ISOLATION AND CHARACTERIZATION OF NOVEL BIOLOGICALLY ACTIVE MOLECULES

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UNDER THE FACULTY OF SCIENCE

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JUNE 2009

DEDICATED TO MY BELOVED PARENTS

June 26, 2009

STATEMENT

I hereby declare that the matter embodied in the thesis entitled, **"Isolation and characterization of novel biologically active molecules"** are results of investigations carried out by me at the Organic Chemistry Section, Chemical Sciences and Technology Division of the National Institute for Interdisciplinary Science and Technology (CSIR), Trivandrum, under the supervision of Dr. Mangalam S. Nair and the same has not been submitted elsewhere for a degree.

In keeping with the general practice of reporting scientific observations, due acknowledgement has been made wherever the work described is based on the findings of other investigators.

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CERTIFICATE

This is to certify that the work embodied in the thesis entitled, **"Isolation and characterization of novel biologically active molecules"** has been carried out by Miss P. S. Hema, under my supervision and the same has not been submitted elsewhere for a degree.

Mangalam S. Nair

(Thesis Supervisor)

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ABBREVIATIONS

AA	:	Antioxidant activity
AAE	:	Ascorbic acid equivalent
A_0	:	Initial absorbance
A_1	:	Final absorbance
ACE	:	*Alpinia calcarata* ethanol extract
ACA	:	*Alpinia calcarata* acetone extract (defatted)
AGE	:	*Alpinia galanga* ethanol extract
AGA	:	*Alpinia galanga* acetone extract
[(AH)]	:	Concentration of antioxidant
$[(AH)_0]$	:	Initial antioxidant concentration
AIDS	:	Acquired immune deficiency syndrome
ALA	:	Alpha- linolenic acid
ATP	:	Adenosine triphosphate
BCE	:	Before the Christian Era
BHA	:	Butylated hydroxy anisole
BHT	:	Butylated hydroxy toluene
CA-4	:	Combretastatin A-4
CAT	:	Catalase
$CDCl_3$	:	Deuterated chloroform
CD_3COCD_3	:	Deuterated acetone
CD_3OD	:	Deuterated methanol
Co A	:	Coenzyme A
CPT	:	Camptothecin
d	:	doublet
dd	:	doublet of doublet
DEPT	:	Distortionless Enhancement by polarization transfer
DHA	:	Docosahexaenoic acid
DMSO	:	Dimethyl sulphoxide
DNA	:	Deoxyribo nucleic acid

$DPPH^{\cdot}$	:	Diphenyl picryl hydrazyl radical
$[DPPH^{\cdot}]$	:	Concentration of $DPPH^{\cdot}$
$[DPPH^{\cdot}]_{REM}$	:	Concentration of remaining $DPPH^{\cdot}$
$[DPPH^{\cdot}]_t$	:	Concentration of $DPPH^{\cdot}$ at time t
$[DPPH^{\cdot}]_0$	:	Initial $DPPH^{\cdot}$ concentration
DR_c	:	Degradation rate of control
DR_s	:	Degradation rate of sample
EC_{50}	:	Effective concentration for 50 % inhibition
EDTA	:	Ethylenediamine tetraacetic acid
EI-MS	:	Electron Impact mass spectroscopy
EPA	:	Eicosapentaenoic acid
FAB	:	Fast atom bombardment
FRAP	:	Ferric reducing antioxidant power
g	:	gram
GAE	:	Gallic acid equivalent
GC-MS	:	Gas chromatography mass spectroscopy
GPx	:	Glutathione peroxidase
GRx	:	Glutathione reductase
GSSG	:	Glutathione (oxidized)
GSH	:	Glutathione
h	:	hour
HIV	:	Human Immunodeficiency Virus
HPLC	:	High performance liquid chromatography
IR	:	Infra red
J	:	Coupling constant
k_{obsd}	:	Pseudo first order rate constant
k_2	:	Second order rate constant
kg	:	kilogram
l	:	litre
LDL	:	Low density lipoprotein

LRMS	:	Low resolution mass spectroscopy
M	:	Molar
m	:	multiplet
MDA	:	Malondialdehyde
mg	:	milli gram
MHz	:	Mega Hertz
ml	:	milli litre
mM	:	milli molar
mmol	:	milli moles
MO	:	Molecular Orbital
MS	:	Mass spectroscopy
MTT	:	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	:	Nicotinamide Adenine disodium salt
NADPH	:	Nicotinamide Adenine Dinucleotide phosphate
NBT	:	Nitro blue tetrazolium chloride
NCI	:	National Cancer Institute
NED	:	N-(1-naphthyl)ethylenediamine dihydrochloride
NMR	:	Nuclear magnetic Resonance
NOSs	:	Nitric Oxide Synthases
OD	:	Optical density
OI	:	Oxidative index
p	:	para
PMS	:	Phenazonium methosulphate
ppm	:	parts per million (mg/l)
PUFA	:	Poly unsaturated fatty acid
q	:	quartet
QE	:	Quercetin equivalent
RDA	:	Retro Diels Alder
RNA	:	Ribonucleic acid
RNS	:	Reactive Nitrogen Species

ROS	:	Reactive Oxygen Species
RSC	:	Radical scavenging capacity
s	:	seconds
s	:	singlet
SAR	:	Structure Activity Relationship
SD	:	Standard deviation
SOD	:	Superoxide dismutase
SRB	:	Sulphorhodamine B
t	:	time
t	:	triplet
TCA	:	Trichloroacetic acid
TLC	:	Thin layer chromatography
TPTZ	:	2,4,6-Tripyridyl-s-triazine
UV	:	Ultra violet
UV-B	:	Ultra violet B (medium wave 280-315 nm)
UV-vis	:	Ultra violet visible
α	:	alpha
β	:	beta
γ	:	gamma
δ	:	delta
µM	:	micromolar
µg	:	microgram
µl	:	microlitre

CONTENTS

Chapter 1

Biologically active Natural Products – An Overview with special reference to anticancer and antioxidant compounds from terrestrial sources

1.1 Introduction

Man has utilized materials from nature for his basic needs like food, clothing, shelter and medicine throughout the ages. Nature has been the source of medicines for the treatment of a wide spectrum of diseases all over the world and across wide spectrum of civilizations. Plant based sophisticated traditional systems of medicines were developed in many parts of the world like Egypt (*Eberus Papyrus* which dated from 1500 BCE documenting over 700 drugs), China (*Chinese materia medica* dating from about 1100 BCE) and India (*Charaka* and *Sushruta Samhitas* from before 1000 BCE) from ancient days. The indigenous people of South America derived medicines and poisons from thousands of plants. The rational development of modern medicine has its roots in such traditional medicines and therapies.

Many such drugs discovered early are still used in the modern system of medicine and many more carry the structural imprint of the parent molecular prototype or natural product which led to their discovery. It has been reported that nearly 120 compounds derived from 90 plant species can be considered as important drugs currently in use in one or more countries, with 77 % of these being derived from plants used in traditional medicine.[1] A large number of therapeutic activities are mediated by these drugs and a host of these drugs currently in use are still obtained from the plants in which they

are synthesized. Examples include steroids, cardiotonic glycosides (*Digitalis* glycosides), anticholinergics (belladonna type tropane alkaloids), analgesics and antitussives (opium alkaloids), antihypertensives (reserpine), cholinergics (physostigmine, pilocarpine), antimalarials (*Cinchona* alkaloids), antigout (colchicine), anesthetic (cocaine), skeletal muscle relaxant (tubocurarine) and anticancer (taxol) agents. Some of the important plant based drugs (**1**-**15**) are shown in Table 1.1.

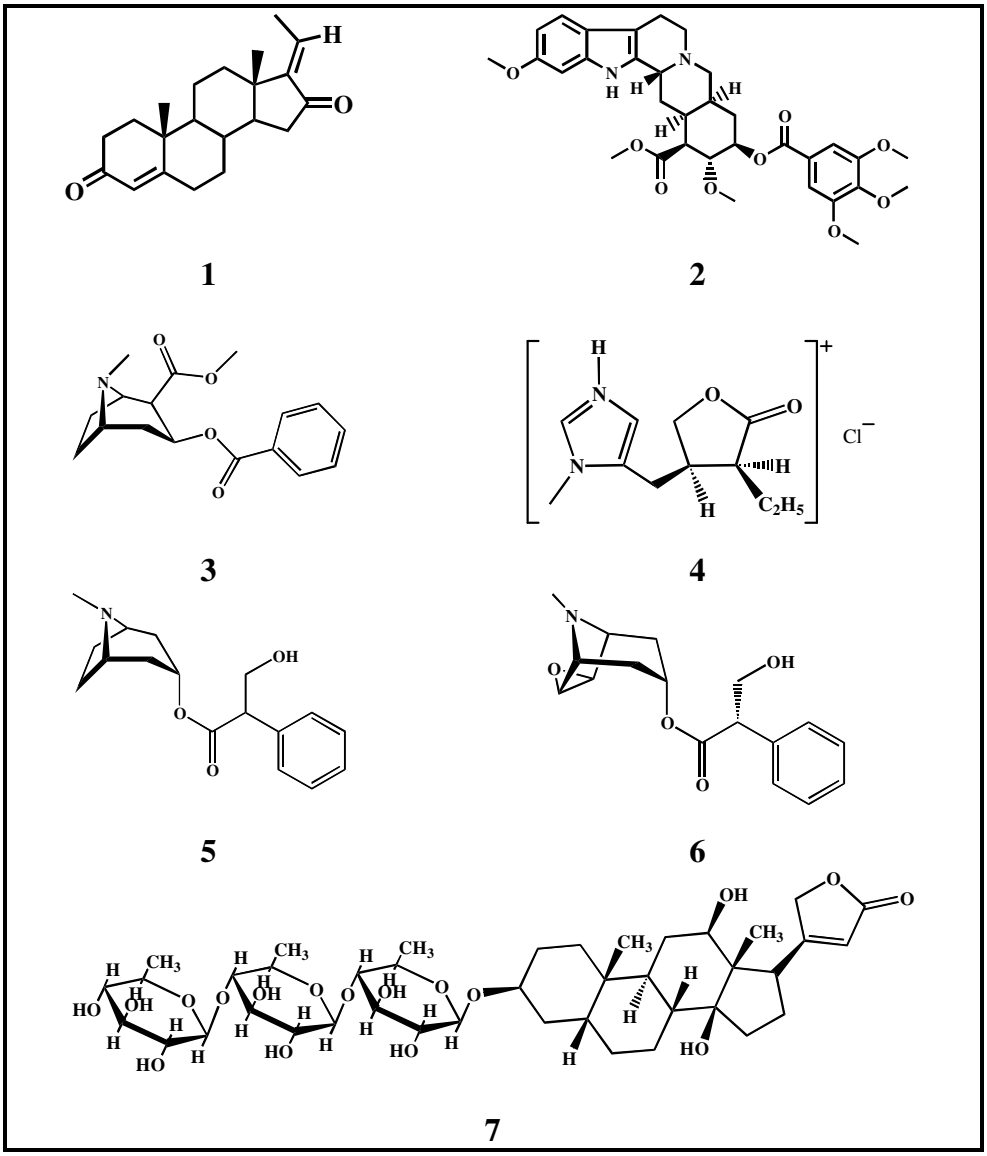
Table 1.1: Prominent plant derived medicinal compounds

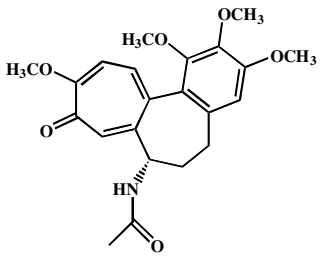
No:	***Name***	***Source***	***Uses***
1	Guggulsterone	*Commiphora mukul*	Lowers cholesterol
2	Reserpine	*Rauwolfia serpentina*	Controls high blood pressure
3	Cocaine	*Erythroxylon coca*	Anesthetic
4	Pilocarpine	*Pilocarpus jaborandi*	Cures glaucoma
5	Atropine	*Atropa belladonna*	Ophthalmic treatment
6	Hyoscine	*Hyoscyamus niger*	Treats nausea
7	Digoxin	*Digitalis lanata*	To treat cardiac disorders
8	Colchicine	*Colchicum autumnale*	Cures rheumatism
9	Emetine	*Psychotria ipecacuanha*	Anti-amoebic
10	Vincristine	*Catharanthus roseus*	Cancer chemotherapy
11	Taxol/Paclitaxel	*Taxus brevifolia*	Cancer chemotherapy
12	Forskolin	*Coleus forskohlii*	Vasodilator
13	Calanolide A	*Calophylum lanigerum*	Anti-HIV agent
14	Quinine	*Cinchona officinalis*	Antimalarial
15	Artemisinin	*Artemesia annua*	Antimalarial

Emergence of modern pharmaceutical industry is an outcome of different activities involving synthetic chemists, natural product chemists, pharmacologists, microbiologists and biochemists etc., which has led to the

development of potent single molecules with highly selective activity for a wide variety of ailments. Synthetic drugs were developed with improved properties as compared to the natural ones they were based on. For e.g., chloroquine, the synthetic anti-malarial drug is much less toxic than quinine **15**, the white crystalline alkaloid extracted from the bark of South-American Cinchona tree. The most fascinating aspect of these plant based drugs is their structural variety as shown in chart 1.1.

Chart 1.1: Structures of some prominent plant based drugs





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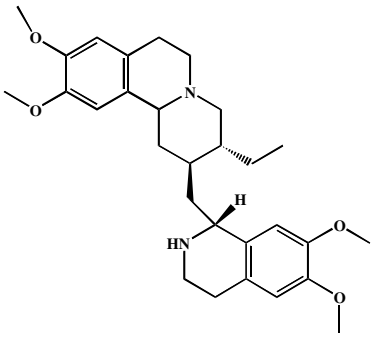

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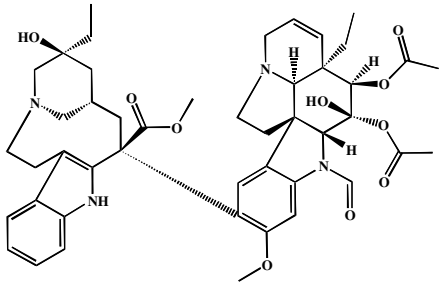

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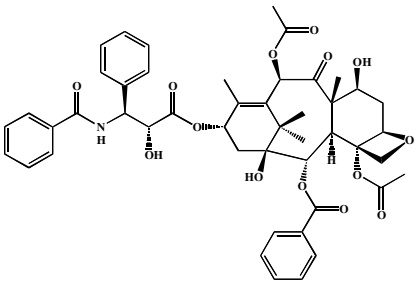

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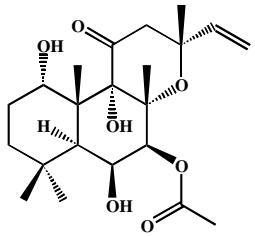

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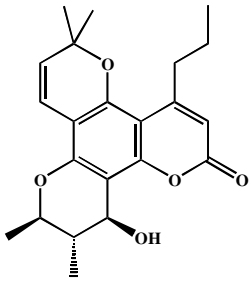

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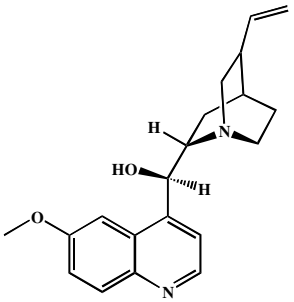

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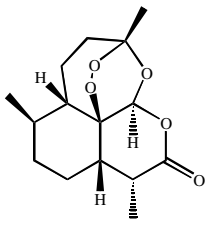

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However, rapid growth of synthetic organic chemistry in the early and mid twentieth century made available a very large number of compounds, and random screening of such chemicals by pharmaceutical companies led to the development of many synthetic drugs like sulphonamides, isoniazids, synthetic anti-psychotics, anti-histamines and synthetic penicillin derivatives etc., which were highly useful. These successes from synthetic therapeutic drugs reduced interest in natural product based drug discovery and many major drug companies almost neglected natural product chemistry in the latter decades. In addition, the clinical efficacy of the botanical medications could not be evaluated/established *de rigour*. Thus, herbal medicines often reflected poor quality control for clinical efficacy.

Currently, the lag phase for botanical medicine is rapidly changing for a number of reasons. Problems with drug-resistant micro-organisms, side effects of modern synthetic drugs and emerging diseases where no medicines are available have stimulated renewed interest in plants as a significant source of new medicines. As a result, considerable research on pharmacognosy, chemistry, pharmacology and clinical therapeutics are being carried out on medicinal plants[2] mainly based on the information from the traditional systems of medicine. A whole range of chronic and difficult to treat diseases such as cancer, cardiovascular diseases, diabetes, rheumatism and AIDS, all require new effective drugs.

1.1.1 Current status

It has been estimated that a large group of world population depends on crude plant drug preparations to tackle various health problems. In India, China and other countries with reputed traditional systems of medicine, plant based therapeutic agents occupy an important niche in health management. The last three decades witnessed new developments in natural products based drugs. Even in the economically developed countries, there is an ever growing interest in natural remedies, which have come to be known as

'phytomedicines'. These preparations are invariably single plant extracts or fractions thereof as distinct from pure chemical entities which may be called molecular drugs. The World Health Organization also has recognized the importance of traditional medicine and has been active in creating strategies, guidelines and standards for botanical medicines.[3]

The mass screening of plants in the search for new drugs is vastly expensive and inefficient. However, such a programme was carried out for obtaining anticancer drugs by National Cancer Institute, USA and this effort led to the discovery of the anticancer drug Taxol (**11**). It would be cheaper and perhaps more productive to re-examine plant remedies described in different traditional medicine texts. i.e., Ethno-pharmacology could be used to identify drugs to alleviate human illness through a thorough analysis of plants known to be used by different human cultures throughout the world. Thus, there is opportunity for multidisciplinary research that joins the forces of natural products chemistry, molecular and cellular biology, synthetic and analytical chemistry, biochemistry and pharmacology to exploit the vast diversity of chemical structures and biological activities of natural products.

The traditional systems of medicine have a relatively organized database and a more exhaustive description of botanical material that can be tested using modern scientific methods. In India, the *Ayurvedic* system of medicine has an important role in the bioprospecting of new medicines.

1.1.2 *Ayurveda*

Ayurveda is an ancient system of health care that is native to the Indian subcontinent and is being practiced for thousands of years.[4] The word *Ayurveda* is a compound of the Sanskrit words *āyus* meaning "life" and *veda*, which refers to "knowledge". Thus, *Ayurveda* roughly translates as the "knowledge of life". According to *Charaka Samhita*, an ancient Indian *Ayurvedic* text on internal medicine written by Charaka, "life" itself is defined

as the "combination of the body, sense organs, mind and soul, the factor responsible for preventing decay and death, which sustains the body over time and guides the processes of rebirth". According to this perspective, *Ayurveda* is concerned with measures to protect *āyus*, which includes healthy living along with therapeutic measures that relate to physical, mental, social and spiritual harmony. *Ayurveda* is also one among the few traditional systems of medicine to contain a sophisticated system of surgery. *Ayurveda* is still being successfully used in many countries. Indian healthcare consists of medical pluralism and *Ayurveda* still remains dominant compared to modern medicine, particularly for treatment of a variety of chronic disease conditions.

Traditional *Ayurvedic* therapeutic formulations draw on an impressive array of plants, many of which have not been scrutinized thoroughly by modern scientific methods. India has about 45,000 plant species and several thousands of them have been found to be of medicinal use. The first *Ayurvedic* herb which attracted international attention was *Rauwolfia serpentina* when it was found that its constituent alkaloid, reserpine **2**[5], had the twin effect of lowering high blood pressure and can act as a tranquilizer. In its traditional usage, this plant has been used for the treatment of snake bites, feverish illnesses and insanity for about 3000 years.[6] In the classical *Ayurvedic* literature, several therapeutically useful plants which act as immunomodulators, memory enhancers, neuroprotectives, antiobesity, antiaging agents, etc., have been described and which are now receiving modern scientific attention.[7] Some recent work in drug development taking advantage of the *Ayurvedic* knowledge relates to the species of *Commiphora* (hypolipidaemic agent), *Picrorhiza* (hepatoprotective), *Bacopa* (memory enhancer), *Curcuma* (anti-inflammatory) and *Ascelpias* (cardiotonic).[8] Numerous molecules have come out of *Ayurvedic* experiential base, examples of which include the rauwolfia alkaloids for hypertension, psoralens from

Psoralea corylifolia in vitiligo, holarrhena alkaloids in amoebiasis, guggulsterons as hypolipidemic agents, *Mucuna pruriens* for Parkinson's disease, piperidines as bioavailability enhancers, baccosides in mental retension, picrosides in hepatic protection, phyllanthins as anti-virals, curcumines in inflammation, withanoloides and many other steroids, lactones and glycosides as immunomodulators.[9]

It is now generally believed that recapitulation and adaptation of this older science to modern drug discovery processes can bring renewed interest to the pharmaceutical world and offer unique therapeutic solutions for a wide range of human disorders. Eventhough time-tested evidences vouch immense therapeutic benefits for *Ayurvedic* herbs and formulations, several important issues are required to be resolved for successful implementation of *Ayurvedic* principles to present drug discovery methodologies. Additionally, clinical examination in the extent of efficacy, safety and drug interactions of newly developed *Ayurvedic* drugs and formulations are required to be carefully evaluated. A reverse-pharmacology approach focusing on the potential of *Ayurvedic* herbs and herbal products for different targets could perhaps bring tremendous leads to *Ayurveda* based drug discovery. Although several novel leads and drug molecules have already been discovered from *Ayurvedic* medicinal herbs, further scientific exploration in this arena along with verification and standardization according to the modern system of medicine is required.

1.2 Natural products as anticancer agents

As the current research work deals mainly with the anticancer and antioxidant activities of natural products, an introduction to both these properties is discussed in detail in the coming sections.

1.2.1 Cancer

Cancer may be considered as one of the worst form of human diseases prevailing now. Modern man is confronted with an increasing incidence of cancer and cancer death annually. Mortality that results even from the common forms of cancer is still unacceptably high.[10] Statistics indicate that men are largely plagued by lung, colon, rectum and prostrate cancer whilst women increasingly suffer from breast, colon, rectum and stomach cancer.[11] The cause for the occurrence of cancer is considered to be one among the three main reasons, viz., incorrect diet, genetic predisposition and via the environment.

Cancer is a disease in which disorder occurs in the normal process of cell division that are controlled by the genetic material (DNA) of the cell. For a cell to replicate, it must:

(1) faithfully reproduce its DNA

(2) manufacture sufficient cellular organelles, membranes, soluble proteins etc., to enable the daughter cells to survive and,

(3) partition the DNA and cytoplasm equally to form two daughter cells.

This process requires a significant amount of feedback control to ensure that the molecular steps are sequentially and correctly oriented. Failure to control the cell cycle is believed to proceed through many stages over a number of years or even decades. The stages of carcinogenesis include initiation, promotion and progression.

1.2.1.1 Carcinogenesis

The substances that initiate cancer in human body are termed as carcinogens. Viruses, chemical carcinogens, chromosomal rearrangements or spontaneous transformations, inactivity of tumor suppressor genes etc., have been implicated as causes of cancer. Genetic predisposition to cancer lends itself to ~20% of cancer cases thus leaving the majority of cancers being

associated with a host of environmental carcinogens.[12] Environmental carcinogens include both natural and manmade chemicals, radiations and viruses. The carcinogens may be divided into genotoxic carcinogens, procarcinogens, epigenetic carcinogens and unclassified carcinogens. Genotoxic carcinogens are those substances that react with nucleic acids. These can be directly acting carcinogens as they can directly affect cellular constituents. Procarcinogens are substances that require metabolic activation to induce carcinogenesis. Epigenetic carcinogens are carcinogens that are not genotoxic (Table 1.2).

Table 1.2: Types of carcinogens[13]

Type	Example
1. Genotoxic carcinogen	
Primary, direct-acting alkylating agents	**Dimethyl sulphate, ethylene imine, β -propiolactone**
2. Procarcinogens	
Polycyclic aromatic hydrocarbons	**Benzo[a]pyrene**
Nitrosamines	**Dimethylnitrosamine**
Hydrazine	**1,2-Dimethylhydrazine**
Inorganic	**Cadmium, Plutonium**
3. Epigenetic carcinogens	
Promotors	**Phorbolesters, saccharin, bileacids**
Solid state	**Asbestos, plastic**
Hormones	**Estrogens**
Immunosuppressants	**Purine analogues**
Cocarcinogens	**Catechol**
4. Unclassified	
Peroxisome proliferators	**Clofibrate, phthalate esters**

Molecular diversity of cancer initiating compounds range from metals to complex organic molecules with large variation in their potencies. The variation in structure and potency suggests that more than one mechanism is involved in carcinogenesis.

Carcinogens in the diet that trigger the initial stage include moulds and aflatoxins (in peanuts and maize), nitrosamines (in smoked meats and other cured products), rancid fats and cooking oils, alcohol and additives and preservatives. A combination of foods may have a cumulative effect and

when incorrect diet is added to a polluted environment, smoking, UV radiation, free radicals, lack of exercise and stress, the stage is set for DNA damage and cancer progression. On the protective side, a diet rich in fruits, vegetables and fibre is associated with a reduced risk of cancer at most sites. The elimination of environmental carcinogens or at least avoiding exposure to them offers the opportunity to prevent most cancers, which is the basis of primary prevention.

One of the most important mechanisms contributing to cancer is considered to be oxidative damage to the DNA. If a cell containing damaged DNA divides before it is repaired, the result is likely to be a permanent genetic alteration constituting a first step in carcinogenesis. Body cells that divide rapidly are more susceptible to carcinogenesis because there is less opportunity for DNA repair before cell division. Mutagenic changes in the components of signaling pathways also lead to cancer.

1.2.1.2 Chemo preventive agents

Chemo preventive agents used in anticancer therapy exert their protective effects in specific stages of multi step carcinogenesis. During the late 1960s and early 1970s, pace setting studies were performed by Dr. Lee W. Wattenberg and his associates at the University of Minnesota in which it was demonstrated that various compounds, especially those associated with fruits and vegetables such as indoles and isothiocyanates could inhibit chemically induced tumors.[14] This was the advent of the "*chemoprophylaxis of carcinogenesis*" and the implications of these observations in terms of human health maintenance became immediately apparent. Subsequently, a series of hallmark studies performed with a myriad of retinoids, Dr. Michael B. Sporn demonstrated that "cancer chemoprevention" was possible.[15] In general terms, cancer chemoprevention may be considered as the prevention of cancer in human populations by ingestion of chemical agents that prevent carcinogenesis.

According to the conventional classification of chemo preventive agents as proposed by Wattenberg,[16] they are of two categories viz., the blocking agents and the suppressing agents. The classification of chemopreventive agents according to their mechanism of action is illustrated in figure 1.1.[17]

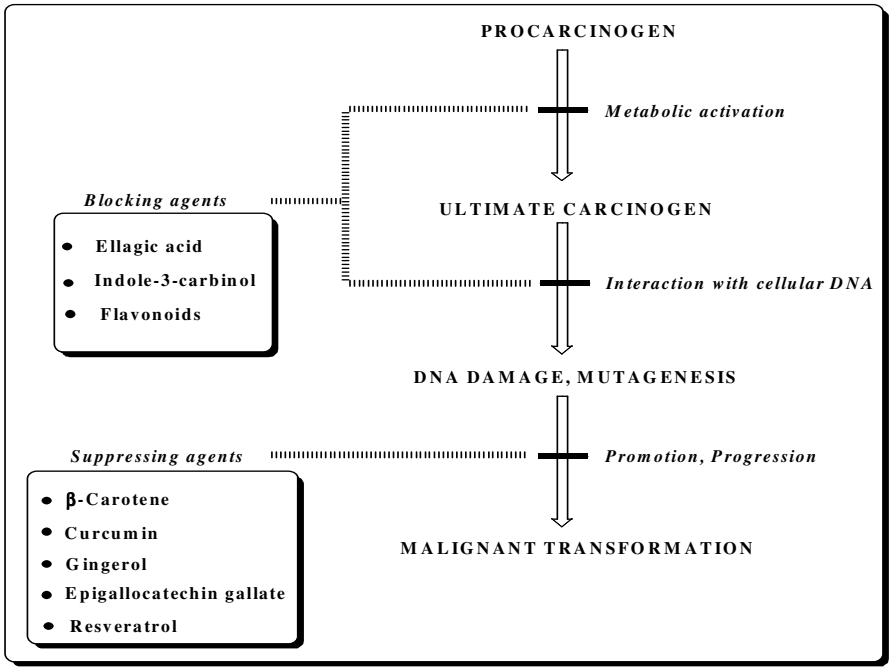
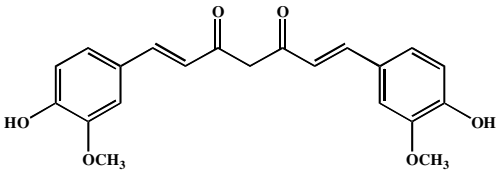


Figure 1.1: Classification of chemo preventive phytochemicals based on their mode of action

Blocking agents are typically those compounds that can inhibit initiation either by inhibiting the formation of carcinogens from (i) precursor molecules, (ii) reactive metabolites from the parent carcinogens and those preventing the ultimate electrophilic and carcinogenic species from interacting with critical cellular target molecules, such as DNA, RNA and proteins. Suppressing agents are considered to inhibit malignant expression of initiated cells, in either the promotion or the progression stage. Certain chemo preventive agents such as curcumin **16** and resveratrol **17** have more than one

defined mechanism of action and hence possess both suppressing and blocking properties.[18]



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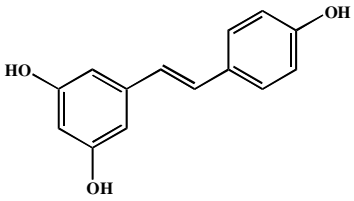

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The fragility of humans for the susceptibility of cancer presents an ongoing challenge for individuals who are involved in the discipline of therapeutic intervention. It is therefore very important for the full recognition of the benefits of disease prevention through therapeutic interventions and/or for aggressive implementation.

A vast variety of chemical compounds have been identified to elicit pronounced chemo preventive effects and many of them are of plant origin that are present naturally in our daily foods or have been used for traditional herbal medication.[19] As such, many herbal medicines, botanicals, dietary supplements and edible plants have all been suggested as potentially important in cancer chemoprevention.[20]

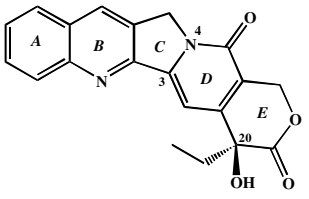
1.2.1.3 Natural products in anticancer therapy

Nature abounds with a rich potential heritage of therapeutic resource that has been exploited for effective and beneficial use against many human cancers, either in the prevention strategy or in therapeutic armamentaria to kill tumor cells. Many of the bioactive natural compounds might have evolved in the plants to counteract natural predators and for self defense. The potential of using natural products as anticancer agents was first recognized in the 1950s by the US National Cancer Institute (NCI) under the leadership of late Dr. Jonathan Hartwell and NCI has since made major contributions to the discovery of new naturally occurring anticancer agents.[21] Several recent

reviews have provided data that document the importance of natural products as a source of bioactive compounds.

Literature studies reveal that many natural products are available as chemo protective agents against commonly occurring cancers. A major group of these compounds are the powerful antioxidants, others are phenolic in nature and the remainder include compounds bearing reactive groups that confer protective properties. Although the mechanism of the protective effect is unclear, the fact that the consumption of fruit and vegetables lowers the incidence of carcinogenesis at a wide variety of sites is broadly accepted. Of the many anticarcinogens already detected in plant foods, the antioxidants vitamin C and E and the provitamin β -carotene have received the most attention.[22] In the last few decades, advances in cancer research have enhanced our understanding of cancer biology and genetics. Most important finding is that genes that control apoptosis have a major effect on malignancy through the disruption of the apoptotic process that leads to tumor initiation, progression and metastasis. Therefore, one mechanism of tumor suppression by natural products may be to induce apoptosis, thereby providing a genetic basis for cancer therapy by natural products. Many naturally occurring antioxidants, fatty acids, amino acids, flavonoids, resveratrol and alkaloids can play an important role in cancer prevention. A large number of plant, marine and microbial sources have been tested as leads and many compounds have survived those tests as potential leads.[23] The chemistry and properties of some of the major plant derived anticancer drugs are discussed below.

Camptothecin



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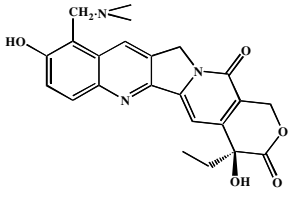

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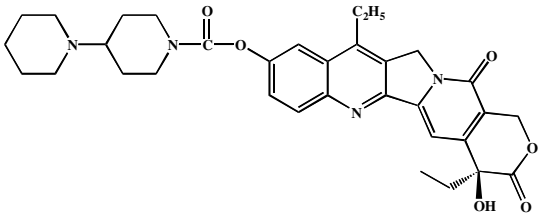


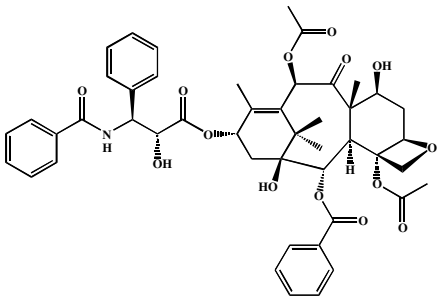
20

The discovery of camptothecin (CPT, **18**) by Wall and Wani as an anticancer drug in the early sixties added an entirely new dimension to the field of chemotherapy. Camptothecin, was first extracted[24] from the stem wood of the Chinese ornamental tree *Camptotheca acuminata*. The molecule became so important and at present the first generation analogues of camptothecin, hycamtin (**19**, topotecan) and camptosar (CPT – II, **20**, irinotecan) are used for the treatment of ovarian and colon cancers. Camptothecin is a member of the quinolinoalkaloid group. It consists of a pentacyclic ring structure that includes a pyrrole (3,4β) quinoline moiety and one asymmetric centre within the α -hydroxy lactone ring with 20(*S*) configuration (ring E). The planar pentacyclic ring structure (rings A–E) was suggested to be one of the most important structural features of this type of compounds. The stereochemistry at C-20 of CPT is very crucial for its activity, as 20(*S*) hydroxyl is active while the corresponding 20(*R*) hydroxyl compound is inactive.[25] One of the major drawbacks observed in the use of CPT analogues in clinical studies was a marked loss of therapeutic activity due to their intrinsic instabilities resulting from the rapid hydrolysis of the lactone ring in the body. Apart form the above drawback, it is a potent cytotoxic agent. It shows anticancer activity mainly for solid tumors. It shows anticancer activity mainly against ovarian, colon and pancreatic cancer cells. But its analogues showed anticancer activity in breast, liver, prostate cancers etc. Camptothecin inhibits DNA topoisomerase I[26] thereby preventing DNA replication. The development of synthetic and semisynthetic strategies has

facilitated the study of the CPT mechanism, as well as the identification of analogues with improved properties.

The most successful derivatives of CPT have been obtained due to modifications of rings A and B. To date, the only CPT analogues approved for clinical use[27] are topotecan (**19**) and irinotecan (**20**). All the anlogues of CPT have proved as potent cytotoxic agents by inhibiting cellular DNA topoisomerase I by a mechanism similar to CPT with similar or better activity. Continued studies on the camptothecin-DNA topoisomerase I interaction in addition to its detailed mechanism of action may suggest new directions in the synthesis of new camptothecins.

Taxol



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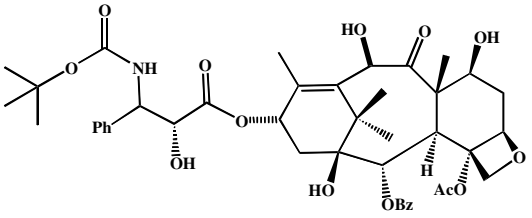


21

Taxol (generic name paclitaxel, trade name taxol, **11**) is a complex polyoxygenated diterpenoid isolated from the pacific yew, *Taxus brevifolia*.[28] Taxol is used for the treatment of refractory ovarian cancer, metastatic breast and lung cancer and Kaposi's sarcoma. Taxotere (docetaxel, **21**), one of its semisynthetic derivatives, is now known as a better anticancer drug than taxol. Taxol has a basic [$9.3.1.0^{3,8}$] pentadecane, tetracyclic ring system. It has a N-benzoyl-β-phenylisoserine side chain attached at the C-13 hydroxyl as an ester linkage. This side chain is essentially required in taxol for anticancer activity and so is the C-2'-hydroxyl. Figure 1.2 depicts some of the interesting structure activity relationship (SAR) shown by taxol.

