction was checked and those fractions whose TLC profiles were alike, pooled together to get 35 different fraction pools. Fraction pool 15 (Fr. 152-206) obtained by eluting the column with 25% ethyl acetate in hexane, contained a major UV active compound. This fraction was subjected to crystallization using dichloromethane-hexane mixture. 450 mg of pure nimbolide (**32**) was obtained as pale yellow crystals. The structure of the compound was confirmed from the spectral data obtained, as shown below.

Pale yellow crystals; m.p. 244-246 ^{o}C, lit.[13] 245-247 ^{o}C

FT-IR (KBr, υ_{max}, cm^{-1}) : 2978, 1778, 1730, 1672, 1433, 1296, 1238, 1192, 1153, 1069, 951, 827, 750

^{1}H NMR (500 MHz, $CDCl_3$) : 7.32 (t, *J* = 1.5 Hz, 1H, H-23), 7.28 (d, *J* = 9.5 Hz, 1H, H-3), 7.22 (s, 1H, H-21), 6.25 (m, 1H, H-22), 5.93 (d, *J* = 10.0 Hz, 1H, H-2), 5.53 (m, 1H, H-15), 4.62 (dd, *J* = 12 .5, 3.7 Hz, 1H, H-6), 4.27 (d, *J* = 3.5 Hz, 1H, H-7), 3.67 (d, *J* = 9.0 Hz, 1H, H-17), 3.54 (s, 3H, -OMe), 3.25 (dd, *J* = 16.3, 5.0 Hz, 1H, H-11a), 3.19 (d, *J* = 12.5 Hz, 1H, H-5), 2.74 (t, *J* = 5.5 Hz, 1H, H-9), 2.38 (dd, *J* = 16.3, 5.5 Hz, 1H, H-11b), 2.22 (dd, *J* = 12.0, 6.5 Hz, 1H, H-16a), 2.10 (m, 1H, H-16b), 1.70 (s, 3H, H-18), 1.47 (s, 3H, H-29), 1.37 (s, 3H, H-30), 1.22 (s, 3H, H-19)



^{13}C NMR (125 MHz, $CDCl_3$) : δ 200.8 (C-1), 175.0 (C-28), 173.0 (C-12), 149.6 (C-3), 144.8 (C-14), 143.2 (C-23), 138.9 (C-21), 136.4 (C-13), 131.0 (C-2), 126.5 (C-20), 110.3 (C-22), 88.5 (C-15), 82.9 (C-7), 73.4 (C-6), 51.8 (-OMe), 50.3 (C-8), 49.5 (C-17), 47.7 (C-5), 45.3 (C-10),

43.7 (C-4), 41.2 (C-16), 41.1 (C-9), 32.1 (C-11), 18.5 (C-29), 17.2 (C-30), 15.2 (C-19), 12.9 (C-18)

HRMS (m/z) : 467.2079 $[(M+H)^+]$; $C_{27}H_{30}O_{7,}$ requires 466.1992

NMR spectral assignments were made on the basis of DEPT-135, 2D NMR and by comparison with literature reports.[14]

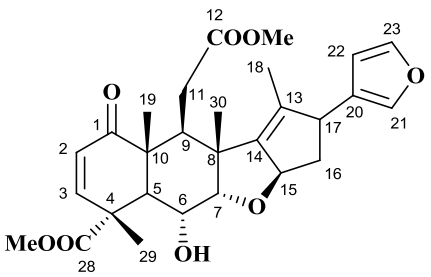
4.7.2.2. Isolation of compound 33 (desacetylnimbin)

The mother liquor left after the crystallization of nimbolide from the above fraction pool (Fr. 152-206) was found to contain another UV active compound in it. Therefore, it was subjected to flash column chromatographic purification using prepacked silica cartridge. Column chromatographic purification followed by crystallization using dichloromethane-hexane solvent system yielded 20 mg of colourless crystals. Compound **33** was confirmed to be desacetylnimbin from the spectral data obtained, as shown below.

Colourless crystals; m.p. 208-210 ^oC, lit.[28] 210-212 ^oC

FT-IR (KBr, υ_{max}, cm^{-1}) : 3574, 1771, 1731, 1681, 1547, 1536, 1435, 1397, 1257, 1228

^1H NMR (500 MHz, $CDCl_3$) : δ 7.33 (t, *J* = 1.5 Hz, 1H, H-23), 7.24 (s, 1H, H-21), 6.41 (d, *J* = 10.5 Hz, 1H, H-3), 6.33 (d, *J* = 1.5 Hz, 1H, H-22), 5.86 (d, *J* = 10.5 Hz, 1H, H-2), 5.55 (m, 1H, H-15), 4.03 (d, *J* = 3.5 Hz, 1H, H-7), 3.93 (m, 1H, H-6), 3.71 (s, 3H, -OMe), 3.68 (m, 1H, H-17), 3.66 (s, 3H. -OMe), 3.40 (d, *J* = 12.0 Hz, 1H, H-5), 2.90 (dd, *J* = 16.5, 5.5 Hz, 1H, H-11a), 2.77 (m, 1H, H-9), 2.20 (m, 2H, H-11b, H-16a), 2.04 (m, 2H, H-16b, -OH), 1.69 (s, 3H, H-18), 1.61 (s, 3H. H-29), 1.29 (s, 3H, H-30), 1.22 (s, 3H, H-19)



^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 201.6 (C-1), 175.6 (C-28), 173.7 (C-12), 148.1 (C-3), 146.8 (C-14), 143.1 (C-23), 139.0 (C-21), 134.9 (C-13), 126.8 (C-20), 126.4 (C-2), 110.4 (C-22), 87.4 (C-15), 86.9 (C-7), 66.2 (C-6), 53.0 (-OMe), 51.7 (-OMe), 49.6 (C-17), 47.8 (C-4), 47.5 (C-10), 47.3 (C-8), 43.9 (C-5), 41.4 (C-16), 39.1 (C-9), 34.4 (C-11), 17.5 (C-30), 17.2 (C-29), 16.4 (C-19), 12.9 (C-18)
HRMS (m/z)	:	499.2343 [$(M+H)^+$]; $C_{28}H_{34}O_8$, requires 498.2254

NMR spectral assignments were made on the basis of DEPT-135, 2D NMR and by comparison with literature reports.[30]

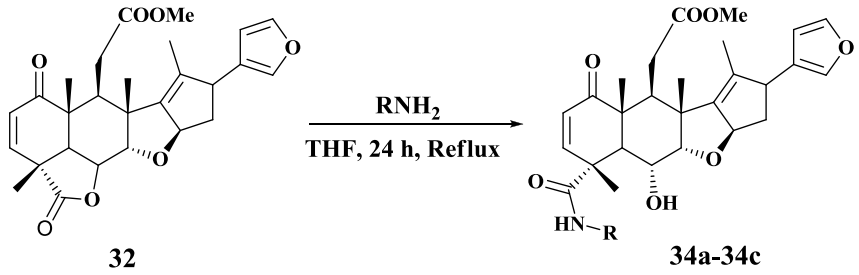
For carrying out the detailed *in vitro* and *in vivo* anticancer studies, nimbolide was required in larger quantities. So the isolation procedure for nimbolide was repeated for four more times to get a total of 2.3 g of pure nimbolide.

4.7.3. Antimicrobial activity of nimbolide and its derivative

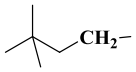
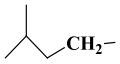
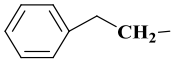
4.7.3.1. Synthesis of amide derivatives of nimbolide (34a-34c)

In a typical experiment, nimbolide (100 mg) was dissolved in dry THF and to this 3.5 equivalents of respective amine was added and the contents were refluxed under stirring for a period of 24 h. Progress of the reaction was monitored using TLC. After completion of the reaction THF was removed under reduced pressure and the product was isolated in good yields using silica gel column chromatography by eluting the column with 50% ethyl acetate in hexane. Three different amide derivatives of nimbolide were synthesized and structures of the compounds were confirmed using various spectroscopic techniques. Amines used were purchased from Sigma Aldrich and general scheme of the reaction, amines used and their respective yields are given in table 4.6.

Table 4.6: General scheme and amines used for the synthesis of amide derivatives



32 **34a-34c**

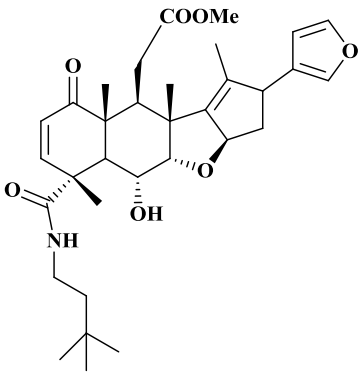
Sl. No.	Amine used	R	Yield (%)
34a	3,3-dimethylbutamine		52
34b	3-methylbutamine		62
34c	2-phenylethylamine		82

Spectral details of Methyl-2-((2*R*,3a*R*,4a*S*,5*R*,6*R*,9a*R*,10*S*,10a*R*)-6-(3,3-dimethylbutyl carbamoyl)-2-(furan-3-yl)-5-hydroxy-1,6,9a,10a-tetramethyl-9-oxo-3,3a,4a,5,5a,6,9,9a, 10,10a-decahydro-2H-cyclopenta[d]naphtho[2,3-b]furan-10-yl)acetate (**34a**)

Pale yellow crystals; m.p. 273-275 ^{o}C

FT-IR (KBr, υ_{max}, cm^{-1}) : 3558, 3380, 2952, 1739, 1680, 1640, 1532, 1442, 1371, 1263, 1163, 1062, 1029

^{1}H NMR (500 MHz, $CDCl_3$) : δ 7.33 (s, 1H), 7.23 (s, 1H), 6.36 (d, *J* = 10 Hz, 1H), 6.32 (s, 1H), 5.87 (d, *J* = 10 Hz, 1H), 5.71 (t, *J* = 5.5 Hz, 1H), 5.54 (t, *J* = 7Hz, 1H), 4.01 (d, *J* = 3 Hz, 1H), 3.94 (m, 1H), 3.67 (brs, 1H), 3.65 (s, 3H), 3.35 (d, *J* = 11.5 Hz, 1H), 3.25 (m, 2H), 2.88 (dd, *J* = 16.5, 5.5 Hz, 1H), 2.78 (t, *J* = 5 Hz, 1H), 2.48 (d, *J* = 10.5 Hz, 1H), 2.22 (dd, *J* = 16.5, 3.5 Hz, 1H), 2.18 (m, 1H), 2.04 (m , 1H), 1.68 (s, 3H), 1.59 (s, 3H), 1.40 (t, *J* = 8 Hz, 2H), 1.29 (s, 3H), 1.24



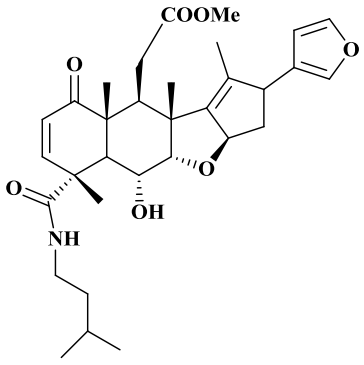
(s, 3H) 0.92 (s, 9H)

^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 202.4, 175.0, 173.6, 149.2, 146.9, 143.1, 139.0, 134.8, 126.9, 126.3, 110.4, 87.7, 86.8, 66.4, 51.7, 49.6, 48.1, 47.4, 47.3, 43.7, 43.0, 41.4, 39.0, 37.2, 34.5, 29.9, 29.2 (3C), 17.5, 16.9, 16.6, 12.9
HRMS (m/z)	:	568.3244 [$(M+H)^+$]; $C_{33}H_{45}NO_7$, requires 567.3196

Spectral details of Methyl-2-((2*R*,3a*R*,4a*S*,5*R*,6*R*,9a*R*,10*S*,10a*R*)-2-(furan-3-yl)-5-hydroxy-6-(isopentylcarbamoyl)-1,6,9a,10a-tetramethyl-9-oxo-3,3a,4a,5,5a,6,9,9a,10,10a-decahydro-2H-cyclopenta[d]naphtho[2,3-b]furan-10-yl)acetate (**34b**)

Pale yellow crystals; m.p. 259-261 ^oC

FT-IR (KBr, υ_{max}, cm^{-1})	:	3561, 3377, 2954, 1739, 1680, 1642, 1531, 1442, 1375, 1262, 1162,1198, 1062, 1029
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 7.33 (s, 1H), 7.23 (s, 1H), 6.36 (d, *J* = 10 Hz, 1H), 6.32 (s, 1H), 5.86 (d, *J* = 10 Hz, 1H), 5.77 (m, 1H), 5.53 (t, *J* = 7 Hz, 1H), 4.01 (d, *J* = 3 Hz, 1H), 3.93 (t, *J* = 4 Hz, 1H), 3.67 (brs, 1H), 3.65 (s, 3H,), 3.35 (d, *J* = 11.5 Hz, 1H), 3.25 (m, 2H), 2.89 (dd, *J* = 17.5, 5.5 Hz, 1H), 2.77 (t, *J* = 5.0 Hz, 1H), 2.46 (d, *J* = 10 Hz, 1H), 2.23 (dd, *J* = 16.5, 4.0 Hz, 1H), 2.17 (m, 1H), 2.04 (m, 1H), 1.69 (s, 3H), 1.60 (m, 1H), 1.59 (s, 3H), 1.39 (m, 2H), 1.29 (s, 3H), 1.24 (s, 3H), 0.91 (d, *J* = 6.5 Hz, 6H)



^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 202.4, 175.0, 173.6, 149.3, 146.9, 143.0, 139.0, 134.8, 126.9, 126.3, 110.5, 87.7, 86.8, 66.4, 51.6, 49.6, 48.1, 47.5, 47.3, 43.7, 41.4, 39.0, 38.7, 38.1, 34.5, 25.9,