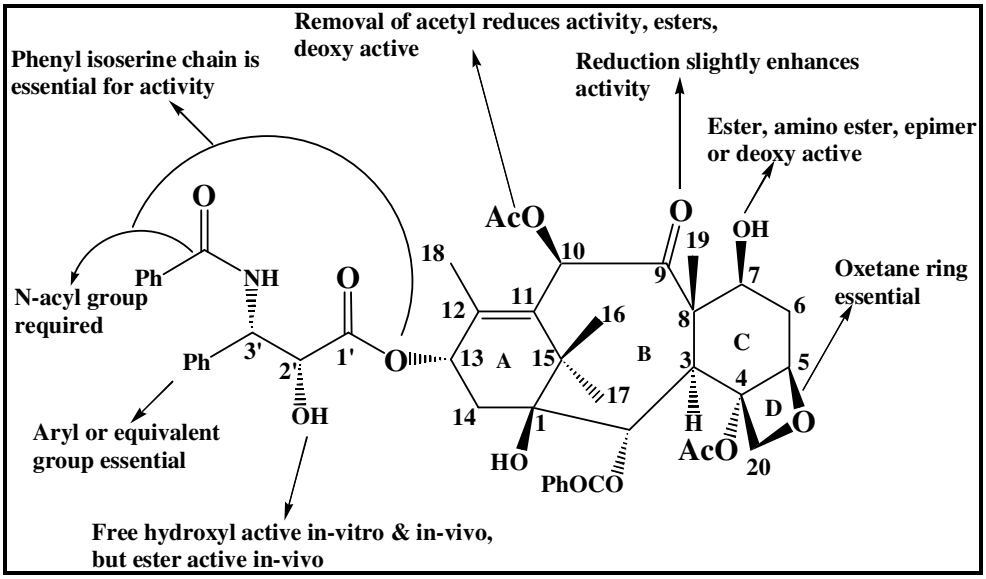
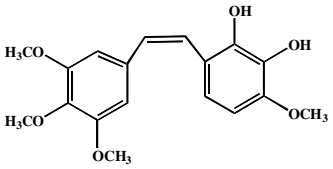


Figure 1.2: A brief description of SAR of taxol

Taxol has a unique mode of action.[29] It acts as a microtubulin stabilizing agent. Tubulin polymerizes to microtubulin which in turn reverts back to tubulin. In a normal case, this process is in equilibrium. Taxol makes a microtubulin bundle larger in size than the normal bundle size required for the process of cell multiplication. Due to this, a defective polymerization occurs and the cells have unnatural bundles of microtubules with the absence of the mitotic spindle. The cancerous cells lack a check point to detect the absence of a spindle and attempts to continue the cell cycle which eventually lead to cell death. Because of this reason, taxol is also referred to as a "spindle poison". A major drawback of taxol is that it has poor bio-availability due to its poor solubility in water.

Taxol is a drug tolerated by its recipients better than any other anticancer drugs used today. Many derivatives of taxol like taxotere **21** and isotaxel having more advantages such as better potency, greater solubility and lesser side effects have been developed.[30]

Combretastatin A-4



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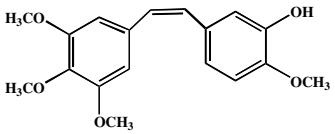

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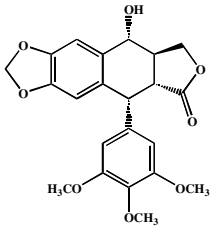
Combretastatins are mitotic agents isolated from the bark of the South African tree *Combretum caffrum*. Combretastatins A-1[31] **22** and A-4[32] **23** were isolated by Petit and coworkers in 1987 and 1989 respectively. Combretastatin A-4 (CA-4) is a simple stilbene that has been shown to compete with colchicines for binding sites on tubulin. Both are stilbene derivatives having two phenyl rings separated by a C-C double bond. It is concluded that a diaryl system separated through a double bond along with a trimethoxy system in one of the rings show cytotoxic activity.[33]

CA-4 is a potent cytotoxic agent which strongly inhibits the polymerization of brain tubulin by binding to the colchicine site. It shows potent cytotoxicity against a wide variety of human cancer cell lines and is also an attractive lead molecule for the development of novel anticancer drugs.[34] CA-4 is an investigation drug of the NCI. The compound is active against colon, lung and leukemia cancers. It is stated that this molecule is the most cytotoxic phytomolecule isolated so far. Varied modified analogues of CA-4 including modification in the functional group, aromatic ring and in the linear alkene have been synthesized and evaluated. [35]

Podophyllotoxin



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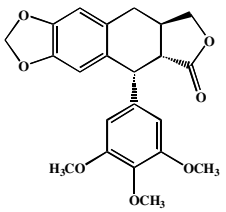

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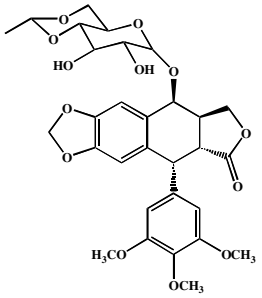

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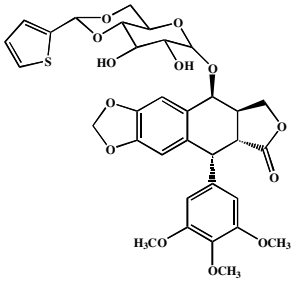


27

Podophyllotoxin (**24**) and deoxypodophyllotoxin (**25**) are two well known naturally occurring aryltetralin lignans. It was first isolated by Podwyssotzki from the North American plant *Podophyllum peltatum* (may Apple).[36] Deoxypodophyllotoxin has been isolated[37] from *Anthriscus sylvestris* and *Pulsatilla koreana*. Two of the semisynthetic derivatives of podophyllotoxin viz., etoposide **26** and teniposide **27** are currently used in frontline cancer chemotherapy against various cancers.[38]

Chemically, podophyllotoxin is an aryltetralin lignan having a lactone ring. The SAR studies reveal that only the A and E rings of this compound is essential for its activity and the D-ring in lactone form enhances the activity. Also, introduction of bulky groups at the C-4 position in ring C enhances the activity.

Podophyllotoxin is effective in the treatment of Wilms tumors, various genital tumors and in non-Hodgkin's and other lymphomas and lung cancer.[39] Synthetic analogues such as epipodophyllotoxin, etoposide and tenetoposide have less toxic side effects than podophyllotoxin.

Over the years, a number of approaches have been developed for the discovery of new molecules for clinical use and as a result of this, a number of anticancer drugs have come out. The main problem with these molecules is the lack of specificity as these drugs also kill the healthy cells. Apart from this, drug resistance is another problem. The development of a safe, economic

and site specific anticancer drug is still a challenge. Perhaps it is necessary to look towards nature for other molecules with novel modes of action in order to tackle this dreadful disease.

1.3 Natural products as antioxidants

1.3.1 Introduction

Oxygen is an element indispensable for life. When cells use oxygen to generate energy, free radicals are created as a consequence of ATP (adenosine triphosphate) production by the mitochondria. A molecule with one or more unpaired electron in its outer shell is called a free radical. Free radicals are less stable than non-radical species, although they are more reactive. Free radicals are formed from molecules *via* (i) the breakage of a chemical bond such that each fragment keeps one electron, (ii) by cleavage of a radical to give another radical and, also *via* (iii) redox reactions. Oxygen free radicals or more generally, reactive oxygen species (ROS), as well as reactive nitrogen species (RNS) are products of normal cellular metabolism. ROS and RNS are the terms collectively describing free radicals and other non-radical reactive derivatives which are also called oxidants.[40]

Free radicals, as mentioned earlier, can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals. This unpaired electron(s) usually gives a considerable degree of reactivity to the free radical. Radicals derived from oxygen represent the most important class of radical species generated in living systems. Molecular oxygen (dioxygen, O_2) has a unique electronic configuration and is itself a radical (Figure 1.3) with unpaired electrons.

These ROS and RNS formed as the by products of normal cellular process, are well recognized for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems. These biological free radicals are highly unstable molecules that have electrons available to react with various organic substrates such as lipids,

proteins and DNA. The harmful effect of free radicals causing potential biological damage is termed oxidative stress and nitrosative stress. In other words, oxidative stress results from the metabolic reactions that use oxygen and represents a disturbance in the equilibrium status of pro-oxidant/antioxidant reactions in living organisms.[41]

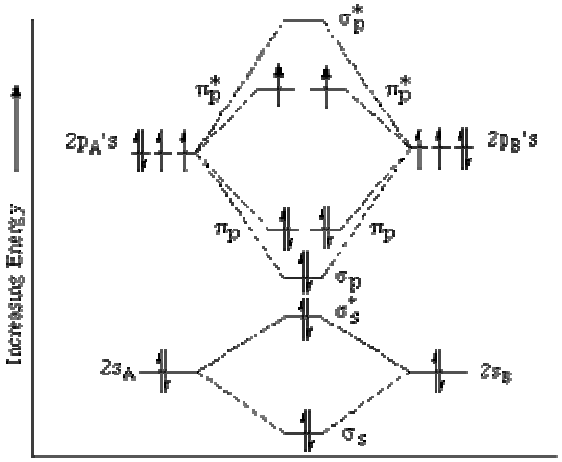


Fig. 1.3: MO diagram of O_2

The excess ROS can damage cellular lipids, proteins or DNA, inhibiting their normal function. Because of this, oxidative stress has been implicated in a number of human diseases as well as in the aging process. The delicate balance between beneficial and harmful effects of free radicals is a very important aspect of living organisms and is achieved by mechanisms called “redox regulation”. The process of “redox regulation” protects living organisms from various oxidative stresses and maintains “redox homeostasis” by controlling the redox status *in vivo*. When free radicals are generated *in vivo*, many antioxidants in the body act by defending the organism from oxidative damage. As a first line of defense, the preventive antioxidants such as peroxidases and metal chelating proteins suppress the generation of free radials. Next, the radical-scavenging antioxidants such as vitamin C and vitamin E scavenge radicals to inhibit the oxidation chain initiation and prevent chain propagation as a second line of defense. This may also include

the termination of a chain by the reaction of two radicals. The repair and *de novo* enzymes act as the third line of defense by repairing damage and reconstituting membranes.[42]

1.3.1.1 Reactive oxygen species (ROS)

The reactive oxygen species include hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), peroxyl ($ROO^{\bullet}$) and lipid peroxyl ($LOO^{\bullet}$). On the other hand, hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3) and lipid peroxide (LOOH), generally called oxidants are not free radicals, but can easily lead to free radical reactions in living organisms. The addition of one electron to dioxygen forms the superoxide anion radical ($O_2^{\bullet-}$).[43] Superoxide anion, arising either through metabolic processes or following oxygen "activation" by physical irradiation, is considered the "primary" ROS and can further interact with other molecules to generate "secondary" ROS, either directly or prevalently through enzyme- or metal-catalysed processes.[44] The production of superoxide occurs mostly within the mitochondria of a cell.[45] The mitochondrial electron transport chain is the main source of ATP in the mammalian cell and thus is essential for life. During energy transduction, a small number of electrons "leak" to oxygen prematurely, forming the oxygen free radical superoxide, which has been implicated in the pathophysiology of a variety of diseases. Another ROS, the hydroxyl radical, $^{\bullet}OH$, is the neutral form of the hydroxide ion. The hydroxyl radical has high reactivity, making it a very dangerous radical with a very short *in vivo* half-life of approximately 9–10 s.[46] Thus when produced *in vivo* $^{\bullet}OH$ reacts close to its site of formation. Under stress conditions, an excess of superoxide releases "free iron" from iron-containing molecules. The released Fe^{2+} can participate in the Fenton reaction, generating highly reactive hydroxyl radical ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$). Thus under stress conditions, $O_2^{\bullet-}$ acts as an oxidant and facilitates

$^{\bullet}OH$ production from H_2O_2 by making Fe^{2+} available for the Fenton reaction. The superoxide radical participates in the Haber–Weiss reaction ($O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^-$) which combines a Fenton reaction and the reduction of Fe^{3+} by superoxide, yielding Fe^{2+} and oxygen ($Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$). Additional reactive radicals derived from oxygen that can be formed in living systems are peroxyl radicals ($ROO^{\bullet}$). The simplest peroxyl radical is $HOO^{\bullet}$, which is the protonated form of superoxide ($O_2^{\bullet-}$) and is usually termed either hydroperoxyl radical or perhydroxyl radical.

1.3.1.2 Reactive nitrogen species (RNS)

Nitric oxide ($NO^{\bullet}$) is a small molecule that contains one unpaired electron on the antibonding $2\pi_y^*$ orbital and is therefore, a radical. $NO^{\bullet}$ is generated in biological tissues by specific nitric oxide synthases (NOSs), which metabolise arginine to citrulline with the formation of $NO^{\bullet}$ *via* a five electron oxidative reaction.[47] $NO^{\bullet}$ is an abundant reactive radical that acts as an important oxidative biological signaling molecule in a large variety of diverse physiological processes, including neurotransmission, blood pressure regulation, defense mechanisms, smooth muscle relaxation and immune regulation. Due to its extraordinary properties, $NO^{\bullet}$ was acclaimed as the "molecule of the year" in *Science Magazine* in 1992.[47c] $NO^{\bullet}$ has a half-life of only a few seconds in an aqueous environment. $NO^{\bullet}$ has greater stability in an environment with a lower oxygen concentration (half life >15 s). However, since it is soluble in both aqueous and lipid media, it readily diffuses through the cytoplasm and plasma membranes. $NO^{\bullet}$ has effects on neuronal transmission as well as on synaptic plasticity in the central nervous system. In the extracellular milieu, $NO^{\bullet}$ reacts with oxygen and water to form nitrate and nitrite anions. Overproduction of reactive nitrogen species is called nitrosative stress. This may occur when the generation of reactive nitrogen species in a system exceeds the system's ability to neutralise and eliminate them.

Nitrosative stress may lead to nitrosylation reactions that can alter the structure of proteins and so inhibit their normal function.

Cells of the immune system produce both the superoxide anion and nitric oxide during the oxidative burst triggered during inflammatory processes. Under these conditions, nitric oxide and the superoxide anion may react together to produce significant amounts of a much more oxidatively active molecule, peroxynitrite anion ($ONOO^-$), which is a potent oxidising agent that can cause DNA fragmentation and lipid oxidation, $NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^-$. This reaction has one of the highest rate constants known for reactions of $NO^{\bullet}$, viz., $7.0\times10^9\ M^{-1}s^{-1}$. Thus $NO^{\bullet}$ toxicity is predominantly linked to its ability to combine with superoxide anions.

These ROS and RNS species play a dual role as both toxic and beneficial compounds. The delicate balance between their two antagonistic effects is clearly an important aspect of life.

1.3.1.3 Generation of free radicals and oxidants

Formation of ROS and RNS can occur in the cells either by enzymatic or by non-enzymatic reactions. Enzymatic reactions generating free radicals include those involved in the respiratory chain, the phagocytosis, the prostaglandin synthesis and the cytochrome P450 system.[48] For example, the superoxide anion radical ($O_2^{\bullet -}$) is generated via several cellular oxidase systems such as NADPH oxidase, xanthine oxidase and peroxidases. Once formed, it participates in several reactions yielding various ROS and RNS such as hydrogen peroxide, hydroxyl radical ($^{\bullet}OH$), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), etc. H_2O_2 (a non radical) is produced by the action of several oxidase enzymes, including aminoacid oxidase and xanthine oxidase. Hydrogen peroxide catalyses the oxidation of hypoxanthine to xanthine and of xanthine to uric acid. Hydroxyl radical ($^{\bullet}OH$), the most reactive free radical *in vivo*, is formed by the reaction of $O_2^{\bullet -}$ with H_2O_2 in the presence of Fe^{2+} or Cu^+ (catalyst) as mentioned in section 1.3.1.1.

Hypochlorous acid (HOCl) is produced by the neutrophil-derived enzyme, myeloperoxidase, which oxidizes chloride ions in the presence of H_2O_2. Nitric oxide radical ($NO^{\bullet}$) is formed in biological tissues from the oxidation of L-arginine to citrulline by nitric oxide synthase.[48b] Free radicals can be produced from non-enzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing radiations. The non nzymatic process can also occur during oxidative phosphorylation (i.e. aerobic respiration) in the mitochondria. ROS and RNS are generated from either endogenous or exogenous sources.

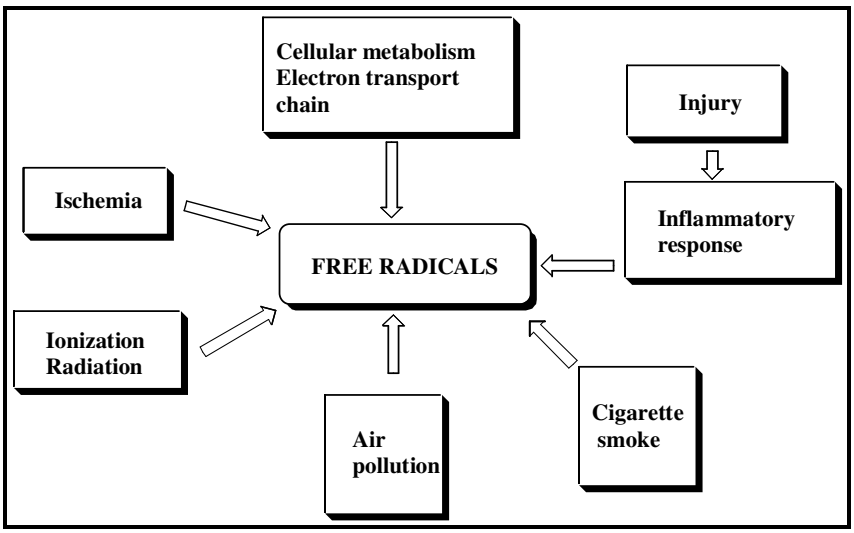


Fig. 1.4: Summary of sources of free radicals

Endogenous free radicals are generated from immune cell activation, inflammation, mental stress, excessive exercise, ischemia, infection, cancer or aging. Exogenous ROS/RNS result from air and water pollution, exposure to ultraviolet radiation, cigarette smoke, alcohol, heavy or transition metals (Cd, Hg, Pb, Fe, As), certain drugs (cyclosporine, tacrolimus, gentamycin, bleomycin), industrial solvents, cooking (smoked meat, used oil, fat), radiation etc (Figure 1.4).[49] After penetration into the body by different routes, these exogenous compounds are decomposed or metabolized into free radicals.

1.3.1.4 Beneficial activities of free radicals and oxidants

At low or moderate concentrations, ROS and RNS are necessary for the maturation process of cellular structures and can act as weapons for the host defense system. Beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in cellular responses to noxia, as for example, in defense against infectious agents and in the function of a number of cellular signaling systems. At low/moderate concentrations ROS invokes induction of a mitogenic response too.[48] Indeed, phagocytes (neutrophils, macrophages, monocytes) release free radicals to destroy invading pathogenic microbes as part of the body's defense mechanism against disease. The importance of ROS production by the immune system is clearly exemplified by patients with granulomatous disease. These patients have defective membrane-bound NADPH oxidase system which makes them unable to produce the superoxide anion radical ($O_2^{\bullet -}$), thereby resulting in multiple and persistent infection. Other beneficial effects of ROS and RNS involve their physiological roles in the function of a number of cellular signaling systems. Their production by non phagocytic NADPH oxidase isoforms play a key role in the regulation of intracellular signaling cascades in various types of nonphagocytic cells including fibroblasts, endothelial cells, vascular smooth muscle cells, cardiac myocytes and thyroid tissue. For example, nitric oxide ($NO^{\bullet}$) is an intercellular messenger for modulating blood flow, thrombosis and neural activity. $NO^{\bullet}$ is also important for nonspecific host defense and for killing intracellular pathogens and tumors. In brief, ROS/RNS at low or moderate levels are vital to human health.

1.3.1.5 Deleterious activities of free radicals and oxidants

Oxidative stress can arise when cells cannot adequately destroy the excess of free radicals formed. In other words, oxidative stress results from an

imbalance between formation and neutralization of ROS/RNS. For example, hydroxyl radical and peroxynitrite in excess can damage cell membranes and lipoproteins by a process called lipid peroxidation. This reaction leads to the formation of malondialdehyde (MDA) and conjugated diene compounds, which are cytotoxic and mutagenic. Lipid peroxidation occurs by a radical chain reaction, i.e. once started; it spreads rapidly and affects a great number of lipid molecules.[49] Proteins may also be damaged by ROS/RNS, leading to structural changes and loss of enzyme activity. Oxidative damage to DNA leads to the formation of different oxidative DNA lesions which can cause mutations. Various oxidative stress induced diseases in humans is summarized in figure 1.5.

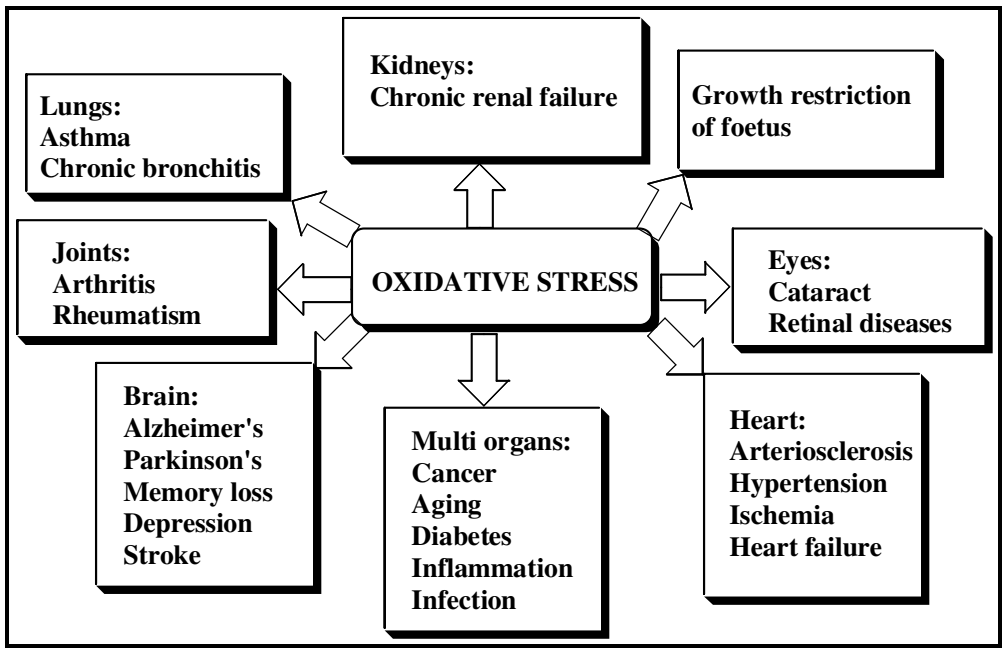


Figure 1.5: Oxidative stress induced diseases in humans

Our body has several mechanisms to counteract these attacks by using DNA repair enzymes and/or antioxidants. If not regulated properly, oxidative stress can induce a variety of chronic and degenerative diseases like cancer, cardiovascular diseases, neurological diseases, pulmonary diseases,

rheumatoid arthritis, nephropathy, ocular diseases, as well as the aging process and some acute pathology (trauma, stroke). Here comes the importance of antioxidants to fight against these oxidative stress induced diseases.

1.3.2 ANTIOXIDANTS

The word antioxidant has become popular in modern society as it gained publicity through mass media coverage of its health benefits. The dictionary[50] definition of antioxidant is rather straight forward viz., "*a substance that opposes oxidation or inhibits reactions promoted by oxygen or peroxides*". A more biologically relevant definition of antioxidant is "*a synthetic or natural substance added to products to prevent or delay their deterioration by action of oxygen in air*'. In biochemistry and medicine, "*antioxidants are enzymes or other substances such as Vit. E or β - carotene that are capable of counteracting the damaging effects of oxidation in animal tissues*".[51] The most important and widely accepted explanation of an antioxidant is that defined by Halliwell and Gutteridge,[40a] as "*any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate.*" Antioxidants fight against the free radicals generated *in vivo*, thus preventing the organism against oxidative damage. Hence, the media attention on their health benefits.

1.3.2.1 Antioxidant classification

Antioxidants in cells can be classified as enzymatic antioxidants and non-enzymatic antioxidants. The major enzymatic antioxidants directly involved in the neutralization of ROS and RNS are: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRx).[40] SOD, the first line of defense against free radicals, catalyzes the dismutation of superoxide anion radical ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2) by reduction. The oxidant formed (H_2O_2) is transformed into

water and oxygen (O_2) by catalase (CAT) or glutathione peroxidase (GPx). The selenoprotein GPx enzyme removes H_2O_2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG). Glutathione reductase, a flavoprotein enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power. Besides hydrogen peroxide, GPx also reduces lipid or nonlipid hydroperoxides while oxidizing glutathione (GSH). The non-enzymatic antioxidants are divided into metabolic antioxidants and nutrient antioxidants. Metabolic antioxidants are endogenous antioxidants, produced by metabolism in the body, such as lipoid acid, glutathione, L-arginine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins, transferrin, etc. Nutrient antioxidants are exogenous antioxidants. They are compounds which cannot be produced in the body and must be provided through foods or supplements, such as vitamin E, vitamin C, carotenoids, trace metals (selenium, manganese, zinc), flavonoids, omega-3 and omega-6 fatty acids, etc.

1.3.2.2 Endogenous Antioxidants

Antioxidants that are produced within the body for defense as a result of normal metabolic processes are called endogenous antioxidants. There is a vast network of intracellular and extracellular antioxidants with diverse roles within each area of defense. As already mentioned, catalase converts H_2O_2 to O_2 and H_2O while superoxide dismutase (SOD) converts the superoxide radical to H_2O_2 and O_2. Some of the antioxidant enzymes exist in several forms. For example, membrane, cytosolic and plasma forms of glutathione peroxidase have been isolated and SOD has membrane, cytosolic and extracellular forms. The levels and locations of these antioxidants must be tightly regulated for cell survival. The antioxidant enzymes, SOD, glutathione peroxidase (GPx) and catalase (CAT), work within the cells to remove most superoxides and peroxides before they react with metal ions to form more reactive free radicals. Peroxidative chain reactions initiated by free radicals

that escaped the antioxidant defenses are terminated by chain-breaking water or lipid soluble antioxidants.[52]

1.3.2.3 Exogenous Antioxidants

Antioxidant compounds supplied through diet is termed as exogenous antioxidants. Diet plays a vital role in the production of the antioxidant defense system by providing essential nutrient antioxidants such as vitamin E, C and β-carotene, other antioxidant plant phenols including flavonoids and essential minerals that form important antioxidant enzymes. Diet also plays an important role in the oxidation process by affecting the substrates that are subject to oxidation. The best example is the oxidation of lipids. Polyunsaturated fatty acids (PUFA) having two or more double bonds are increasingly susceptible to free radical attack as the number of double bonds increases. Antioxidants available at the site of radical attack break the chain of oxidation by being preferentially oxidized by the attacking radical, thereby preventing oxidation of the adjacent fatty acid.

1.3.3 Antioxidant Process

When an antioxidant destroys a free radical, this antioxidant itself becomes oxidized. Therefore, the antioxidant resources must be constantly restored in the body. Thus, while in one particular system an antioxidant is effective against free radicals, in other systems the same antioxidant could become ineffective. Also, in certain circumstances, an antioxidant may even act as a pro-oxidant e.g., it can generate toxic ROS/RNS.[49a] The antioxidant process can function in one of two ways: chain-breaking or prevention. When a radical releases or steals an electron, a second radical is formed. This exerts the same action on another molecule and the process continues until either the free radical formed is stabilized by a chain-breaking antioxidant (vitamin C, E, carotenoids, etc.), or it simply disintegrates into an inoffensive product. The classic example of such a chain reaction is lipid peroxidation which will be discussed in detail in Chapter 2.

For the preventive way, antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase prevent oxidation by reducing the rate of chain initiation, e.g., either by scavenging initiating free radicals or by stabilizing transition metal radicals such as copper and iron. The groups of antioxidants and their actions are presented in figure 1.6.

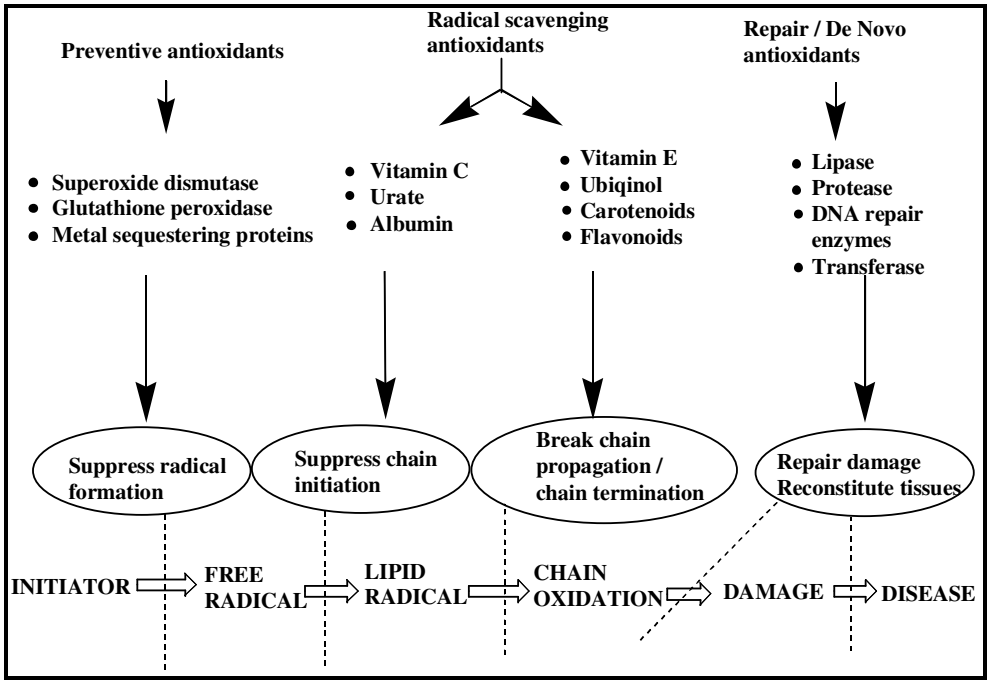


Fig 1.6: Antioxidant groups and actions

Certain compounds have shown antioxidant properties *in vitro*, but uncertain *in vivo*. Such compounds include bilurubin, α-keto acids, melatonin, coenzyme Q, lipoic acid, carnosine, anserine and melanins. A majority of compounds have proved their role in the antioxidant defense mechanisms either directly or indirectly in human system. These include both the endogenous and the exogenous antioxidants.

The major components of the antioxidant defense system with their mode of antioxidant action are summarized in Table 1.3.

Table 1.3: Major components of antioxidant defense system

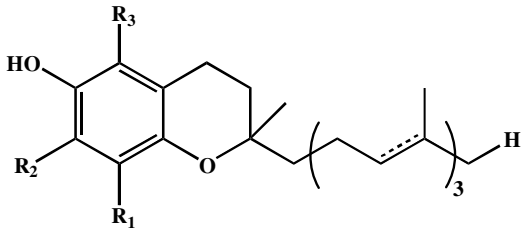
Components	Antioxidant action
Enzymes	
Superoxide dismutase (SOD)	Removal of superoxide radical
Catalase	Reduction of H_2O_2 to water
Glutathione peroxidase	Reduction of H_2O_2 to water
Thioredoxin	Reduction of peroxides
Metal ion sequestration	
Metallothionein	Chelates Zn, Ag, Cu, Cd, Hg
Phytochelatins	Chelates Cd, Zn, Cu
Transferrin	Chelate Fe
Albumin	Chelates Fe and Cu
Low molecular mass (endogenous)	
Urate	Scavenges NO_2
Low molecular mass (endogenous)	
Ascorbic acid	Spares tocopherol, scavenges free radicals
Vitamin E	Scavenges peroxyl radicals, most important chain breaking inhibitor of lipid peroxidation
Carotenoids	*In vivo* antioxidant role uncertain
Plant phenols	Suggested, but not proven to inhibit LDL oxidation *in vivo*

1.3.4 NATURAL PRODUCTS AS ANTIOXIDANTS

Natural antioxidants (from the diet) play an important role in helping endogenous antioxidants for the neutralization of oxidative stress. Nutrient antioxidant deficiency is considered to be among the causes of numerous chronic and degenerative pathologies. Each nutrient is unique in terms of its structure and antioxidant function.[53] The properties of some of the important exogenous (natural) antioxidants are summarized below.

Vitamin E

Vitamin E is a fat-soluble vitamin with high antioxidant potency. It is a chiral compound with eight stereoisomers: α, β, γ, δ tocopherol and α, β, γ, δ tocotrienol (with double bonds in side chain). α-Tocopherol is the most bioactive form in humans.[54] As it is fat-soluble, α-tocopherol safeguards cell membranes from damage by free radicals. Its antioxidant function mainly resides in the protection against lipid peroxidation.



α-tocopherol, $R_1 = R_2 = R_3 = CH_3$
α-tocotrienol, $R_1 = R_2 = R_3 = CH_3$

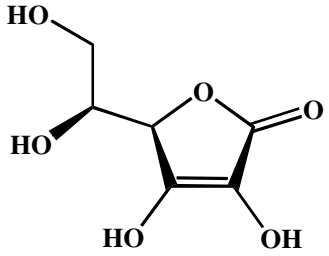
γ-tocopherol, $R_1 = R_2 = CH_3$ $R_3 = H$
γ-tocotrienol, $R_1 = R_2 = CH_3$ $R_3 = H$

β-tocopherol, $R_1 = R_3 = CH_3$; $R_2 = H$
β-tocotrienol, $R_1 = R_3 = CH_3$; $R_2 = H$

δ-tocopherol, $R_1 = R_2 = R_3 = H$
δ-tocotrienol, $R_1 = R_2 = R_3 = H$

Vitamin E has been proposed for the prevention against colon, prostate and breast cancers, some cardiovascular diseases, ischemia, cataract, arthritis and certain neurological disorders. The dietary sources of vitamin E are vegetable oils, wheat germ oil, whole grains, nuts, cereals, fruits, eggs, poultry, meat etc.

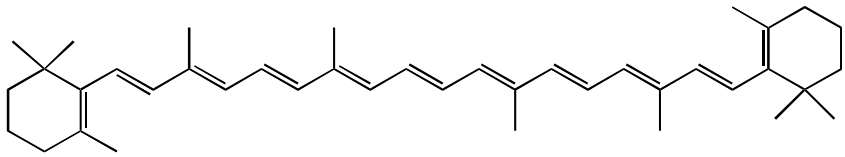
Vitamin C



Vitamin C, also known as ascorbic acid is a water-soluble vitamin. It is essential for collagen, carnitine and neurotransmitters biosynthesis.[55] Health benefits of vitamin C are as antioxidant, anti-atherogenic, anti-carcinogenic and as an immunomodulator. The positive effect of vitamin C resides in

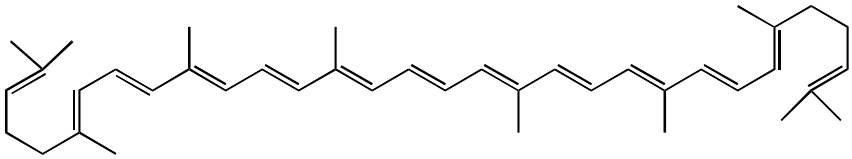
reducing the incidence of stomach cancer and in preventing lung and colorectal cancer. Vitamin C works synergistically with vitamin E to quench free radicals and also regenerates the reduced form of vitamin E. Natural sources of vitamin C are acidic fruits such as lemon and orange, green vegetables, tomatoes etc.

β-Carotene



β-Carotene is a fat soluble member of the carotenoids which are considered pro-vitamins because they can be converted to active vitamin A. β-Carotene is converted to retinol, which is essential for vision. It is a strong antioxidant and is the best quencher of singlet oxygen.[56] β-Carotene is present in many fruits, grains, oil and vegetables (carrots, green plants, squash, spinach etc.).