22.5, 22.4, 17.5, 16.9, 16.6, 12.9

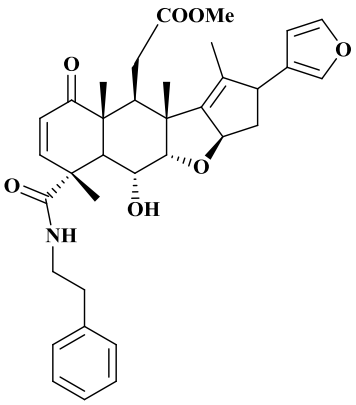
HRMS (m/z) : 576.2942 [$(M+Na)^+$]; $C_{32}H_{43}NO_7$, requires 553.3040

Spectral details of Methyl-2-((2*R*,3*aR*,4a*S*,5*R*,6*R*,9a*R*,10*S*,10a*R*)-2-(furan-3-yl)-5-hydroxy-1,6,9a,10a-tetramethyl-9-oxo-6-(phenethylcarbamoyl)-3,3a,4a,5,5a,6,9,9a,10, 10a-decahydro-2H-cyclopenta[d]naphtho[2,3b]furan-10-yl) acetate (**34c**)

Pale yellow crystals; m.p. 202-204 ^oC

FT-IR (KBr, υ_{max}, cm^{-1}) : 3547, 3379, 2929, 1735, 1678, 1648, 1529, 1444, 1374, 1262, 1163, 1062, 1026

^1H NMR (500 MHz, $CDCl_3$) : δ 7.31 (m, 3H), 7.21 (m, 4H), 6.33 (m, 1H), 6.24 (d, *J* = 10 Hz, 1H), 5.86 (t, *J* = 5 Hz, 1H), 5.80 (d, *J* = 10 Hz, 1H), 5.53 (m, 1H), 4.00 (d, *J* = 3.5 Hz, 1H), 3.93 (dd, *J* = 12.0, 3.0 Hz, 1H) 3.67 (s, 1H), 3.64 (s, 3H), 3.56 (m, 1H), 3.45 (m, 1H), 3.34 (d, *J* = 11.5 Hz, 1H), 2.88 (dd, *J* = 15.0, 5.5 Hz, 1H), 2.82 (m, 2H), 2.21 (m, 3H), 2.05 (m, 2H), 1.51 (s, 3H), 1.28 (s, 3H), 1.26 (s, 3H), 1.21 (s, 3H)



^{13}C NMR (125 MHz, $CDCl_3$) : δ 202.4, 175.1, 173.6, 149.2, 146.9, 143.1, 139.0, 138.9, 134.8, 128.9 (2C), 128.7 (2C), 126.9, 126.6, 126.2, 110.5, 87.7, 86.8, 66.4, 51.6, 49.6, 48.1, 47.5, 47.4, 43.6, 41.5, 41.4, 39.0, 35.4, 34.5, 17.5, 16.8, 16.5, 12.9

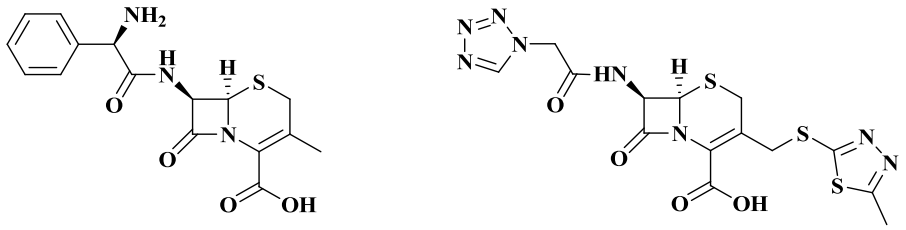
HRMS (m/z) : 588.2930 [$(M+H)^+$]; $C_{35}H_{41}NO_7$, requires 587.2883

4.7.3.2. Test microorganisms

The following four major wound associated medically important bacteria were used in the present study - *Staphylococcus aureus, Staphylococcus epidermis* MTCC 435, *Klebsiella pneumoniae* MTCC 109 and *Pseudomonas aeruginosa* MTCC 2642. The study was also carried out on the Methicillin resistant *Staphylococcus aureus* (MR-*S. aureus*) ATCC 43300. All the strains were cultured at 37 °C on nutrient agar (NA) medium and stored at 4 °C. All the test microorganisms except MR-*S. aureus* were purchased from Microbial Type Culture Collection Centre, IMTECH, Chandigarh, India. MR-*S. aureus* was gifted by Dr. Pratap Chandran, Associate Professor, SDV College of Arts and Applied Science, Alappuzha, Kerala.

4.7.3.3. Antibiotics used

The following two first generation cephalosporin antibiotics purchased from Sigma-Aldrich, USA, were used in this study–Cefalexin (97% pure) and Cefazolin (98% pure). Structures of the compounds are given in fig. 4.19.



Cefalexin **Cefazolin**

Figure 4.19: Chemical structure of antibiotics used

4.7.3.4. Determination of the *in vitro* synergistic activity by checkerboard assay

The antimicrobial effects of different combinations of two or more antimicrobial agents were assessed using the checkerboard test.[78] The antimicrobial assays were performed with the two natural limonoids and three amide derivatives of nimbolide in combination with antibiotics. The inocula were prepared from colonies that had been grown on Mueller Hinton agar (MHA) overnight. The final bacterial concentration after inoculation was 2×10^5 CFU/mL. The MIC was determined after 24 h incubation at 37 °C. The MIC was defined as the lowest concentration of the test compounds, alone or in combination with antibiotics, visibly inhibiting the growth of bacteria by measuring the OD at 600 nm using a microplate reader (Bio-rad, USA). Each experiment was repeated thrice. To assess the *in vitro* interaction of combinations of

test compounds (A) and antibiotics (B), the results obtained were further analyzed using the fractional inhibitory concentration (FIC) index. The fractional inhibitory concentration and the fractional bactericidal concentration are mathematical expressions of the effect of the combination of antibacterial agents. The interaction of two compounds was regulated as synergy, indifferent or antagonistic on the basis of the FIC index values. The FIC values were calculated as follows: FIC of test compounds (FIC_A) = (MIC of A in combination with B)/ (MIC of A alone), where A and B were mixed in the ratio 7:3.

FIC of antibiotics (FIC_B) = (MIC of B in combination with A)/ (MIC of B alone), where A and B were mixed in the ratio 4:6

The sum of fractional inhibitory concentration indices (FICI) of two compounds (A and B) in the combination was calculated as follows:

$FIC_A + FIC_B = FICI$.

A synergistic relationship was defined as FIC index ≤ 0.5, an additive relationship was defined as 0.5 < FIC index ≤ 1.0, an indifferent relationship was defined as 1.0 < FIC index ≤ 4.0, and an antagonistic relationship was defined as FIC index >4.0.

4.7.3.5. Killing curve determination

Killing curve determination was carried out in order to confirm the antibacterial and synergistic activities of the two natural limonoids and three amide derivatives of nimbolide when used singly and in combination with antibiotics. The viabilities of bacteria after exposure to these agents alone and in combination at seven distinct times (0, 2, 4, 6, 12, 24 and 48 h) were counted. The assay was followed by the previously described method with some modifications.[79] Concisely, inocula (5×10^5 CFU/mL) were exposed to the test compounds either singly or in combination with antibiotics. Aliquots (0.1 mL) of each exposed time were removed from tubes and diluted in normal saline as needed to enumerate 30–300 colonies. The diluted cultures were platted and spread thoroughly on plates containing MHA. After incubating at 35 °C for 18 h, the growing colonies were counted. The lowest detectable limit for counting is 10^3 CFU/mL. The experiment was performed in triplicate; data are shown as mean ± SD.

4.8. Conclusion

The major bioactive limonoid, nimbolide, was isolated from *A. indica* leaves along with the minor compound desacetylnimbin. Anticancer activity studies of nimbolide on colon cancer cell lines were carried out using both *in vitro* and *in vivo* methods. It has been found that the compound inhibited proliferation, induced apoptosis and suppressed NF-kB activation and NF-kB–regulated tumorigenic proteins in colorectal cancer cells. *In vivo* studies revealed that nimbolide (at 5 and 20 mg/kg body weight), injected intraperitoneally after tumor inoculation significantly decreased the volume of colorectal cancer xenografts. The nimbolide-treated xenografts exhibited significant downregulation in the expression of proteins involved in tumor cell survival, proliferation, metastasis and angiogenesis. The limonoid nimbolide was found to be bioavailable in the blood plasma and tumor tissues of treated mice (*Clin. Cancer Res.*, **2013**, *19*, 4465-4476).

Antibacterial effect of nimbolide, desacetylnimbin and the amide derivatives of nimbolide and their synergistic effect when in combination, with first generation cephalosporin antibacterial agents were recorded. Nimbolide showed the highest synergistic effect with the tested antibiotics, followed by desacetylnimbin. Amide derivatives were found to be less potent when compared to the two natural limonoids. Interestingly these natural limonoids with antibiotics recorded significant synergy against MR-*S. aureus*, an important drug resistant bacteria. These results may be applied in the near future in alternative therapies for the diseases caused by wound associated pathogenic strains (*RSC Adv.*, **2015**, *5*, 89503-89514).

4.9. References

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Chapter 5

Isolation and Anticancer Activity Studies of the Major Sesquiterpene Lactones from *Elephantopus scaber*

5.1. Introduction

Asteraceae is one of the largest and wide spread families of flowering plants consisting of more than 1,620 genera and 23,600 species of herbs, shrubs and trees.[1] Elephantopus is one of the important genus belonging to Asteraceae family. It is distributed all over the world and consists of approximately 32 species, including *E. angustifolius, E. carolinianus, E. mollis, E. scaber, E. tomentosus* etc. However, among these, *Elephantopus scaber* (fig. 5.1) is the only species known to occur in India.

Figure 5.1: *Elephantopus scaber*

E. scaber is commonly known as 'Prickly-leaved elephant's foot' in English, 'Anachuvadi' in Malayalam and 'Gojihva' in Sanskrit. It is a rigid herb with large obviate, oblong, radical leaves forming a rosette and numerous flower heads.[2] The whole plant is used for medicinal purposes. In India, it is extensively used in traditional forms of medicine to reduce fever and to eliminate bladder stones as well as to treat nephritis, edema, chest pain, scabies and leukemia.[3,4] The root is given to arrest vomiting, diarrhoea and bruised leaves boiled in coconut oil are applied to ulcers and eczema.[5] In Chinese medicine, the extract of this plant is used as an antidiuretic, antiviral and antibacterial agent as well as in the treatment of hepatitis, bronchitis, cough associated with pneumonia and joint pain.[6] People in India use the decoction of whole plant to treat gonorrhoea and colic pain. In Nepal, the plant is used to treat blood vomiting in tuberculosis. In Thailand, it is used in the treatment of cough and in Taiwan it is used to treat hepatitis.[7]

5.1.1. Phytochemistry of *Elephantopus scaber*

Sesquiterpene lactones are one of the characteristic constituents of the family Asteraceae and their principal characteristic is the presence of α,β-unsaturated-γ-lactone.[8] Both alcoholic and chloroform extracts of *E. scaber* has been reported to contain cytotoxic germacranolide type sesquiterpene lactones in it.[9,10] Isodeoxyelephantopin, deoxyelephantopin,[11] molephantinin,[12] 11,13-dihydro deoxyelephantopin,[13] 17,19-dihydrodeoxyelephantopin, iso-17,19-dihydrodeoxy elephantopin,[10] scabertopinol,[14] scabertopin, isoscabertopin,[15] etc., are the important germacranolide type sesquiterpene lactones isolated from *E. scaber* (structures of the compounds are shown in chart 5.1) and elescaberin[16] is the elamanolide type sesquiterpene lactone isolated from the plant. Sesquiterpene lactones have received considerable attention for their cytotoxic and antitumor properties in recent years.[17] Apart from sesquiterpene lactones, there are many flavonoids, flavonoid esters and triterpenes present in this plant.[18, 19]

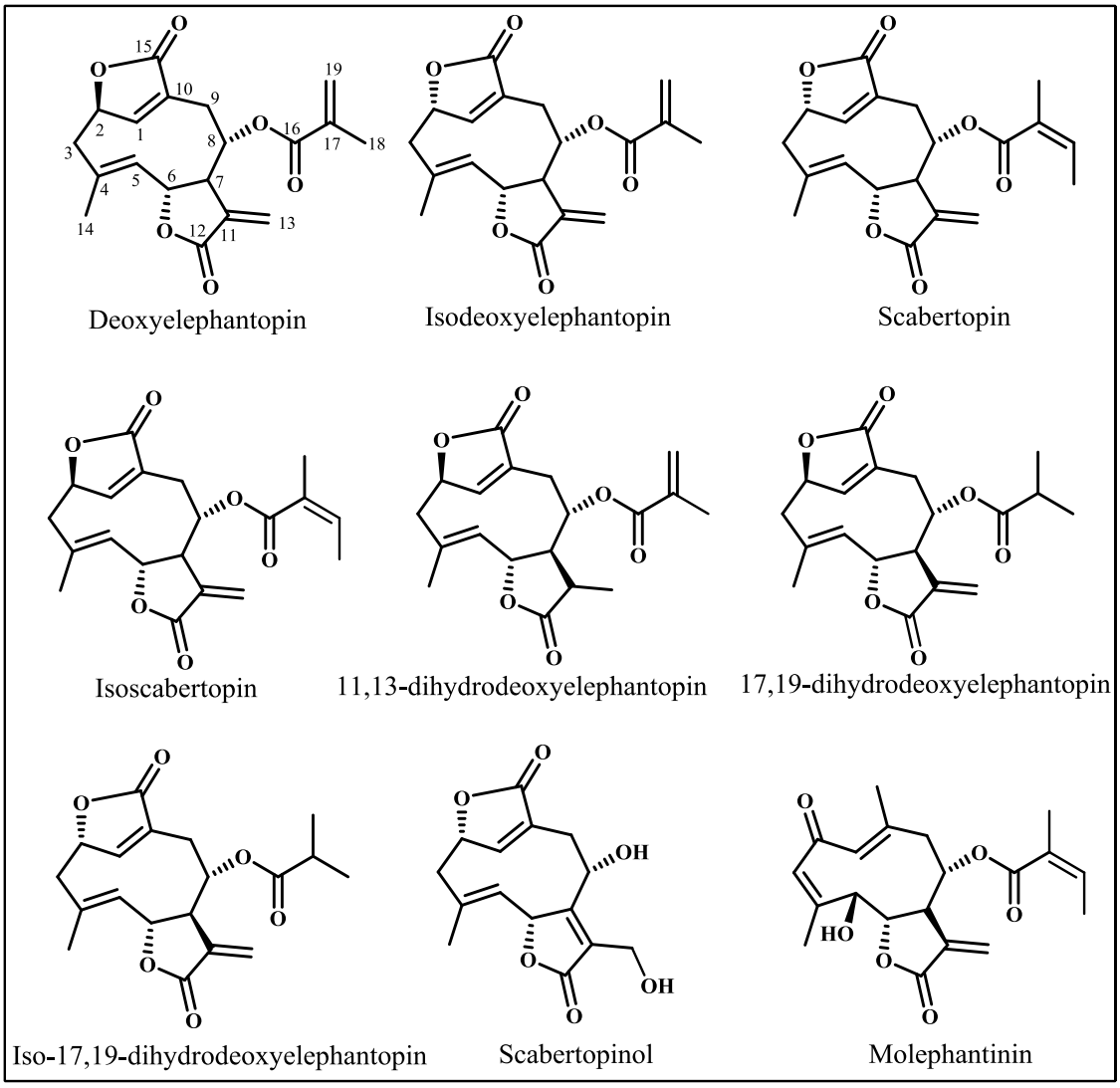
5.1.2. Literature reports on anticancer activity studies of deoxyelephantopin and isodeoxyelephantopin

Previous studies have indicated that both isodeoxyelephantopin and deoxyelephantopin possess potential anti-tumor effect on several cancer cell lines such as cervical carcinoma - HeLa, colon carcinoma - Caco-2 and hepatocarcinoma-SMMC7721.[15] Deoxyelephantopin was found to be very effective against breast cancer (TS/A, MDA-MB-231 and MCF-7)[20] and nasopharyngeal cell lines (CNE).[21] Earlier reports from our laboratory suggested that both isodeoxyelephantopin and deoxyelephantopin acted through blocking the tumor necrosis factor (TNF)-induced nuclear factor-κB (NF-κB) activation. Isodeoxyelephantopin was found to be much more potent than deoxyelephantopin and the suppression was not cell type specific. Isodeoxyelephantopin potentiates apoptosis, inhibits invasion and abolishes osteoclastogenesis through suppression of NF-κB activation and NF-κB-regulated gene expression.[22] In a different study, both these compounds have been found to reduce the viability of murine fibroblast cell lines (L-929) in a dose and time dependent manner.[23]

Anticancer activities of these compounds could be attributed to the presence of α-methylene-γ-lactone system, which can form stable adducts through Michael type addition reaction with biological nucleophiles, especially with the cysteine residue of proteins (which contains the thiol group). Furthermore, the cytotoxic activities of these

compounds are enhanced by the presence of conjugated ester side chain at C-8 position, which is one of the alkylating centres in the molecule.[8]

Chart 5.1: Structures of germacranolide type sesquiterpene lactones isolated from *E. scaber*



5.2. Aim and scope of the present study

The above discussion clearly portrays that isodeoxyelephantopin and deoxyelephantopin are emerging as promising leads for the development of new chemotherapeutic agents in the fight against cancer. Due to our continuous interest in the area of anticancer activities of these two sesquiterpene lactones, isolation and further anticancer activity studies of isodeoxyelephantopin and deoxyelephantopin from *E. scaber* were also carried out during this Ph.D. period.

5.3. Isolation and characterization of major compounds from *Elephantopus scaber*

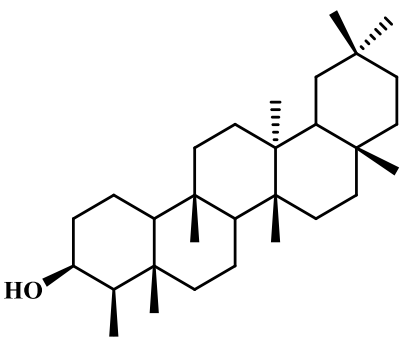
5.3.1. Collection of plant material and extraction

About 2 kg of *E. scaber* as whole plants were collected from Palode in Thiruvananthapuram district, Kerala. The plant material was then cleaned, dried and coarsely powdered. Approximately 1 kg of the powdered material was subjected to repeated extraction using chloroform (3 L × 3) at room temperature. The total extract was then concentrated under reduced pressure to yield about 28 g of the crude extract.

5.3.2. Isolation and characterization of phytochemicals

After studying the TLC, 28 g/(total) of the extract was subjected to column chromatographic purification using silica gel (100-200 mesh). Column elution was started using 100% hexane and increase in polarity was carried out by increasing the amount of ethyl acetate in the mixture. Different fractions of approximately 150 mL each were collected. A total of 388 fractions were thus collected. TLC of each fraction was checked and those fractions whose TLC profiles were alike were pooled together.

TLC of fractions 50-69 obtained by eluting the column with 5% ethyl acetate in hexane showed an iodine active spot along with some other impurities. These fractions were mixed together and then re-purified using silica gel column chromatography (100-200 mesh). Column elution was started using 100% hexane. Subfractions 24-30 obtained by eluting the column with 8% ethyl acetate in hexane on crystallization yielded 210 mg of compound **35**. Colourless crystals of compound **35** thus obtained was characterized as the phytosterol, **epifriedelanol** on the basis of various spectral data, the single crystal X-ray analysis (fig.5.2) and on comparison with data reported in literature.[24,25,26] The structure of the compound is shown below.



Compound **35 - Epifriedelanol**

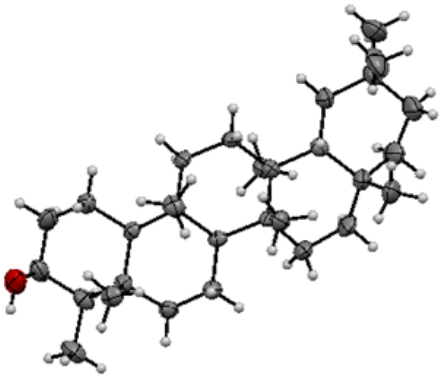
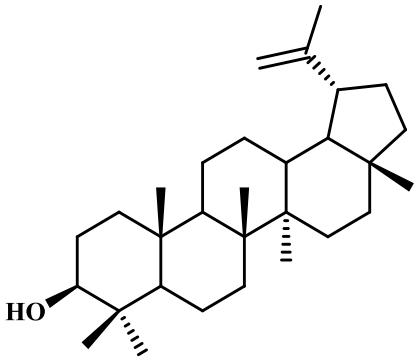


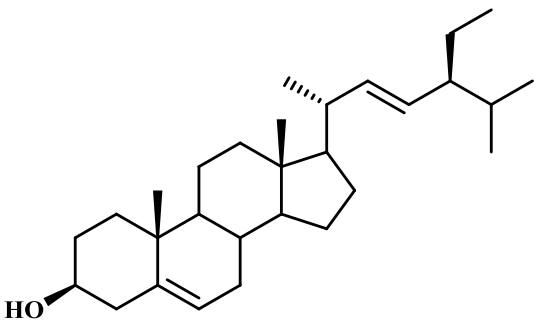
Figure 5.2: ORTEP diagram of epifriedelanol

TLC of fractions 70-78 obtained by eluting the column with 10% ethyl acetate in hexane showed the presence of a single compound, which on crystallization in hexane yielded 157 mg of compound **36** as white crystals. This was characterized as the phytosterol, **lupeol**, using various spectroscopic data and on comparison with data reported in literature.[27] The structure of the compound is shown below.



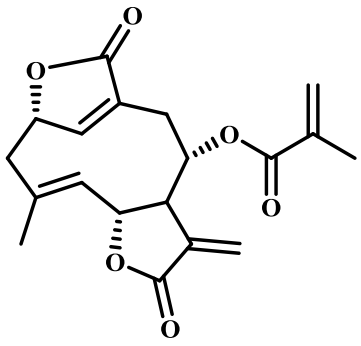
Compound **36 - Lupeol**

TLC of fractions 79-89 obtained by eluting the column with 10% ethyl acetate in hexane showed the presence of iodine absorbing spots in it. This fraction was subjected to another column chromatographic separation, using a different solvent system, for further purification. Column elution was started using 100% dichloromethane (DCM). Subfractions 14-19 obtained by eluting the column with 100% DCM on crystallization yielded 127 mg of compound **37.** The compound was found to be the phytosterol, **stigmasterol**, by using various spectroscopic techniques and by comparison with data reported in literature.[28] The structure of the compound is shown below.



Compound **37 - Stigmasterol**

Fractions 265-287 obtained by eluting the column with 40% ethyl acetate in hexane was found to contain a UV active compound with minor impurities. This fraction on further purification using flash column chromatography followed by crystallization of the compound yielded 232 mg of white needle like crystals of compound **38**. The IR spectrum of the compound showed absorption at 1765, 1750 and 1714 cm^{-1}, which indicated the presence of three different types of carbonyl groups as in an *α*-methylene-*γ*-lactone, *α,β*- unsaturated lactone and an *α,β*-unsaturated ester respectively. The absorptions at 3075, 1637, and 885 cm^{-1} indicated the presence of exocyclic double bond. ^{1}H NMR spectrum (fig. 5.3) showed two doublets (with very small *J* value), each integrating for three protons, at δ 1.93 and 1.79 suggesting the presence of two methyl groups attached to olefinic carbons. Signals at δ 7.13 and 5.38 could be ascribed to the protons of *α,β*- unsaturated lactone. Doublets at δ 6.21 and 5.65 could be attributed to the methylene protons of *α*-methylene-*γ*-lactone ring system. Methylene protons of methacrylate ester side chain resonated at δ 6.15 and 5.67. Another olefinic proton at H-5 resonated as a multiplet at δ 6.15. From the ^{13}C NMR spectrum (fig. 5.4), it was evident that the compound contained nineteen carbons, of which the signals at δ 174.3, 169.5 and 166.6 could be attributed to carbonyl carbons of an *α*-methylene-*γ*-lactone, *α,β*-unsaturated lactone and *α,β*-unsaturated ester moiety respectively. The spectrum also showed signals attributed to the eight olefinic carbons at δ 149.2, 135.5, 135.3, 134.0, 131.6, 126.9, 125.5 and 123.2. The signals at δ 79.5, 78.7 and 74.1 were suggestive of carbon attached to oxygen atoms. Mass spectrum of the compound showed molecular ion peak at m/z 367.1166, which is the $(M+Na)^{+}$ peak. Consolidating all the spectral details and on comparison with the literature,[11] compound **38** was found to be **isodeoxyelephantopin**. The structure of the compound is as given below.