Lycopene



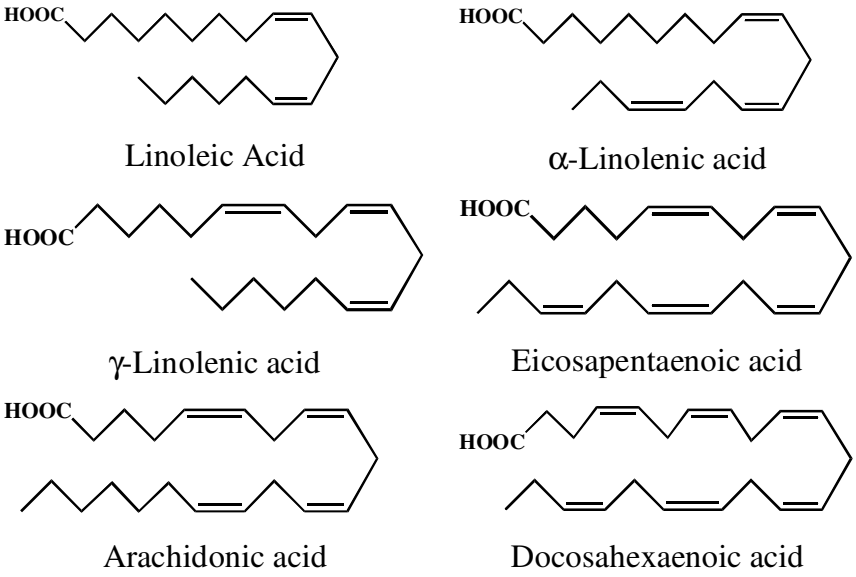
Lycopene, a carotenoid, possesses antioxidant and antiproliferative properties.[57] Lycopene has been found to be very protective, particularly for prostate cancer. The major dietary source of lycopene is tomatoes, with the lycopene in cooked tomatoes, tomato juice and tomato sauce included, being more bio-available than that in raw tomatoes.

Selenium (Se)

Se is a trace mineral found in soil, water, vegetables (garlic, onion, grains, nuts and soybean), sea food, meat, liver and yeast.[58] It forms the active site of several antioxidant enzymes including glutathione peroxidase. At low

dose, health benefits of Se are antioxidant, anti-carcinogenic and as an immunomodulator. Selenium is also necessary for the thyroid function. In China, people in the area with Se poor soil have developed a fatal cardiomyopathy called *Keshan* disease which was cured with Se supplement.

Omega-3 and omega-6 fatty acids



They are essential long-chain polyunsaturated fatty acids because the human body cannot synthesize them.[59] Therefore, they are only derived from food. Omega-3 fatty acids can be found in fatty fish (salmon, tuna, halibut, sardines, pollock), algae, walnut, nut oils and flaxseed. There are three major dietary types of omega-3 fatty acids: eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and alpha-linolenic acid (ALA). EPA and DHA are abundant in fish and are directly used by the body; while ALA is found in nuts and has to be converted to DHA and EPA by the body. Dietary sources of omega-6 fatty acids (linoleic acid) include vegetable oils, nuts, cereals, eggs, poultry. It is important to maintain an appropriate balance of omega-3s and omega-6s in the diet, as these two substances work together to promote health. Omega-3s reduce inflammation and prevent chronic ailments such as heart disease, stroke, memory loss, depression, arthritis, cataract and cancer.

Omega-6s improve diabetic neuropathy, eczema, psoriasis, osteoporosis and aid in cancer treatment.

Flavonoids

Flavonoids have been reported to prevent or delay a number of chronic and degenerative ailments such as cancer, cardiovascular diseases, arthritis, aging, cataract, memory loss, stroke, Alzheimer's disease, inflammation and infection. The main natural sources of flavonoids include green tea, grapes (red wine), apple, cocoa (chocolate), *Ginkgo biloba*, soybean, curcuma, berries, onion, broccoli, etc.

As the major work of this thesis deals with the flavonoids and their biological activities, a detailed introduction on flavonoids is portrayed here.

1.4 FLAVONOIDS – An introduction

Flavonoids are a group of more than 4000 polyphenolic compounds that occur naturally in foods of plant origin. These compounds possess a common phenylbenzopyrone structure. Flavonoids are most commonly divided into six sub-classes, based on the connection position of B and C rings as well as the degree of saturation, oxidation and hydroxylation of the C ring as flavonols, flavones, flavanones, flavan-3-ols (or catechins), isoflavones and anthocyanidines.[60] The basic structure with numbering system of flavonoids and the structures of the different types of flavonoids are given in figures 1.7 and 1.8 respectively. The subclasses and dietary sources of flavonoids with a few representative examples are given in Table 1.4.

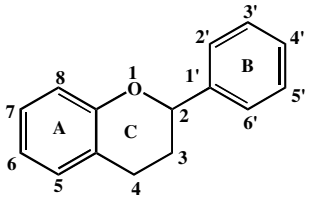


Fig 1.7: Basic flavonoid structure

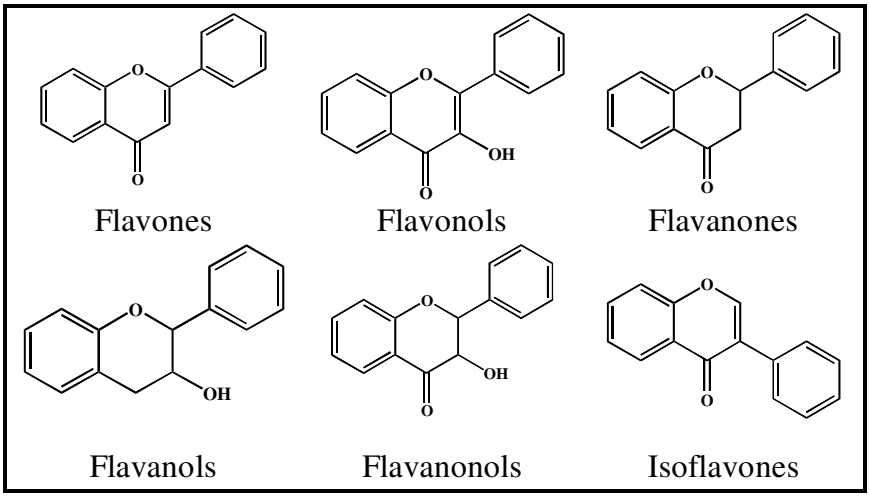


Figure 1.8: Chemical structures of the flavonoid family

Table 1.4: Subclasses and dietary sources of flavonoids

Flavonoid subgroup	Major food sources	Representative flavonoids
Flavones	Parsley, thyme	Apigenin, chrysin, luteolin
Flavonols	Onions, cherries, apples, tomato, tea, red wine	Kaempferol, myricetin, quercetin, rutin
Flavanones	Oranges, grapefruit	Eriodictyol, hesperitin, naringenin
Flavanols	Apples, tea	Catechin, gallocatechin
Flavanonols	Lemon	Taxifolin
Isoflavones	Soyabeans, legumes	Daidzen, genistein, glucitein, formononetin

Flavonoids have probably existed in the plant kingdom for over one billion years. They are synthesized by all vascular plants and are present in practically all dietary sources like fruits, nuts, vegetables, herbs, whole grains etc. It is estimated that the human intake of all flavonoids is a few hundreds of milligrams per day.[61] Additionally, flavonoids are found in several medicinal plants and herbal remedies containing flavonoids have been used in folk

medicine around the world. Within the plant, flavonoids are involved in electron transport during photosynthesis, serve as antioxidants against the pro-oxidant effects of ultraviolet light and act against bacterial, fungal and viral pathogens as well as some insect predators.[62]

Resistance of plants to UV-B (280 – 315 nm) may take many forms, but one type of resistance lies in the flavonoid pigments, which are known to be almost universally present in green leaves. These flavonoids generally absorb in the 280-315 nm region and thus are capable of acting as UV filters, thereby protecting the underlying photosynthetic tissues from damage. One of the undisputed functions of flavonoids and related polyphenols is their role in protecting plants against microbial invasion. This not only involves the presence of flavonoids in plants as constitutive agents but also their accumulation as phytoalexins in response to microbial attack.[63] There is an ever increasing interest in plant flavonoids for treating human diseases and especially for controlling the immunodeficiency virus which is the causative agent of AIDS. The presence of a phenolic group in a natural flavonoid would be expected to provide antimicrobial activity and the addition of further phenolic groups might be expected to enhance this activity.[64]

1.4.1 Biosynthesis of flavonoids

All flavonoids derive their 15-carbon skeletons from two basic metabolites, malonyl-CoA and *p*-coumaroyl-CoA.[65] Basically, flavonoids are derivatives of 1,3-diphenylpropan-1-one (C6–C3–C6). The crucial biosynthetic reaction is the condensation of three molecules of malonyl-CoA with one molecule of *p*-coumaroyl-CoA to a chalcone intermediate. Chalcones and dihydrochalcones are classes of flavonoids that consist of two phenolic groups which are connected by an open three carbon bridge. Derived from the chalcone structure, a flavonoid-class containing three rings, the flavanones, can be formed. Here, the three-carbon bridge is part of an additional heterocyclic six-membered ring that involves one of the phenolic

groups on the adjacent ring. Based on these flavanones all other flavonoid-classes are generated, including isoflavones, flavanols, anthocyanidines, flavonols and flavones (Figure 1.9).

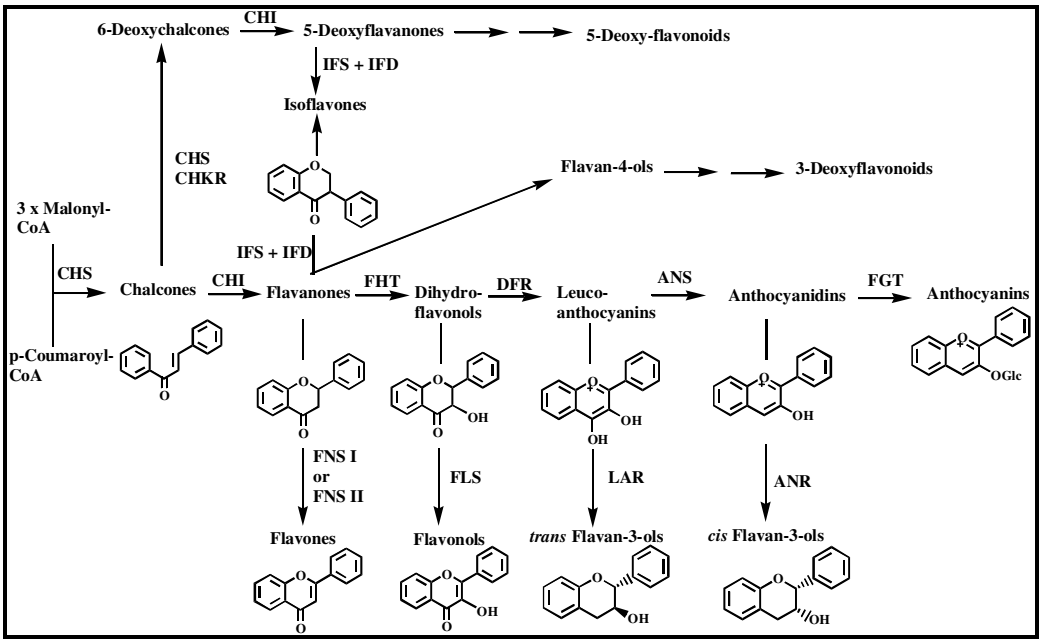


Figure 1.9: Scheme of general flavonoid pathway.
Enzymes are abbreviated as follows: CHS, chalcone synthase; CHKR, chalcone polyketide reductase; CHI, chalcone isomerase; FHT, flavanone 3-β-hydroxylase; DFR, dihydroflavonol-4-reductase; ANS, anthocyanidin synthase; FGT, flavonoid glycosyltransferase; FNS, flavone synthase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; IFS, isoflavone synthase; IFD, isoflavone dehydratase.

1.4.2 Biological activity of flavonoids

Flavonoids display a remarkable spectrum of biological activities including those that might be able to influence processes that are deregulated during cancer development. These include, for example, antiallergic, anti-inflammatory, antioxidant, antimutagenic, anticarcinogenic and modulation of enzymatic activities.[66] They may therefore have beneficial health effects and can be considered possible chemopreventive or therapeutic agents against cancer.[67]

1.4.3 Flavonoids in anticancer therapy

Impressive epidemiological evidence exists for the protective effect of flavonoids against cancer. A large number of such epidemiological studies suggest that high flavonoid intake may be correlated with a decreased risk of cancer.[68] Recently, population based studies on the protective effects of flavonoids against cancer conducted in Shanghai, Finland and Hawai have also provided evidence for the protective role of flavonoids against cancer.[69] Studies on the potential anticancer activity of flavonoids in diverse cell systems have demonstrated their ability to inhibit carcinogenesis *in vitro* and substantial evidence indicates that they can also do so *in vivo*.[70] For example, Hirano *et al*[70d] examined anticancer efficacy of 28 flavonoids on human acute myeloid leukemia cell line HL-60 and compared with those of four clinical anticancer agents. Eight of the 28 flavonoids showed considerable suppressive effects on HL-60 cell growth. Kuntz *et al*[70e] screened more than 30 flavonoids for their effects on cell proliferation and potential cytotoxicity in human colon cancer cell lines Caco-2 and HT-29. Almost all compounds displayed antiproliferative activity without cytotoxicity. There was no obvious SAR in the antiproliferative effects either on the basis of subclasses or with respect to kind or position of substituents within a class. An array of 55 flavones having a variety of substituents was evaluated by Cushman and Nagarathnam[70f] for cytotoxicity in five cancer cell cultures, A-549 lung carcinoma, MCF-7 breast carcinoma, HT-29 colon adenocarcinoma, SKMEL-5 melanoma and MLM melanoma. Fifteen of the 55 flavone derivatives were significantly active against at least one of these cell cultures. In addition, seven of the 27 citrus flavonoids examined were observed to inhibit the proliferation of tumor cells, while being less active against normal human cells.[70g]

Flavonoids may inhibit carcinogenesis by affecting the molecular events in the initiation, promotion and progression stages. Animal studies and investigations using different cellular models suggested that certain

flavonoids could inhibit tumor initiation as well as tumor progression.[71] The encouraging results of anticancer effects in preclinical studies have stimulated the clinical trials of some flavonoids in human beings. Based on the studies *in vivo* and *in vitro*, many mechanisms of action have been proposed. These include, carcinogen inactivation, antiproliferation, cell cycle arrest, induction of apoptosis and differentiation, inhibition of angiogenesis, antioxidation and reversal of multi drug resistance or a combination of these mechanisms.

Flavonoids may interfere in several of the steps that lead to the development of malignant tumors, including protecting DNA from oxidative damage, inhibiting carcinogen activation and activating carcinogen detoxifying systems. Dietary phenolics have also been shown to act as prooxidants in systems containing redox-active metals. i.e., In the presence of O_2, transition metals such as copper (Cu) and iron (Fe) catalyze the redox cycling of phenolics, leading to the formation of reactive oxygen species (ROS) and phenoxyl radicals that can damage DNA, lipids and other biological molecules.[72] Thus phenolic antioxidants can be both pro-oxidative and antioxidative (Figure 1.10), which suggests that flavonoids/phenolics also have the potential to lead to oxidative risk. Therefore, consumption of large amounts of flavonoids in the form of a concentrated supplement might not be considered safe until their *in vivo* potential for oxidative stress is evaluated fully.

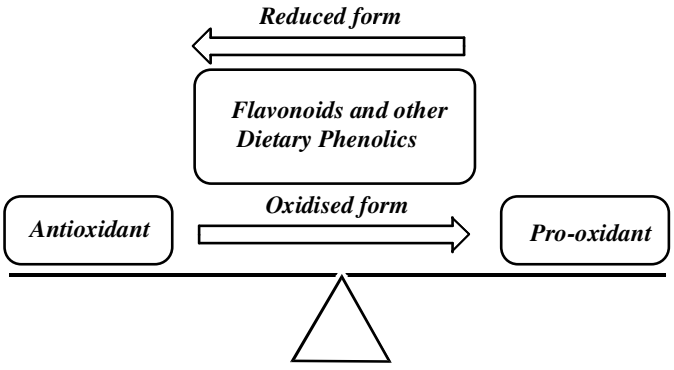


Fig. 1.10: Diagram representing the balance between antioxidant and pro-oxidant characteristics of flavonoids

1.4.3.1 Pro-oxidant behavior and anticancer property

The beneficial effects of flavonoids in cancer therapy have often been linked to their ability to act as antioxidants, which includes their reducing capacities and ROS-scavenging capabilities.[73] The chemopreventive properties of flavonoids are generally believed to reflect their ability to scavenge endogenous ROS. However, the pro-oxidant action of plant-derived phenolics rather than their antioxidant action may be an important mechanism for their anticancer and apoptosis-inducing properties, as ROS can mediate apoptotic DNA fragmentation.[74] Certain properties of dietary phenolic compounds, such as binding and cleavage of DNA and the generation of ROS in the presence of transition metal ions, are similar to those of known anticancer drugs. Another mechanism proposed for the anticancer and tumor cell apoptosis-inducing properties of flavonoids is that their pro-oxidant phenoxyl radicals cause mitochondrial toxicity by collapsing the mitochondrial membrane potential. Apoptosis (programmed cell death) is required to maintain a balance between cell proliferation and cell loss. Misregulation of this balance can lead to malignant transformation, whereas induction of apoptosis suppresses the development of cancer.[75] Various diet-derived compounds, e.g., resveratrol **17**, have been shown to induce apoptosis in malignant cells and provide a promising natural strategy to prevent cancer.[76] Pro-oxidant activity is thought to be directly proportional to the total number of hydroxyl groups present especially in the flavonoid B-ring. Glycosylation and methylation of OH groups attenuate the pro-oxidant behavior of flavonoids.[77]

While these experiences strengthen the notion that flavonoids could be useful as anticancer agents, to date only few clinical studies have demonstrated that these bioflavonoids retain anticancer properties in humans *in vivo*. Considering the fact that many chemotherapeutic agents against tumor cells kill without sparing normal cells remains a major obstacle and

development of multidrug resistance further limits chemotherapy in cancer, the promising results on flavonoids have stimulated further study of these compounds for cancer chemoprevention and chemotherapy.

1.4.4 Flavonoids as antioxidants

Owing to the incomplete efficiency of our endogenous defense systems and the existence of some physiopathological situations (cigarette smoke, air pollutants, UV radiation, high polyunsaturated fatty acid diet, inflammation, ischemia/reperfusion, etc.) in which ROS are produced in excess and at the wrong time and place, dietary antioxidants are needed for diminishing the cumulative effects of oxidative damage over the life span.[78] According to Halliwell and Gutteridge[40a], mechanisms of antioxidant action can include (1) suppressing reactive oxygen species formation either by inhibition of enzymes or chelating trace elements involved in free radical production; (2) scavenging reactive oxygen species; and (3) upregulating or protecting antioxidant defenses. Flavonoids inhibit the enzymes responsible for superoxide anion production, such as xanthine oxidase and protein kinase C.[79] Flavonoids have been also shown to inhibit cyclooxygenase, lipoxygenase, microsomal monooxygenase, glutathione *S*-transferase, mitochondrial succinoxidase and NADH oxidase, all involved in reactive oxygen species generation.[80] A number of flavonoids efficiently chelate trace metals, which play an important role in oxygen metabolism. Free iron and copper are potential enhancers of reactive oxygen species formation, as exemplified by the reduction of hydrogen peroxide with generation of the highly aggressive hydroxyl radical, $H_2O_2 + Fe^{2+}$ (Cu^{+}) $\longrightarrow$ $^{\cdot}OH + OH^{-} + Fe^{3+}$ (Cu^{2+}) or by the copper-mediated LDL (low-density lipoprotein) oxidation, LH $\longrightarrow$ $L^{\cdot}$ $\longrightarrow$ $LOO^{\cdot}$, where LH represents LDL. Nevertheless, it has to be remembered that these metal ions are essential for many physiological functions, as constituents of hemoproteins and cofactors of different enzymes, including those involved (iron for catalase, copper for ceruloplasmin and Cu,Zn-

superoxide dismutase) in the antioxidant defense.[81] The proposed binding sites for trace metals to flavonoids are the catechol moiety in ring B, the 3-hydroxyl, 4-oxo groups in the heterocyclic ring and the 4-oxo, 5-hydroxyl groups between the heterocyclic and the A rings (Figure 1.11).

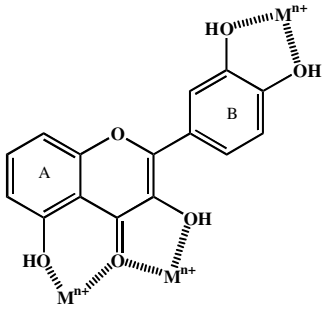


Figure 1.11: Binding sites for trace metals

However, the major contribution to metal chelation is due to the catechol moiety.[82] Due to their lower redox potentials, flavonoids are thermodynamically able to reduce highly oxidizing free radicals, such as superoxide, peroxyl, alkoxyl and hydroxyl radicals by hydrogen atom donation, via the reaction, Fl-OH + $R^{\cdot}$ ⟶ Fl-$O^{\cdot}$ + RH , where $R^{\cdot}$ represents superoxide anion, peroxyl, alkoxyl and hydroxyl radicals.[83] The aroxyl radical (Fl-$O^{\cdot}$) may react with a second radical, acquiring a stable quinone structure (Figure 1.12).

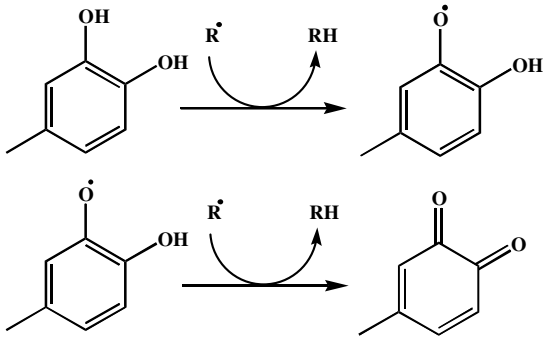


Figure 1.12: Scavenging of ROS ($R^{\cdot}$) by flavonoids

The aroxyl radicals could interact with oxygen, generating quinones and superoxide anion, rather than terminating chain reactions. The last

reaction may take place in the presence of high levels of transient metal ions and is responsible for the sometimes undesired pro-oxidant effect of flavonoids.[84] Thus, the overall capacity of flavonoids to act as antioxidants depends not only on the redox potential of the couple Fl-O$^{\cdot}$/Fl-OH but also on possible side reactions of the aroxyl radical. Scavenging of superoxide is particularly important, because this radical is ubiquitous in aerobic cells and, despite its mild reactivity, is a potential precursor of the aggressive hydroxyl radical in the Fenton and Haber-Weiss reactions. Besides scavenging, flavonoids also stabilize free radicals involved in oxidative processes by complexing with them.[85]

1.4.4.1 Structural features and antioxidant activity of flavonoids

The antioxidant activity of flavonoids and their metabolites *in vitro* depends upon the arrangement of functional groups about the nuclear structure.[86] Consistent with most polyphenolic antioxidants, both the configuration and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity. Free radical scavenging capacity is primarily attributed to the high reactivities of hydroxyl substituents that participate in Fl-OH + R$^{\cdot}$ $\longrightarrow$ Fl-O$^{\cdot}$ + RH reaction. The B-ring hydroxyl configuration is the most significant determinant of scavenging of ROS and RNS.[87] Hydroxyl groups on the B-ring donate hydrogen and an electron to hydroxyl, peroxyl and peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoid radical. A 3',4'-catechol structure in the B-ring as in quercetin **28**, strongly enhances lipid peroxidation inhibition. This arrangement is a salient feature of the most potent scavengers of peroxyl, superoxide and peroxynitrite radicals.[88] The significance of other hydroxyl configurations is less clear, but beyond increasing total number of hydroxyl groups, A-ring substitution correlates little with antioxidant activity. Compared to the B-ring hydroxylation pattern, the impact of the A-ring

arrangement on antioxidant activity is of questionable significance. The flavonoid heterocycle contributes to antioxidant activity by (i) the presence of a free 3-OH and (ii) permitting conjugation between the aromatic rings. The closed C-ring itself may not be critical to the activity of flavonoids, given that chalcones are active antioxidants.[89] Free radical scavenging by flavonoids is highly dependent on the presence of a free 3-OH. Flavonoids with a 3-OH and 3',4'-catechol are reported to be 10- fold more potent than ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one; a known RNS scavenger) against peroxynitrite. The torsion angle of the B-ring with respect to the rest of the molecule strongly influences free radical scavenging ability. Flavonols and flavanols with a 3-OH are planar, while the flavones and flavanones, lacking this feature, are slightly twisted. Planarity permits conjugation, electron dislocation and a corresponding increase in flavonoid phenoxyl radical stability. Removal of a 3-OH abrogates coplanarity and conjugation, thereby compromising scavenging ability.[90]

Substitution of 3-OH by a methyl or glycosyl group completely abolishes the activity. It is postulated that B-ring hydroxyl groups form hydrogen bonds with the 3-OH, aligning the B-ring with the heterocycle and A-ring. Eliminating this hydrogen bond effects a minor twist of the B-ring, compromising electron delocalization capacity. Due to this intramolecular hydrogen bonding, the influence of a 3-OH is potentiated by the presence of a 3',4'-catechol,[91] explaining the potent antioxidant activity of flavan-3-ols and flavon-3-ols that possess the latter feature.

The differences in antioxidant activity between polyhydroxylated and polymethoxylated flavonoids are most likely due to differences in both hydrophobicity and molecular planarity. Suppression of antioxidant activity by *O*-methylation[92] may reflect steric effects that perturb planarity. Flavonoids with a 2–3 double bond in conjugation with a 4-carbonyl group exhibit stronger antioxidant activity.[93] The majority of research supports that

flavonoids lacking one or both of these features are less potent antioxidants than those with both elements. Conjugation between the A and B rings permits a resonance effect of the aromatic nucleus that lends stability to the flavonoid radical and is therefore critical in optimizing the phenoxyl radical-stabilizing effect of a 3',4'-catechol. The premise that flavanols are more effective free radical scavengers than flavones[94] may be ascribed to the greater number of hydroxyl groups and 3-OH in the former. Aglycones are more potent antioxidants than their corresponding glycosides.[95] i.e., glycosylation in a flavonoid decreases antioxidant activity.

Tea and soy flavonoid are also one among the major sources of dietary antioxidants. Tea is rich in antioxidant polyphenols such as catechins, flavonols, theaflavins and thearubigins. Tea flavonoids have many health benefits. Tea flavonoids reduce the oxidation of low-density lipoprotein, lowers the blood levels of cholesterol and triglycerides. Soy flavonoids (isoflavones) can also reduce blood cholesterol and can help to prevent osteoporis. Soy flavonoids are also used to ease menopausal symptoms.

1.5 Spices as antioxidants

Spices and herbs that are widely used in ethnic foods are major natural antioxidants. Oxidation processes caused by ROS are a major cause of deterioration of various food products. Oxidation of food products is associated with loss of quality. Significant changes can occur in flavour, colour and texture and finally, can lead to loss of nutritive value or complete spoilage. In order to prevent these processes, antioxidants are used. The use of synthetic antioxidants in food products like butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) is under strict regulation owing to uncertainty about their safety.[96] Therefore there is a demand for the discovery of safe antioxidants especially from natural origin and many groups are currently actively involved in the search for natural antioxidants.

Since ancient times, spices like ginger and turmeric have been added to different types of food to improve their flavour. Some of the early scientific investigations carried out by Sehti and Aggarwa1,[97] reported the improved storage stability of groundnut oil after the addition of different spices. Chipault *et al*[98] investigated the antioxidant activity of several spices. Since the early work of Chipault *et al,* the interest in the antioxidative activity of spices has increased and has led to a large amount of data/information about the compounds present in them and the mechanisms involved. Because of their strong antioxidant and antimicrobial properties which are more appreciable than many currently used natural and synthetic antioxidants, there is continuous efforts among industry and scientific circles in the study of spices and herbs. These advantageous properties are due to many substances, including some vitamins, flavonoids, terpenoids, carotenoids, phytoestrogens, minerals, etc., which render spices/herbs or their antioxidant components the ability to function as preservative agents in food.[99] Apart from the antioxidants like β -carotene, tocopherols, vitamin C etc., there are specific compounds that are characteristic to each of the aromatic herbs and spices. Some examples of specific antioxidants from spices include biflorin **29**, eugenol **30** and eugenyl acetate in clove;[100] carnosol **31**, carnosic acid **32**, rosmanol **33**, rosmaridiphenol, rosmadial and rosmariquinone **34** and various methyl and ethyl esters of these substances in rosemary and sage;[101] diarylheptanoid, gingerol **35** and zingerone **36** in ginger;[102] curcumin **16** and tetradehydrocurcumin **37** in turmeric;[103] flavonoids, ferulic acid **38**, piperine **39**, phenolic amide feruperine **40** in black pepper;[104] thymol **41** and carvacrol **42** in essential oils from *Algerian origanum*[105] etc (Chart 1.2). Modern health conscious consumers often ask for natural ingredients, free of synthetic additives. Therefore, the application of natural antioxidants will probably gain even more interest the future and it will be necessary to study their effect and interactions in more detail. It is of interest to note that in Kerala, several

species of Zingiberaceae are used as spices and also as important constituents in formulations under the *Ayurvedic* system of medicines.

1.6 The Zingiberaceae: General and Botanic Aspects

The Zingiberaceae is a large family of rhizomatous plants originating from Asia and far-east which has been cultivated for centuries. It is one of the largest families of the plant kingdom.[106] Plants belonging to Zingiberaceae are distributed mostly in tropical and subtropical areas. It is an important natural source that provides many useful products for food, spices, medicines, dyes and perfumes to man. Zingiberaceae plants are perennial rhizomatous herbs. Leaves are simple and distichous. Inflorescence is terminal on the leafy shoot or on the lateral shoot. Flowers are delicate, ephemeral and highly modified. All parts of the plant are aromatic and the fruits are capsules. All Zingiberaceae are ground plants mostly growing in damp and humid shady places. However, few rare species can tolerate full exposure to the sun while a small number grow at high elevation also.

Zingiberaceae comprises of 53 genera and 1400 species. In India, 21 genera and 190 species are found.[107] In the North Eastern region of India, 19 genera and 70 species are present. In South India, the family is represented by 11 genera and 60 species. Zingiberaceae family shows high endemism in India. Two genera and 70 species are endemic to India. The name Zingiber, originated from the Greek word '*Zingiberis*' which in turn originated from the Sanskrit word '*Singa Verni*' meaning 'deer horn shaped' presumably in allusion to the sprouting rhizomes. There is another argument that the word Zingiber originated from the Malayalam word of ginger–"*Inchiver*". The rhizomes of these plants are fleshy with nodes and internodes and sometimes with scaly leaf sheaths.

Zingiberaceae is broadly divided into four tribes[108] namely,

- HEDYCHIEAE
- ZINGIBEREAE

- ALPINIEAE and
- GLOBBAE

The species under each tribe is given in Table 1.5.

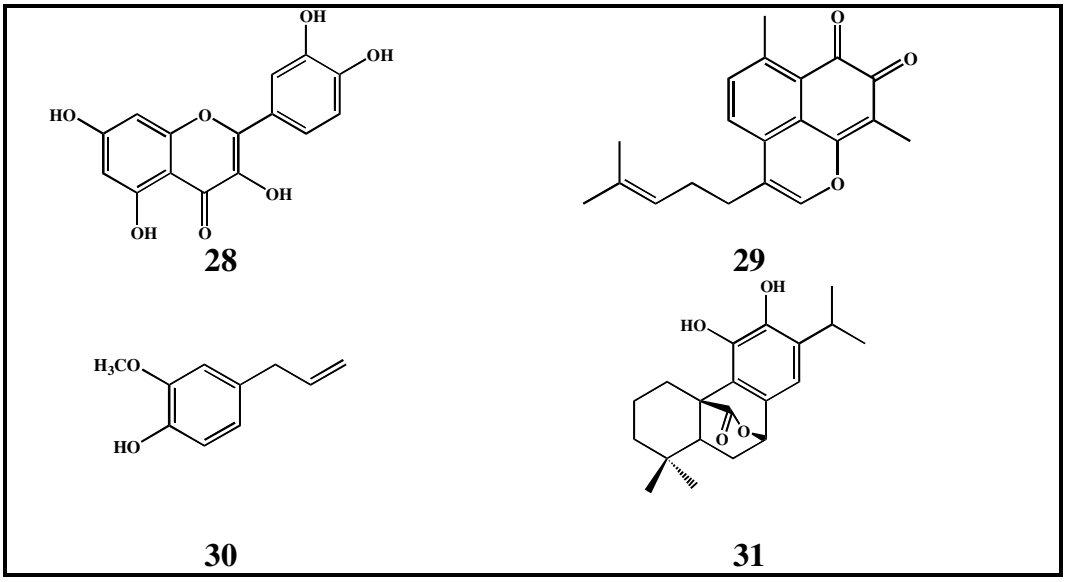
Table 1.5: Classification of the Zingiberaceae family

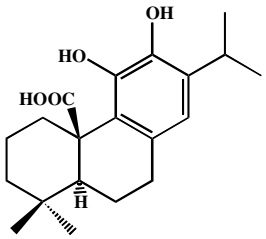
Tribe	***Type of species***
HEDYCHIEAE	○ *Bosenbergia* ○ *Caulokaempferia* ○ *Cautleya* ○ *Curcuma* ○ *Curcumorpha* ○ *Haniffia* ○ *Hedychium* ○ *Kaempferia* ○ *Scaphochlamys* ○ *Stahlianthus*
ZINGIBEREAE	• *Zingiber*
ALPINIEAE	▪ *Alpinia* ▪ *Amomum* ▪ *Elettariopsis* ▪ *Etlingera* ▪ *Geostachys* ▪ *Hornstedtia* ▪ *Pomereschia*
GLOBBEAE	✓ *Gagnepainia* ✓ *Globba*

Zingiberaceae is also considered as an economically important family[109] as the source of important spice plants such as *Curcuma domestica*, *Curcuma longa* (turmeric), *Elettaria cardamomum* (cardamom) and *Zingiber officinale* (ginger). While most *Hedychium* species are cultivated as ornamental plants for their flowers, few *Alpinia* are grown for their rhizomes that are used as spices and in traditional medicines. Various pharmacological properties of many species of Zingiberaceae are reported.[110] Ginger and turmeric are widely used in Indian system of medicine. Ginger, the rhizome of *Zingiber officinale* is one of the most familiar spices and one of the most frequently used medicinal plant in traditional medicinal systems of India and

South East Asia. It is used as carminative, stimulant and in the treatment of gastrointestinal and respiratory diseases in India. The rhizomes have antipyretic effect. Zingiberene **43** (Chart 1.2), a major compound from the rhizome of *Zingiber officinale* has antiviral, antiulcer and antifertility effects.[111] Numerous chemical investigations of the flavor and bioactive compounds of ginger have led to the isolation of a large number of terpenoids and phenylalkanoids.[112] Many Zingibers are used by tribals for treatment of several ailments. The plants exhibit much diverse pharmacological and insecticidal acitivites. For eg., curcumin **16**, the yellow bioactive component of turmeric has been shown to have a wide spectrum of biological actions.[113] These include its antiinflammatory, antioxidant, anticarcinogenic, antimutagenic, anticoagulant, antifertility, antidiabetic, antibacterial, antifungal, antiprotozoal, antiviral, antifibrotic, antivenom, antiulcer, hypotensive and hypocholesteremic activities. Its anticancer effect is mainly mediated through induction of apoptosis. Its anti-inflammatory, anticancer and antioxidant roles may be clinically exploited to control rheumatism, carcinogenesis and oxidative stress-related pathogenesis. Clinically, curcumin has already been used to reduce post-operative inflammation.