Compound **38 - Isodeoxyelephantopin**

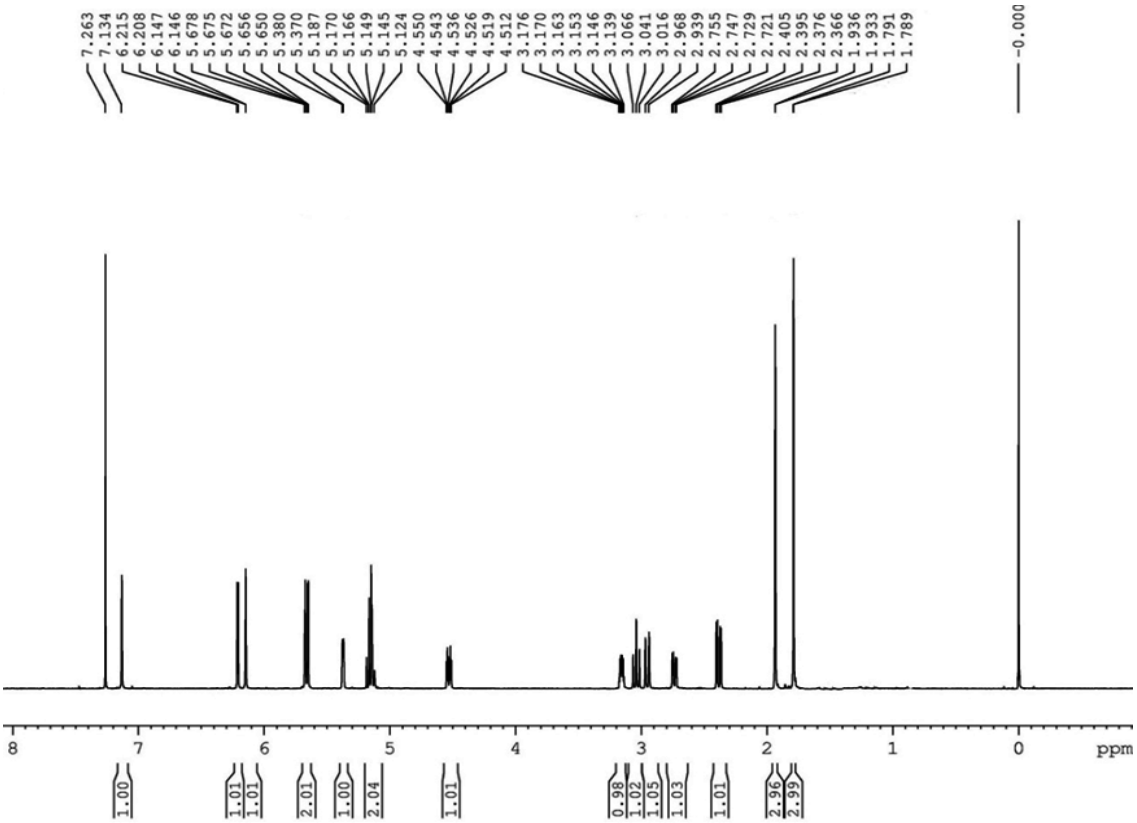


Figure 5.3: ^{1}H NMR spectrum of isodeoxyelephantopin

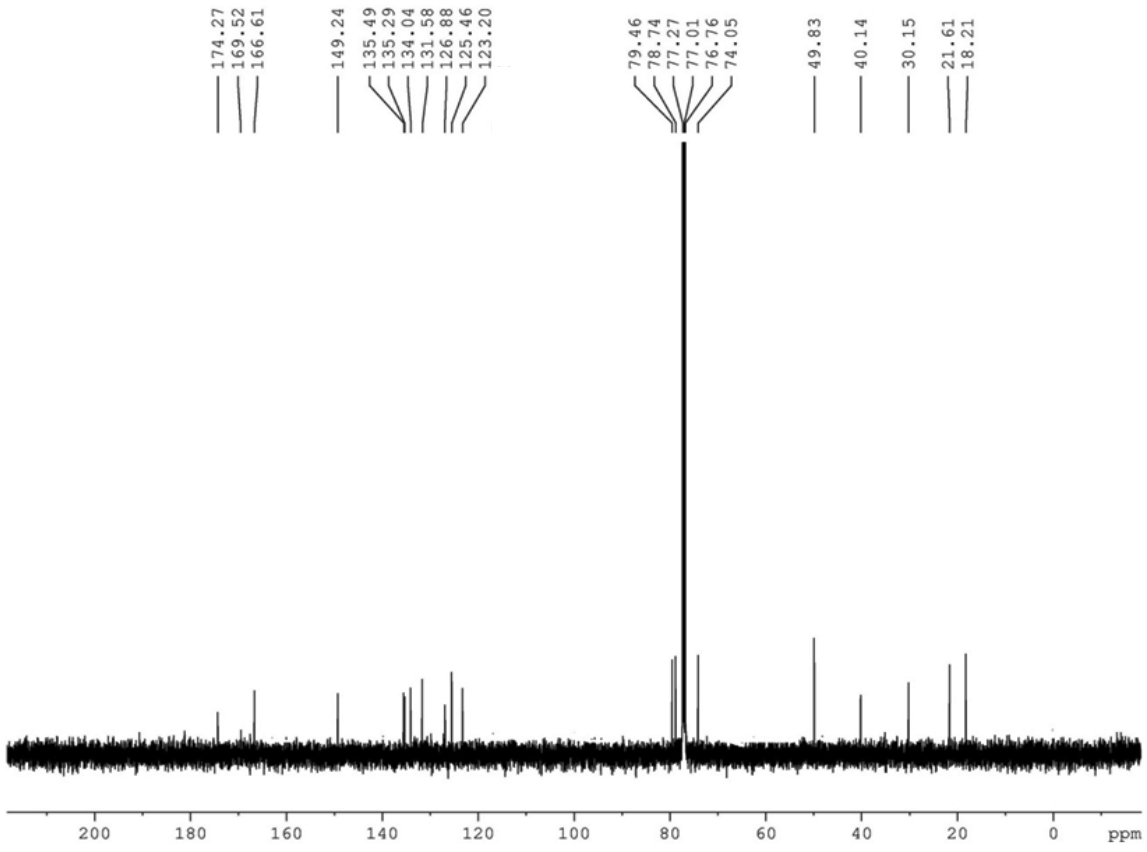
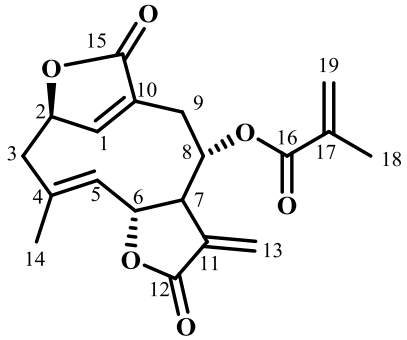


Figure 5.4: ^{13}C NMR spectrum of isodeoxyelephantopin

Fractions 310-332 obtained by eluting the column with 45% ethyl acetate in hexane was found to contain a UV active compound which on crystallization yielded 164 mg of compound **39** as colourless needle like crystals. IR spectrum of compound **39** was similar to that of compound **38**. It showed absorption at 1770 cm^{-1} indicating the presence of *α*-methylene-*γ*-lactone. The peak at 1751cm^{-1} could be ascribed to the carbonyl group in *α,β*-unsaturated lactone. The *α,β*-unsaturated ester carbonyl group gave peak at 1707cm^{-1}. Exocyclic olefin peaks were also observed at 3099, 1632 and 927 cm^{-1}. Two doublets each integrating for three protons at δ 1.93 and 1.85 in the ^{1}H NMR spectrum (fig. 5.5) suggested the presence of two methyl groups attached to olefinic carbons, which was again confirmed by the peaks at δ 20.1 and 18.2 in the ^{13}C NMR spectrum (fig. 5.6). Signals at δ 7.08 and 5.46 could be attributed to the protons of *α,β*-unsaturated lactone. Methylene protons of *α*-methylene-*γ*-lactone ring and methacrylate ester side chain resonated at δ 6.23, 5.65 and δ 6.14, 5.66 respectively. H-5 proton resonated as a multiplet at δ 4.77. Eight olefinic carbons were confirmed by the peaks at δ 153.5, 136.0, 135.6, 134.1, 133.7, 128.4, 126.7 and 123.7 in the ^{13}C NMR spectrum. The peaks at δ 172.5, 169.4 and 166.4 confirmed the presence of *α*-methylene-*γ*-lactone, *α,β*-unsaturated lactone and ester moiety respectively. The peaks at δ 81.4, 78.0, and 71.5 ppm could be attributed to the carbons attached to oxygen. Mass spectrum of the compound showed molecular ion peak at m/z 367.1164, which is the $(M+Na)^{+}$ peak. From the IR and ^{1}H NMR spectrum, it was clear that both compounds **38** and **39** contain *α*-methylene-*γ*-lactone system, *α, β*- unsaturated lactone and a methacrylate ester side chain. The ^{1}H NMR spectrum of compound **39** showed an upfield shift of H-5 and H-7 due to the effect of *β* oriented oxygen atom at C-2. Thus from the comparison of spectral value reported in literature,[11] compound **39** was confirmed to be **deoxyelephantopin**, which is a stereoisomer of isodeoxyelephantopin. The structure of the compound is given below.



Compound **39 - Deoxyelephantopin**

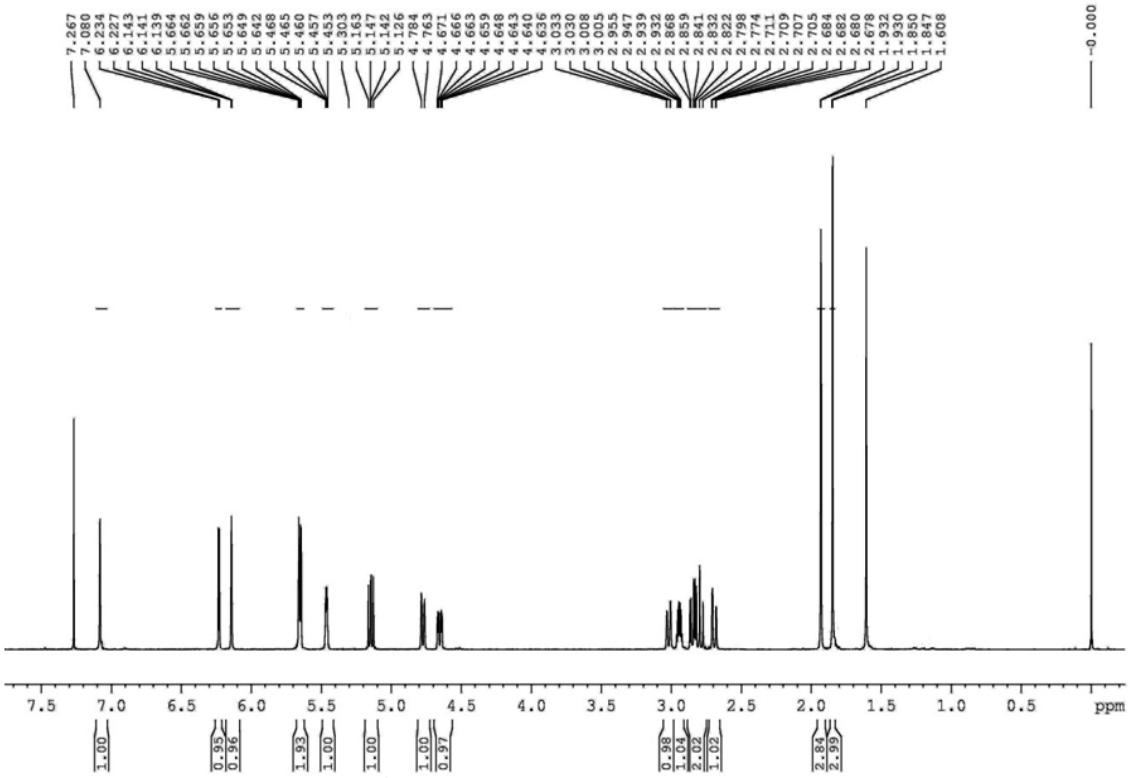


Figure 5.5: ^{1}H NMR spectrum of deoxyelephantopin

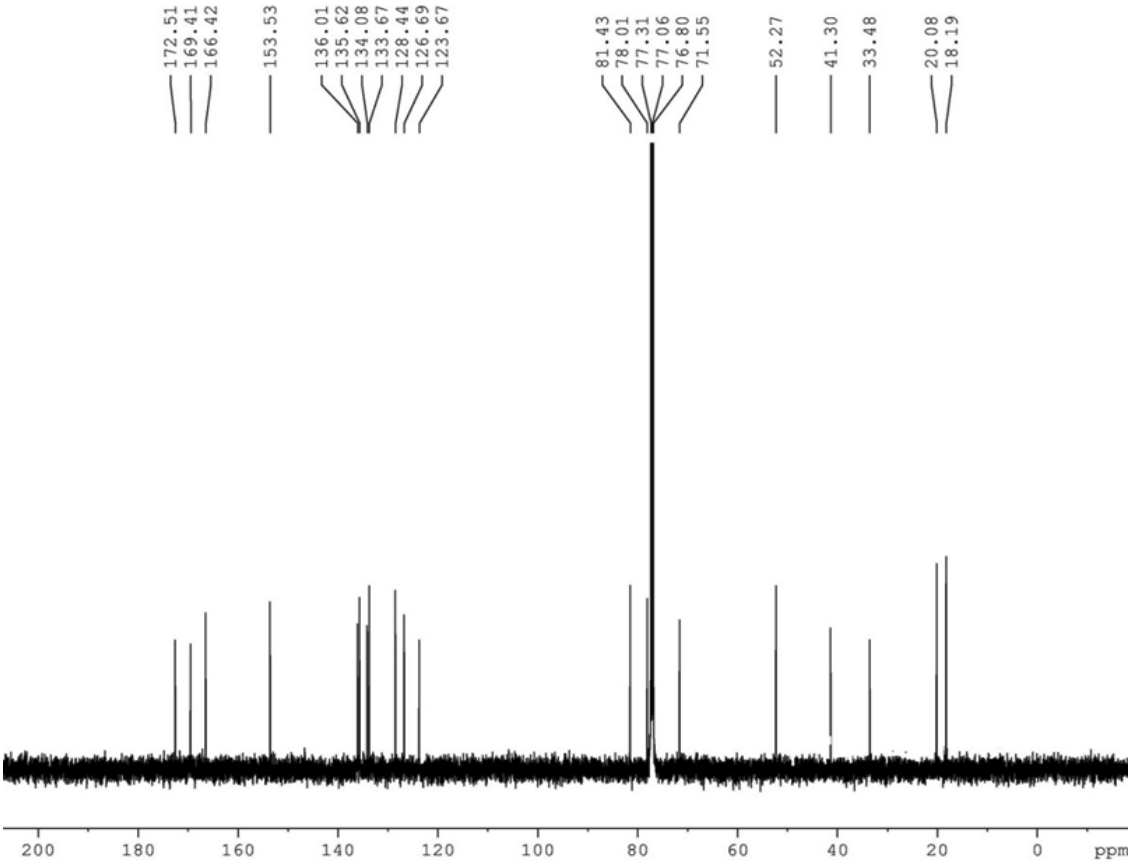


Figure 5.6: ^{13}C NMR spectrum of deoxyelephantopin

5.4. Anticancer activity studies of deoxyelephantopin and isodeoxyelephantopin

As explained in section 5.1.2 of this chapter, the two sesquiterpene lactones, deoxyelephantopin and isodeoxyelephantopin were found to have significant anticancer activities against various cancer cell lines. Therefore we were interested in exploring the anticancer efficacy of these compounds against other important human cancer cell lines. *These studies were done in collaboration with Dr. Remani P and Dr. Farha A K, Division of Cancer Research, Regional Cancer Centre, Thiruvananthapuram, Kerala.*

5.4.1. Anticancer activity studies of deoxyelephantopin

Effects of deoxyelephantopin (DOE) against lung adenocarcinoma (A549) and cervical carcinoma (SiHa) cell lines were evaluated and it has been found that the compound shows very good *in vitro* anticancer activity against both these cell lines. IC_{50} values were found to be 12.29 and 4.14 µg/mL against A549 and SiHa cell lines respectively. In both these cell lines, the compound showed inhibition of colony formation in a dose and time dependent manner. Photographs of colonies taken 14 days after the deoxyelephantopin treatment of the cells for 2 h are shown in fig. 5.7.

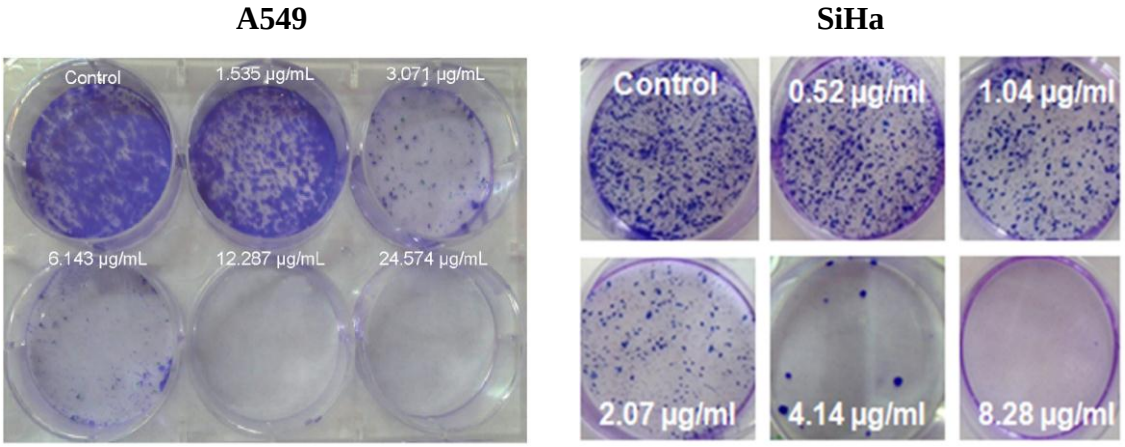


Figure 5.7: Effect of deoxyelephantopin on colony formation after 14 days

Induction of apoptosis by DOE in A549 and SiHa cell lines were analyzed using morphological changes observed using inverted microscope (fig. 5.8A) and by staining assays such as acridine orange/ethidium bromide (fig. 5.8B) and Hoechst 33342 staining (fig. 5.8C). Results indicated that in both the cell lines, DOE acts through apoptosis mediated pathway.

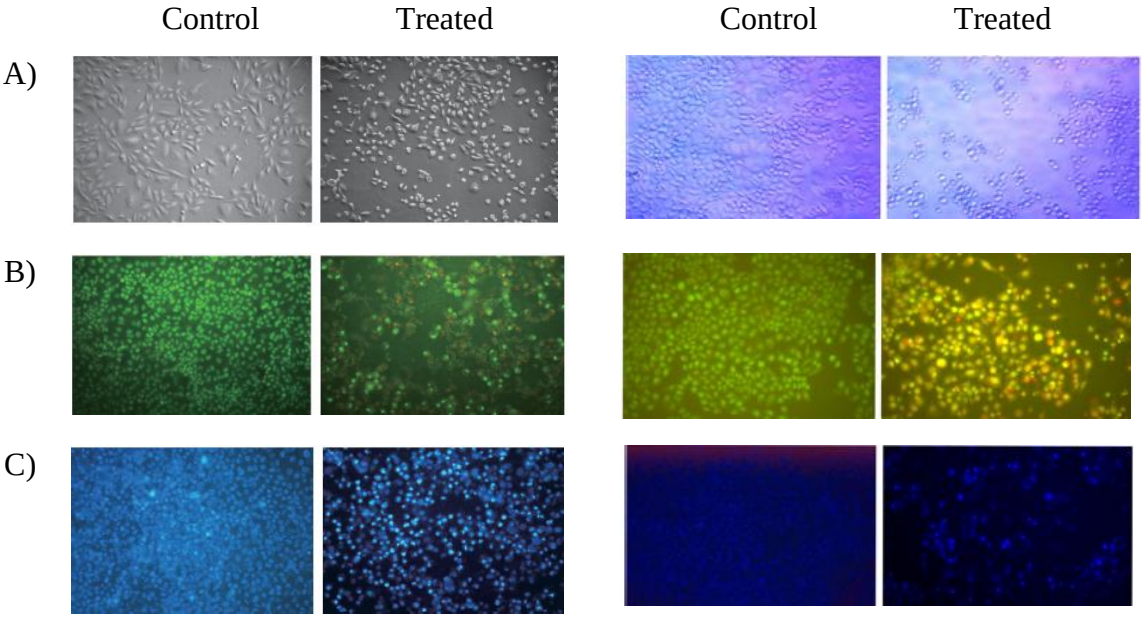


Figure 5.8: Morphological analysis on treatment with DOE A) Inverted microscopy B) Acridine orange/ ethidium bromide staining C) Hoechst 33342 staining

For quantification of apoptosis, cells cultured with or without DOE for 48 h were subjected to flow cytometric analysis using FITC Annexin V-FITC/propidium iodide (PI) staining (fig. 5.9). In Annexin V-FITC/PI staining, a small proportion of A549 cells were in the early stage of apoptosis (1.7%) and the majority being in the late stage of apoptosis (27.4%). In the case of SiHa cell lines majority of apoptotic cells were found in the late apoptotic stage (20.2%).

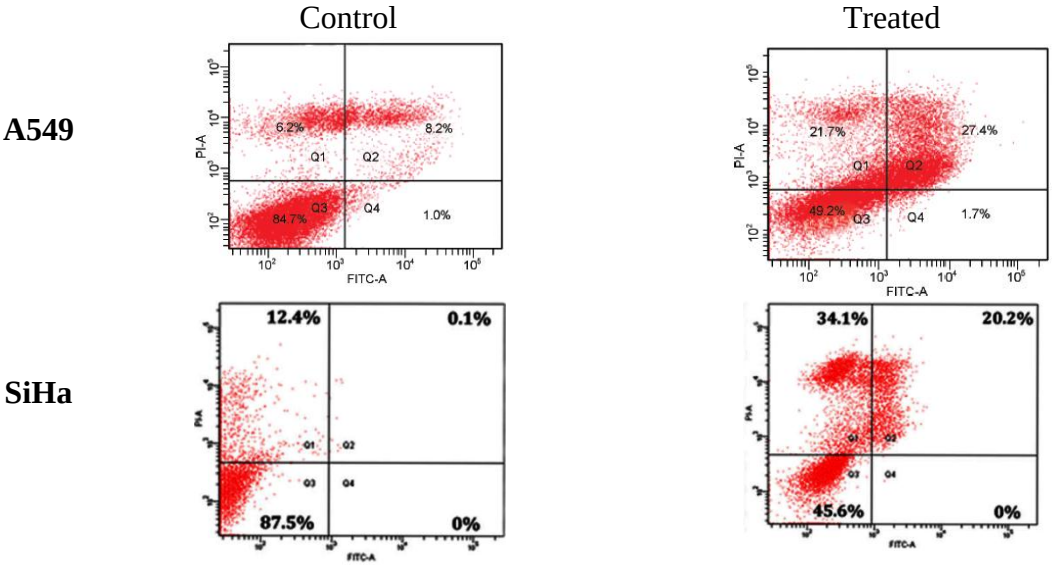


Figure 5.9: Annexin V-FITC and PI staining after DOE treatment

The effect of DOE on cell cycle regulation was examined using cell cycle analysis by flow cytometry. The compound induced cell cycle arrest at the G2/M phase at 48 h in both the cell lines. In these cell lines deoxyelephantopin initiated apoptosis through the activation of both the cell surface death receptor (extrinsic) pathway and the mitochondria mediated (intrinsic) pathway. The extrinsic pathway is initiated by cleaved caspase-8 whereas in the intrinsic pathway, cleaved caspase-9 is responsible for initiating apoptosis. In the case of SiHa, the G2/M cell cycle arrest by DOE was associated with decreased levels of cell regulatory proteins such as cyclin B1 and Cdc2 with an increased level of p21Waf1/Cip1 and p53. In addition to cell cycle arrest, apoptosis as well as modulation and suppression of different signaling molecules involved in biochemical pathways such as MAPKs, PI3K, and p-STAT3 were also seen in SiHa cells after they were treated with deoxyelephantopin.[29,30]

In vitro anti-metastatic effects of deoxyelephantopin on lung adenocarcinoma (A549) cells were also evaluated. Results indicated that the compound significantly decreased the metastatic potential. Anti-metastatic effect is associated with the down-regulation of NF-κB, IκBα MMP-2, MMP-9, uPA and uPAR and up-regulation of TIMP-2 at the transcript level. Deoxyelephantopin also suppressed the activation of ERK1/2 and Akt, but promoted the activation of JNK and p38 and resulted in the inhibition of metastasis (fig.5.10).[31]

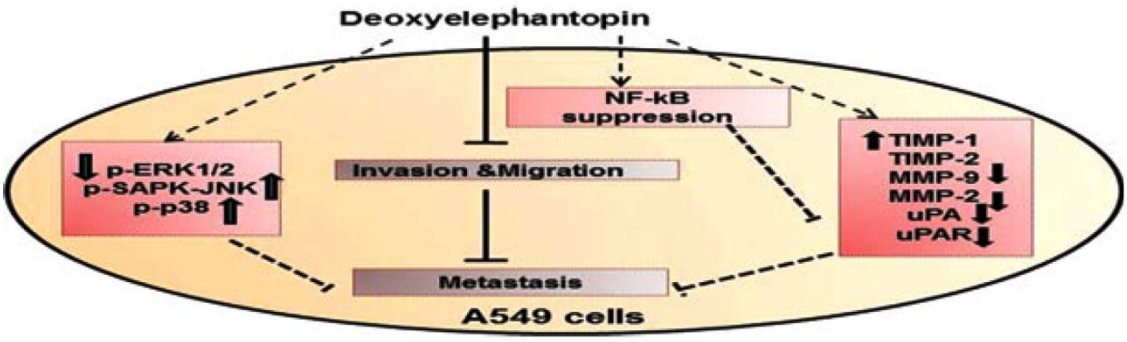


Figure 5.10: Proposed model for anti-metastatic activity of deoxyelephantopin

5.4.2. Anticancer activity studies of isodeoxyelephantopin

In vitro anticancer activities of isodeoxyelephantopin (IDOE) were tested against three different cell lines viz., human nasopharyngeal epidermoid carcinoma (KB), breast carcinoma (T47D) and lung carcinoma (A549) cell lines. IC_{50} values were found to be 3.94, 1.30 and 10.46 μg/mL respectively.

Morphological features of IDOE treated cells viewed under inverted microscopy as well as after staining with acridine orange/ethidium bromide (AO/EB) and Hoechst 33342 revealed the features of apoptosis such as membrane blebbing, cell shrinkage and chromatin condensation after 48 h of treatment (fig.5.11).

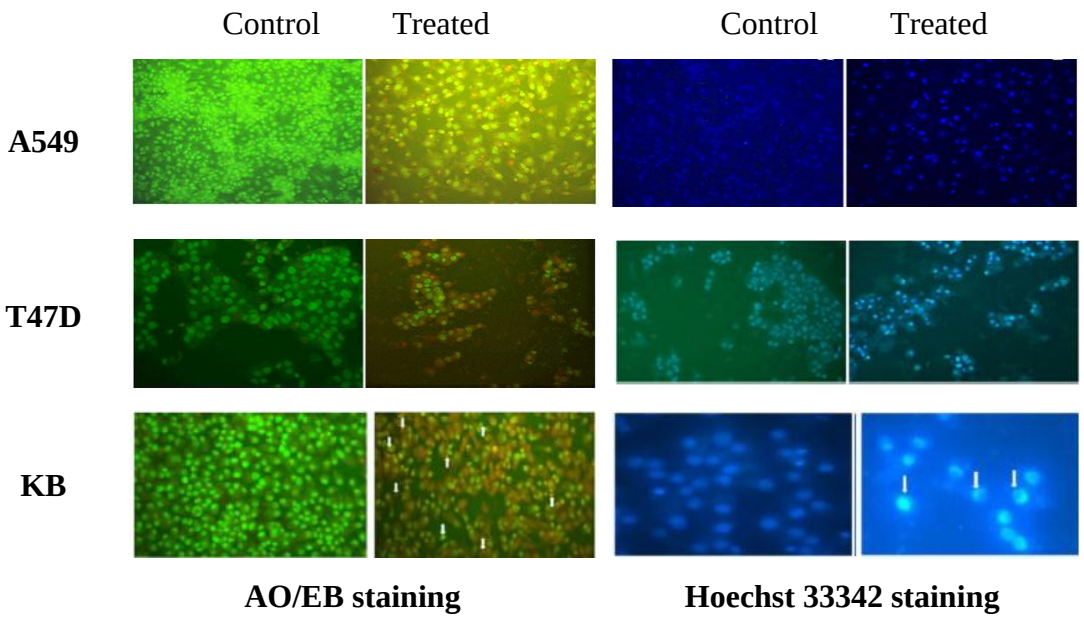


Figure 5.11: Morphological analysis on treatment with IDOE by acridine orange/ ethidium bromide staining and Hoechst 33342 staining

Quantification of apoptosis was carried out using Annexin V-FITC/PI staining. Results indicated that in all the three tested cell lines, percentage of apoptotic cells after treatment with IDOE increased in a dose and time dependent manner. IDOE induced cell cycle arrest at the G2/M phase and furthermore, in all these tested cell lines the compound induced caspase-3-mediated apoptosis.[32,33]

These findings indicate that both deoxyelephantopin and isodeoxyelephantopin have very good *in vitro* cytotoxic effects against various human cancer cell lines and can be considered as promising candidates for the development of new chemotherapeutic agents for the treatment of cancer.

5.5. Experimental

General experimental procedures are reported in chapter 2 of this thesis.