Chart 1.2





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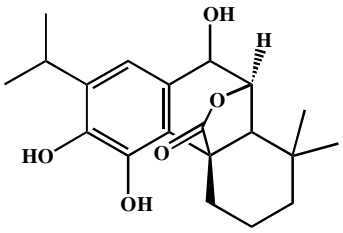

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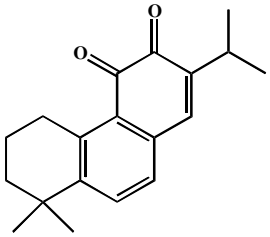

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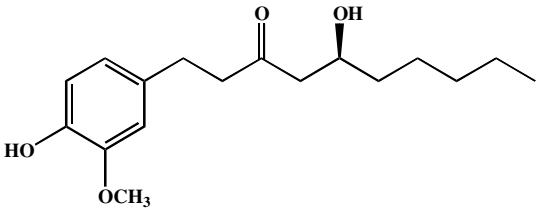

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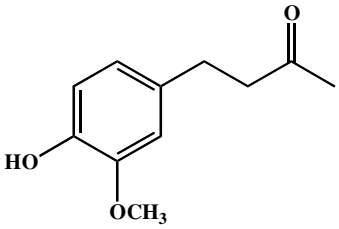

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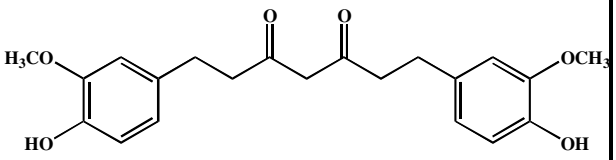

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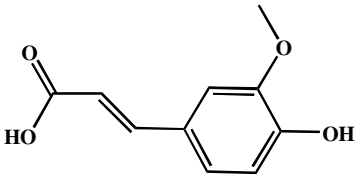

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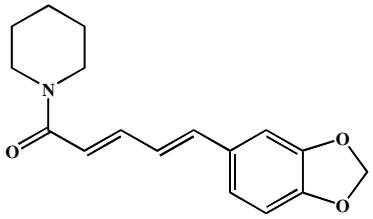

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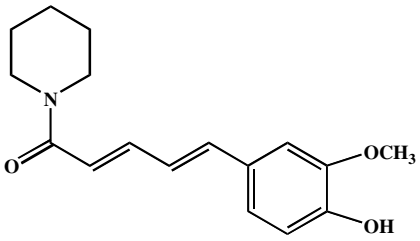

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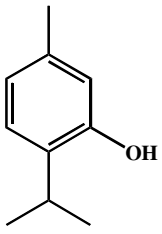

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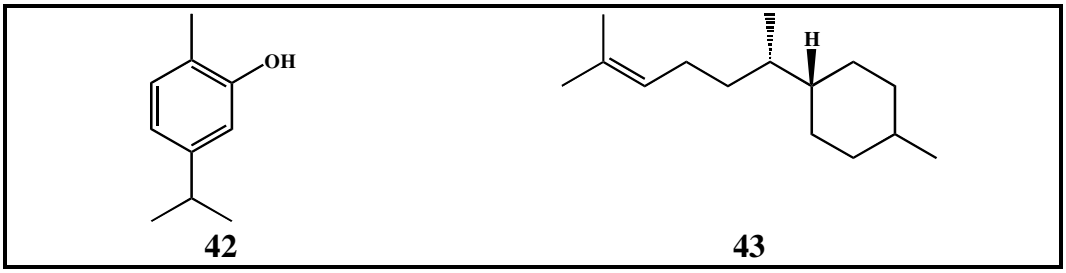

42 **43**

A literature search on Zingiberaceae showed that a variety of biologically active compounds belonging to different structural types are present in them. These structural types includes flavonoids, flavonols, steroidal saponins, hydroxyl phenyl alkanones, phenolic esters, labdane type diterpenoids, stilbenes, mono and bicyclic sesquiterpenes, diarylheptanoids, phenylbutanoids and their dimers, carabrane type sesquiterpenes, oxygenated bisabolanes, chalcones, steroid glycosides, triterpenoids, cyclohexane diepoxides etc.

1.7 Objectives and Organization of the thesis

The preceding pages clearly portray the importance of natural products and the heightened awareness about the potential of medicinal plants used in traditional systems of medicine in the bioprospecting for drugs, drug leads and neutraceuticals. *Ayurvedic* therapeutic formulations are used extensively in India and have proven efficacy for treatment of chronic diseases such as rheumatism, atherosclerosis etc. Since several Zingiberaceae plants are used in such formulations, and it is now well known that reactive oxygen species play a major role in the initiation of these chronic diseases, it appeared timely and relevant to study these plants in detail. Among the important Zingiberaceae plants, only *Zingiber officinale* and *Curcuma longa* have been studied by many groups. Other genuses of this family, which are also of much importance are *Alpinia* and *Kaempferia*. Accordingly, a detailed investigation of the three plants, *Alpinia galanga*, *Alpinia calcarata* and *Kaempferia pulchra* have been undertaken during this PhD programme.

It is impossible to give a comprehensive overview of natural products in a brief manner. But an attempt has been made to bring out the importance of biologically active natural products with special reference to anticancer and antioxidant compounds from terrestrial plants in Chapter 1.

Natural product chemistry has undergone a renaissance in the last few decades. Sophistication in separation and analytical techniques has added momentum to the discovery of new molecules. Even though the principal motivation for searching for new substances remains one of discovering new pharmacologically useful materials, the field of "chemosystematics" has also played a major role in the isolation and structural elucidation of a large number of natural products.

A detailed discussion on the genus *Alpinia* of the Zingiberaceae family with special emphasis on the phytochemicals present in them is given as a preamble to Chapter 2. *Alpinia galanga* commonly known as 'greater galangal' is the subject matter of Chapter 2, describing the compounds isolated from the rhizomes and their biological activity (anticancer and antioxidant activities). Chapter 3 deals with the phytochemical investigation and biological activity studies of the rhizomes of *Alpinia calcarata.* In Chapter 4, phytochemical investigation of *Kaempferia pulchra* belonging to the genus *Kaempferia* is discussed. The search for biologically active compounds especially anticancer compounds from natural products prompted us to investigate the leaves of the most important and common Indian medicinal plant *Azadirachta indica* (Neem). This resulted in the isolation of nimbolide from its leaves which is discussed in the final chapter, viz., Chapter 5. Here, a short discussion of the plant *Azadirachta indica*, the compound nimbolide isolated from the leaves and its anticancer and the antioxidant properties are discussed.

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Chapter 2

Phytochemical Investigation and Biological Activity Studies on *Alpinia galanga*

As discussed in the introductory chapter, there is growing interest in the exploration of biologically active medicinal plants for their phytochemical constituents. Plants belonging to the Zingiberaceae family occupy an important place in the *Ayurvedic* system of medicine. Many Zingiberaceae plants have been investigated for phytochemical constituents and evaluated for their biological activities. Two of the most studied plants include *Zingiber officinale* belonging to the genus *Zingiber* and *Curcuma longa* belonging to the genus *Curcuma*. *Alpinia* is another important genus of Zingiberaceae family and many plants under this genus are used in traditional systems of medicine. We embarked upon a study of the phytochemistry and biological activities of the genus *Alpinia*. Two plants from the genus *Alpinia* viz., *Alpinia galanga* and *Alpinia calcarata* were selected for detailed study as they are extensively used in the *Ayurvedic* system of medicine. Our findings of the phytochemical constituents and biological activities of these plants form the subject matter of chapter 2 and chapter 3 respectively. Literature survey on Zingiberaceae plants in general and *Alpinieae* specifically was carried out to understand the current status of knowledge of the genus *Alpinia* and the various species in it which is summarized in the following introduction.

2.1 Introduction – genus *Alpinia*

Alpinia is the largest and most widespread genus in the Zingiberaceae with nearly 230 species occurring in India, Sri Lanka, China, all of South East Asia and Japan, the Pacific as far as Fiji, Samoa and the Caroline Islands as

well as Australia.[1] *Alpinia* is a genus of the tribe *Alpinieae* belonging to the family Zingiberaceae. This tribe consists of perennial evergreen herbs, in which an abscission layer between the rhizome and the leafy shoots is lacking, the plane of distichy of the leaves is transverse to the direction of growth of the rhizome and the lateral staminodes of the flowers are small, reduced to swellings at either side of the base of the labellum, or are entirely absent. Most species grow in low to mid elevation forests and form clumps with stems from 1–3 m height. Some species are found in forests up to 2000 m above sea level in New Guinea and the Sulawesi island in Indonesia. However, very few are tolerant of frost. The most northerly species is *Alpinia japonica* which survives north of Tokyo where the winters can be severe. Several species are important ornamentals as potted plants, landscape accents and cut flowers and at least one (*A. zerumbet*) is naturalized in tropical regions around the world. In Asia, especially China[2], several *Alpinias* are used for medicinal purposes and in cooking.

The generic name *Alpinia* was first used by Linnaeus for *Alpinia racemosa*, a neotropical species. Many Asiatic species were added to *Alpinia*, while later, authors tended to refer American species to *Renealmia*. Schumann finalized these taxonomic concepts and subsequently *Alpinia* Roxb. was conserved for the Asiatic species with *Alpinia galanga* (L.) Willd. as its type.[3]

Virtually all species flower terminally on the leafy shoots. Infrageneric classifications of *Alpinia* have been based on inflorescence and flower characters. Much variation exists in these features as is clear from figure 2.1. Extra floral nectaries are absent and the fruit is usually spherical and indehiscent or fleshy.[4] The rootstocks are rhizomatous with tall and leafy stems. *Alpinias* require rich soil and shady environment and are propagated by the divisions of rootstocks.

In India, about seventeen species of *Alpinia* are found[5] in which the most common and widely cultivated are *A. galanga*, *A. calcarata*, *A.*

conchigera, *A. malaccensis*, *A. nigra*, *A. zerumbet* and *A. officinarum*. *A. smithiae* has been reported from South India as an endangered species native to the evergreen forests in the Western Ghats of Kerala.[6] The medicinally important species are *A. galanga* that provides the drug 'Greater Galangal' and *A. officinarum* which is known as 'Lesser Galangal'. *A. calcarata* is also a medicinally important specie which is considered to have the same medicinal properties that of *A. galanga* and is used extensively in Kerala for *Ayurvedic* medicinal preparations.[9]

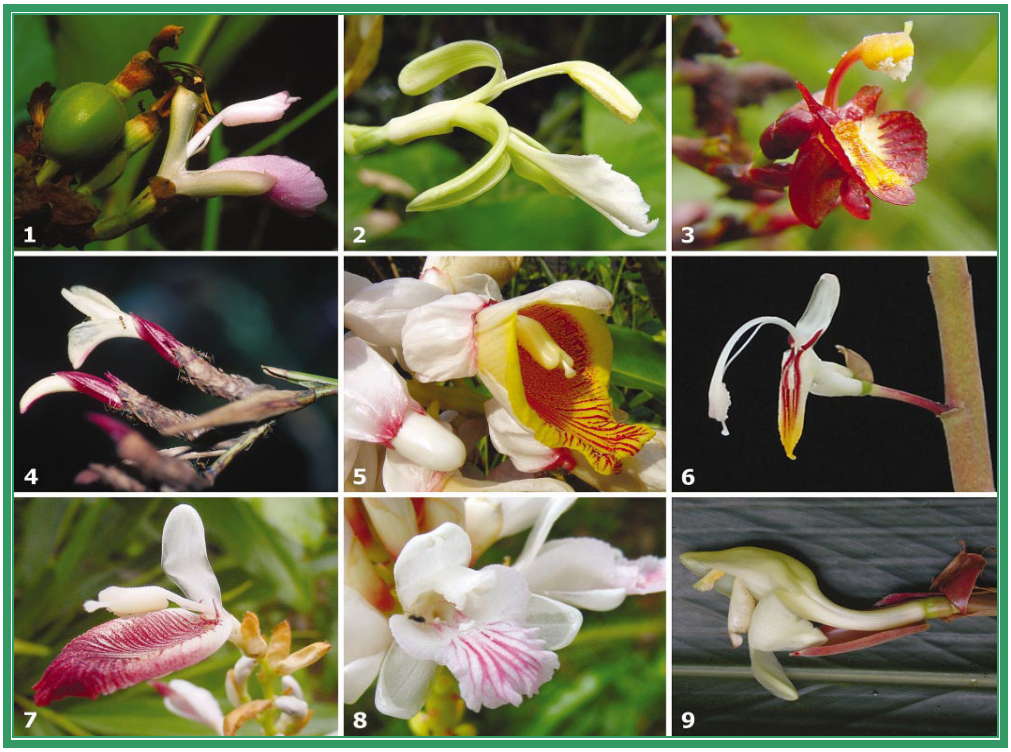

Figure 2.1: Representative floral types of the major groups of *Alpinia*: 1. *Alpinia nigra*; 2. *A. galanga*; 3. *A. conchigera*; 4. *A. carolinensis*; 5. *A. zerumbet*; 6. *A. guangdongensis*; 7. *A. calcarata*; 8. *A. oxyphylla*; 9. *A. elegans*.

A literature survey on the chemical composition and biological activities of the various *Alpinia* species reported so far is discussed briefly

here and the isolated phytochemical constituents are given in Tables 2.1- 2.7 and their structures in charts 2.1-2.7 as shown below.

Species	**Table No.**	**Chart No.**
Alpinia blepharocalyx	2.1	2.1
Alipina calcarata	2.2	2.2
Alpinia chinensis		
Alpinia conchigera		
Alpinia densibracteata	2.3	2.3
Alpinia flabellata		
Alpinia formosana		
Alpinia galanga	2.4	2.4
Alpinia henryi		
Alpinia japonica		
Alpinia javanica		
Alpinia jiangfeng		
Alpinia katsumadai	2.5	2.5
Alpinia malaccensis		
Alpinia mutica		
Alpinia nutans		
Alpinia officinarum	2.6	2.6
Alpinia oxyphylla		
Alpinia pinnanensis	2.7	2.7
Alpinia rafflesiana		
Alpinia speciosa		
Alpinia tonkinensis		
Alpinia zerumbet		

Alpinia allughas is a less studied specie of the genus *Alpinia* which grows in the Himalayan region. A preliminary report on the essential oil composition was reported by Purohit and Devi as early as 1976.[7a] Following that, the essential oil compositon was analysed by Prakash *et al*[7b] which led to the identification of twenty two constituents from the rhizomes and forty two constituents from the leaves of *A. allughas* of which β- pinene was found to be the major constituent. In an experiment conducted by Chirangini *et al*[7c], the medicinal use of the rhizomes as dietary agent was confirmed by evaluating its antioxidant capacity.

The seeds of *A. blepharocalyx* are used as a stomachic in South-West China.[8a] This plant contains the largest number of arylheptanoids among the *Alpinia* plants investigated so far. The arylheptanoids isolated were unique in their structure with a flavanone or a chalcone moiety in the molecule (**44-81**).[8] Apart from the diarylheptanoids, phenolic compounds (**82-103**) and other glycosides were also isolated from the seeds.[8k] The nitric oxide inhibitory activity,[8b,h] antiplatelet[8e] and the antiproliferative activity[8g,m] of the isolated diarylheptanoids are also reported. The volatile oil from the rhizomes of *A. blepharocalyx* was found to contain methyl ester of cinnamic acid as the major constituent.[8o]

Table 2.1: Phytochemical constituents isolated from *A. blepharocalyx*

Plant	Compound	No.
Alpinia blepharocalyx	Calyxin A	44
	Calyxin B	45
	Calyxin C	46
	Calyxin D	47
	Calyxin E	48
	Calyxin F	49

	Table 2.1 continued…	
	Calyxin G	50
	Calyxin H	51
	Calyxin I	52
	Calyxin J	53
	Calyxin K	54
	Calyxin L	55
	Calyxin M	56
	Epicalyxin B	57
	Epicalyxin C	58
	Epicalyxin D	59
	Epicalyxin F	60
	Epicalyxin G	61
	Epicalyxin H	62
	Epicalyxin I	63
	Epicalyxin J	64
	Epicalyxin K	65
	Epicalyxin M	66
	Blepharocalyxin A	67
	Blepharocalyxin B	68
	Blepharocalyxin C	69
	Blepharocalyxin D	70
	Blepharocalyxin E	71
	Deoxycalyxin A	72
	6-Hydroxycalyxin F	73
	Neocalyxin A	74
	Neocalyxin B	75
	(3*S*,5*S*)-3-Hydroxy-5-methoxy-1-(4-hydroxy-	

	Table 2.1 continued…	
	phenyl)-7-phenyl-6*E*-heptene	76
	(3*S*,5*R*)-3-Hydroxy-5-methoxy-1-(4-hydroxy-phenyl)-7-phenyl-6*E*-heptene	77
	(3*S*,5*S*)-3-Hydroxy-5-ethoxy-1-(4-hydroxy-phenyl)-7-phenyl-6*E*-heptene	78
	(3*S*,5*R*)-3-Hydroxy-5-ethoxy-1-(4-hydroxy-phenyl)-7-phenyl-6*E*-heptene	79
	(3*S*)-Methoxy-1,7-bis(4-hydroxyphenyl)-6*E*-heptene-5-one	80
	1,7-Bis(4-hydroxyphenyl)hepta-4*E*,6*E*-dien-3-one	81
	Alpinetin	82
	Cardamonin	83
	Helichrysetin	84
	1,7-Bis(4-hydroxyphenyl)-3-hydroxy-1,3-heptadien-5-one	85
	1,7-Bis(4-hydroxyphenyl)-3-hydroxy-1,3,6-heptatrien-5-one	86
	4'-Hydroxydehydrokawain	87
	5,6-Dehydrokawain	88
	4',7-Dihydroxy-5-methoxyflavanone	89
	p-Hydroxybenzaldehyde	90
	1,2-Dihydrobis(de-*O*-methyl)-curcumin	91
	(3*S*,5*S*)-3,5-Dihydroxy-1,7-bis(4-hydroxy-phenyl)heptane	92
	2',6'-Dimethoxy-4,4'dihydroxychalcone	93
	4,4'-Dihydroxychalcone	88

	Table 2.1 continued...	
	5-*O*-Methylnaringenin	94
	β-Sitosterol glucoside	95
	Methyl-*p*-hydroxycinnamate	96
	Methyl-*p*-hydroxycinnamyl ketone	97
	p-Hydroxycinnamic acid	98
	Phloroglucinol	99
	(3*S*,7*R*)-5,6-Dehydro-1,7-bis-(4-hydroxyphenyl)-4”-de-*O*-methylcentrolobine	100
	(3*S*,5*S*,6*S*,7*R*)-5,6-Dihydroxy-1,7-bis-(4-hydroxyphenyl)-4”-de-*O*-methylcentrolobine	101
	(3*S*,5R,6*S*,7*R*)-5,6-Dihydroxy-1,7-bis-(4-hydroxyphenyl)-4”-de-*O*-methylcentrolobine	102
	(3*S*,5*S*,6*R*,7*R*)-5,6-Dihydroxy-1,7-bis-(4-hydroxyphenyl)-4”-de-*O*-methylcentrolobine	103

Chart 2.1: Structures of the phytochemical constituents isolated from *A. blepharocalyx*

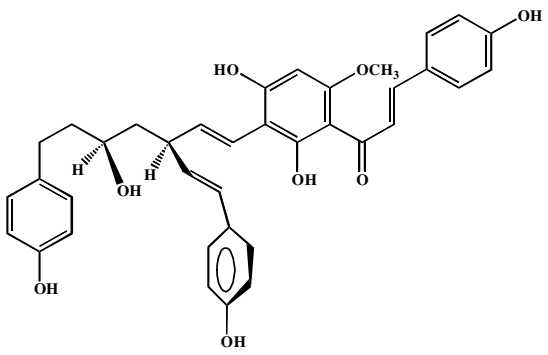

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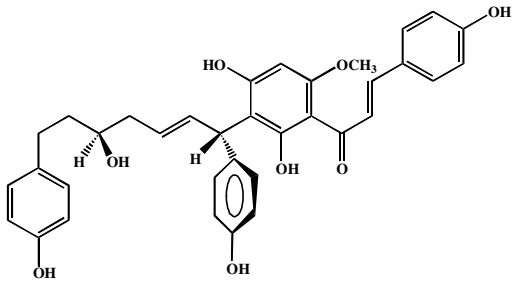
Chart 2.1 continued…



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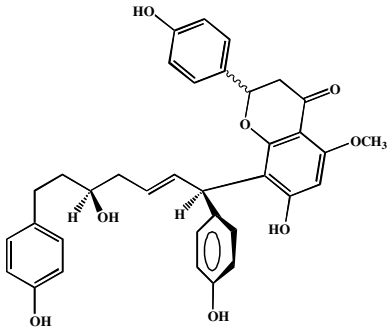
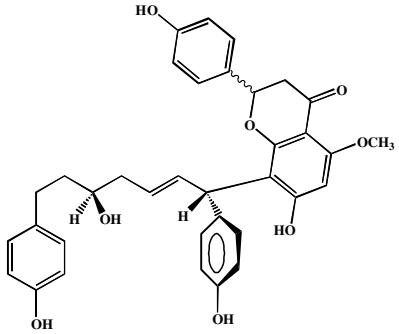
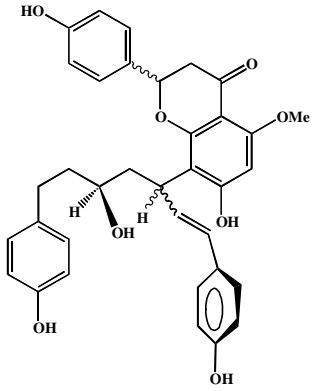


46, 58 (epimers)



47, 59 (epimers)



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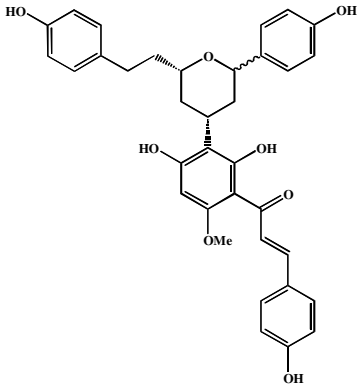

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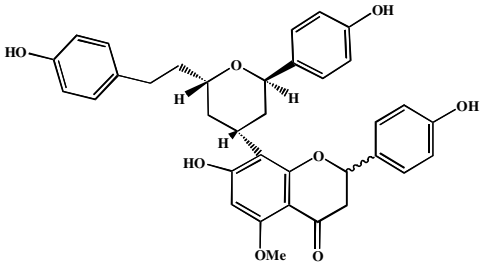

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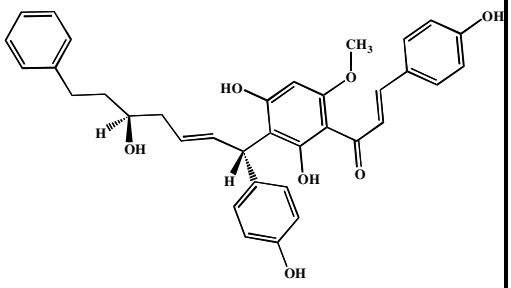

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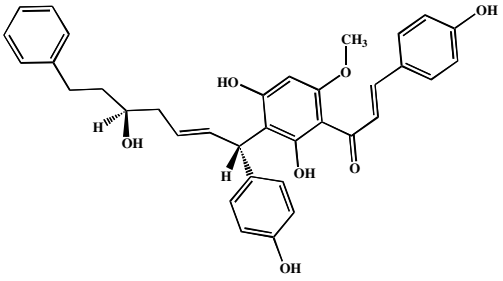

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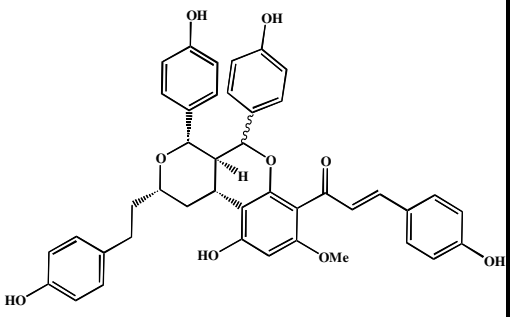
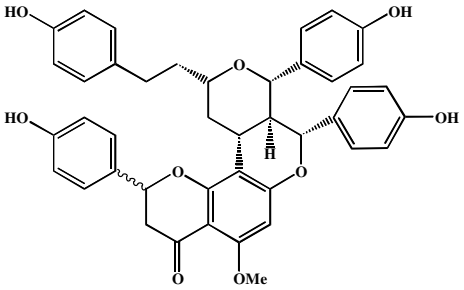
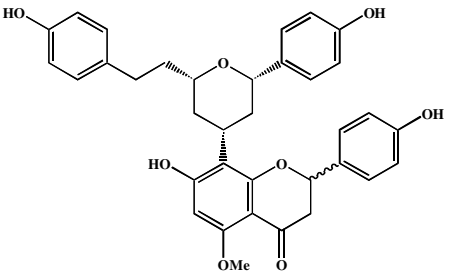


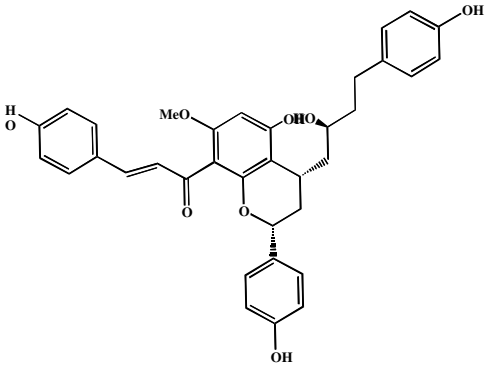
52, 63 (epimers)



53, 64 (epimers)



54, 65 (epimers)



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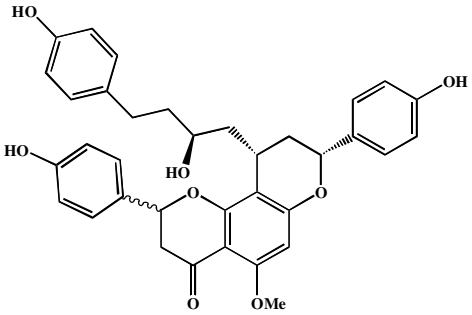
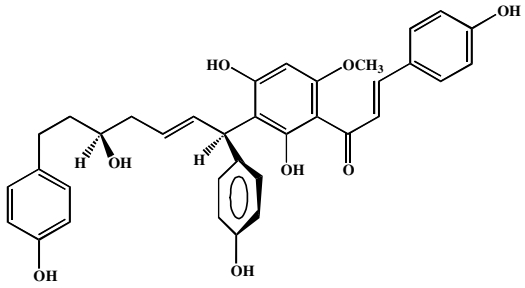


56, 66 (epimers)



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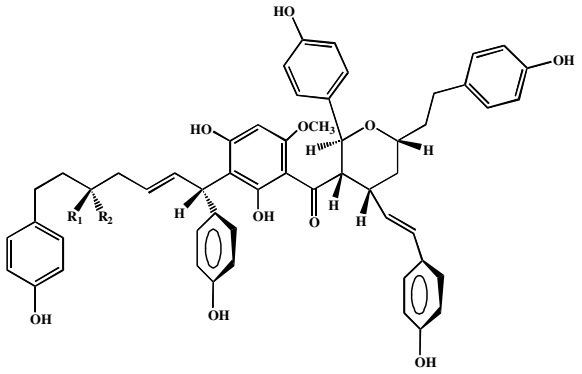
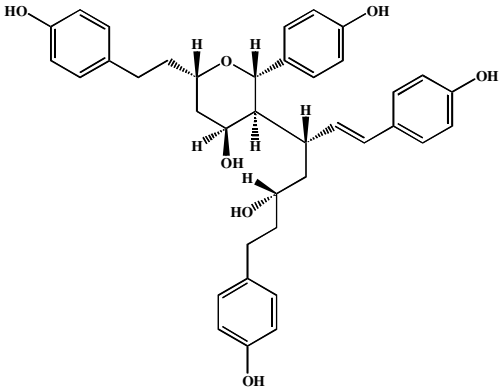


67; $R^1 = H, R^2 = OH$

68; $R^1 = OH, R^2 = H$



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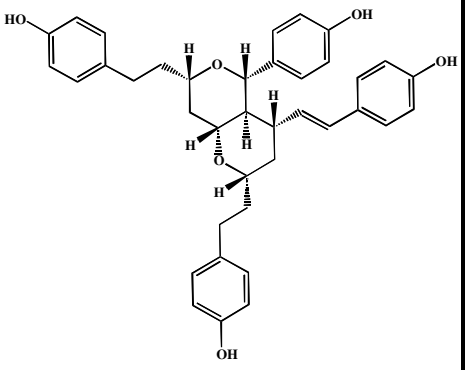

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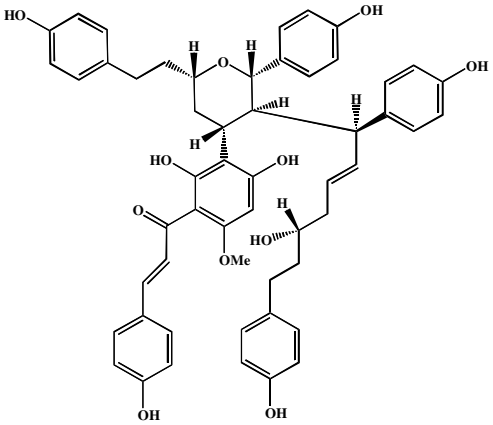

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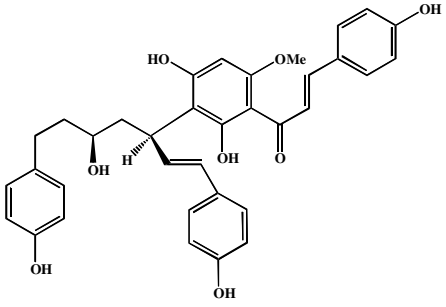

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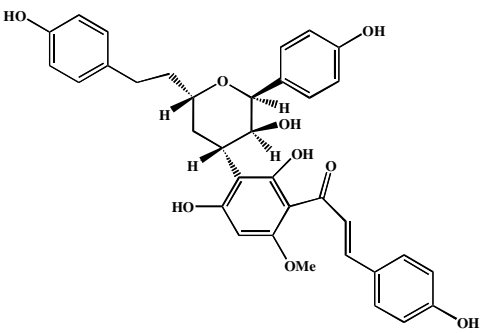
Chart 2.1 continued…



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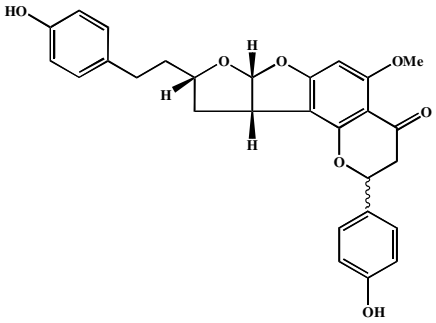
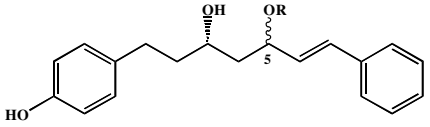
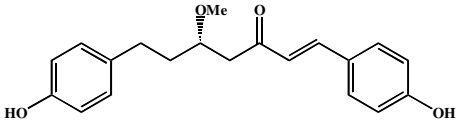


74, 75 (isomers)



	R	C-5		R	C-5
76;	Me	*S*;	**77;**	Me	*R*
78;	Et	*S*;	**79;**	Et	*R*



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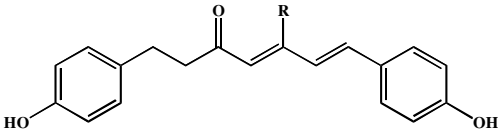
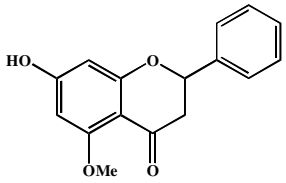


81; R = H
91; R = OH



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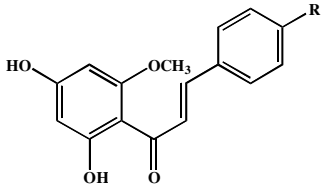
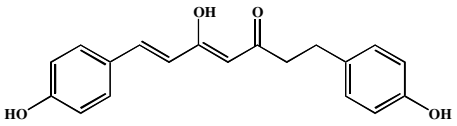


83; R = H
84; R = OH



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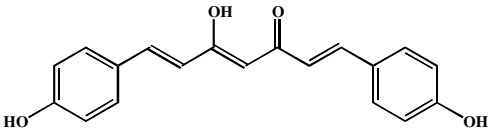

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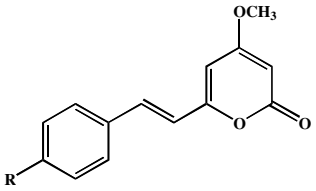
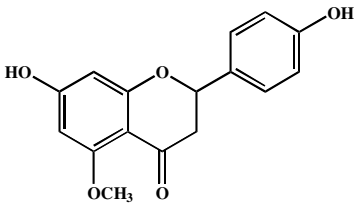


87; R = OH
88; R = H

Chart 2.1 continued…



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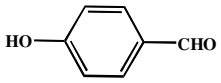

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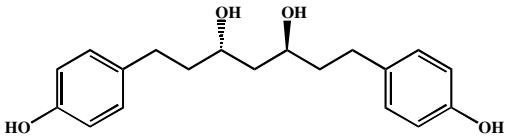

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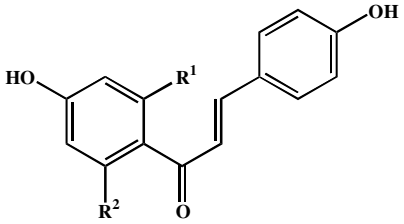
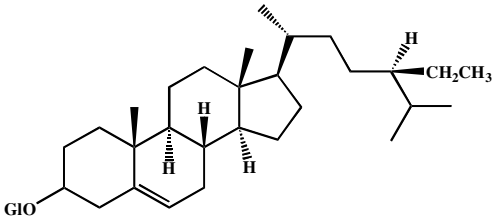


93; $R^1 = R^2 = OMe$
94; $R^1 = R^2 = H$



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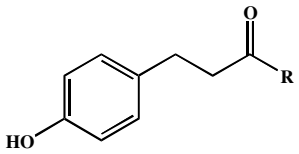
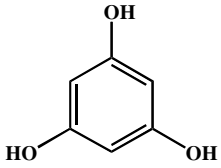


96; R = OMe, **97;** R = Me,
98; R = OH



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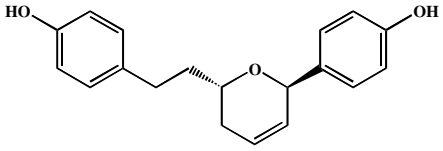

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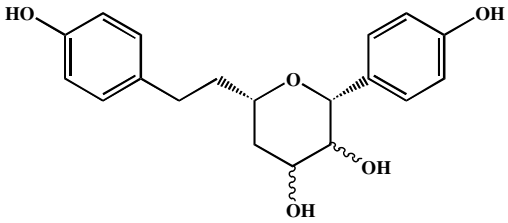


101; 5S, 6S
102; 5R, 6S
103; 5S, 6R

A. calcarata is a slender, rhizomatous herb, often cultivated for medicinal purposes in East and South India.[9] The compounds isolated from *A. calcarata* include the calcaratarins **104-110** given in Table 2.2 with their structures depicted in chart 2.2. A detailed description on the literature reports of this plant is given in Chapter 3; section A, as study of *A. calcarata* forms the subject matter of that chapter.

A. carinata is a less studied specie under this genus and is found in North India. Singh *et al*[10] have evaluated the constituents of the leaf oil of *A. carinata* from North India. Sixty two components were identified, of which β - pinene, terpinen-4-ol, 1,8-cineole and α-pinene were the major components.

A. chinensis is a perennial herb found in the ravines and shaded woodlands of China. In Hong Kong, it is used in traditional medicine as an antiasthmatic and analgesic. A number of sesquiterpene hydrocarbons were identified from the essential oil of the leaves[11a] and flowers of *A. chinensis*.[11b] An investigation by Sy and Brown[11c] on the aerial parts of the plant has led to the isolation of ten labdane diterpenes **119-128**. Among these, alpinia epoxide (**119**) was found to be the major component constituting 1.16% fraction weight of the plant material. The absolute stereochemistry of the epoxide was confirmed by applying the Mosher ester techniques.[11d]

A. conchigera is a herb growing up to 1m in height.[9] It commonly grows in damp and open spaces. It is reported to produce the smallest flowers among the *Alpinia* species found in peninsular Malaysia.[12] In India, the plant is found in West Bengal, where a poultice of the boiled leaves and rhizomes is used for treating rheumatism.[9] The rhizomes are used in traditional medicine to relieve gastrointestinal disorders, arthritis and a variety of other ailments.[13] The rhizomes are also used as an appetizer, analgesic and anti-inflammatory agent. The rhizomes are used in Malaysia as a condiment and is also made into a wine. The essential oil of the rhizomes have been evaluated.[14a,f] In a recent report, the essential oil of the dried leaves, pseudostem and the

rhizomes of *A. conchigera* were tested for antifungal and antibacterial activity and weak inhibitions against the tested microorganisms were obtained.[14b] Yu *et al*[14c] reported the detection of anti-inflammatory phenylpropanoid derivatives in the fruits of *A. conchigera* collected from China. From the rhizome extract, one new and four known diarylheptanoids (**133**-**137**) along with two flavonoids - galangin (**138**) and kaempferide (**139**) were identified.[14d] Lee *et al*[14e] analyzed the biological activity of the chalcone analog cardamomin (**83**) from *A. conchigera* and found that cardamomin could be used for the intervention of NF-κB dependent pathological condition such as inflammation.