5.5.1. Extraction

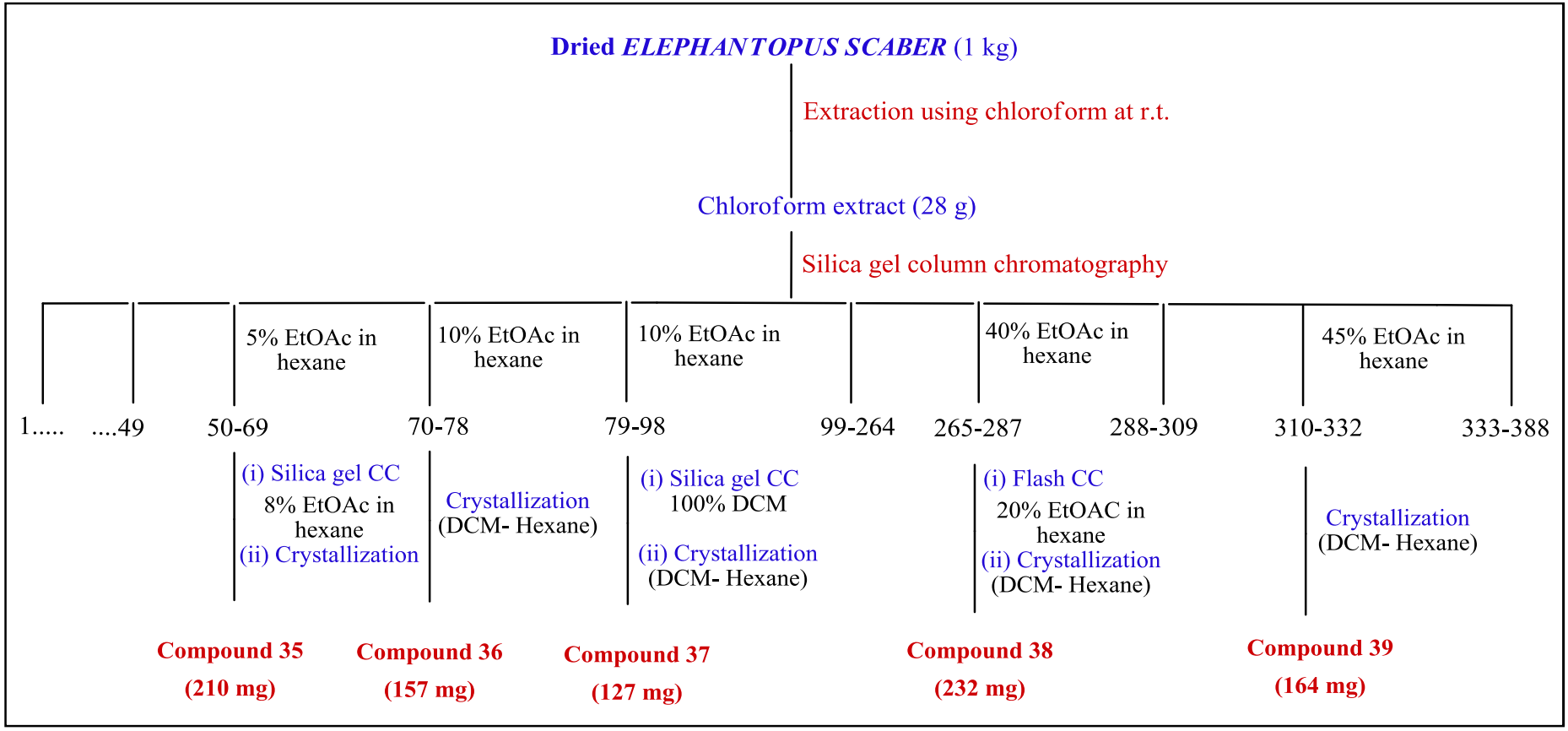
About 2 kg of fresh *E. scaber,* as whole plants, were collected from Palode in Thiruvananthapuram district, Kerala. A voucher specimen (TBGT-25419) was deposited in the herbarium of Jawaharlal Nehru Tropical Botanical Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram. The plant material was then cleaned and dried in an air oven maintained at 45 °C for five days. Dried material was coarsely powdered and weighed. The dried material weighing approximately 1 kg was then subjected to extraction using chloroform (3 L) for 24 hours at room temperature. The process was repeated thrice. The collected chloroform extract was concentrated under reduced pressure using Büchi rotary evaporator to remove the solvent, which provided 28 g of the crude extract.

5.5.2. Isolation of compounds

Thin layer chromatography of the chloroform extract was carried out using solvents of different polarity starting from 100% hexane to 70% ethyl acetate in hexane. After studying the TLC, the chloroform extract was subjected to column chromatographic purification. 28 g of the chloroform extract was loaded on top of the column packed with 500 g of silica gel and hexane. Column elution was started using 100% hexane. The polarity was increased gradually by adding desired amount of ethyl acetate to hexane. Final elution was carried out by using 100% ethyl acetate.

Different fractions of approximately 150 mL were collected in conical flasks. A total of 388 fractions were thus collected. TLC of each fraction was checked and those fractions whose TLC profiles were alike were pooled together to get 46 different fraction pools. Each of these pooled fractions was concentrated by removing the solvent under reduced pressure using Buchi rotary evaporator. Pictorial representation for the isolation of compounds from *E. scaber* is given in chart 5.2.

Chart 5.2: Pictorial representation for the isolation of compounds from *E. scaber*



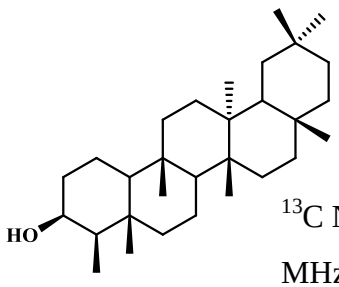
5.5.2.1.1. Isolation of compound 35

TLC of fractions 50-69 obtained by eluting the column with 5% ethyl acetate in hexane showed an iodine active spot along with some other impurities. These fractions were mixed together and repurified again using silica gel column chromatography. Column elution was started using 100% hexane. Subfractions 24-30 obtained by eluting the column with 8% ethyl acetate in hexane on crystallization yielded 210 mg of colourless crystals. It was characterized as **epifriedelanol** on the basis of various spectral data shown below, single crystal X-ray structure and on comparison with data reported in literature.[24,26]

Colourless crystals; m.p. 280-282 °C, lit.[26] 281.2-282.6 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 3474, 2929, 2866, 1440, 1383, 1257, 1173, 1120, 1087, 1046, 1000, 945, 917,784

^{1}H NMR (500 MHz, $CDCl_3$) : δ 3.74 (s, 1H), 1.90 (m, 1H), 1.73 (m, 1H), 1.58-1.25 (m, 22H), 1.17 (s, 3H), 1.01 (s, 3H), 0.99 (s, 3H), 0.99 (s, 3H), 0.96 (s, 3H), 0.94 (s, 3H), 0.93 (s, 3H), 0.91 (m, 2H), 0.86 (s, 3H)

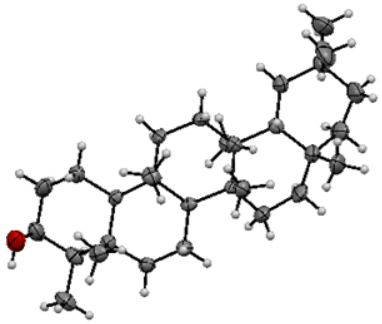


^{13}C NMR (125 MHz, $CDCl_3$) : δ 72.8, 61.3, 53.2, 49.2, 42.8, 41.7, 39.7, 39.3, 38.4, 37.8, 37.1, 36.1, 35.5, 35.3, 35.2, 35.0, 32.8, 32.3, 32.1, 31.8, 30.6, 30.0, 28.2, 20.1, 18.6, 18.2, 17.5, 16.4, 15.8, 11.6

HRMS (m/z) : 427.2168 $[(M-H)^{+}]$; $C_{30}H_{52}O$, requires 428.4018

5.5.2.1.2. Crystal parameters of compound 35

Chemical formula moiety	:	$C_{30}H_{52}O$
Chemical formula sum	:	$C_{30}H_{52}O$
Chemical formula weight	:	428.73
Symmetry cell setting	:	Monoclinic



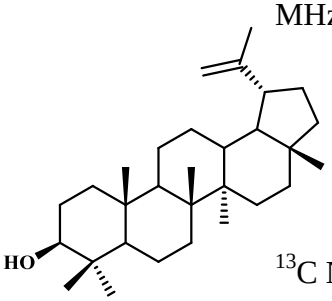
Symmetry space group name	:	C 2
Cell length a	:	13.4652 (14)
Cell length b	:	6.418 (7)
Cell length c	:	29.6310 (10)
Cell angle alpha	:	90
Cell angle beta	:	92.171
Cell angle gamma	:	90
Cell volume	:	2559 (3)
Cell formula units Z	:	4
Cell measurement temperature	:	301 (2)
Cell measurement reflns used	:	2249
Cell measurement theta min	:	3.0
Cell measurement theta max	:	27.5

5.5.2.2. Isolation of compound 36

TLC of fractions 70-78 obtained by eluting the column with 10% ethyl acetate in hexane showed a prominent iodine absorbing spot along with some minor impurities. This fraction on crystallization in DCM-hexane mixture yielded 157 mg of white crystals. The compound was characterized as **lupeol** on the basis of various spectral data as given below and on comparison with literature.[27]

Colourless crystals; m.p. 214-216 °C, lit.[34] 214-215 °C

FT-IR (KBr, υ_{max}, cm^{-1})	:	3375, 3071, 2946, 2863, 1720, 1640, 1459, 1379, 1268, 1190, 1098, 1036, 978, 884
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 4.68 (s, 1H), 4.56 (s, 1H), 3.18 (dd, *J* = 10.7, 5.4 Hz, 1H), 2.37 (m, 1H), 1.9 (m, 1H), 1.68 (s, 3H), 1.66-1.09 (m, 20H), 1.03 (s, 3H), 0.97 (s, 3H), 0.94 (s, 3H), 0.83 (s, 3H), 0.79 (s, 3H), 0.76 (s, 3H), 0.70 (s, 3H)
^{13}C NMR (125 MHz, $CDCl_3$)	:	δ 150.9, 109.3, 79.0, 55.3, 50.4, 48.3, 47.9, 42.9, 42.8, 40.8, 39.9, 38.8, 38.7, 38.0, 37.1, 35.6, 34.3, 29.8, 28.0, 27.4, 27.3,



25.1, 20.9, 19.3, 18.3, 18.0, 16.1, 15.9, 15.3, 14.5

HRMS (m/z) : 427.3561 $[(M+H)^+]$; $C_{30}H_{50}O$, requires 426.3862

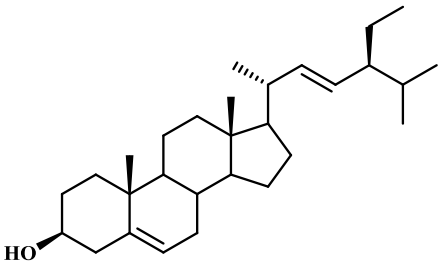
5.5.2.3. Isolation of compound 37

Fractions 79-89 obtained by eluting the column with 10% ethyl acetate in hexane showed the presence of iodine active spots in it. This fraction was subjected to column chromatographic separation for further purification. Column elution was started using 100% DCM. Subfractions 14-19 obtained by eluting the column with 100% DCM on crystallization using DCM-hexane mixture yielded 127 mg of compound **37.** It was characterized as **stigmasterol** based on the various spectral data obtained, as shown below and on comparison with literature.[28]

Colourless crystals; m.p. 164-166 °C, lit.[35] 167-169 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 3275, 2938, 2893, 2860, 1742, 1447, 1377, 1192, 1101, 154, 1016, 963, 834, 800

^{1}H NMR (500 MHz, $CDCl_3$) : δ 5.35 (m, 1H), 5.15 (dd, *J* = 15.0, 9.0 Hz, 1H), 5.02 (dd, *J* = 15.0, 9.0 Hz, 1H), 3.53 (m, 1H), 2.26 (m, 2H), 1.99 (m, 3H), 1.84 (m, 2H), 1.71-1.06 (m, 18H), 1.02 (d, *J* = 6.5 Hz, 3H), 1.01 (s, 3H), 0.85 (d, *J* = 6.5 Hz, 3H), 0.82 (t, *J* = 7.0 Hz, 3H), 0.80 (d, *J* = 6.5 Hz, 3H), 0.70 (s, 3H)



^{13}C NMR (125 MHz, $CDCl_3$) : δ 140.7, 138.3, 129.3, 121.7, 71.8, 56.9, 55.9, 51.2, 50.2, 42.3, 42.2, 40.5, 39.7, 37.3, 36.5, 31.9 (2C), 31.6 (2C), 28.9, 25.4, 24.4, 21.2, 21.1 (2C), 19.4, 19.0, 12.2, 12.0

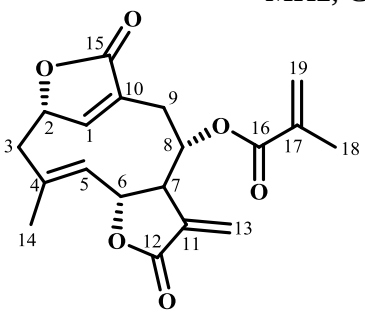
HRMS (m/z) : 411.3633 $[(M-H)^+]$; $C_{29}H_{48}O$, requires 412.370

5.5.2.4. Isolation of compound 38

Fractions 265-287 obtained by eluting the column with 40% ethyl acetate in hexane showed a major component with UV absorption, along with minor impurities. This fraction was further subjected to purification using flash column chromatography in a combiflash RF 150 UV/Vis. instrument using a prepacked cartridge. Column elution was started using 20% ethyl acetate in hexane. Subfractions 26-36 on crystallization in DCM-hexane mixture yielded 232 mg of colourless needle like crystals of compound **38**. The compound was characterized as **isodeoxyelephantopin** based on the spectral data obtained as shown below and on comparison with the values reported in literature.[11]

Colourless crystals; m.p. 157-159 °C, lit.[11] 158-160 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 3075, 2968, 1765, 1750, 1714, 1637, 1443, 1390, 1320, 1262, 155, 1086, 983, 951



^{1}H NMR (500 MHz, $CDCl_3$) : δ 7.13 (s, 1H, H-1), 6.21 (d, *J* = 3.5 Hz, 1H, H-13a), 6.15 (d, *J* = 0.5 Hz, 1H, H-19a), 5.67 (t, *J* = 1.5 Hz, 1H, H-19b), 5.65 (d, *J* = 3.0 Hz, 1H, H-13b), 5.38 (d, *J* = 5.0 Hz, 1H, H-2), 5.15 (m, 2H, H-5, H-6), 4.53 (m, 1H, H-8), 3.18-2.93 (m, 3H, H-3a, H-7, H-9a), 2.74 (dd, *J* = 13.0, 4.0 Hz, 1H, H-9b), 2.39 (dd, *J* = 14.5, 5.0 Hz, 1H, H-3b), 1.93 (d, *J* = 1.5 Hz, 3H, H-18), 1.79 (d, *J*=1 Hz, 3H, H-14)