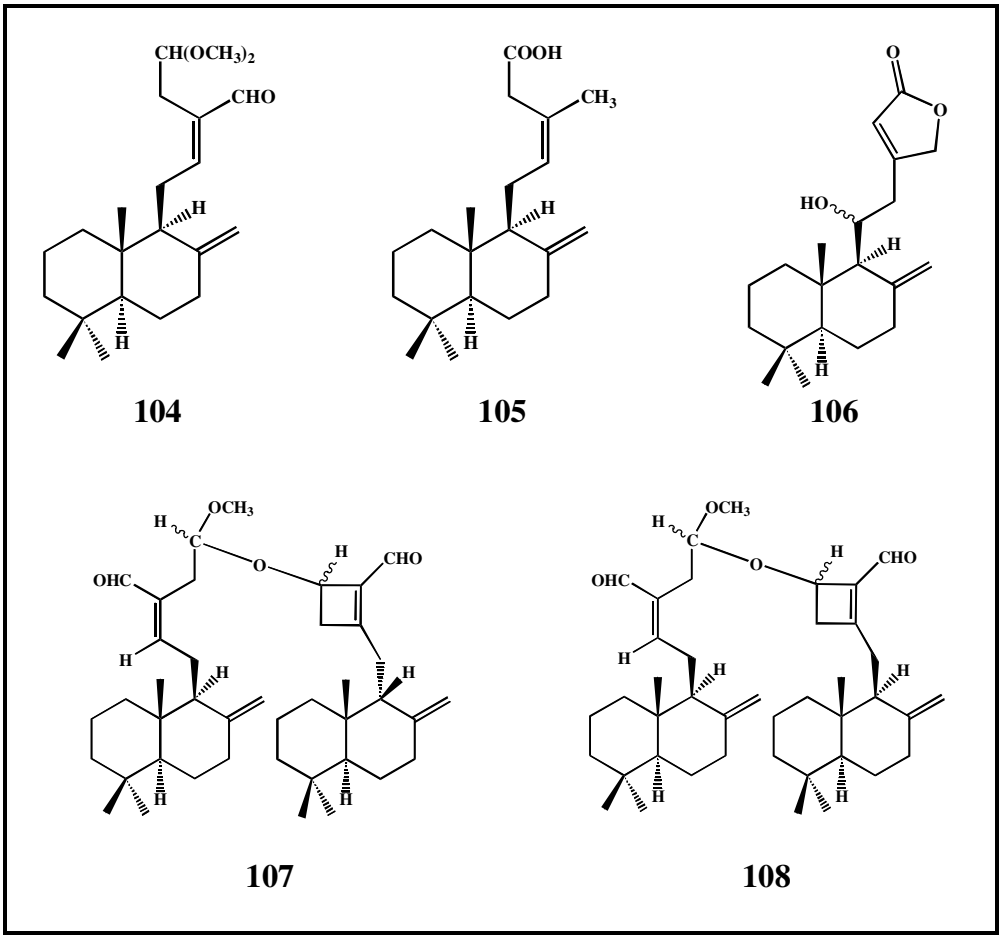
Table 2.2: Phytochemical constituents isolated from *A. calcarata*, *A. chinensis* and *A. conchigera*

Plant	Compound	No.
Alpinia calcarata	Calcaratarin A	104
	Calcaratarin B	105
	Calcaratarin C	106
	Calcaratarin D	107
	Calcaratarin E	108
	Calcaratarin G	109
	Calcaratarin H	110
	γ-Bicyclohomofarnesol	111
	(*E*)-15,16-Bisnorlabda-8(17),11-dien-13-one	112
	Labda-8(17),11,13-trien-15(16)-olide	113
	(*E*)-Labda-8(17),12-dien-15-ol-16-al	114
	Zerumin A	115
	Isocoronarin D	116
	Shyobunone	117

	Table 2.2 continued...	
	7-Methoxycoumarin	118
Alpinia chinensis	14ξ,15-Epoxylabda-8(17),12-dien-16-al (*E*)	119
	Labda-8(17),12-diene-15,16-dial (*E*)	120
	Coronarin E	121
	15-Hydroxylabda-8(17),11,13-trien-16-al (*E*,*E*)	122
	15,16-Epoxylabda-8(17),11,13-trien-16-ol (*E*)	123
	15-Hydroxylabda-8(17),11,13-trien-16,15-olide (*E*)	124
	14ξ,15-Dihydroxylabda-8(17),12-dien-16-al (*E*)	125
	12ξ,15-Dihydroxylabda-8(17)-13-dien-16-al (*E*)	126
	15-Hydroxy-11ξ,14ξ-peroxylabda-8(17),12-dien-16-al	127
	Coronarin C	128
Alpinia conchigera	Chavicol acetate	129
	1-Hydroxychavicol acetate	130
	4-Acetoxycinnamyl alcohol	131
	4-Acetoxycinnamyl acetate	132
	1,7-Diphenyl-3,5-heptanedione	133
	1,7-Diphenyl-5-hydroxy-3-heptanone	134
	5-Hydroxy-7-(4'-hydroxy-3'-methoxyphenyl)-1-phenyl-3-heptanone	135
	1,7-Diphenylhept-4-en-3-one	136
	7-(4'-Hydroxy-3'-methoxyphenyl)-1-phenylhept-4-en-3-one	137
	3,5,7-Trihydroxyflavone (Galangin)	138

	Table 2.2 continued...	
	3,5,7-Trihydroxy-4'-methoxyflavone (Kaempferide)	139
	Cardamomin	83
	Nonacosane	140
	β-Sitosterol	141
	1'-Acetoxychavicol acetate	142
	1'-Acetoxyeugenol acetate	143

Chart 2.2: Structures of the phytochemical constituents isolated from *A. calcarata*, *A. chinensis* and *A. conchigera*



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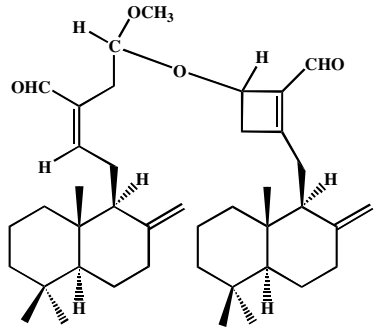

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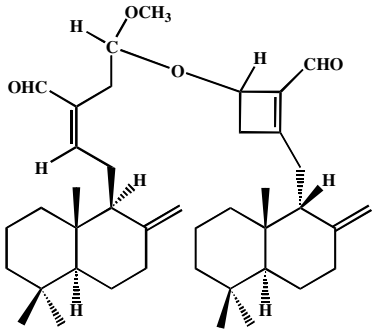

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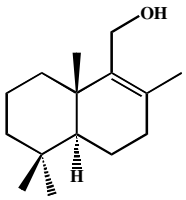

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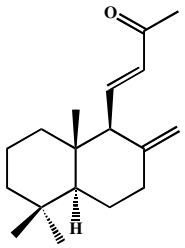

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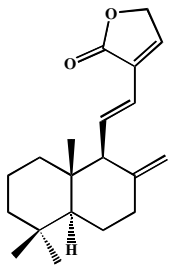

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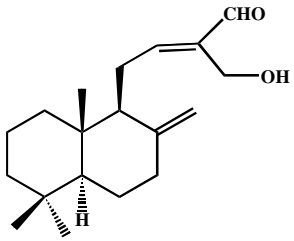

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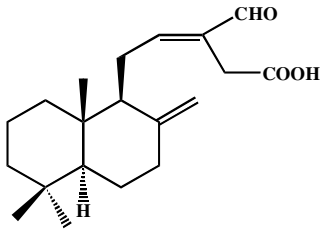

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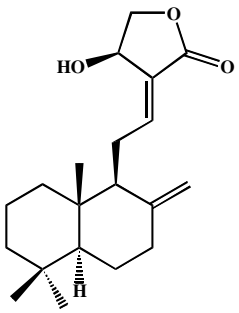

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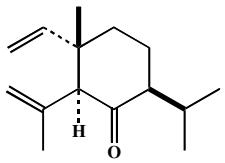

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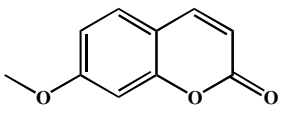

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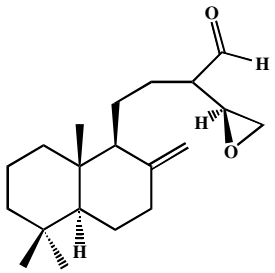

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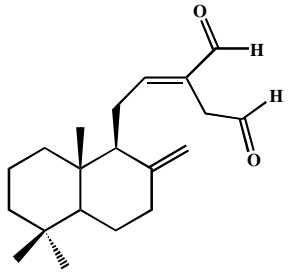

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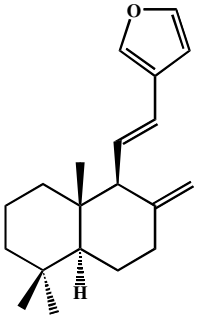

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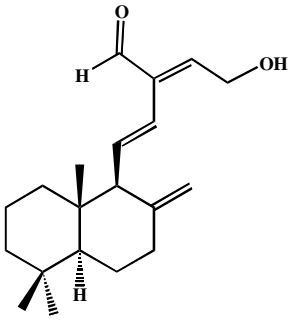

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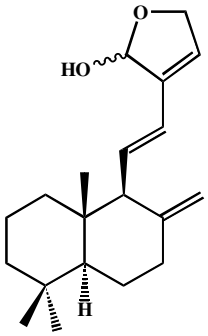

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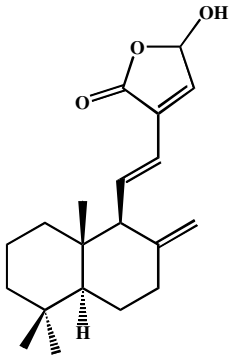

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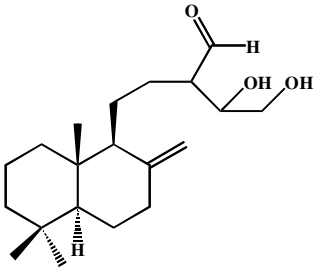

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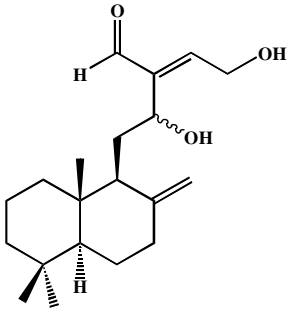

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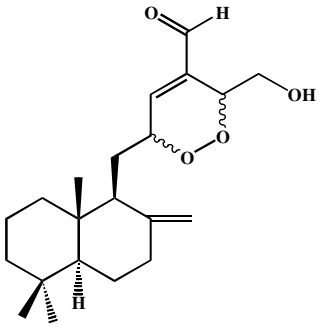

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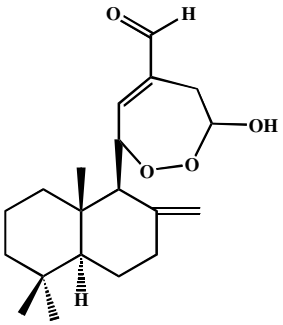

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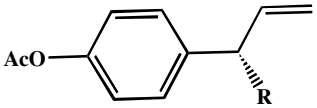
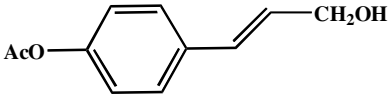


129; R = H
130; R = OH



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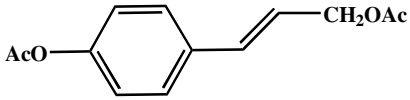

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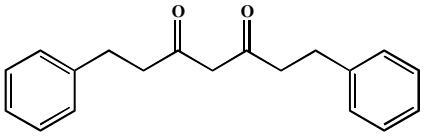

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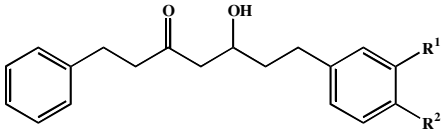
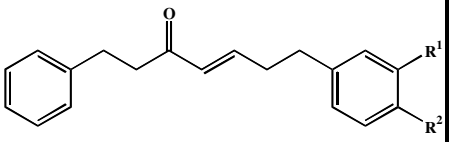
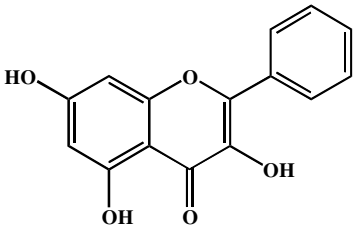


134; $R^1 = R^2 = H$
135; R^1 = OH, R^2 = OMe



136; $R^1 = R^2 = H$
137; R^1 = OMe, R^2 = OH



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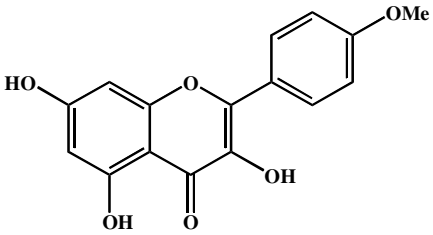

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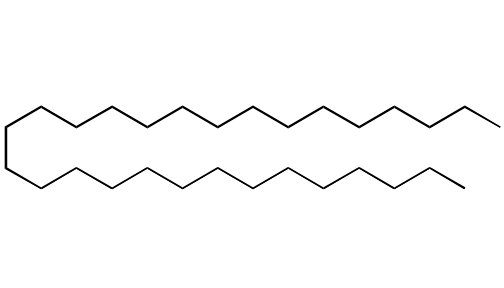

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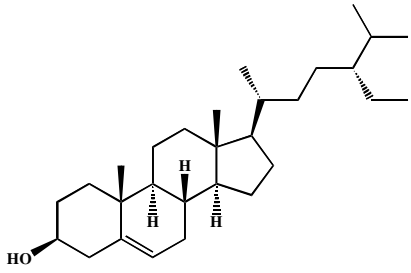

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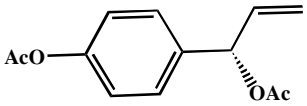

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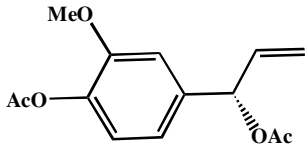


143

A. densibracteata is a perennial herb found mostly in the shady woodlands of Hong Kong. The aerial parts yielded oxygenated bisabolane sesquiterpenes (**144**, **145**, **152**-**155**) and four oxygenated monoterpenes (**146**, **147**, **149**, **150**) along with other known bisabolanes and menthones.[15]

A. flabellata is a rare specie of *Alpinia* found in Japan.[16a] It is used in foodstuffs and traditional medicines in Japan.[16b] The presence of a new cyclohexane derivative viz., aflabene (**162**) has been reported from the roots of *A. flabellata* by Mori *et al* in 1978.[16c] Masuda *et al*[16d] reported the isolation of a phenylbutanoid dimer (**163**) along with 2',4',5'-trimethoxyphenylbutadiene (**164**) and aflabene from the acetone extract of the fresh rhizomes of *A. flabellata*. Apart from the medicinal use of the rhizomes, the leaves of this plant have been used as a wrapping and flavoring material for foods in the southern part of Japan. Therefore investigation on the chemical composition of the leaves of *A. flabellata* was carried out by Kikuzaki *et al*[16e] which led to the isolation of three new phenylbutanoid dimers (**165**-**167**) along with three known compounds (**168**-**170**). The same group have also reported the isolation and characterization of the

phenylbutanoid (**171**) and a novel labdane diterpene adducted by a phenyl butanoid (**172**) from the leaves.[16f] Each of these isolated compounds possess a 2,4,5-trimethoxyphenyl moiety in the molecule. Kikuzaki *et al*[16g] reported the isolation of two new flavonol-phenylbutanoid adducts (**173** and **174**) from the leaves.

The diterpene (*E*)-Labda-8(17),12-diene-15-ol-16-al (**114**) was isolated form the rhizomes of *A. formosana*, together with six known compounds (**112**, **178**-**182**).[17] The isolation of these compounds from *A. formosana* which are identical to the compounds isolated from *A. speciosa* has led to the conclusion that chemotaxonomically both these plants are closely related in the genus *Alpinia*.

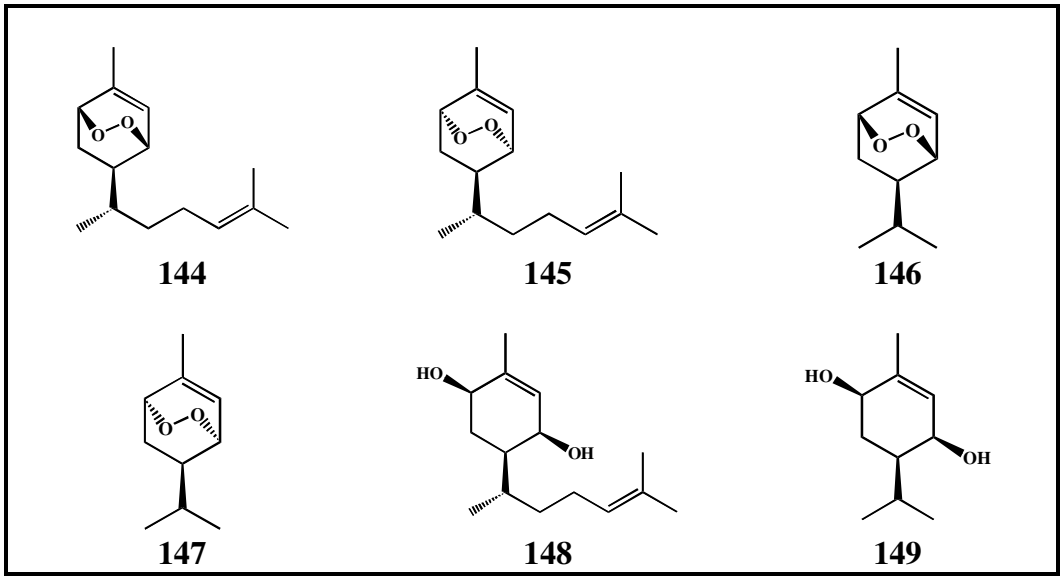
Table 2.3: Phytochemical constituents isolated from *A. densibracteata*, *A. flabellata* and *A. formosana*

Plant	Compound	No.
Alpinia densibracteata	(1*S*,4*R*,6*R*)-1,4-Epidioxy-bisabola-2,10-diene	144
	(1*R*,4*S*,6*R*)-1,4-Epidioxy-bisabola-2,10-diene	145
	(1*S*,4*R*,6*R*)-1,4-Epidioxy-*p*-menth-2-ene	146
	(1*R*,4*S*,6*R*)-1,4-Epidioxy- *p*-menth-2-ene	147
	1,4-Dihydroxybisaboladiene	148
	(1*S*,4*R*,6*R*)-*p*-Menthenediol	149
	(1*R*,4*S*,6*R*)-*p*-Menthenediol	150
	(*S*)-α-Curcumene	151
	3-Hydroxy-11-hydroperoxy-bisabola-1,9-diene	152
	3-Hydroxy-10-hydroperoxy-bisabola-1,10-diene	153
	4-Hydroxy-11-hydroperoxy-bisabola-1,3(15),9-triene	154

	Table 2.3 continued...	
	3,4-Dihydroxy-bisabola-1,10-diene	155
	3,4-Dihydroxy-*p*-menth-1-ene	156
	2α-Cinnamoyl cineole	157
	2β-Cinnamoyl cineole	158
	α-*trans*-Bergamotene	159
	Sesquiphellandrene	160
	Zingiberene	161
Alpinia flabellata	Alflabene	162
	(±)-*trans*-3-(2,4,5-Trimethoxyphenyl)-4-[(*E*)-2,4,5-trimethoxystyryl]-cyclohexene	163
	2',4',5'-Trimethoxyphenylbutadiene	164
	cis-1-(2,4,5-Trimethoxy-*E*-styryl)-2-(2,4,5-trimethoxy-*Z*-styryl)cyclobutane	165
	trans-1-(2,4,5-Trimethoxy-*E*-styryl)-2-(2,4,5-trimethoxy-*Z*-styryl)cyclobutane	166
	1,2-Bis(2,4,5-trimethoxy-*Z*-styryl)-cyclobutane	167
	2,4,5-Trimethoxybenzaldehyde	168
	2,4,5-Trimethoxycinnamaldehyde	169
	3,5-Dihydroxy-7,4'-dimethoxyflavone	170
	4-Hydroxy-2-(2,4,5-trimethoxyphenyl)-2*E*-butenal	171
	rel-Labd-12-en-15(16)-olid-7-one-8*R*-spiro-1'-[2*S*-(2,4,5-trimethoxyphenyl)-3-cyclohexene]	172
	rel-5-Hydroxy-7,4'-dimethoxy-2"*S*-(2,4,5-trimethoxy-*E*-styryl)tetrahydrofuro[4"*R*,5"*R*:2,3] flavanonol	173

	Table 2.3 continued...	
	rel-5-Hydroxy-7,4'-dimethoxy-3"*S*-(2,4,5-trimethoxy-*E*-styryl)tetrahydrofuro[4"*R*,5"*R*:2,3] flavanonol	174
	2,4,5-Trimethoxybenzoic acid	175
	2,4,5-Trimethoxycinnamic acid	176
	5-Hydroxy-3,7,4'-trimethoxyflavone	177
Alpinia formosana	(*E*)-Labda-8(17),12-diene-15-ol-16-al	114
	(*E*)-15,16-Bisnorlabda-8(17),11-diene-13-one	112
	Furopelargone B	178
	Humulene epoxide II	179
	Methyl-*trans*-cinnamate	180
	Dihydro-5,6-dehydrokawain	181
	Dihydroflavokawin B	182

Chart 2.3: Structures of the phytochemical constituents isolated from *A. densibracteata*, *A. flabellate* and *A. formosana*



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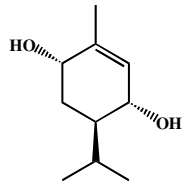

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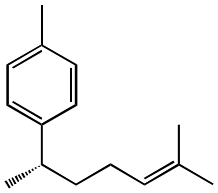

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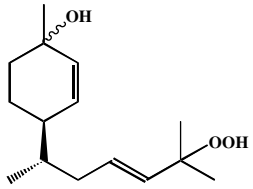

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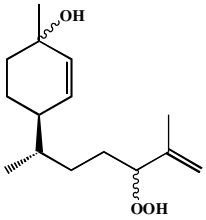

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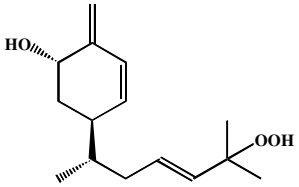

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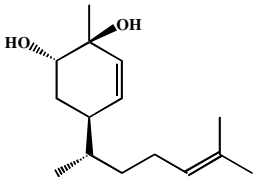

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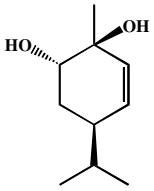

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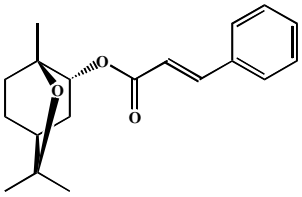

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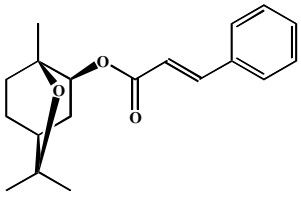

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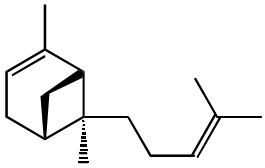

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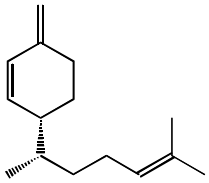

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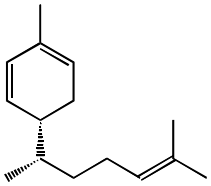

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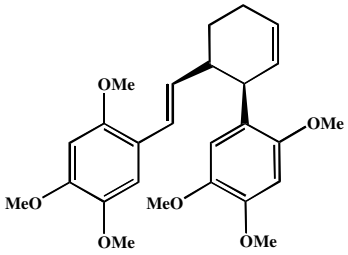

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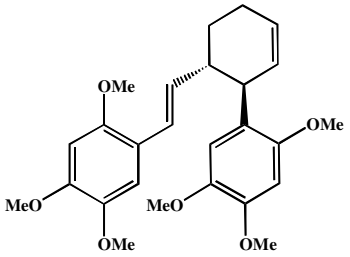

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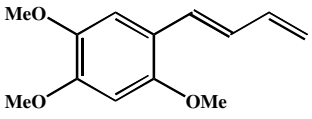

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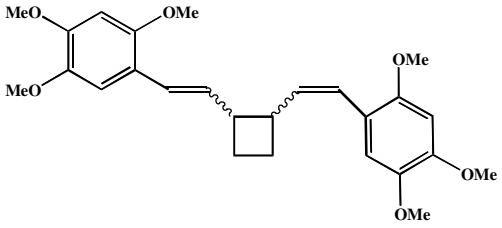

165; *cis*
166; *trans*

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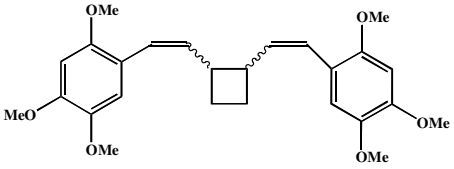

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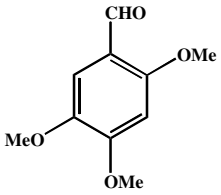

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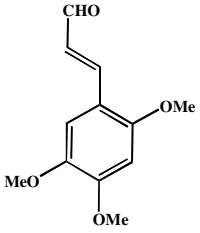

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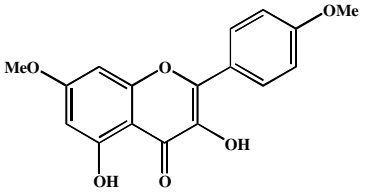

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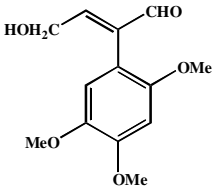

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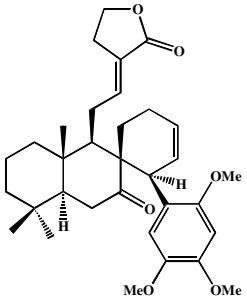

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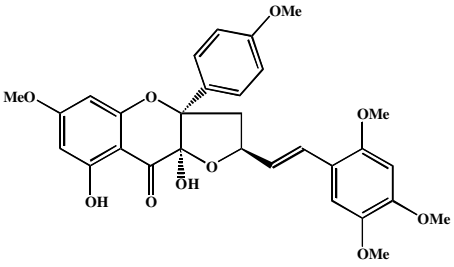

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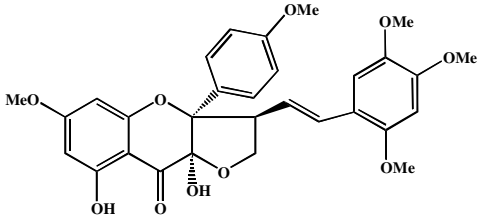

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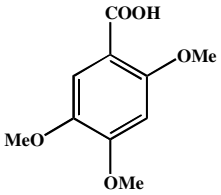

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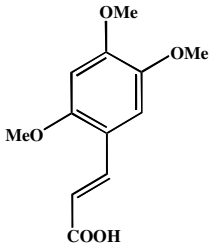

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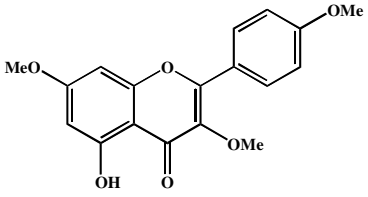

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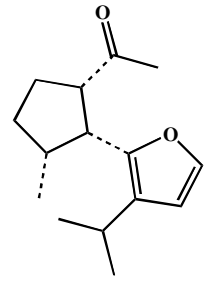

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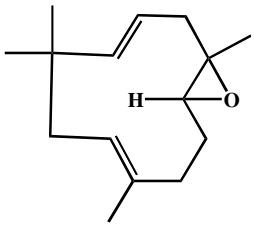

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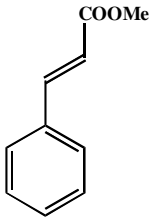

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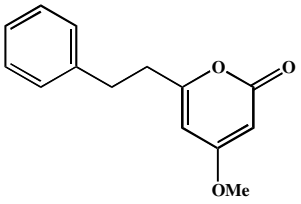

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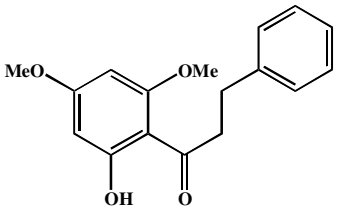


182

A. galanga is a very important medicinal plant of the genus *Alpinia*. Several biologically active compounds have been isolated from it. As the present chapter deals with the chemistry and biological activity of *A. galanga*, a detailed description of the plant is provided in section 2.2.1.

The chemical composition of the essential oil from *A. henryi* rhizomes collected from Vietnam has been studied and was found to contain 1,8-cineole as the major compound.[18a] The vasorelaxant effects of cardamomin (**83**) and alpinetin (**82**) isolated from *A. henryi* rhizomes was examined by Wang *et al*.[18b] They also showed that in *A. henryi*, cardamomin is present in the *trans* and alpinetin is found in the *S* configuration.

The seeds of *A. japonica* are used as an aromatic stomachic under the name, "*Izu-shukusha*" in Japan. Flavonoids such as alpinone (**205**), izalpinin (**206**), kumatakenin (**207**), rhamnocitrin (**208**) and monoterpenes (**209**-**210**) have been isolated from the seeds by Kimura and coworkers.[19a] They also reported the presence of sesquiterpene alcohols in the seed essential oil.[19b]

The sesquiterpenes **212**-**215** called agarofurans as well as the peroxy compound **222** have been isolated from the rhizomes of *A. japonica*.[19c-e] Some of these sesquiterpenes and their derivatives were found to posess spasmolytic activities.[19f]

The rhizomes of *A. jianganfeng* are used in traditional Chinese medicine to cure rheumatism.[20a] Four compounds (**141**, **224**-**226**) as shown in Table 2.4 were isolated from the rhizomes.[20b]

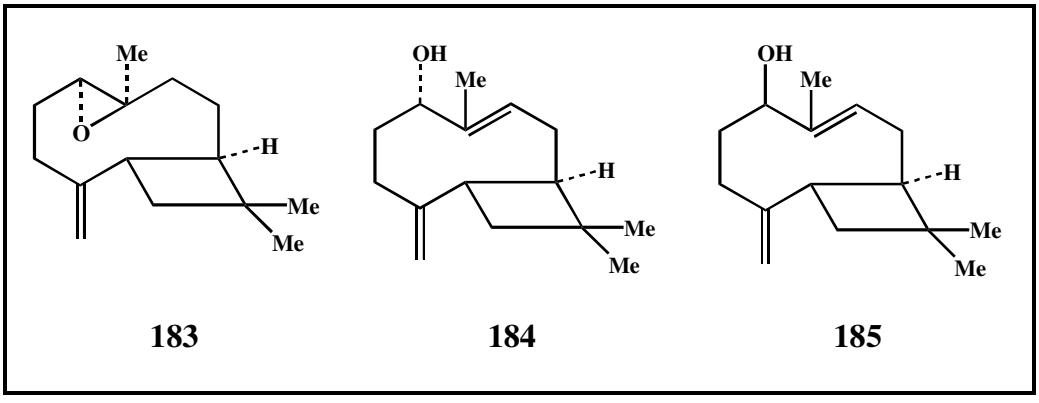
Table 2.4: Phytochemical constituents isolated from *A. galanga*, *A. henryi*, *A. japonica*, *A. javanica* and *A. jiangfeng*

Plant	Compound	No.
Alpinia galanga	1'-Acetoxychavicol acetate	142
	1'-Acetoxyeugenol acetate	143
	Caryophyllene oxide	183
	Caryophellenol-I	184
	Caryophellenol-II	185
	trans-*p*-Hydroxycinnamaldehyde	186
	[Di-(*p*-hydroxy-*cis*-styryl)]methane	187
	Galanal A	188
	Galanal B	189
	Galanolactone	190
	(*E*)-8(17),12-Labddiene-15,16-dial	120
	(*E*)-8β(17)-Epoxylabd-12-ene-15,16-dial (Aframodial)	191
	trans-*p*-Coumaryl diacetate	192
	trans-Coniferyl diacetate	193
	4-Hydroxybenzaldehyde	90
	trans-2-Acetoxy-1,8-cineole	194

	Table 2.4 continued...	
	trans-3-Acetoxy-1,8-cineole	195
	cis-3-Acetoxy-1,8-cineole	196
	cis-2-Acetoxy-1,8-cineole	197
	(1*R*,2*R*,4*S*)-*trans*-2-Hydroxy-1,8-cineole-β-D-glucopyranoside	198
	(1*S*,2*S*,4*R*)-*trans*-2-Hydroxy-1,8-cineole-β-D-glucopyranoside	199
	(1*R*,3*S*,4*S*)-*trans*-3-Hydroxy-1,8-cineole-β-D-glucopyranoside	200
	Methyleugenol	201
	Chavicol-β-D-glucopyranoside	202
	trans-p-Hydroxycinnamyl acetate	203
	trans-p-Coumaryl alcohol	204
Alpinia henryi	Alpinetin	82
	Cardamomin	83
Alpinia japonica	Alpinone	205
	Izalpinin	206
	Kumatakenin	207
	Rhamnocitrin	208
	Camphor	209
	Cineole	210
	Alpiniol	211
	4α-Hydroxydihydroagarofuran	212
	3α,4α-Oxidoagarofuran	213
	3β,4β-Oxidoagarofuran	214
	α-Agarofuran	215

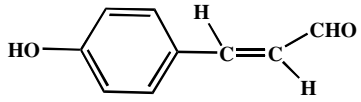
	Table 2.4 continued...	
	β-Eudesmol	216
	$\Delta^{9(10)}$-Eremophilen-11-ol	217
	10-*epi*-γ-Eudesmol	218
	Dihydroagarofuran	219
	Hanamyol	220
	Alpinolide	221
	Hanalpinol	222
	Furopelargone A	223
	Furopelargone B	198
	Humulene epoxide II	179
Alpinia javanica	Labda-8(17),12-diene-15,16-dial	120
	Coronarin E	121
Alpinia jiangfeng	Kaempferol-3-*O*-glucuronide	224
	Docosanoic acid	225
	3-Hydroxy-stigmast-5-en-7-one	226
	β-Sitosterol	141

Chart 2.4: Structures of the phytochemical constituents isolated from *A. galanga*, *A. henryi*, *A. japonica*, *A. javanica* and *A. jiangfeng*



183 **184** **185**

Chart 2.4 continued…



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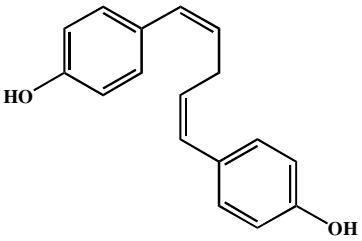

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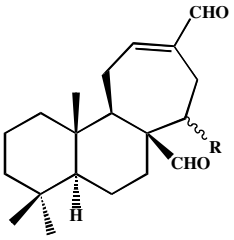
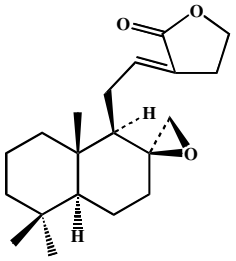


188; R = OH

189; R = OH



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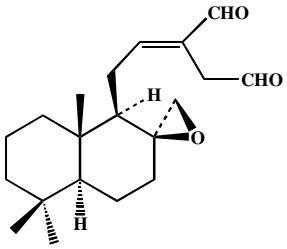

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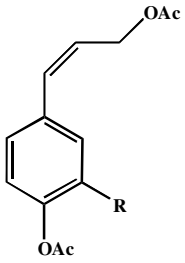
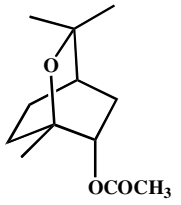


192; R = H

193; R = OMe



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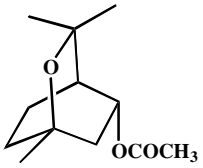

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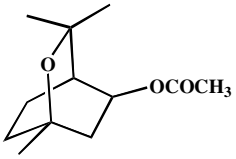

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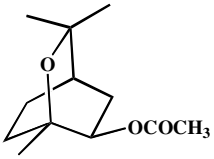

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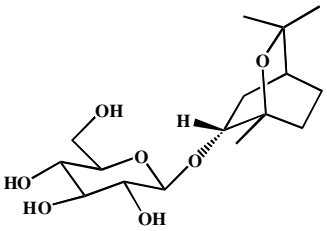

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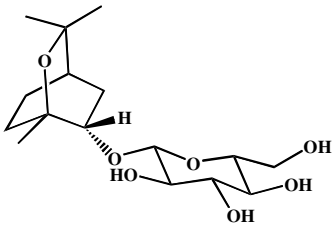

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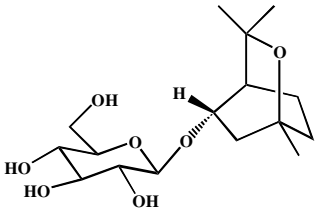