^{13}C NMR (125 MHz, $CDCl_3$) : δ 174.3 (C-15), 169.5 (C-12), 166.6 (C-16), 149.2 (C-1), 135.5 (C-17), 135.3 (C-4), 134.0 (C-11), 131.6 (C-10), 126.9 (C-19), 125.5 (C-5), 123.2 (C-13), 79.5 (C-2), 78.7 (C-6), 74.1 (C-8), 49.8 (C-7), 40.1 (C-3), 30.1 (C-9), 21.6 (C-14), 18.2 (C-18)

HRMS (m/z) : 367.1166 [$(M+Na)^+$]; $C_{19}H_{20}O_6$, requires 344.1260

NMR spectral assignments were made on the basis of DEPT-135, 2D NMR analysis and on comparison with the literature reports.[11]

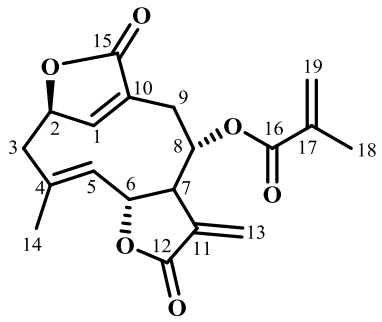
5.5.2.5. Isolation of compound 39

Fractions 310-332 obtained by eluting the column with 45% ethyl acetate in hexane showed a major spot, which is UV active, along with some minor impurities. This fraction was subjected to crystallization in DCM-hexane solvent system for further purification. 164 mg of colourless crystals of compound **39** thus obtained was characterized as **deoxyelephantopin** based on the spectral data obtained as shown below and on comparison with the values reported in literature.[11]

Colourless crystals; m.p. 196-198 °C, lit.[11] 196-199 °C

FT-IR (KBr, υ_{max}, cm^{-1}) : 3099, 2962, 1770, 1751, 1707, 1632, 1432, 1295, 1201, 1170, 1070, 927

^{1}H NMR (500 MHz, $CDCl_3$) : δ 7.08 (s, 1H, H-1), 6.23 (d, *J* = 3.5 Hz, 1H, H-13a), 6.14 (t, *J* = 1.0 Hz, 1H, H-19a), 5.66 (m, 1H, H-19b), 5.65 (d, *J* = 3.0 Hz, 1H, H-13b), 5.46 (m, 1H, H-2), 5.14 (dd, *J* = 10.5, 8.0 Hz, 1H, H-6), 4.77 (d, *J* = 10.5 Hz, 1H, H-5), 4.65 (m, 1H, H-8), 3.01 (m, 1H, H-9b), 2.94 (m, 1H, H-7), 2.86-2.77 (m, 2H, H-3a, H-9a), 2.70 (m, 1H, H-3b), 1.93 (d, *J* = 1.0 Hz, 3H, H-18), 1.85 (d, *J* = 1.5 Hz, 3H, H-14)



^{13}C NMR (125 MHz, $CDCl_3$) : δ 172.5 (C-15), 169.4 (C-12), 166.4 (C-16), 153.5 (C-1), 136.0 (C-17), 135.6 (C-4), 134.1 (C-11), 133.7 (C-5), 128.4 (C-10), 126.7 (C-19), 123.7 (C-13), 81.4 (C-6), 78.0 (C-2), 71.5 (C-8), 52.3 (C-7),

41.3 (C-3), 33.5 (C-9), 20.1 (C-14), 18.2 (C-18)

HRMS (m/z) : 367.1164 [$(M+Na)^+$]; $C_{19}H_{20}O_6$, requires 344.1260

NMR spectral assignments were made on the basis of DEPT-135, 2D NMR analysis and on comparison with the literature reports.[11]

5.6. Conclusion

Two major sesquiterpene lactones deoxyelephantopin and isodeoxyelephantopin were isolated in good yield from the chloroform extract of *Elephantopus scaber*. Three phytosterols viz., epifriedelanol, lupeol as well as stigmasterol were also obtained during the course of isolation. *In vitro* anticancer activities of deoxyelephantopin and isodeoxyelephantopin were evaluated against various human cancer cell lines and from the promising results obtained, it can be concluded that these compounds have enormous potential to induce cell death through apoptosis mediated pathway (*Nat. Prod. Res.*, **2015,** *29* (24), *2*341-2345; *Cell Biol. Toxicol.*, **2014**, *30* (6), 331-343; *Chin. Med.* **2014**, *9*, 14; *Asian J. Pharm. Clin. Res.* **2013**, *6*, 51-56; *J. Integr. Med.* **2013**, *11*, 269-277).

5.7. References

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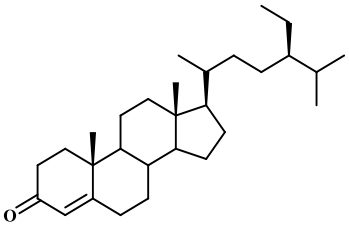
Summary and Conclusion

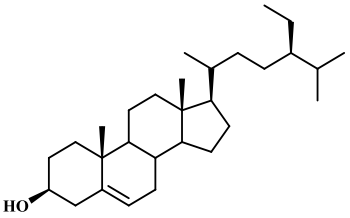
Plants have always played a pivotal role as the prime source of drugs and drug leads for the treatment of a broad spectrum of diseases. Plant based natural products and their semi-synthetic analogues contributed largely to the modern drug discovery process as well. Plants, which find extensive use in various traditional systems of medicine across the globe have exclusively provided a considerable number of drug leads and are still an attractive source of novel chemical compounds with lead potential. *Ayurveda*, the well known traditional system of medicine practiced in India, uses a plethora of medicinal plants for the preparation of various drug formulations. From the estimated 45,000 plant species available in India, only a small percentage has been investigated in terms of its phytochemical and pharmacological point of view. In this context, it appeared judicious to isolate and characterize biologically active compounds from plants which are used as drugs in *Ayurveda* since they have a higher chance of containing novel bioactive compounds in them. Accordingly, this thesis involves the phytochemical investigation and bioactivity studies of four important medicinal plants viz., *Chonemorpha fragrans* (Apocynaceae family), *Piper cubeba* (Piperaceae family), *Azadirachta indica* (Meliaceae family) and *Elephantopus scaber* (Asteraceae family).

Chapter 1 of the thesis gives a brief introduction about the importance of natural products in modern drug discovery process. The important natural products/natural product derived molecules, from various sources, which are now in clinical use for the treatment of different diseases are highlighted in this chapter. A detailed description of plant-derived compounds giving special emphasis to plant-derived anticancer agents is also included in this chapter.

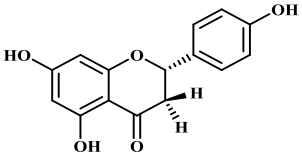
Chapter 2 of the thesis deals with the detailed phytochemical investigation and bioactivity studies on the medicinal plant *Chonemorpha fragrans*, belonging to Apocynaceae family. Literature review on the genus *Chonemorpha* and biosynthesis of pregnane type steroidal alkaloids are given in the introductory part of this chapter. Nine non-alkaloid constituents viz., sitostenone (**1**), *β*–sitosterol (**2**), naringenin (**3**), aromadendrin (**4**), matairesinol (**5**), vanillic acid (**6**), ferulic acid (**7**), protocatechuic acid (**8**) and sitosterol-3-*O*-*β*-D-glucopyranoside (**9**) were isolated and characterized from the acetone extract of *C. fragrans* roots. Except *β*–sitosterol all other compounds are being reported for the first time from *C. fragrans.*



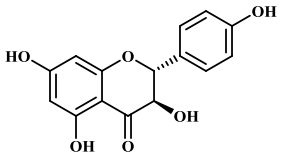
Sitostenone (**1**)



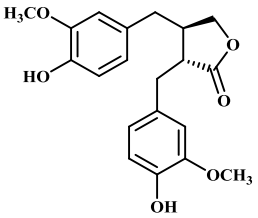
β–sitosterol (**2**)



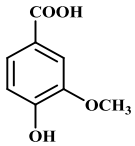
Naringenin (**3**)



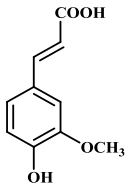
Aromadendrin (**4**)



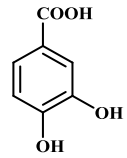
Matairesinol (**5**)



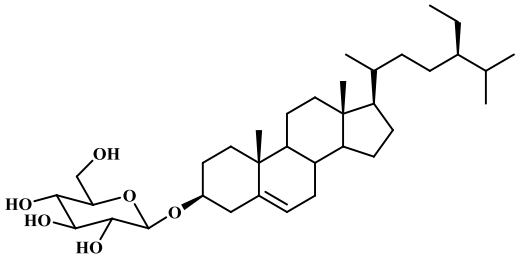
Vanillic acid (**6**)



Ferulic acid (7)

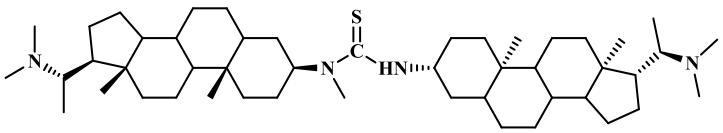


Protocatechuic acid (**8**)

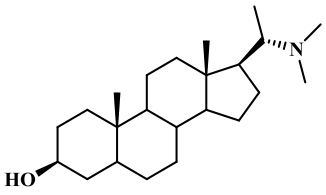


Sitosterol-3-*O*-*β*-D-glucopyranoside (**9**)

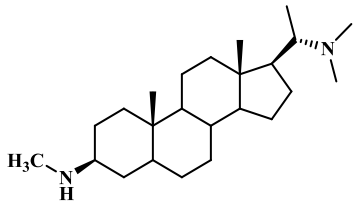
Five pregnane type steroidal alkaloids viz., japindine (**10**), deaminooxochonemorphine (sarcorucinine D, **11**), *N*-methylchonemorphine (dictyophlebine, **12**), chonemorphine (**13**), and *N*-formylchonemorphine (**14**) were isolated from the ethanol extract of *C. fragrans* roots. Among these alkaloids deaminooxochonemorphine is being reported for the first time from the plant and from Apocynaceae family.



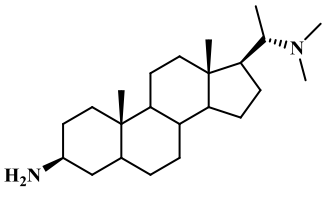
Japindine (**10**)



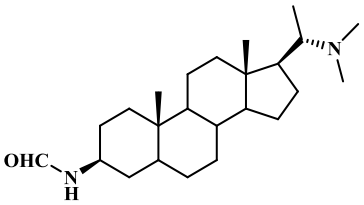
Deaminooxochonemorphine (**11**)



N-methylchonemorphine (**12**)



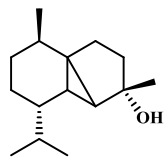
Chonemorphine (**13**)



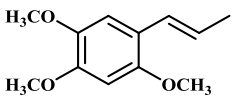
N-formylchonemorphine (**14**)

Chemical indices of both acetone and ethanol extracts of *C. fragrans* roots were evaluated and from the result obtained it has been found that *C. fragrans* acetone extract possesses high phenolic and flavonoid content as well as high antioxidant capacity compared to *C. fragrans* ethanol extract. Preliminary radical scavenging capacities of the extracts as well as isolated phenolic compounds, determined using DPPH radical scavenging assay revealed that protocatechuic acid shows highest activity among the various phenolic compounds isolated and among the two extracts *C. fragrans* acetone extract has better activity compared to the ethanol extract. *In vitro* antibacterial activity of the alkaloids isolated were tested against four different Gram-positive and five different Gram-negative pathogens. Deaminooxochonemorphine (DOC, also known as sarcorucinine D, **11**) showed very good activity against all the tested organisms. Best result for DOC was obtained against *Salmonella typhi* and the compound was found be non-toxic up to 100 μM concentration against normal human cell lines.

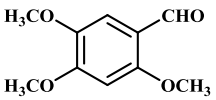
Chapter 3 is divided into two parts. The first part (3A) deals with the isolation and structural elucidation of phytochemicals from the seeds of the medicinal plant *Piper cubeba* (belonging to Piperaceae family). A brief outline on the phytochemistry of important medicinal plants belonging to the Piper species viz., *P. nigrum, P. longum, P. brachystachyum* and *P. betle* as well as *P. cubeba* are given in the introductory part of this chapter. Phytochemical investigation of *P. cubeba* seeds led to the isolation of fourteen compounds namely; cubebol (**15**), *α*-asarone (**16**), 2,4,5-trimethoxy benzaldehyde (**17**), podoandin (**18**), hinokinin (**19**), cubebin (**20**), yatein (**21**), (5*α*,8*α*)-2-oxo-1-(10),3,7,(11)-guaiatrien-12,8-olide (**22**), cubebininolide (**23**), clusin (**24**), dihydrocubebin (**25**), 2,4,5-trimethoxybenzoic acid (**26**), cubebinin (**27**) and dihydroclusin (**28**). Among these compounds, podoandin and 2,4,5-trimethoxybenzoic acid are being reported for the first time from *P. cubeba*.