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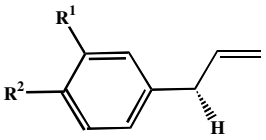
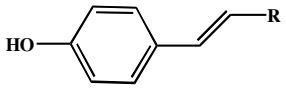


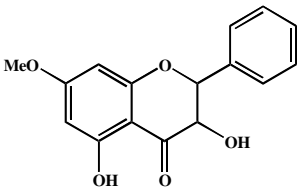
201; R^1 = OMe, R^2 = OH

202; R^1 = H, R^2 = OGlc



203; R = CH_2OAc

204; R = CH_2OH



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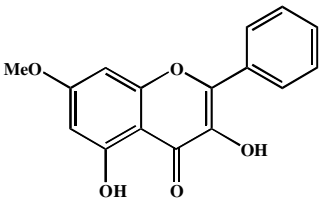

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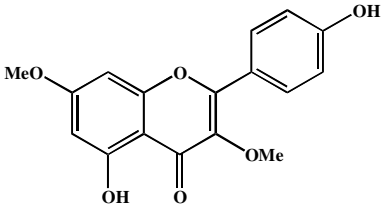

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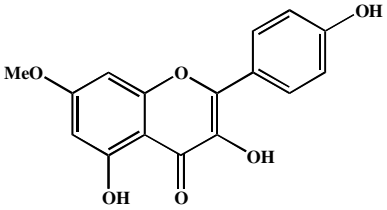

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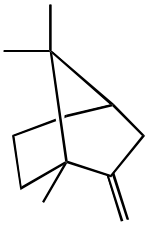

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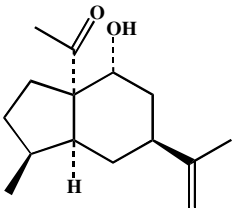

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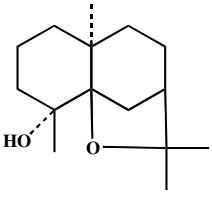

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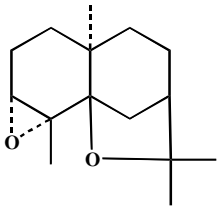

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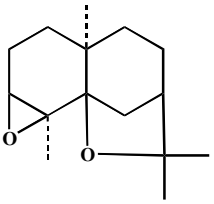

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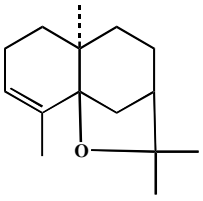

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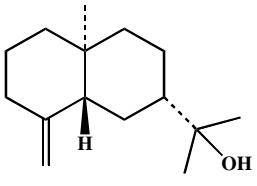

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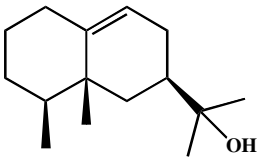

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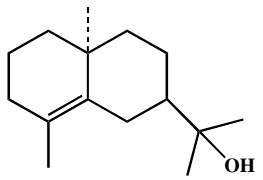

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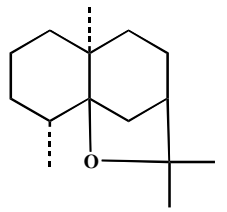

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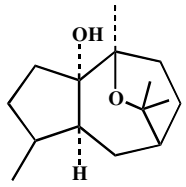

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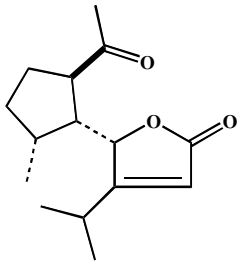

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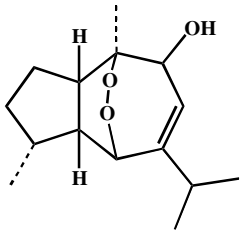

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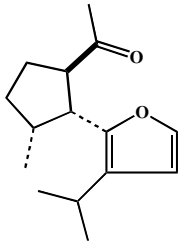

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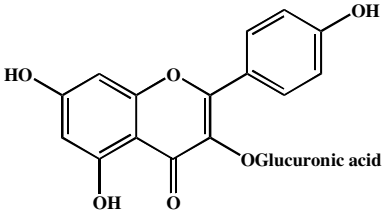

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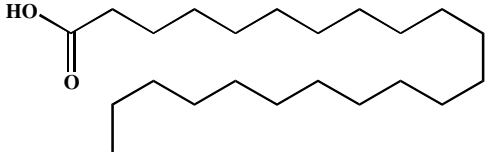

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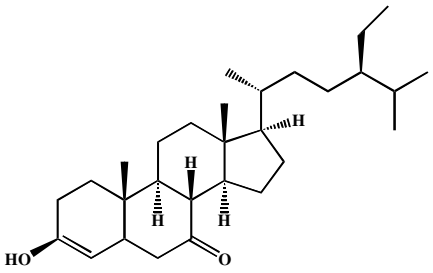


226

A. katsumadai is native to Hainan island in southern China, but is widely distributed in shaded woodland in Hong Kong. It is used in traditional Chinese medicine as an antiemetic and for the treatment of stomach disorders.[21a] The essential oil constituents of *A. katsumadai* was evaluated by Nan *et al.*[21b] A variety of diarylheptanoids (**237**-**240**, **242**, **243**, **251**-**253**),[21c] chalcones such as cardamomin and helichrysetin (**83** and **235**), flavonoids like

alpinetin (**82**), pinocembrin (**236**), 7,4'-dihydroxy-5-methoxyflavone (**89**)[21d] monoterpenes[21e] and sesquiterpenoids[21f] were isolated from the seeds whereas, from the aerial parts of *A. katsumadai*, the stilbenes **245**-**250** were isolated.[21g] Two antiemetic diarylheptanoids katsumadain A (**255**) and katsumadain B (**256**) as well as a biphenylpropanoid katsumadin (**257**) were isolated from the seeds.[21h,k,l] The antioxidant activity[21i] and antibacterial activity[21j] of methanol extract of *A. katsumadai* seed were also evaluated. A recent study reported the isolation of a pair of unique isomeric sesquiterpene-chalcone conjugates sumadin A (**254 A**) and sumadin B (**254 B**) with unprecedented skeletons from the seeds of *A. katsumadai*.[21m]

A. malaccensis is a perennial herb growing widely in the tropical and subtropical regions.[22a] In India, it is found in the terrain region of eastern Himalayas, West Bengal, Assam and Meghalaya and in the cool and shady places of Eastern Ghats, Andhra Pradesh and in Hassan district of Karnataka. In Indonesia, the rhizomes are employed to cure sores. The fruits are used with salt as an emetic. In Malaysia, the plant is employed for various medicinal purposes.[9] The essential oil of the rhizomes contains methylcinnamate and paraffin as the major constituents.[22b] A phytochemical investigation carried out by Nuchinpa and Apichart revealed the presence of 5,6-dehydrokawain (**88**), coronarin E (**121**), coronarin A (**258**), (*E*)-8(17),12-labdadiene-15,16-dial (**120**), hedyforrestin (**259**), cardamomin (**83**), pinocembrin (**236**) and alpinetin (**82**) in the rhizomes.[22c]

A. mutica is a Malaysian specie growing wild in the southern part of peninsular Malaysia.[23a] The Malays infuse the rhizomes for use as a stomachic.[23b] The study of the essential oil composition of the rhizomes revealed that the major components of the essential oil were camphor, 1,8-cineole and borneol.[23c] Sirat *et al* reported the presence of α-bergamotene (**159**), β-bisabolene (**260**), stigmasta-5-en-3-ol (**261**), stigmasta-5,22-dien-3-ol (**262**) including pinocembrin (**236**) in the rhizomes of *A. mutica*.[23d]

A. nigra is found in the wet places all over India, forming dense jungles in the sub-Himalayan region. The rhizomes are aromatic and medicinal. The juice of the rhizome is used in the treatment of gout and colic. The major components of the essential oil of the rhizomes were found to be caryophyllene oxide, geraniol, eudesmol, citronellyl acetate, citronellol, α-phellandrene and geranyl acetate.[9] Roy and Tandon in 1999 has reported the flukicidal activity of *A. nigra* against the trematode *Fasciolopsis buski* in humans.[24]

The methylene chloride extract of *A. nutans* was investigated for its antioxidant constituents. 5,6-dehydrokawain (**88**), flavokawin B (**263**), 1,7-diphenyl-5-hydroxy-6-hepten-3-one (**237**), pinocembrin (**236**), stigmasterol (**264**) and β-sitosterol (**141**) were isolated from the extract.[25a] Cytogenetic evaluation of the effects of aqueous extracts of *A. nutans* on onion root tip cells performed by Dias and Takahashi showed that they exhibited toxicity at higher concntrations.[25b]

Table 2.5: Phytochemical constituents isolated from *A. katsumadai*, *A. malaccensis*, *A. mutica* and *A. nutans*

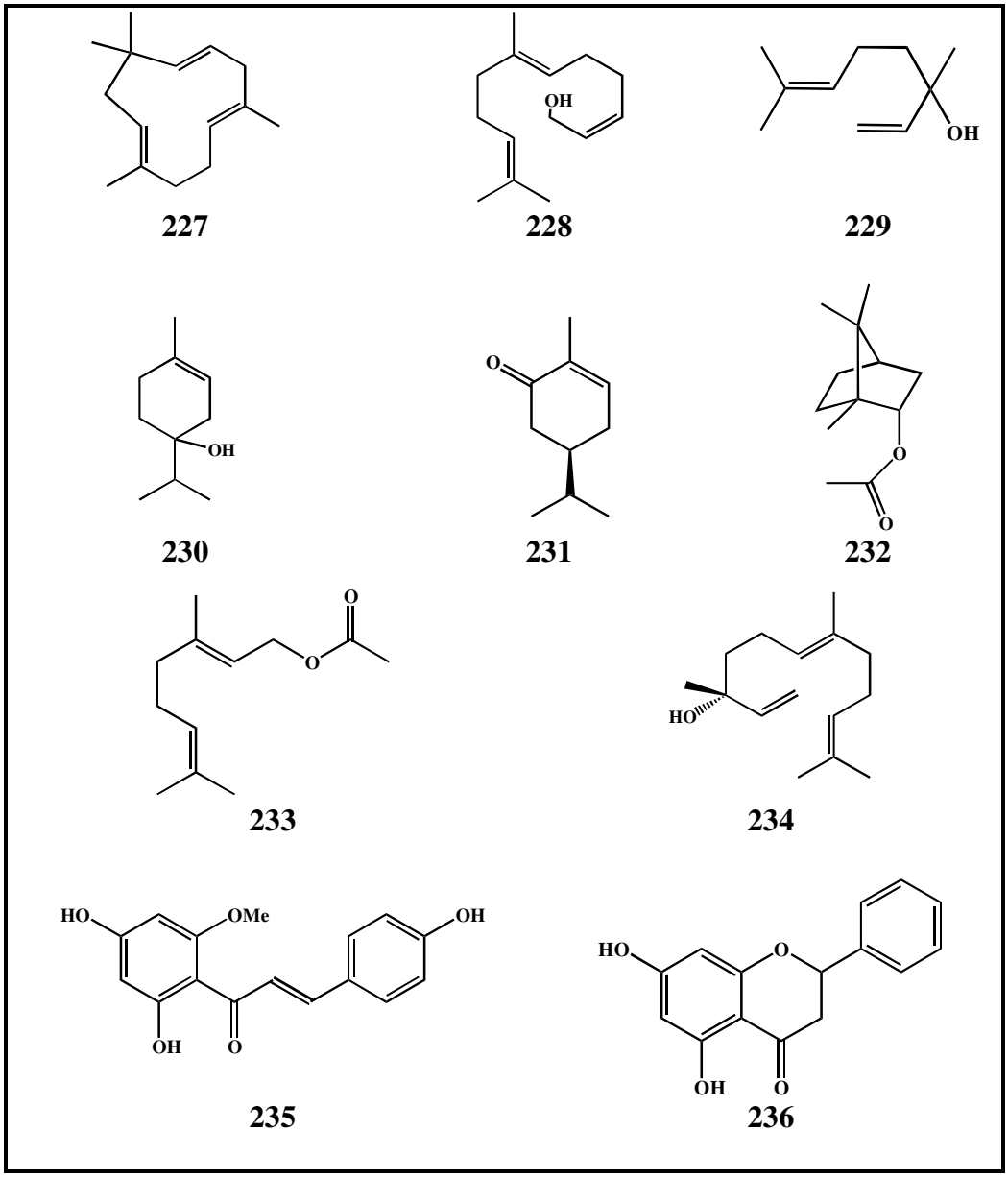
Plant	Compound	No.
Alpinia katsumadai	1,8-Cineole	210
	α-Humulene	227
	trans, trans-Farnesol	228
	Linalool	229
	Camphor	209
	Terpinen-4-ol	230
	Carvotanacetone	231
	Bornyl acetate	232
	Geranyl acetate	233

	Table 2.5 continued...	
	Methyl cinnamate	180
	Nerolidol	234
	Alpinetin	82
	Cardamomin	83
	Helichrysetin	235
	Pinocembrin	236
	(5*R*)-*trans*-1,7-Diphenyl-5-hydroxy-6-hepten-3-one	237
	(3*S*,5*S*)-*trans*-1,7-Diphenyl-3,5-dihydroxy-1-heptene	238
	trans-1,7-Diphenyl-5-hydroxy-1-heptene	239
	trans, trans-1,7-Diphenyl-5-hydroxy-4,6-heptadiene-3-one	240
	trans-Cinnamaldehyde	241
	(3*S*,5*R*)-3,5-Dihydroxy-1,7-diphenyl-heptane	242
	trans, trans-1,7-Diphenyl-4,6-heptadiene-3-one	243
	Galanolactone	190
	(*E*)-8(17),12-Labddiene-15,16-dial	120
	(*E*)-8β(17)-Epoxylabd-12-ene-15,16-dial (Aframodial)	191
	(*E*)-1-(1-Terpinen-4-olyl)-3-methoxystilbene	244
	(*E*)-3-Methoxy-5-hydroxystilbene	245
	Pinosylvin	246
	(*E*)-3,5-Dimethoxystilbene	247
	(*E*)-3,5-Dihydroxy-12-methoxystilbene	248
	(*Z*)-3-Methoxy-5-hydroxystilbene	249
	(*Z*)-3,5-Dihydroxystilbene	250

	Table 2.5 continued…	
	5-Hydroxy-1-(4'-hydroxyphenyl)-7-phenyl-hepta-6-en-3-one	251
	5-Hydroxy-1,7-diphenyl-hepta-6-en-3-one	252
	trans-3,5-Dihydroxy-1,7-diphenyl-hept-1-ene	253
	Sumadin A	254 A
	Sumadin B	254 B
	Katsumadain A	255
	Katsumadain B	256
	Katsumadin	257
	7,4'-Dihydroxy-5-methoxyflavone	89
Alpinia malaccensis	5,6-Dehydrokawain	88
	Coronarin E	121
	Coronarin A	258
	(*E*)-8(17),12-Labdadiene-15,16-dial	120
	Hedyforrestin	259
	Cardamomin	83
	Pinocembrin	236
	Alpinetin	82
Alpinia mutica	α-Bergamotene	159
	β-Bisabolene	260
	Stigmasta-5-en-3-ol	261
	Stigmasta-5,22-dien-3-ol	262
	Pinocembrin	236
Alpinia nutans	5,6-Dehydrokawain	88
	Flavokawin B	263
	1,7-Diphenyl-5-hydroxy-6-hepten-3-one	237
	Pinocembrin	236

	Table 2.5 continued...	
	Stigmasterol	264
	β-Sitosterol	141

Chart 2.5: Phytochemical constituents isolated from *A. katsumadai*, *A. malaccensis*, *A. mutica* and *A. nutans*



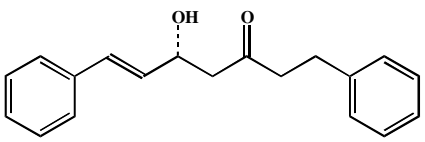
227 **228** **229**

230 **231** **232**

233 **234**

235 **236**

Chart 2.5 continued…



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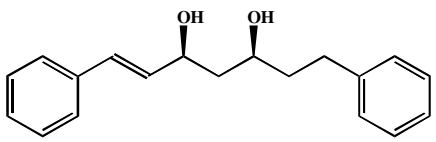

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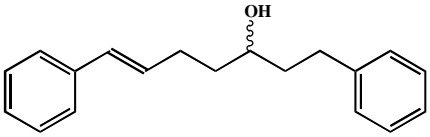

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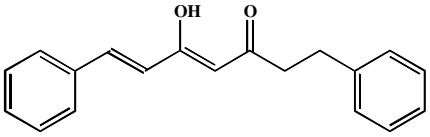

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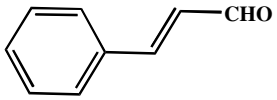

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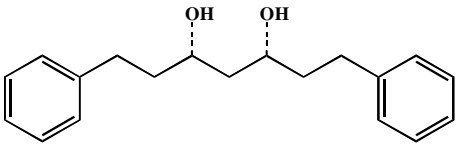

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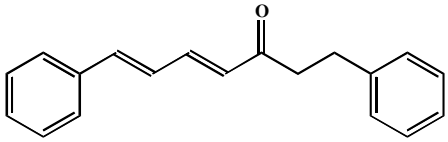

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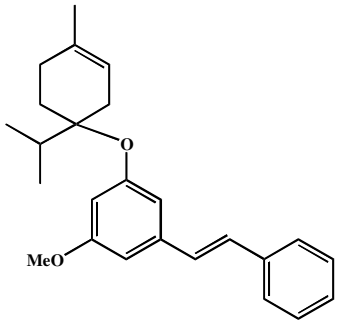

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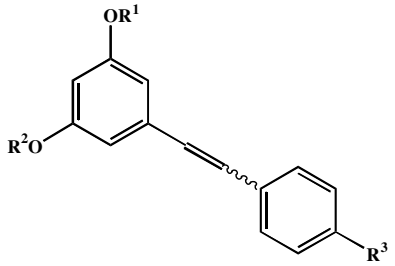
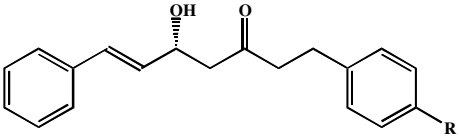


245; R^1 = Me, R^2 = R^3 = H
246; R^1 = R^2 = R^3 = H (*trans*)
247; R^1 = R^2 = Me; R^3 = H
248; R^1 = R^2 = H, R^3 = OMe
249; R^1 = Me, R^2 = R^3 = H
250; R^1 = R^2 = R^3 = H (*cis*)



251; R = OH
252; R = H

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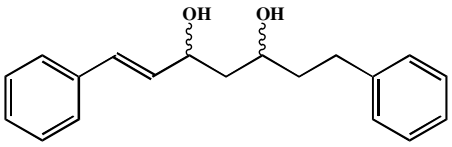

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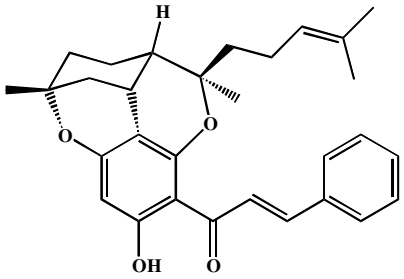

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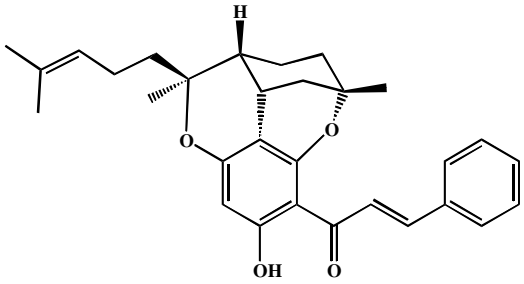

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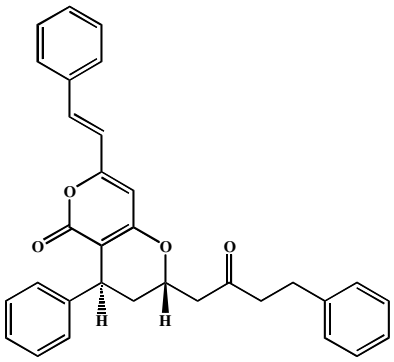

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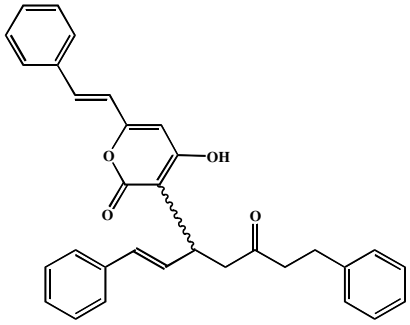

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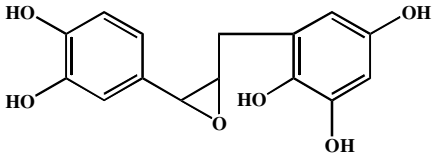

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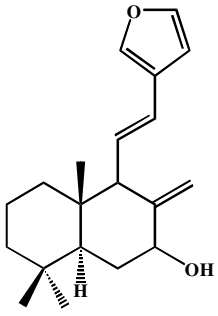

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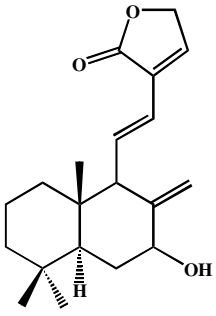

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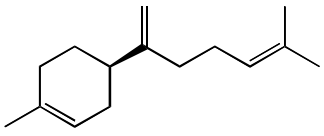

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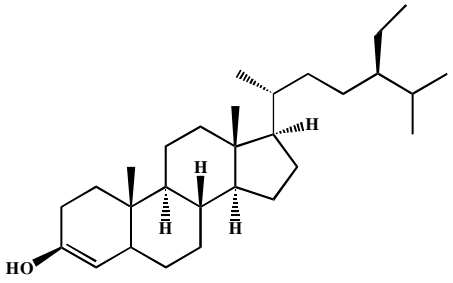

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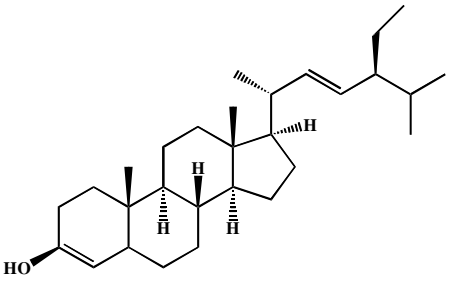

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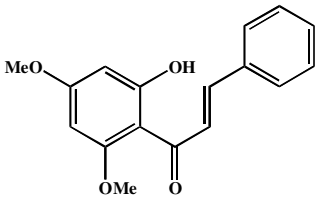

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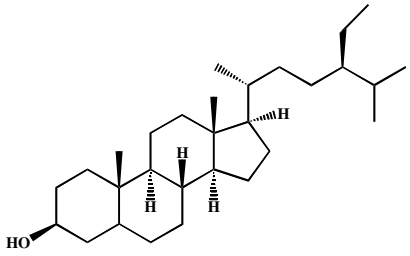


264

A. officinarum is a Chinese herbal drug extensively used as an aromatic, stomachic, analgesic and antiemetic.[26a] It is generally known as 'Lesser galangal'. The components of the essential oil of the rhizomes as well as its antimicrobial activity has been reported.[26b,c] Due to its importance in Chinese medicines, its phytochemical constituents and biological activity has been extensively explored as is evident from the references cited below. Several compounds including flavonoids like galangin (**138**), kaempferide (**139**), kaempferol (**274**), quercetin (**275**), 3-methylethergalangin (**288**) and pinobanksin (**296**), diarylheptanoids (**134**, **136**, **265**-**273**, **276**-**278**, **299**-**306**, **308**), sterols, glycosides (**279**-**287**) and phenylpropanoids (**289**-**295**) have been isolated from the rhizomes of *A. officinarum.*[26d-y] The diarylheptanoids isolated showed various biological activities such as inhibitory effect against prostaglandin biosynthesis,[26g] anti-inflammatory property,[26m] antiemetic activity,[26k] cytotoxicity,[26v,w,x] pancreatic lipase inhibition[26n] and inhibition of

nitric oxide production in lipopolysaccharide activated macrophages.[26r] The phenylpropanoids **289**-**295** isolated showed antioxidative activity.[26o] A study by Li and Tian[27] revealed the presence of fatty acid synthase inhibitors in the rhizomes. The rhizome extract also showed antimicrobial,[28] anti-inflammatory and anti-tumor activites[29] and cancer chemopreventive activities.[30] The vasorelaxation effect of the flavonoids from the rhizome extract of *A. officinarum* was evaluated by Hye *et al*.[31]

A. oxyphylla has been used in traditional oriental medicine and has been shown to contain phenolic compounds referred to as 'yakuchinones' that have diarylheptanoid structure.[32a] The crude drug *yakuchi* in Japan is prepared from the fruits of *A. oxyphylla*. This plant has been used for the treatment of gastrointestinal disturbances in Japan and other oriental countries.[32b] Study of the volatile oil from the fruits of *A. oxyphylla* resulted in the identification of sixty four compounds.[32c] The development of novel skin permeation enhancers from the sesquiterpenes of the essential oil has been reported by Fang *et al.*[32d] The suppression and induction of apoptosis by the fruit extract is also reported.[32a] The diarylheptanoids yakuchinone A (**312**) and yakuchinone B (**313**) from *A. oxyphylla* possessed anti-tumor promotional properties which may contribute to its chemo preventive potential.[32e] An insecticidal sesquiterpene nootkatone (**314**), 14-norcadinane type sesquiterpenes - oxyphyllenodiol A (**315**) and Oxyphyllenodiol B (**316**) and 11,12,13-trinoreudesmane type sesquiterpenes - Oxyphyllenone A (**317**) and Oxyphyllenone B (**318**) with nitric oxide production inhibitory activity were isolated from the methanolic extract of the kernels of *A. oxyphylla*.[32f,g] The fruits also showed neuroprotective effect and antioxidant activity.[32h,i] The bioassay guided fractionation of the methanolic extract resulted in the isolation of the insecticidal diaryheptanoid **312**.[32j] Protocatechuic acid (**311**) from *A. oxyphylla* was found to be effective against neurotoxicity, hydrogen peroxide induced oxidative cell death, promotes migration of human adipose

tissue derived stromal cells to regenerate injured or degenerative tissues and protects cells against oxidative damage and reduces oxidative stress *in vivo*.[32k-n] The isolation of a sesquiterpene nootkatol (**320**) possessing calcium antagonistic property was reported by Shoji *et al*.[32o] In other studies antioxidative and pungent diarylheptanoids (**312** and **324**), sesquiterpene **319**, flavones tectochrysin (**321**) and chrysin (**322**), steroids (**141** and **323**) and mixed fatty acids were isolated from the fruits.[32p-s]

Table 2.6: Phytochemical constituents isolated from *A. officinarum* and *A. oxyphylla*

Plant	Compound	No.
Alpinia officinarum	1,7-Diphenylhept-4-en-3-one	136
	1,7-Diphenylhept-5-hydroxy-3-heptanone (Dihydroyashabushiketol)	134
	7-(4”-Hydroxy-3”-methoxyphenyl)-1-phenylhept-4-en-3-one	265
	5-Hydroxy-7-(4”-hydroxy-3”-methoxyphenyl)-1-phenyl-3-heptanone	266
	7-(4”-Hydroxy-3”-methoxyphenyl)-1-phenyl-3,5-heptadione	267
	5-Methoxy-7-(4”-Hydroxy-3”-methoxyphenyl)-1-phenyl-3-heptanone	268
	5-Hydroxy-7-(4”-hydroxyphenyl)-1-phenyl-3-heptanone	269
	7-(4”-Hydroxyphenyl)-1-phenyl-4-hepten-3-one	270
	5-Methoxy-7-(4”-hydroxyphenyl)-1-phenyl-3-heptanone	271
	5-Methoxy-1,7-diphenyl-3-heptanone	272

	Table 2.6 continued...	
	5-Hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-3-heptanone [Hexahydrocurcumin]	273
	Galangin	138
	Kaempferide	139
	Kaempferol	274
	Quercetin	275
	(3*R*,5*R*)-1-(4-Hydroxyphenyl)-7-phenyl-3,5-heptanediol	276
	5-Hydroxy-1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)-3-heptanone	277
	5-Hydroxy-7-(4-hydroxy-3-methoxyphenyl)-1-(4-hydroxyphenyl)-3-heptanone	278
	(1*R*,3*S*,4*S*)-*trans*-3-Hydroxy-1,8-cineole β-D-glucopyranoside	279
	3-Methyl-but-2-en-1-yl β-D-glucopyranoside	280
	Benzyl - β-D-glucopyranoside	281
	1-*O*-β-D-glucopyranosyl-4-allylbenzene (chavicol- β-D-glucopyranoside)	282
	1-Hydroxy-2-*O*-β-D-glucopyranosyl-4-allylbenzene	283
	1-*O*-β-D-Glucopyranosyl-2-hydroxy-4-allylbenzene (demethyleugenol - β-D-glucopyranoside)	284
	1-*O*-(6-*O*-α-L-Rhamnopyranosyl-β-D-glucopyranosyl)-2-hydroxy-4-allylbenzene (demethyleugenol β-rutinoside)	285

	Table 2.6 continued...	
	1-*O*-(6-*O*-α-L-Rhamnopyranosyl-β-D-glucopyranosyl)-4-allylbenzene (chavicolβ-rutinoside)	286
	1,2-Di-*O*-β-D-Glucopyranosyl-4-allylbenzene	287
	3-Methylethergalangin	288
	(*E*)-*p*-Coumaryl alcohol γ-*O*-methyl ether	289
	(*E*)-*p*-Coumaryl alcohol	290
	(4*E*)-1,5-Bis(4-hydroxyphenyl)-1-methoxy-2-(methoxymethyl)-4-pentene	291
	(4*E*)-1,5-Bis(4-hydroxyphenyl)-1-ethoxy-2-(methoxymethyl)-4-pentene	292
	(4*E*)-1,5-Bis(4-hydroxyphenyl)-1-[(2*E*)-3-(4-acetoxyphenyl)-2-propenoxy]-2-(methoxy-methyl)-4-pentene	293
	(4*E*)-1,5-Bis(4-hydroxyphenyl)-2-(methoxy-methyl)-4-pentene-1-ol	294
	(4*E*)-1,5-Bis(4-hydroxyphenyl)-2-(hydroxy-methyl)-4-pentene-1-ol	295
	Pinobanksin	296
	3-Phenylpropanoic acid	297
	Zingerone	298
	trans, *trans*-1(3'-Methoxy-4'-hydroxyphenyl)-7-phenyl-5-ol-4,6-dien-3-heptanone	299
	trans, *trans*-1,7-Diphenyl-5-ol-4,6-diene-3-heptanone	240
	(5*S*)-5-Hydroxy-7-(3,4-dihydroxyphenyl)-1-phenyl-3-heptanone	300

	Table 2.6 continued...	
	(5*R*)-5-Hydroxy-7-(3-methoxy-4,5-dihydroxy-phenyl)-1-phenyl-3-heptanone	301
	(5*R*)-5-Hydroxy-1-(3,4-dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-3-heptanone	302
	7-(3,4-Dihydroxyphenyl)-1-(4-hydroxy-3-methoxyphenyl)-4-en-3-heptanone	303
	Alpinoid A	304
	Alpinoid B	305
	Alpinoid C	306
	Alpinoid D	307
	Alpinoid E	308
	Alpinoside A	309
	β- Sitosterol	141
	1,7-Diphenyl-5-ol-3-heptone	134
	Emodin	310
	3,4-Dihydroxybenzoic acid	311
Alpinia oxyphylla	Yakuchinone A	312
	Yakuchinone B	313
	Nootkatone	314
	Oxyphyllenodiol A	315
	Oxyphyllenodiol B	316
	Oxyphyllenone A	317
	Oxyphyllenone B	318
	Valencene	319
	Nootkatol	320
	Tectochrysin	321
	Chrysin	322

	Table 2.6 continued...	
	β – Sitosterol	141
	Daucosterol	323
	Protocatechuic acid	311
	1-(4'-Hydroxy-3'-methoxyphenyl)-7-phenyl-3-hept-1-ene	324

Chart 2.6: Structures of the phytochemical constituents isolated from *A. officinarum* and *A. oxyphylla*

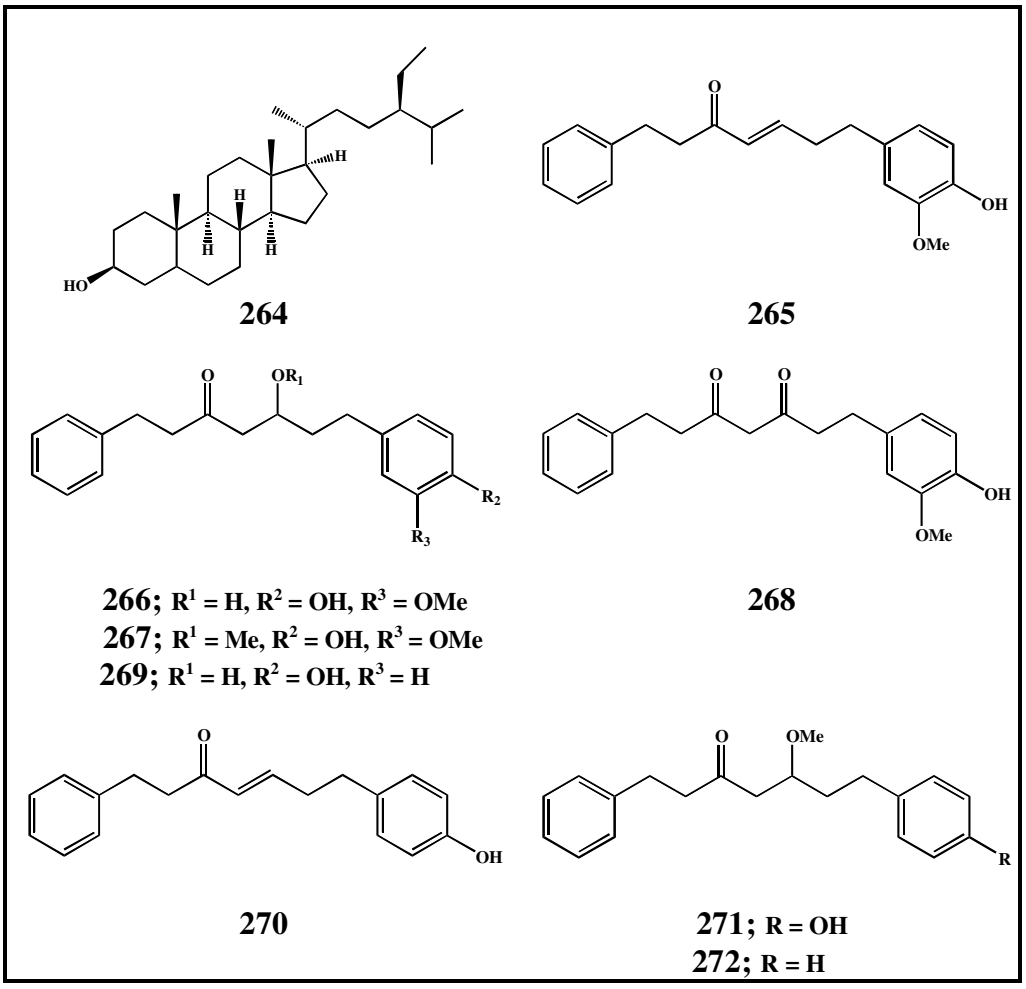
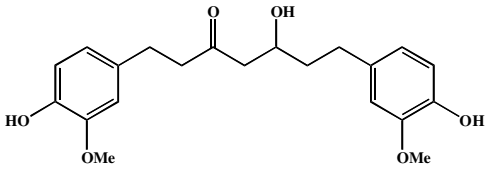


Chart 2.6 continued…



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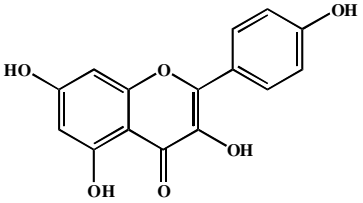

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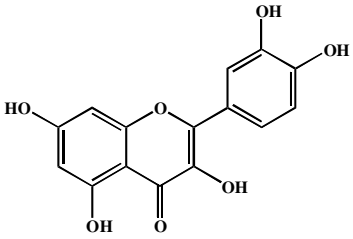

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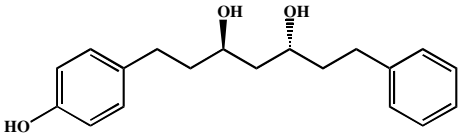

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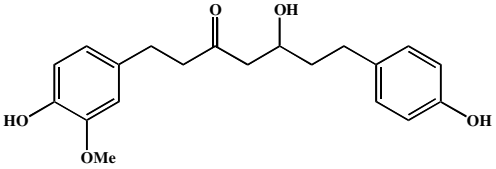

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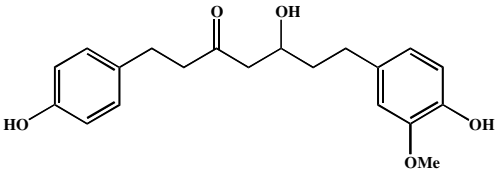

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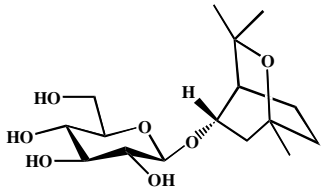

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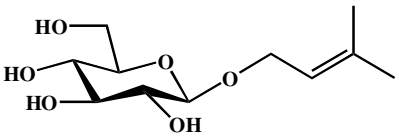

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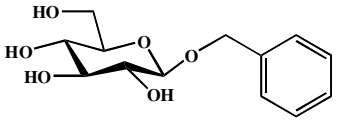

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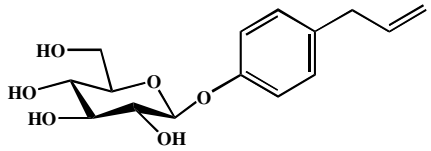

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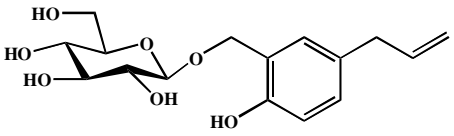

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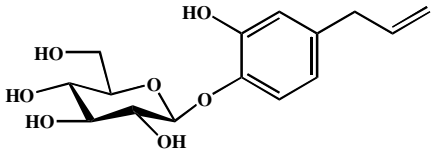

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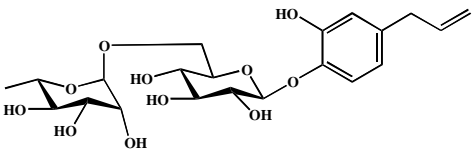

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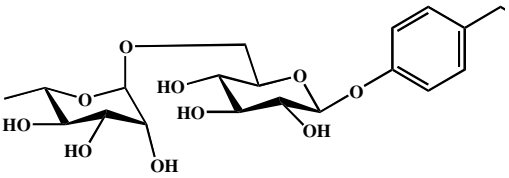

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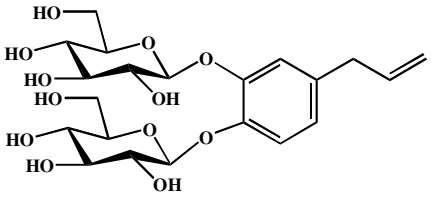

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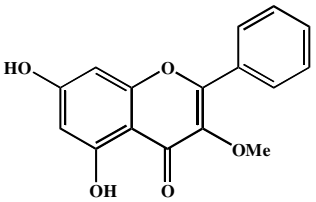

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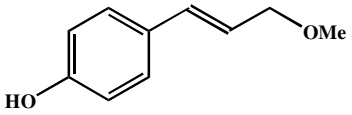

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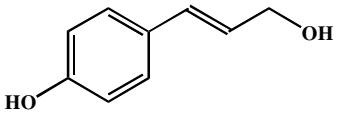

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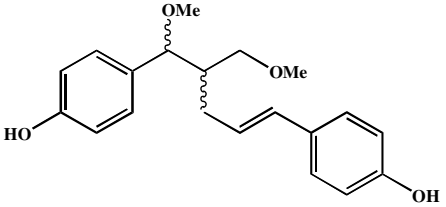

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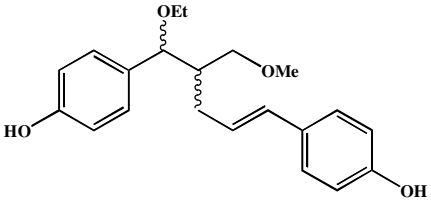

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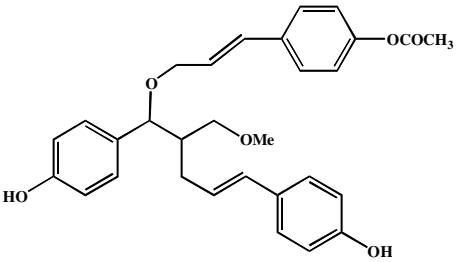

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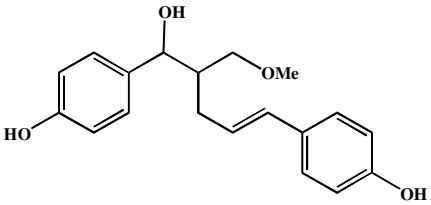

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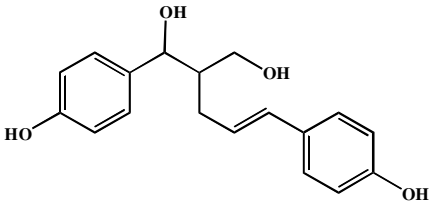

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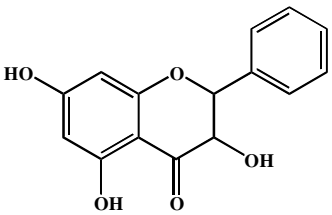

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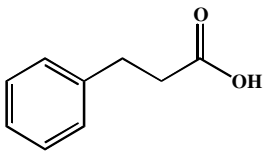

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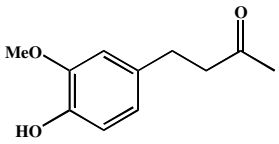

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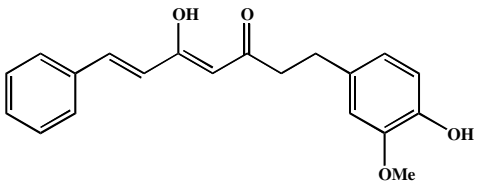

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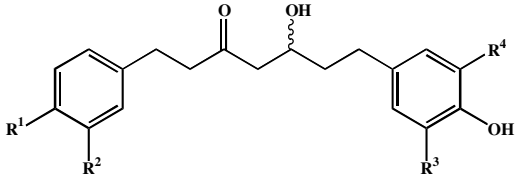
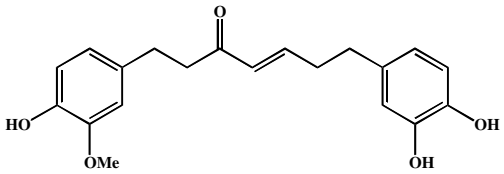


300; R^1 = H, R^2 = H, R^3 = OH, R^4 = H, 5-OH = *S*

301; R^1 = H, R^2 = H, R^3 = OMe, R^4 = OH, 5-OH = *R*

302; R^1 = OH, R^2 = OH, R^3 = OMe, R_4 = H, 5-OH = *R*



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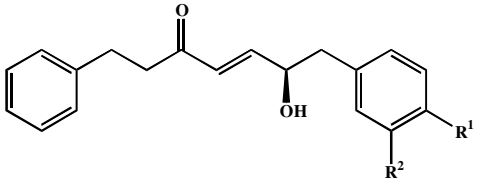
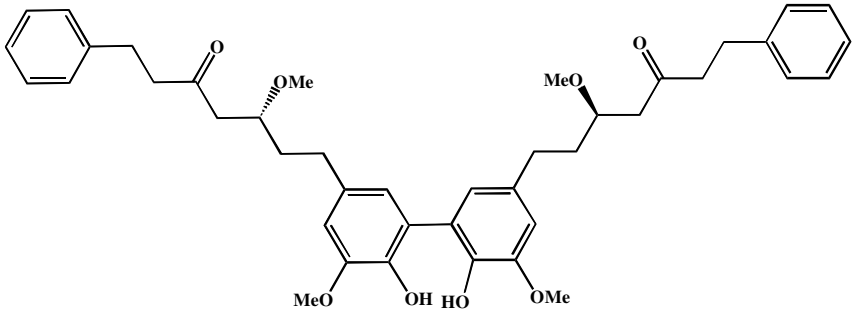


305; R^1 = OH, R^2 = OMe

306; R^1 = H, R^2 = H



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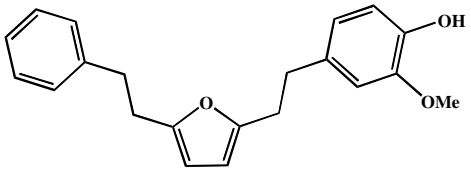

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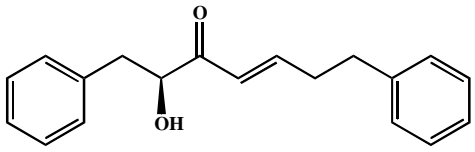

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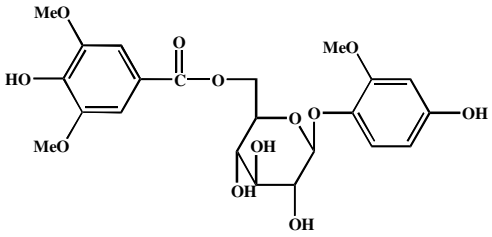

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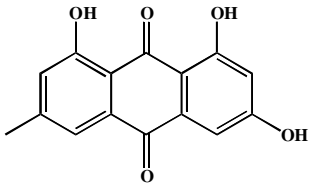

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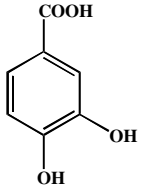

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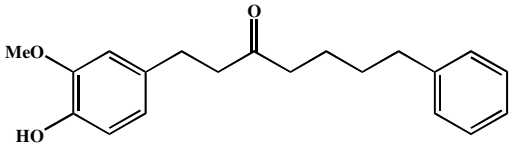

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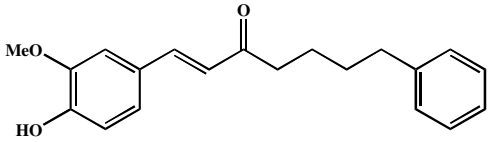

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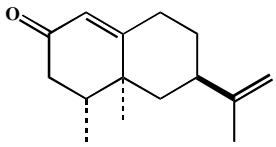

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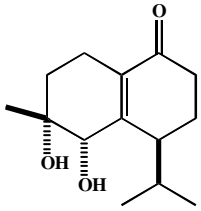

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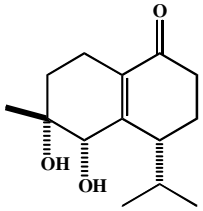

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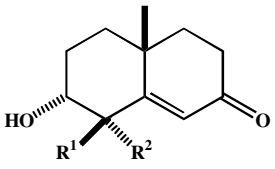
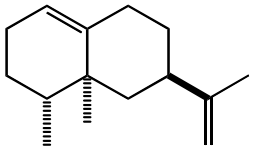


317; R^1 = OH, R^2 = Me
318; R^1 = Me, R^2 = OH



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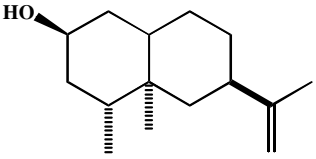

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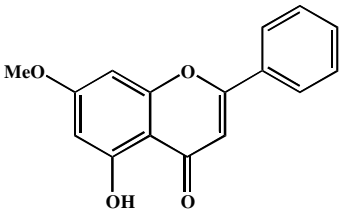

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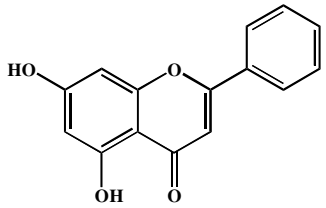

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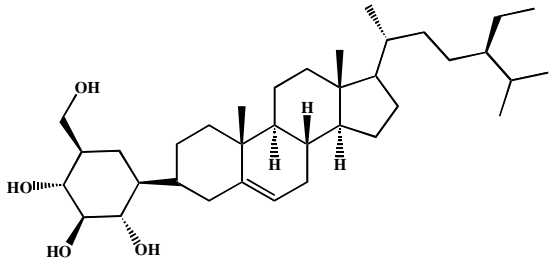

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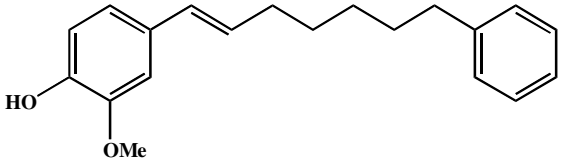


324

A. pinnanensis is usually distributed on mountain slopes, under forest shades etc., at 800-1300 m above the sea level.[33a] The diarylheptanoids alpinnanins A-C (**325**-**327**), (3*S*,5*S*)-*trans*-3,5-dihydroxy-1,7-diphenyl-1-heptene (**238**) chalcones (**328** and **329**), flavanones (**82** and **89**) and steroids (**264** and **141**) were isolated from the rhizomes of *A. pinnanensis* grown in Vietnam.[33b]

A. rafflesiana, like *A. mutica* is a Malaysian specie growing in the southern part of peninsular Malaysia.[23a] The constituents of the rhizomes was studied by Sirat *et al*.[23d] Several DPPH˙ free radical scavengers like 2',3',4',6'-tetrahydroxychalcone (**330**), cardamomin (**83**) and (-)-pinosrobin

(**331**) were isolated from the fruits of *A. rafflesiana*.[34a] Cardamomin isolated from the fruits exhibited anti-inflammatory properties.[34b]

A. speciosa is a plant appreciated for its medicinal properties in Japan. It is used as a diuretic to control hypertension, spasmolytic, cardiovascular, anti-inflammatory, bacteriostatic and fungistatic.[35a] The seeds of *A. speciosa* have been used as an aromatic stomachic in Japan.[35b] An examination of literature shows that the essential oil of *A. speciosa* has been the subject of a number of studies.[35c-h] Labdane diterpenes (**120** and **332**) were isolated from the seeds.[35i] The isolation of antioxidant ferulic acid glycosidic esters (**334** and **335**) from the rhizomes was reported by Masuda *et al*.[35j] The pharmacological and toxicological evaluation of *A. speciosa* performed by Mendoncea *et al* showed positive results.[35k]

Rhizomes of *A. tonkinensis* are commonly used as a spice and as an aromatic drug in China. They are also used to treat stomach-ache as a digestive.[36a] Qin *et al*[36b] analysed the chemical constituents of the rhizome oil by GC-MS. Several flavonoids like 4',5,7-trimethylquercetin (**340**), 3-methylkaempferol (**341**), rhamnocitrin (**208**), quercetin (**275**), 2,3-dihydro-4',7-di-*O*-methylkaempferol (**344**), 4',7-dimethylkaempferol (**345**), 5-hydroxy-3',4',7-trimethoxyflavanone (**346**), kumatakenin (**347**), 4',5,7-trimethoxyflavanol (**348**), ombuine (**349**) and kaempferol (**274**) as well as cinnamyl derivatives and other compounds were isolated from the rhizomes of *A. tonkinensis*.[36c-f]

A. zerumbet occurs in India in the eastern Himalayas from West Bengal onwards.[9] The seeds of *A. zerumbet* have a strong aromatic odour and is a well known crude drug used as a stomachic in China and Japan.[37a] The rhizomes of *A. zerumbet* are used as a substitute for *A. galanga* and even for ginger. The essential oil of the rhizomes is active against the fungus *Candida albicans*. It is traditionally used for the treatment of cardiovascular hypertension and as an antispasmodic agent.[37b] Cardamomin and alpinetin

have been isolated from its seeds.[37c] Furthermore, kava pyrones **181** and **88** and some phenolics (**351**-**354**) have been isolated from the leaves and rhizomes.[37d-f] The labdanic diterpenes zerumin A (**115**), zerumin B (**350**), (*E*)-15,16-bisnorlabda-8(17),11-diene-13-one (**112**) and coronarin E (**121**) have also been isolated from the seeds of *A. zerumbet*.[37g] The tea of the leaves of *A. zerumbet* is popularly utilized by people due to its hypotensive, diuretic and antiulcerogenic properties.[37d] The flavonoids isolated from the leaves were found to possess antihypertensive property.[37h] The presence of kavapyrones in the leaves were determined by GC-MS.[37i] The biological activity of the flavonoids and kavapyrones from the leaves of *A. zerumbet* was evaluated by Mpalantinos *et al.*[37d] The leaf essential oil was found to have antinociceptive effect on mice.[37j] The essential oil, total phenolics, kavapyrones and antioxidant and antibacterial activities of the leaves, flowers and rhizomes has been investigated by several groups.[37k-m] An extraction protocol to obtain essential oils, dihydro-5,6-dehydrokawain and enriched antioxidant phenolic extracts from the leaves and rhizomes was developed by Tawata *et al.*[37n] A report by Lin *et al*[37o] demonstrated that *A. zerumbet* potentially elevates high density lipoprotein cholesterol level in Hamsters.

Table 2.7: Phytochemical constituents isolated from *A. pinnanensis*, *A. rafflesiana*, *A. speciosa*, *A. tonkinensis* and *A. zerumbet*

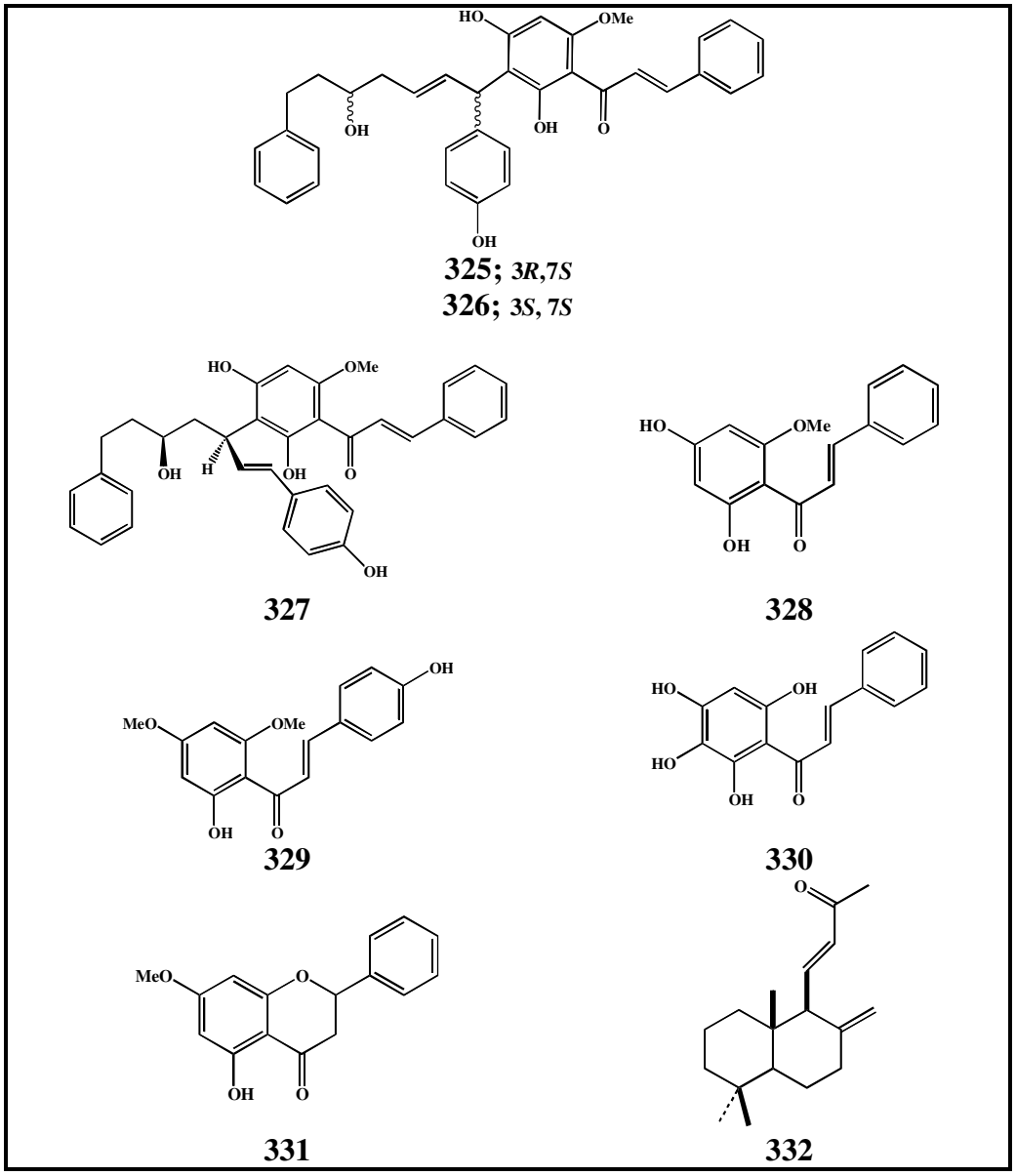
Plant	Compound	No.
Alpinia pinnanensis	Alpinnanin A	325
	Alpinnanin B	326
	Alpinnanin C	327
	2',4'-Dihydroxy-6'-methoxychalcone	328
	4',6'-Dimethylchalconaringenin	329
	Alpinetin	82

	Table 2.7 continued...	
	Naringenin-5-*O*-methyl ether	89
	(3*S*,5*S*)-*trans*-3,5-Dihydroxy-1,7-diphenyl-1-Heptene	238
	Stigmasterol	264
	β- Sitosterol	141
Alpinia rafflesiana	Flavokawin B	263
	Pinocembrin	236
	5,6-Dehyrokawain	88
	Methylcinnamate	180
	1,7-Diphenyl-5-hydroxy-6-heptene-3-one	237
	2',3',4',6'-Tetrahydroxychalcone	330
	Cardamomin	83
	(-)-Pinostrobin	331
Alpinia speciosa	Alpinetin	82
	Cardamomin	83
	Labda-8(17),12-diene-15,16-dial (*E*)	120
	15,16-Bisnorlabda-8(17)-11-dien-13-one	332
	(+)-Epicatechin	333
	Ethyl-4-*O*-feruloyl-β-glucopyranoside	334
	4-Hydroxy-3-methoxyphenyl-4-*O*-feruloyl-β-glucopyranoside	335
	β-Eudesmol	216
	Nerolidol	234
	Humulene epoxide II	179
	4α-Hydroxydihydroagarofuran	212
Alpinia tonkinensis	2α-(*p*-Hydroxycinnamoyl)cineole	336

	Table 2.7 continued...	
	(*E*)-15-Nor-16-oxo-8(17),12-labdadiene	337
	trans-Cinnamyl methyl ester	88
	4(15)-Cadinene-6,10-diol	338
	β- Sitosterol	141
	trans- Cinnamic acid	339
	4',5,7-Trimethylquercetin	340
	3-Methylkaempferol	341
	Rhamnocitrin	208
	Quercetin	275
	15,16-Epoxy-8(17),13(16),14-labdatrien-12ξ-ol	342
	2'-Hydroxy-4',6'-dimethoxychalcone	343
	2,3-Dihydro-4',7-di-*O*-methylkaempferol	344
	4',7-Dimethylkaempferol	345
	5-Hydroxy-3',4',7-trimethoxyflavanone	346
	Kumatakenin	347
	4',5,7-Trimethoxyflavanol	348
	Ombuine	349
	Kaempferol	274
	Zerumin A	115
	Zerumin B	350
	(*E*)-15,16-Bisnorlabda-8(17),11-diene-13-one	112
	Coronarin E	121
	Rutin	351
	Kaempferol-3-*O*-rutinoside	352
	Kaempferol-3-*O*-glucuronide	224
	(+)-Catechin	353
	Alpinetin	82

	Table 2.7 continued...	
	(-)-Epicatechin	354
	Dihydro-5,6-dehydrokawain	181
	5,6-Dehydrokawain	88

Chart 2.7: Structures of the phytochemical constituents isolated from *A. pinnanensis*, *A. rafflesiana*, *A. speciosa*, *A. tonkinensis* and *A. zerumbet*



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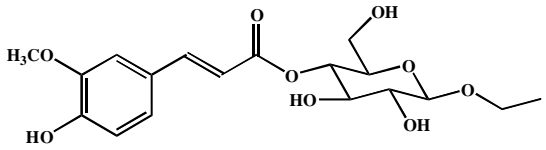

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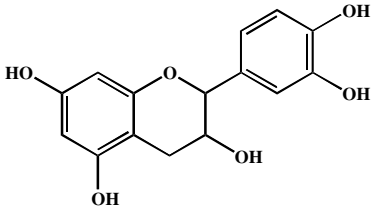

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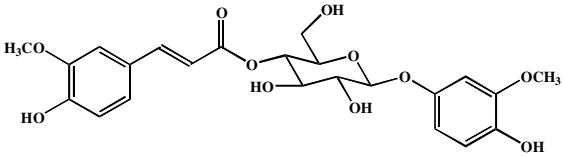

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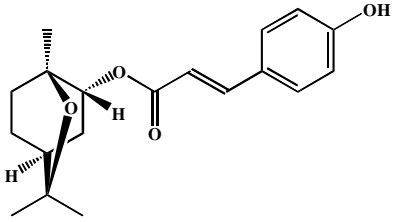

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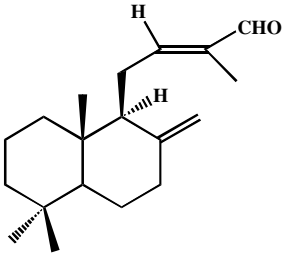

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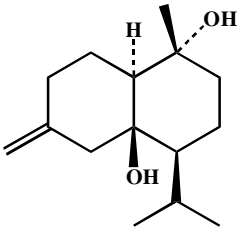

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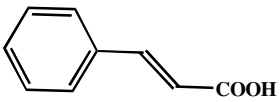

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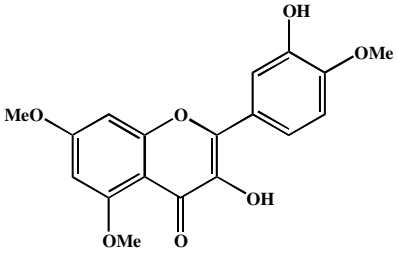

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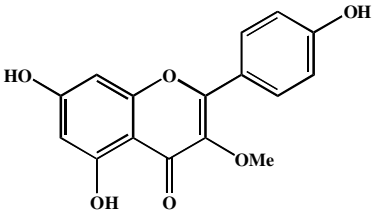

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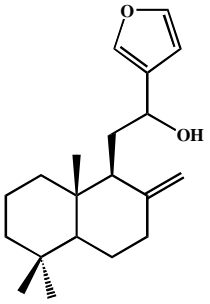

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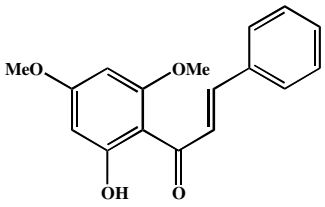

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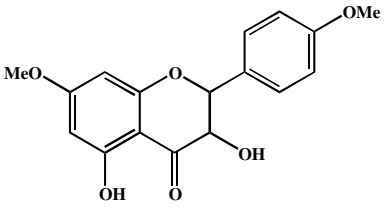

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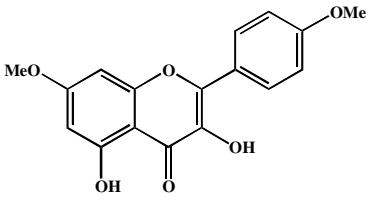

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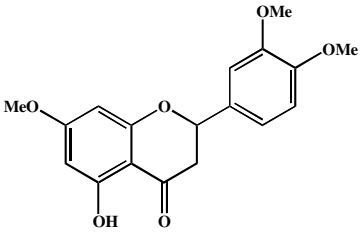

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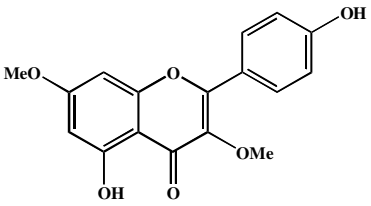

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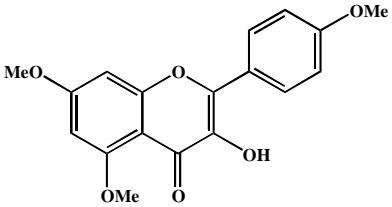

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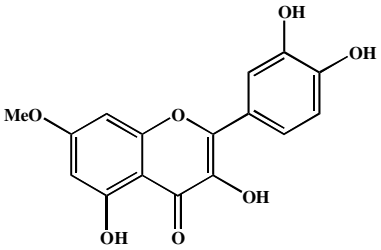

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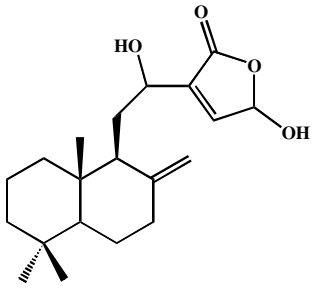

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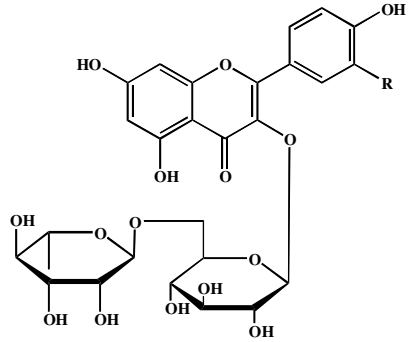

351; R = OH, 352; R = H

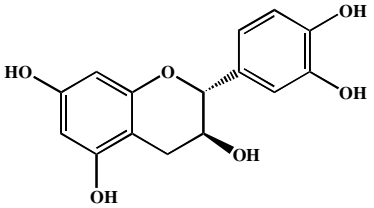

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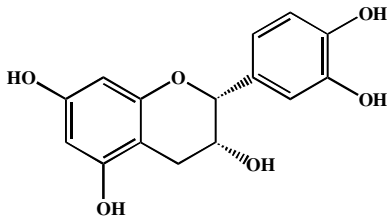
Chart 2.7 continued…



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2.2 Aim and scope of the present investigation

Figure 2.2: *Alpinia galanga* plant

Alpinia galanga (Zingiberaceae) is a herb, known as 'Greater galangal' growing to 1.8-2.4 m in height with tuberous aromatic rootstock, occurring throughout India. It is native to Indonesia, but has now become naturalized in many parts of India and the South-East Asian countries. It is widely cultivated in East Bengal, South India and South-East Asia for its rhizomes. The plant is also common in peninsular Malaysia. Leaves are oblong-lanceolate, acute, glabrous, 30-60 cm long, flowers are greenish white in dense-flowered 30 cm long panicles and seeds are capsules orange or red and globose. *A. galanga* is

known as *Kulinjan* in Hindi, *Aratha* in Malayalam and *Rasna* as well as *Sugandhamula* in Sanskrit. The *Ayurvedic* Formulary of India identifies *rasna* as *Pluchea lanceolate* C. B. Clarke and recommends *Alpinia galanga* (L.) Willd. as a substitute for it specifying the parts to be used in different formulations. In Kerala, the rhizome of both *A. galanga* and/or *A. calcarata* is used as *rasna* in *Ayurvedic* preparations.[39] Some of these medicinal preparations are *Rasnadi kashayam* (*kashayam/quatham*), *Maharasnadi kashayam*, *Rasnadasamoola kashayam*, *Rasna panchakam*, *Rasnasapthakam*, *Rasnarendaadi* etc., (see "*Sahasrayogam*" for details). In South and South-East Asia, the aromatic rhizomes are used as a spice and as an ingredient in traditional medicines. The dried rhizome provides the drug "Greater galangal". The rhizome is 2.5-10.0 cm long, thick and is reddish brown externally and light orange-brown inside. It has a tough fibrous fracture and a spicy pungent taste. The rhizomes are collected during late summer or early autumn. Rhizomes of other *Alpinia* species, *viz*., *A. calcarata*, *A. conchigera*, *A. mutica* and *A. nigra* are sometimes substituted for the genuine drug. The drug is used in rheumatism and bronchial catarrh. It is considered as a tonic, stomachic, carminative and stimulant and is used as a fragrant adjunct to complex preparations and also in cough and digestive mixtures. The drug has an expectorant action and is used in the treatment of many respiratory ailments, especially in children. It has an anti-spasmodic effect which alleviates asthma and also exhibits diuretic properties. The drug is also beneficial in gastro-intestinal affections.[39,40] The rhizomes are used as a condiment in Indonesia. In Kerala, they are used as a spice in seasoning fish and also in pickling.[9] The rhizomes are reported to be employed for insecticidal purposes as the essential oil of rhizomes has a high knock-down effect against houseflies.[9] The flowers are eaten raw or in pickles in some parts of Indonesia. The herb is reported to posess anti-tuberculosis property.[39]

The seeds are considered stomachic. They are prescribed in China for colic, diarrhea and vomiting.

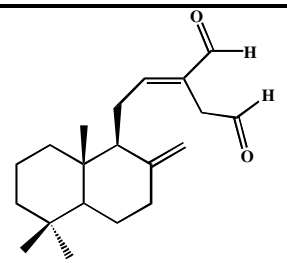
The extensive use of *A. galanga* rhizomes in the preparation of *Ayurvedic* medicines prompted us to study this plant in detail for its phytochemical constituents and antioxidant activity. Accordingly, a detailed literature survey on *A. galanga* was carried out and is discussed below.

2.2.1 *Alpinia galanga* – Literature survey

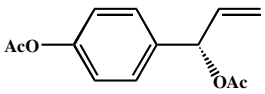
Fresh rhizomes of *A. galanga* on steam distillation is reported to yield essential oil with a peculiar strong and spicy odour and contained methyl cinnamate, cineole, camphor and δ-pinene. It is known to be carminative and in moderate doses has an anti-spasmodic action on involuntary muscle tissue. It is reported to be a central nervous system depressant and possess bactericidal properties. The oil is also used in perfumery industry.[9] De Pooter *et al*[41] has determined the essential oil composition of *A. galanga* from Malaysia and identified forty compounds from the rhizome essential oil with 1,8-cineole as the major component. The essential oil composition of *A. galanga* rhizomes from the lower Himalayan region was evaluated by Raina *et al*[42] and the rhizome oil was found to contain 1,8-cineole and β-pinene as the major constituents among 59 compounds identified from the rhizomes. Jirovetz *et al*[43] analysed the essential oil of the leaves, stems, rhizomes and roots of *A. galanga* from South India. The leaves yielded essential oil containing mostly methyl cinnamate. The constituents of the rhizome and seed oil of *A. galanga* from Malaysia was reported later on by Jantan *et al*[44] who identified 1,8-cineole as the major constituent of the rhizome oil along with sesquiterpenoids, β -bisabolene, (*Z*,*E*)-farnesol, β -caryophyllene and (*E*)- β -farnesene in appreciable amounts. The rhizome essential oil also showed antifungal activity.[45] The volatile oil of the rhizomes of *A. galanga* from South India[46] and Sri Lanka[47] are reported to have 1,8-cineole and zerumbone as the major constituents.

Many biologically active compounds have been isolated from *A. galanga*. In an earlier report, two potent anti-ulcer compounds, viz., 1'-Acetoxychavicol acetate (**142**) and 1'-Acetoxyeugenol acetate (**143**) were isolated from the seeds of *A. galanga*. Besides that, three sesquiterpenes (**183**, **184** and **185**) along with many other compounds were also isolated from the seeds.[48] Later on Morita and Itokawa reported the isolation of cytotoxic and antifungal diterpenes Galanal A (**188**), Galanal B (**189**), Galanolactone (**190**), the dialdehyde **120** and Aframodial (**191**) from the seeds.[49] The structures of the known phytochemical constituents isolated from the seeds of *A. galanga* are depicted in chart 2.8.