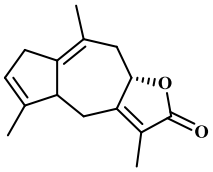
Cubebol (**15**),



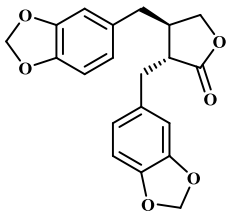
α-asarone (**16**)



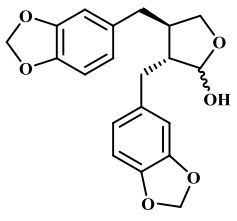
2,4,5-trimethoxy benzaldehyde (**17**)



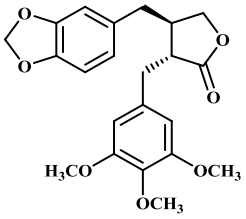
Podoandin (**18**)



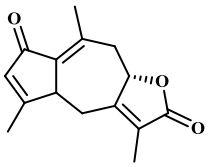
Hinokinin (**19**)



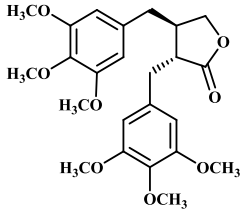
Cubebin (**20**)



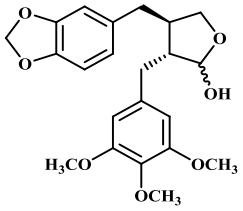
Yatein (**21**)



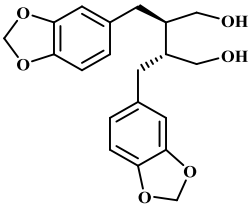
(5*α*,8*α*)-2-oxo-1-(10), 3,7,(11)-guaiatrien-12,8-olide (**22**)



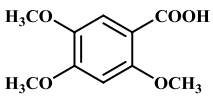
Cubebininolide (**23**)



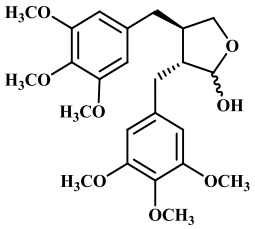
Clusin (**24**)



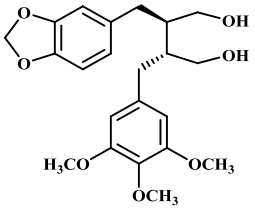
Dihydrocubebin (**25**)



2,4,5-trimethoxy benzoic acid (**26**)

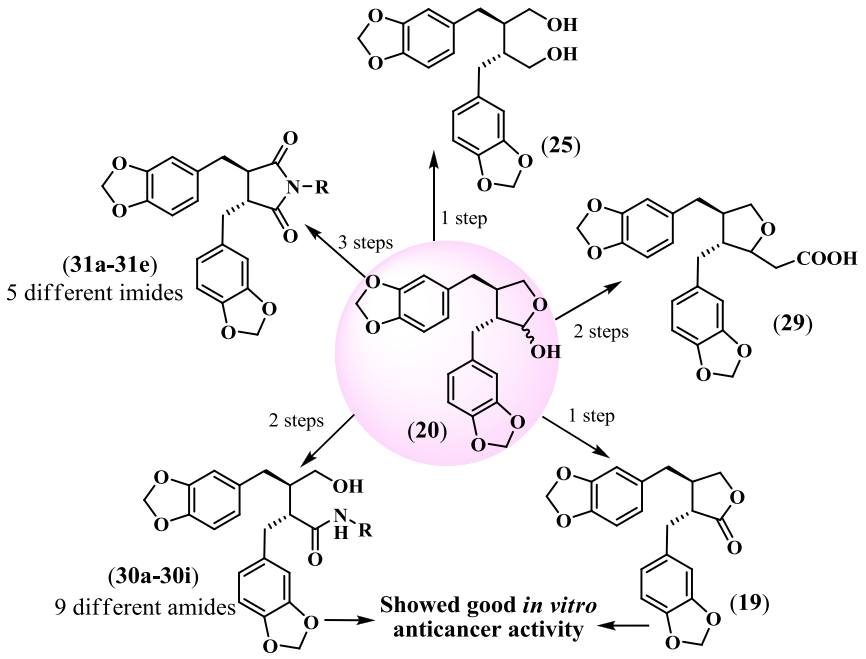


Cubebinin (**27**)

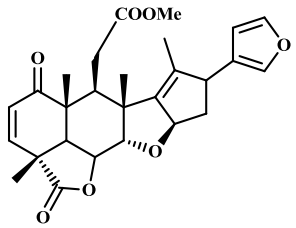


Dihydroclusin (**28**)

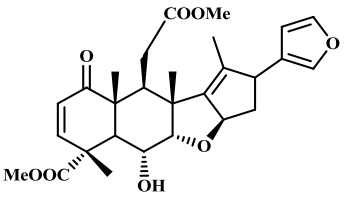
Synthetic transformation of cubebin, the major compound isolated from *P. cubeba* seeds, as well the anticancer activities of cubebin and its derivatives are explained in the second part of chapter 3 (3B). Five different types of derivatives (a total of 17), with varying functionalities, were synthesized and tested for their *in vitro* anticancer activity against six human cancer cell lines using MTT assay. Cubebin as well as its derivatives containing lactone and amide groups showed significant anticancer activity. Morphological analysis indicated that these compounds act through apoptosis mediated pathway of cell death and we expect that these results will pave new paths in the development of novel anticancer agents by the derivatization of (-)-cubebin.



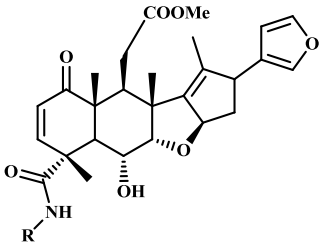
Chapter 4 explains the isolation of nimbolide from *Azadirachta indica* (commonly known in India as Neem; Family: Meliaceae) and evaluation of its anticancer and antibacterial potentials. Nimbolide (**32**) was isolated in good yield from *A. indica* leaves along with the minor compound desacetylnimbin (**33**). *In vitro* and *in vivo* anticancer efficacy of nimbolide against colon cancer were evaluated and it has been found that nimbolide inhibited proliferation, induced apoptosis and suppressed NF-kB activation and NF-kB–regulated tumorigenic proteins in colorectal cancer cell lines.



Nimbolide (**32**)



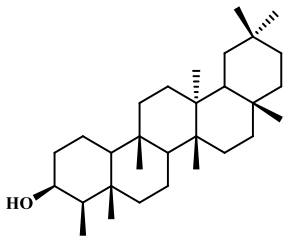
Desacetylnimbin (**33**)



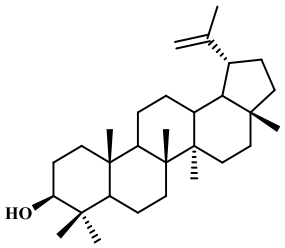
34a-34c

Antibacterial effect of nimbolide, desacetylnimbin and three different amide derivatives of nimbolide (**34a-34c**) and their synergistic effect when in combination with antimicrobial agents were recorded. Nimbolide showed the highest antimicrobial potential and maximum synergistic effect with the tested antibiotics (cefazolin and cefalexin), followed by desacetylnimbin. Amide derivatives were found to be less potent when compared to the two natural limonoids.

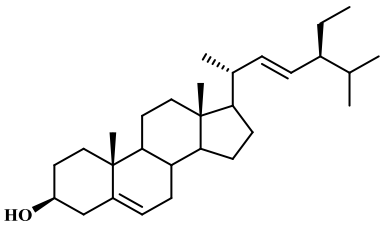
Elephantopus scaber belonging to 'Asteraceae' family is widely used in various traditional systems of medicine. Phytochemical investigation of *E. scaber* led to the isolation of five compounds viz., epifriedelanol (**35**), lupeol (**36**), stigmasterol (**37**), isodeoxyelephantopin (**38**) and deoxyelephantopin (**39**).



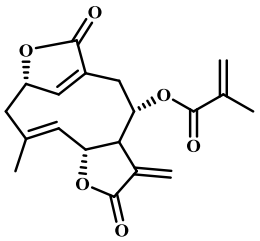
Epifriedelanol (**35**)



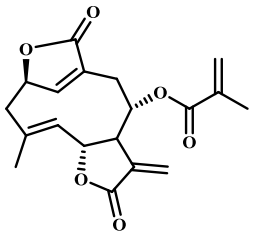
Lupeol (**36**)



Stigmasterol (**37**)



Isodeoxyelephantopin (**38**)



Deoxyelephantopin (**39**)

Cytotoxic effects of deoxyelephantopin and isodeoxyelephantopin on various human cancer cell lines were studied. It has been found that these compounds show promising anticancer activity. Details of the isolation, characterization and results obtained for the anticancer activity studies are given in chapter 5 of the thesis.

In conclusion, detailed phytochemical investigation of *C. fragrans,* belonging to Apocynaceae family and *P. cubeba*, belonging to Piperaceae family were carried out. Fourteen compounds each were isolated from each of these plants. Eight non-alkaloid constituents except *β*–sitosterol and the alkaloid, deaminooxochonemorphine are being reported for the first time from *C. fragrans* whereas podoandin and 2,4,5-trimethoxy benzoic acid are being isolated for the first time from *P. cubeba* seeds. Nimbolide, isolated from *A. indica* leaves has been shown to have very good *in vitro* and *in vivo* anticancer activity against colon cancer. Synergistic antimicrobial studies of nimbolide, desacetylnimbin and amide derivatives of nimbolide revealed that nimbolide possesses highest activity, followed by desacetylnimbin. Apart from that, two sesquiterpene lactones viz., isodeoxyelephantopin and deoxyelephantopin isolated from *E. scaber* have been shown to have very good anticancer activity against various cancer cell lines of interest.

List of Publications

1. **Dhanya, S. R.;** Farha, A. K.; Arya R. M.; Arun, K. B.; Remani, P.; Nisha, P.; Sunil, V. and Nair, M. S. Anticancer activity studies of cubebin isolated from *Piper cubeba* and its synthetic derivatives. *Bioorganic and Medicinal Chemistry Letters,* **2016,** *26* (7), 1767-1771.

2. **Dhanya, S. R.;** Kumar, S. N.; Sankar, V.; Raghu, K. G.; Kumar, B. S. D. and Nair, M. S. Nimbolide from *Azadirachta indica* and its derivatives plus first-generation cephalosporin antibiotics: a novel drug combination for wound-infecting pathogens. *RSC Advances*, **2015**, *5*, 89503-89514.

3. Farha, A. K.; **Dhanya, S. R.;** Nair, M. S. and Remani, P. Anti-metastatic effect of deoxyelephantopin from *Elephantopus scaber* in A549 lung cancer cells *in vitro*. *Natural Product Research*, **2015,** *29(24), 2*341-2345.

4. Farha, A. K.; **Dhanya, S. R.**; Nair, M. S.; Geetha, B. S.; Latha, P. G. and Remani, P. Deoxyelephantopin impairs growth of cervical carcinoma SiHa cells and induces apoptosis by targeting multiple molecular signaling pathways. *Cell Biology and Toxicology*, **2014**, *30(6),* 331-343.

5. Farha, A. K.; Geetha, B. S.; Nair, M. S.; **Dhanya, S. R.**; Latha, P. G. and Remani, P. Isodeoxyelephantopin from *Elephantopus scaber* (Didancao) induces cell cycle arrest and caspase-3-mediated apoptosis in breast carcinoma T47D cells and lung carcinoma A549 cells. *Chinese Medicine*, **2014**, *9*, 1-9.

6. Gupta, S. C.; Prasad, S.; **Dhanya S. R.**; Nair, M. S.; Yin-Yuan Mo and Aggarwal, B. B. Nimbolide, a limonoid triterpene, inhibits growth of human colorectal cancer xenografts by suppressing the proinflammatory microenvironment. *Clinical Cancer Research*, **2013**, *19*, 4465-4476.

7. Farha, A. K.; Geetha, B. S.; Nair, M. S.; **Dhanya, S. R.**; Latha, P. G. and Remani, P. Apoptosis mediated cytotoxicity induced by isodeoxyelephantopin on nasopharyngeal carcinoma cells. *Asian Journal of Pharmaceutical and Clinical Research*, **2013,** *6 (Suppl 2)*, 51-56.

8. Farha, A. K.; Geetha, B. S.; Nair, M. S.; **Dhanya, S. R.**; Latha, P. G.; Sujathan, K. and Remani, P. Antineoplastic effects of deoxyelephantopin, a sesquiterpene lactone from *Elephantopus* scaber, on lung adenocarcinoma (A549) cells. *Journal of Integrative Medicine*, **2013,** *11(4)*, 269-277.

9. **Dhanya, S. R.;** Kumar, S. N.; Sankar, V. and Nair, M. S. Chemical constituents from Chonemorpha fragrans roots and antibacterial activity studies of sarcorucinine D and other steroidal alkaloids. (To be communicated to *RSC Advances*).

Papers Presented at Conferences

1. Sajin Francis, K.; **Dhanya, S. R.** and Mangalam S. Nair. Isolation of heterocyclic compounds and bioactivity studies on the medicinal plant *Azadirachta indica.* Poster No. POS- 133, 3rd International Conference on Heterocyclic Chemistry, held at Department of Chemistry, University of Rajasthan, Jaipur on December 10-13, 2011.

2. **Dhanya, S. R.;** Arya, R. M.; Farha, A. K.; Remani P. and Mangalam S. Nair. Isolation, Characterisation and Bioactivity Studies on *Piper Cubeba*. Poster No. PP-31, National Symposium on Transcending Frontiers in Organic Chemistry-2014, held at CSIR-NIIST, Trivandrum during October 9-11, 2014.

3. Hema, P. S.; **Dhanya, S. R**.; Priya Rani.; Raghu, K. G. and Mangalam S. Nair. Anti- diabetic cyclohexane diepoxides from the rhizomes of *Kaempferia pulchra*. Poster No. P-30, 5th International Conference on Drug Development for Orphan/Neglected Diseases held at CDRI, Lucknow on February 26-28, 2013.

4. **Dhanya, S. R**.; Hema. P. S.; Suchithra, M. V. and Mangalam S. Nair. Chemotaxonomy of *Alpinia galanga* & *Alpinia calcarata* and comparison of their antioxidant activities. [OP-28], Three-Day National Seminar on Frontiers In Chemistry (NSFC-2012), organized by Department of Chemistry, University of Kerala, Trivandrum on April 25-27, 2012. (**Best Paper Award**).