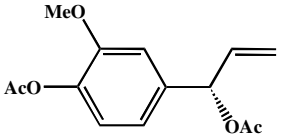
Chart 2.8: Known phytochemical constituents isolated from the seeds of *A. galanga*



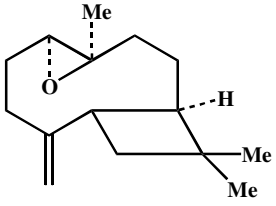
(*E*)-8(17),12-labddiene-15,16-dial
(**120**)



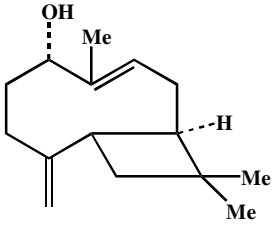
1'-Acetoxychavicol acetate
(**142**)



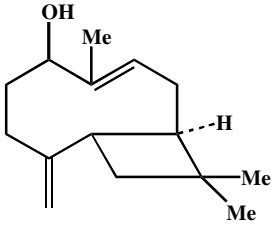
1'-Acetoxyeugenol acetate
(**143**)



Caryophyllene oxide
(**183**)

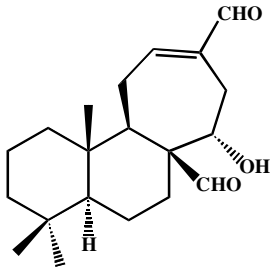


Caryophellenol-I
(**184**)

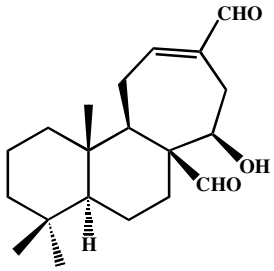


Caryophellenol-II
(**185**)

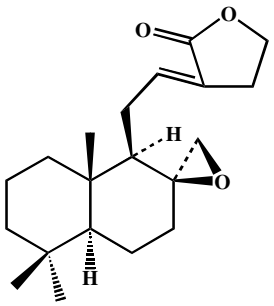
Chart 2.8 continued…



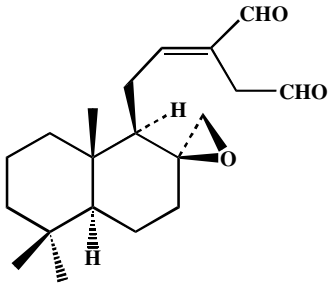
Galanal A
(**188**)



Galanal B
(**189**)



Galanolactone
(**190**)

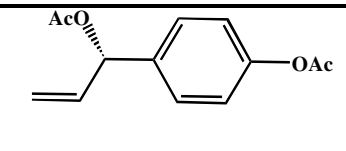


Aframodial
(**191**)

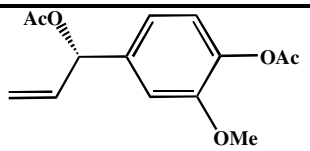
Barik *et al*[50] reported the isolation and characterisation of two new phenolic constituents (**186** and **187**) from the rhizomes of *A. galanga*. Xanthine oxidase inhibitors such as *trans-p*-coumaryl diacetate (**192**), *trans*-coniferyl diacetae (**193**), 4-Hydroxybenzaldehyde (**90**), 1'-Acetoxychavicol acetate (**142**) and 1'-acetoxyeugenol acetate (**143**) were also isolated from the rhizomes.[51] The chavicol acetates **142** and **143** were isolated both from the seeds and rhizomes of *A. galanga*. Kubota *et al*[52] identified four isomers of acetoxycineoles (**194** - **197**) as the odorous components of the rhizomes of *A. galanga*. The substituted cineoles **194** and **197** exhibited woody and sweet aromas respectively whereas, **195** and **196** gave sweet floral and camphoraceous aromas. Of these, *trans*-2-acetoxy-1,8-cineole (**194**) seemed to have the strongest qualitative effect on the characteristic flavour of *A. galanga* rhizomes. The enantiomeric purity and odour characteristics of the

acetoxy cineoles were also determined.[53] In a report by Yang *et al*[54], 1'-Acetoxychavicol acetate (**142**) was identified as the pungent principle of *A. galanga* rhizomes. Apart from the acetoxy cineoles, their glycosides (**198** - **200**) also were isolated as unique aroma components from the rhizomes.[55] The structures of the known phytochemical constituents isolated from the rhizomes of *A. galanga* are depicted in chart 2.9.

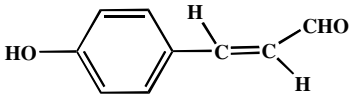
Chart 2.9: Known phytochemical constituents isolated from the rhizomes of *A. galanga*



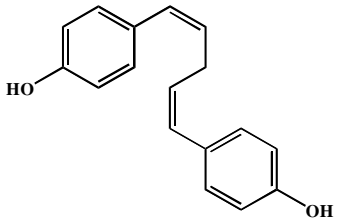
1'-Acetoxychavicol acetate
(**142**)



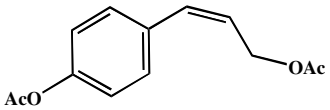
1'-Acetoxyeugenol acetate
(**143**)



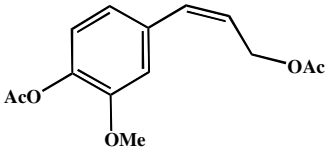
trans-p-hydroxycinnamaldehyde
(**186**)



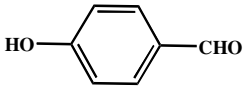
[Di-(*p*-hydroxy-*cis*-styryl)]methane
(**187**)



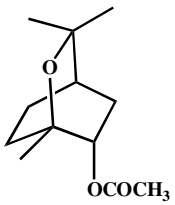
trans-p-coumaryl diacetate
(**192**)



trans-p-coniferyl diacetate
(**193**)

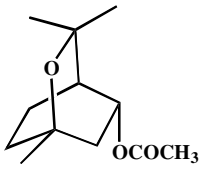


4-Hydroxybenzaldehde
(**90**)

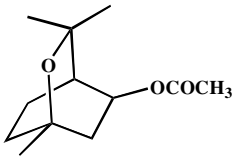


trans-2-Acetoxy-1,8-cineole
(**194**)

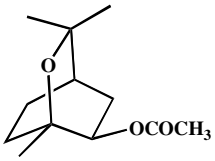
Chart 2.9 continued…



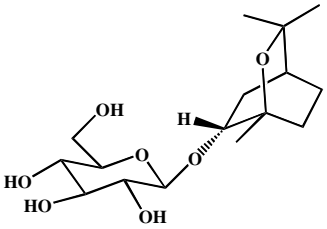
trans-3-Acetoxy-1,8-cineole
(**195**)



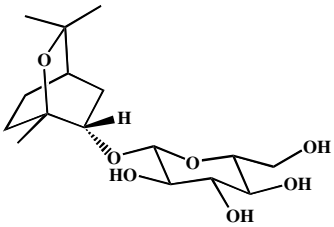
cis-3-Acetoxy-1,8-cineole
(**196**)



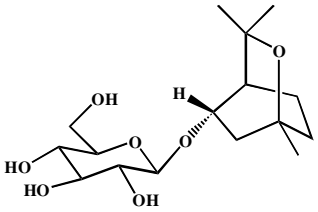
cis-2-Acetoxy-1,8-cineole
(**197**)



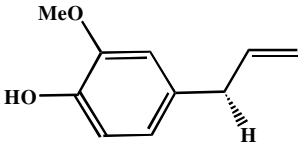
trans-2-Hydroxy-1,8-cineole β-D-glucopyranoside
(**198**)



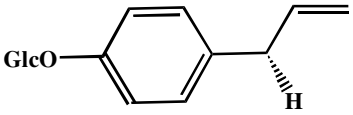
trans-2-hydroxy-1,8-cineole β-D-glucopyranoside
(**199**)



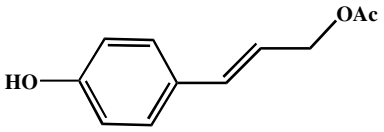
trans-3-hydroxy-1,8-cineole β-D-glucopyranoside
(**200**)



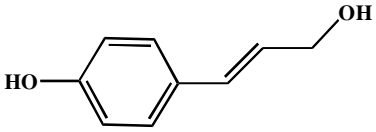
Methyleugenol
(**201**)



Chavicol-β-D-glucopyranoside
(**202**)



trans-p-hydroxycinnamyl acetate
(**203**)



trans-p-coumaryl alcohol
(**204**)

The ability of the rhizomes to reduce blood glucose level was confirmed by evaluating the hypoglycaemic activity.[56] The potent antiallergic activity of **142** and **143** were evaluated by Matsuda *et al*.[57] A study by Bendjeddou *et al*[58] showed that the water soluble polysaccharide extract of *A. galanga* has immunostimulating activity. The ethanolic extract of the rhizomes exhibited excellent phytotoxicity against the aquatic plant *Lemna minor*.[59] The extract also showed good antituberculous activity, cytotoxicity, anti-inflammatory activity, antimicrobial activity, antifungal activity, gastric antisecretory activity, anti-ulcer activity and cytoprotective property.[60] The extract also showed antioxidant activities and the ethanol extract of the rhizomes acted as radical scavenger and also as a lipoxygenase inhibitor.[61] The efficacy of the rhizomes as a possible antioxidant source for meat and meat products was evaluated by Cheah and Hasim.[62] In an evaluation of the antioxidant properties of some exclusive species of Zingiberaceae grown in Manipur, the methanol extract of *A. galanga* rhizome was shown to have strong $DPPH^{\cdot}$ free radical scavenging activity.[63] The antioxidant property of *A. galanga* rhizomes in cooked ground pork was also evaluated.[64]

The survey of literature showed that most of the biological activity studies were done only on crude extract. Also, isolation of polar constituents such as flavonoids from the rhizomes has not been carried out and only few investigations have been carried out on the antioxidant properties of *A. galanga*. Since *Ayurvedic* system of medicine employs only the rhizomes of *A. galanga*, it is of interest to look in to the constituents of the rhizomes in detail and to check the rhizome extract as well as its constituents for biological activity such as anticancer and antioxidant activity. Hence we aimed at the isolation of bioactive flavonoids from the rhizomes of *A. galanga*. Moreover the biological activity of the isolated flavonoids such as the anticancer activity and detailed study on the antioxidant activity of the rhizome extract as well as the individual isolated compounds was carried out.

2.3 Essential oil composition of *A. galanga* rhizomes

As mentioned earlier, the essential oil composition of *A. galanga* rhizomes has been studied by several groups. However, it is well known that essential oil composition can vary depending on climatic condition of the source material. Therefore, it was felt that the essential oil composition of *A. galanga* rhizome grown in Kerala should be studied too. Accordingly, the dried rhizome (100 g) of the material collected from the medicinal plant garden of Ayurvedic Research Institute, Trivandrum was coarsely powdered and was subjected to hydrodistillation in a Clevenger type apparatus. The oil obtained (0.1%) was analyzed on an analytical Shimadzu QP-2010 GC/MS. Most of the constituents were identified by comparison of their mass spectra with those from the library of standard, viz., the Wiley library of standards. The major constituents found to be present in the rhizome essential oil are given in Table 2.8.

Table 2.8: Major essential oil constituents of the rhizomes of *A. galanga*

No.	Compound	Percentage (%)
1	γ-Cadinene	11.64
2	α-Terpineol	9.55
3	α-Farnesene	8.32
4	1,8-Cineole	8.16
5	*trans*-β-caryophyllene	4.01
6	2-Norpinene	3.68
7	2-Butanone,4-phenylbenzyl acetone	2.89
8	Viridifloral	2.39
9	α-Bergamotene	2.22

10	δ-Cadinene	2.19
11	α-Humulene	1.81
12	Camphor	1.45
13	(*E*) Methyl-*p*-methoxycinnamate	1.15
14	Gurjunene	1.03
15	Valencene	0.9
16	α-Cedrol	0.9
17	Germacrene B	1.03
18	δ-Cadinol	0.84
19	δ-Ledol	0.91
20	α-Santalol	0.69
21	Juniper camphor	0.82
22	α-Ylangene	0.49
23	α-Guaiene	1.91
24	Benzoic acid-2-methyl propylester	0.48

The major component of the oil was found to be γ-cadinene with α-terpineol, α-farnesene and 1,8-cineole also present in fairly larger amounts. Apart from that, the oil contained methyl-*p*-methoxy cinnamate and camphor. From the oil analysis, it was found that the percentage of 1,8-cineole was slightly lower in the present sample as compared to the earlier literature reports on the crude drug. Earlier study[43] also reported the presence of fenchyl acetate, camphor and methyl cinnamate as the major constituents from the rhizome essential oil whereas in our study γ-Cadinene, α-Terpineol, α-Farnesene, and 1,8-Cineole were the major constituents from the rhizome essential oil. Also, cinnamic ester derivative is found as methyl-*p*-methoxycinnamate in the present study whereas literature reports showed the presence of methyl cinnamate in the rhizome essential oil.

2.4 Isolation and Characterization of compounds from the hexane extract of *A. galanga* rhizomes

2.4.1 Plant material and extraction

The medicinal plant, *A. galanga* is widely grown in the state of Kerala, India and the rhizomes for this study was obtained from the Ayurvedic Research Institute, Poojappura, Trivandrum and was identified by their resident botanist. A total of 690 g of the coarsely powdered dried rhizomes were extracted with hexane (500 ml x 3) followed by acetone (500 ml x 3) using a Soxhlet extraction apparatus for 24 h each. The hexane extracts were combined and the removal of solvent under reduced pressure in a rotary evaporator yielded 20 g of crude hexane extract. Similarly, the acetone extracts were combined and the removal of solvent under reduced pressure in a rotary evaporator yielded 25 g of acetone extract. However, this acetone extract was found to undergo extensive polymerization on keeping for a few weeks. Therefore, fresh rhizomes were again collected, dried and 250 g of the powdered rhizomes were extracted with acetone at room temperature (250 ml x 3) which yielded 6.8 g of the acetone extract after removal of solvent at 40 °C under reduced pressure. Thin layer chromatography (TLC) was performed on the extracts using solvent mixtures of different polarities.

2.4.2 Isolation and characterization of major compounds from hexane extract

The hexane extract (19 g) was then subjected to careful column chromatography using silica gel (400 g, 100-200 mesh) starting with 100 % hexane as eluant and thereafter gradually raising the polarity (hexane:ethyl acetate; 100:0 to 0:100) based up on the separation observed after examining the fractions by TLC. A total of 165 fractions of 80-90 ml each were collected. They were further pooled together according to similarities in TLC into ten major fraction pools. The initial fraction pools were found to have many less polar compounds (presumably mixtures of the essential oil

components). The sixth fraction pool (fractions 83-98; 1.435 g) was found to contain a UV-active compound as the major component. This fraction was purified by column chromatography using hexane-ethyl acetate mixture (98:2–95:5) to yield compound **I** as off-white needles (90 mg; m.p: 206-207 °C) on crstallisation. It was analyzed by various spectroscopic techniques.

An alcoholic solution of compound **I** responded positively towards Shinoda test and imparted an intense green colour with ferric chloride solution indicating that **I** is a flavonoid derivative. UV-vis spectroscopy serves as an important tool in the structural elucidation of the flavonoids. Usually, the UV spectra of flavonoids will have two major absorption maxima, one in the range 240-285 nm (band II absorption due to A-ring benzoyl system) and the other in the range 300-400 nm (band I absorption due to B ring cinnamoyl system) (Figure 2.3).

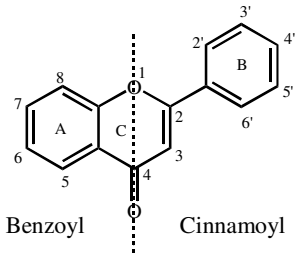
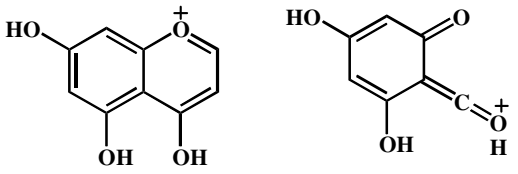


Figure 2.3

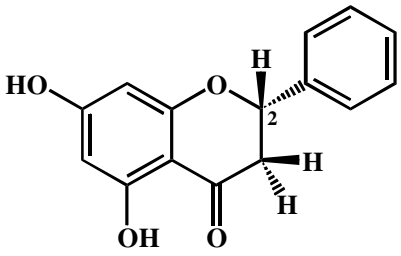
The UV spectra of flavanones are readily distinguished from those of other flavonoids in that they exhibit a low intensity band I absorption which often appears as a shoulder to band II peak.[65] The UV spectrum of **I** (Figure 2.4) was suggestive of a flavonoid with a flavanone skeleton from the low intensity band I peak (326 nm) appearing as a shoulder to band II peak (289 nm). It is reported that the UV spectra of these flavonoids are unaffected by changes in the oxygenation and substitution patterns in the B-ring.[65] However increased oxygenation and substitution in the A ring results in a bathochromic shift in the band II absorption. In the case of compound **I**, the band II absorption is seen at 289 nm (bathochromic shift) indicating oxygenation of

the A ring. The substitution in B-ring cannot be surmised from the UV spectrum. The IR spectrum of compound I showed absorption at 3428 cm^{-1} (hydroxyl) and 1631 cm^{-1} (carbonyl). The ^{1}H NMR spectrum (Figure 2.5) showed a sharp singlet at δ 12.01 indicating the presence of the phenolic 5-OH group[65]. This sharp peak is the result of the strong hydrogen bonding between the carbonyl group at the C4 position and the 5-OH group. The five proton multiplet between δ 7.45-7.37 indicated the B-ring aromatic protons. Thus, it could be concluded that the B-ring of compound **I** is unsubstituted. A singlet at δ 6.01 integrating for two protons indicated that the protons were at position 6 and 8, with hydroxyl groups at positions 5 and 7 in the A-ring.[65] This also supported the bathochromic shift of the band II absorption observed in the UV spectrum. Proton attached to C2 (see structure **I**) appeared as doublet of a doublet at δ 5.43 (J_{trans} = 12.9 Hz; J_{cis} = 3.0 Hz). The two sets of signals (which on expansion were found as doublets of doublets) centered at δ 3.09 and 2.83 could be attributed to the two protons at C3, adjacent to the carbonyl group. Each of these protons gave doublets of doublets due to spin-spin interaction with each other (J = 17.2 Hz) and with the proton in the second position (J = 3.1 Hz). In the ^{13}C NMR spectrum (Figure 2.6), the signal at δ 195.8 confirmed the presence of carbonyl carbon. The signal at δ 79.2 could be attributed to C2 whereas the signal at δ 43.3 to the methylene carbon (C3) adjacent to the carbonyl group. Furthermore, the ^{13}C NMR spectrum clearly showed 15 carbon signals at δ 195.8, 164.6, 164.3, 163.2, 138.2, 128.9 (3C), 126.1 (2C), 103.2, 96.8, 95.5, 79.2 and 43.3 indicating the fifteen carbons as in structure **I**. The mass spectrum (Figure 2.7), of **I** was most informative and was typical of flavanone as it showed $[M+H]^+$ peak, $[M - (B\text{-ring})]^+$ (structure **A**) as well as the Retro Diels Alder (RDA) reaction product ions (**B**) with m/z peaks at 257, 179 and 153 respectively.



(**A**) (**B**)

Based on all the spectral data and by comparing the values with those reported in the literature[23d], the structure of compound **I** was confirmed as 5,7-Dihydroxyflavanone – commonly known as **Pinocembrin**. Pinocembrin has been reported from many species of *Alpinia* like *A. malaccensis*[22c], *A. rafflesiana*[23d], *A. katsumadai*[21c] and *A. nutans*.[25a] However, this is the first report of pinocembrin (**I**) from *A. galanga*.



I

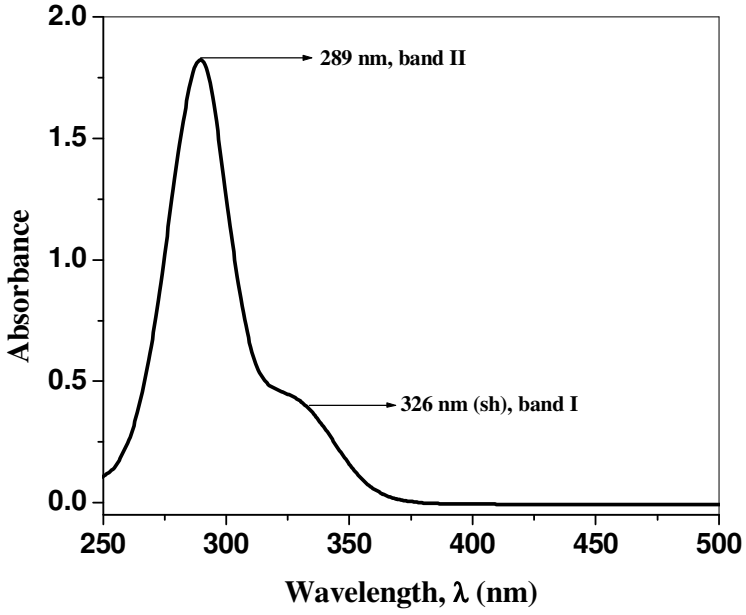


Figure 2.4: UV spectrum of compound **I**

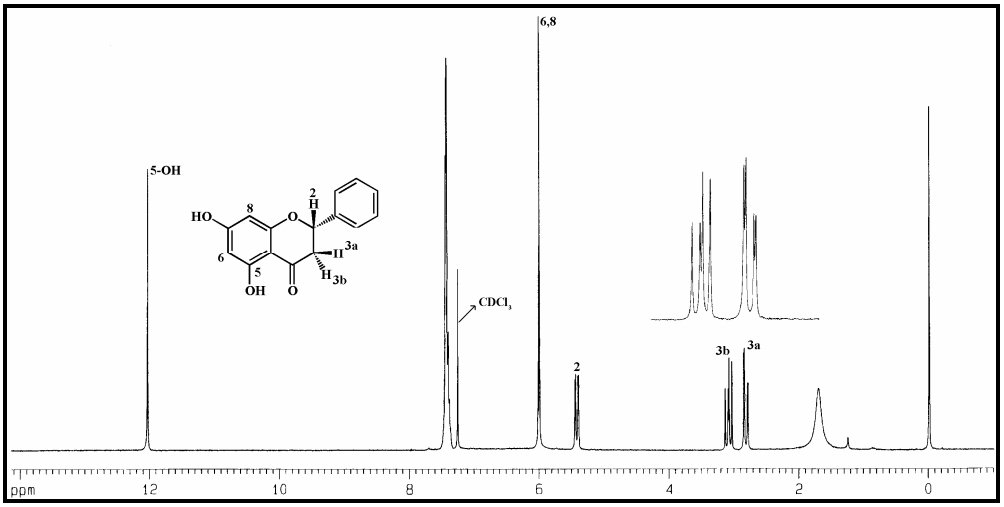


Figure 2.5: ^{1}H NMR spectrum of compound **I**

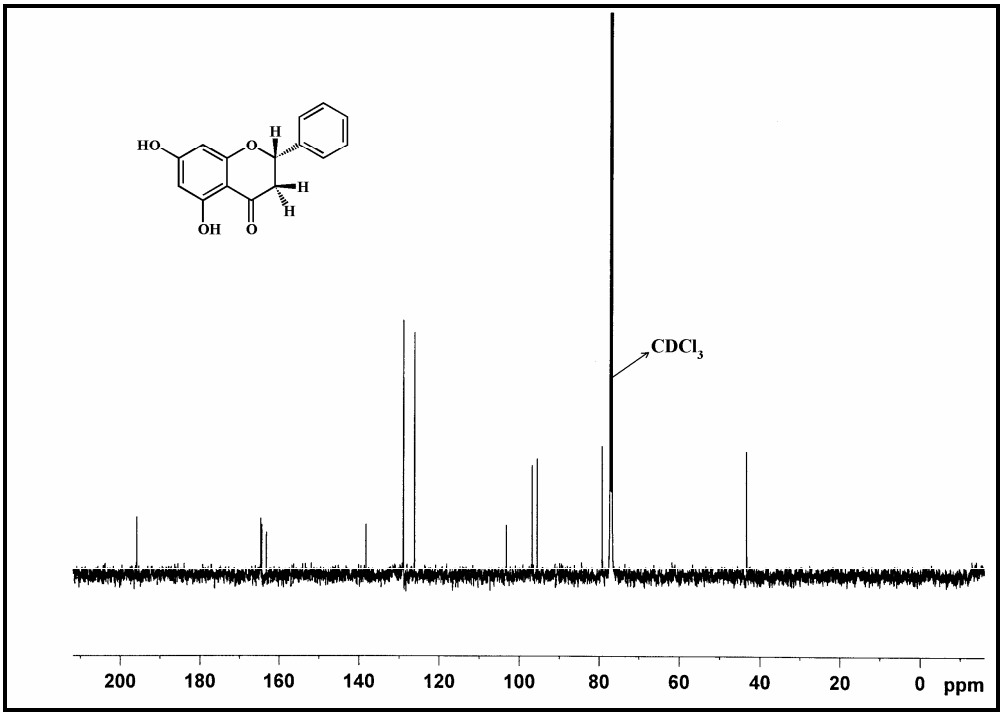


Figure 2.6: ^{13}C NMR spectrum of compound **I**

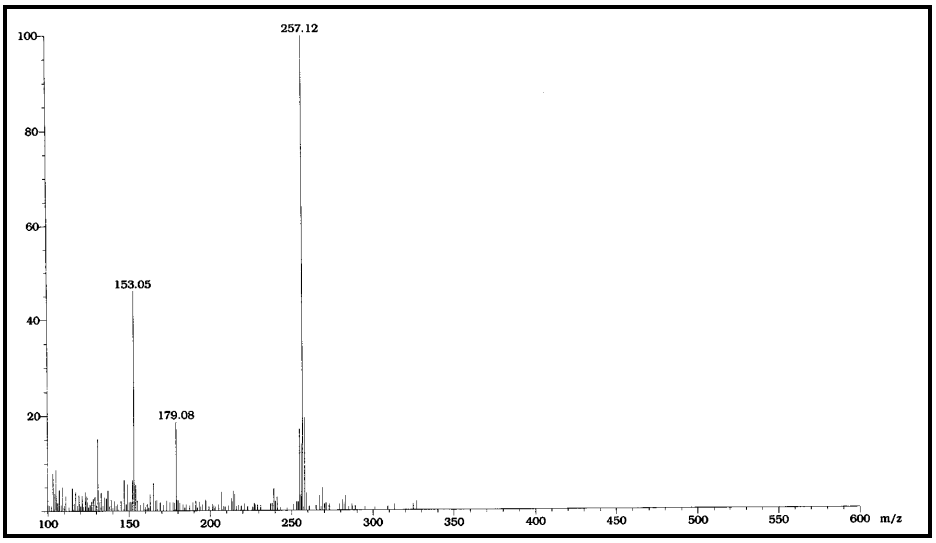
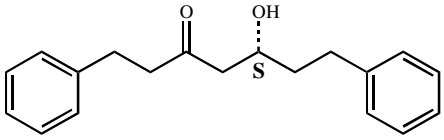


Figure 2.7: Mass spectrum of compound **I**

The seventh fraction pool (fractions 99-109; 1.014 g) indicated the presence of another major compound in TLC. This fraction on column chromatographic separation with hexane - ethyl acetate mixture (95:5-90:10), yielded colourless needle like crystals of compound **II** (220 mg; mp. 52-53 °C). Structural elucidation was carried out with the help of various spectral studies. In the IR spectrum of **II**, absorptions at 3335 cm^{-1} and 1723 cm^{-1} were characteristic of the –OH and >C=O groups respectively. ^{1}H NMR spectrum (Figure 2.8) showed the presence of ten aromatic protons between δ 7.19-7.06. A multiplet observed at δ 3.96 was attributed to the proton adjacent to –OH group. Signals between δ 2.83-2.43 indicated the presence of eight aliphatic $–CH_2–$ protons through integration. A broad multiplet between δ 1.76-1.56 showed the presence of two methylene protons. In the ^{13}C NMR spectrum (Figure 2.9a), the signal at δ 211.0 clearly indicated the presence of carbonyl carbon, whereas the signal at δ 66.8 could be attributed to the carbon bearing an –OH functionality. Presence of five sets of methylene groups could be assumed since signals appeared at δ 49.2, 44.9, 38.0, 31.6 and 29.4. Further, presence of nineteen carbon atoms can be confirmed from the

nineteen signals in the ^{13}C NMR spectrum at δ 211.0, 141.7, 140.6, 128.5 (2C), 128.4 (2C), 128.3 (2C), 128.2 (2C), 126.1, 125.8, 66.8, 49.2, 44.9, 38.0, 31.6 and δ 29.4. The DEPT-135 NMR spectrum (Figure 2.9b) also indicated the presence of five $-CH_2-$ groups and one -CH- group in the aliphatic region. Mass spectrum (Figure 2.10) showed molecular ion peak at m/z 283.1 $[M+H]^+$, from which the molecular formula was found to be $C_{19}H_{22}O_2$. From all the above data as well as comparison with the literature reports,[26f] the structure of compound **II** was assigned as 1,7-Diphenyl-5-hydroxy-3-heptanone as shown here. The same compound was isolated earlier from *A. officinarum*[26f] and *A. conchigera*[14d] and named as dihydroyashabushiketol. Specific rotation value showed that the compound possessed *S* configuration at the OH centre with a specific rotation of +14.31° similar to the literature reports. Eventhough diarylheptanoids are commonly found in most of the *Alpinia* species, presence of the diarylheptanoid **II** in *A. galanga* rhizomes is being reported for the first time.



II

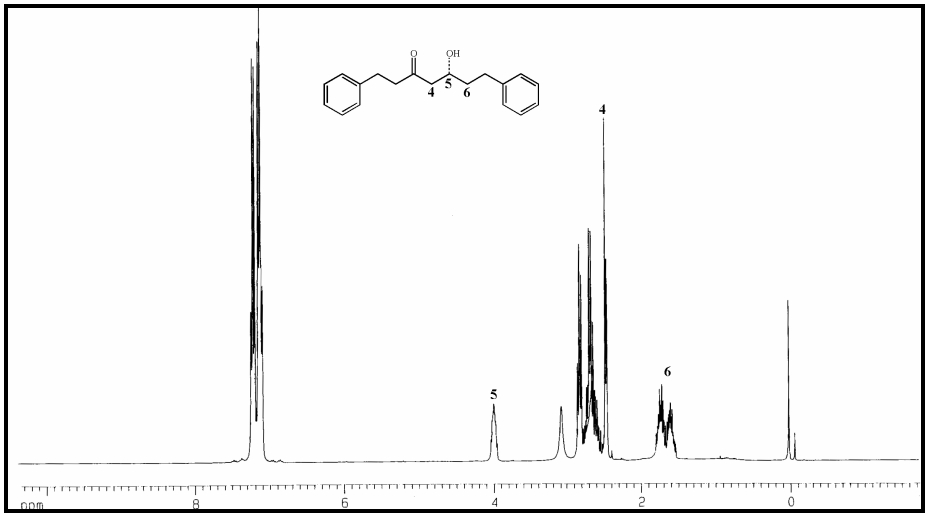


Figure 2.8: ^1H NMR Spectrum of compound **II**

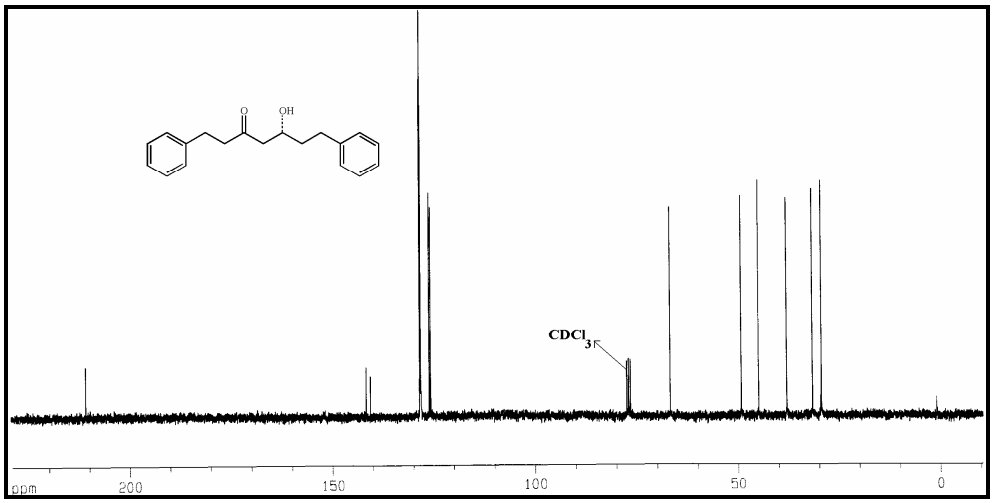


Figure 2.9a: ^{13}C NMR spectrum of compound **II**

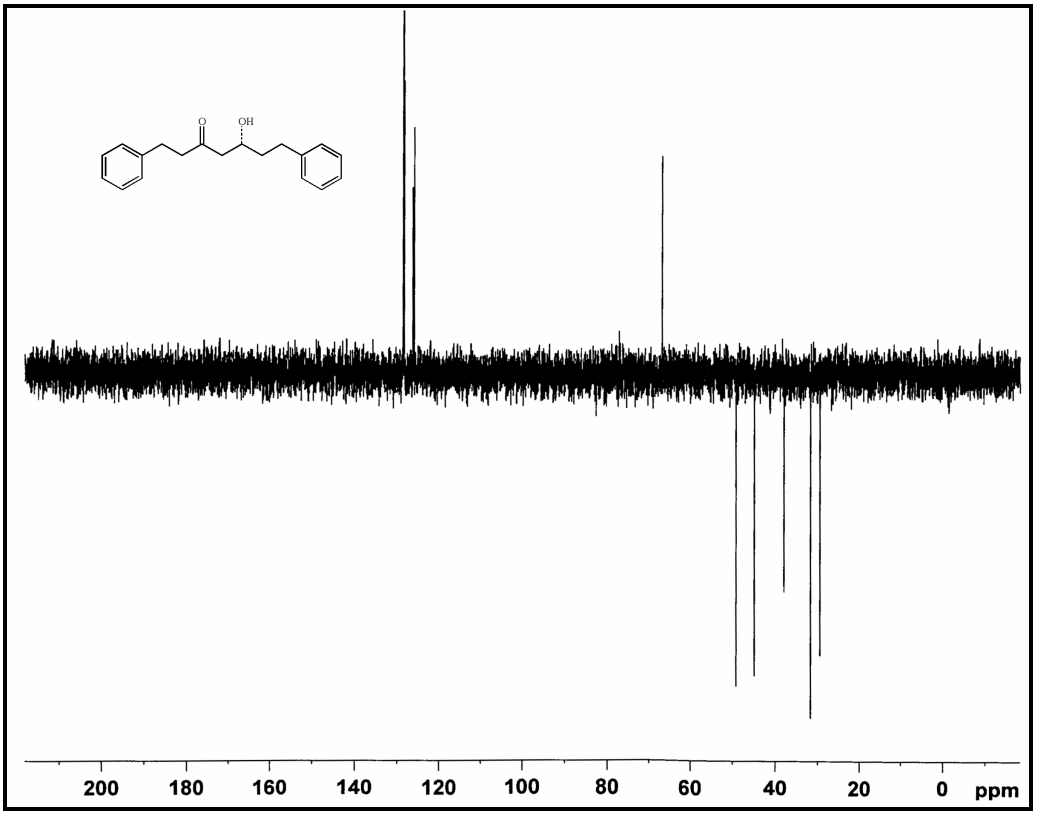


Figure 2.9b: DEPT-135 NMR spectrum of compound **II**

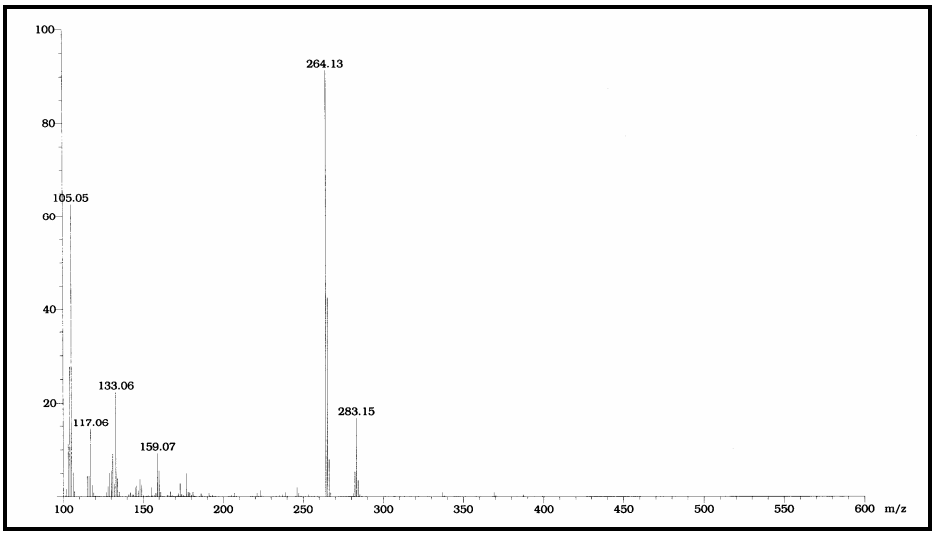


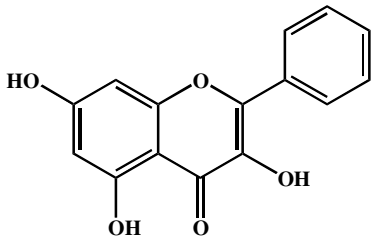
Figure 2.10: Mass spectrum of compound **II**

2.4.3 Isolation of compounds from acetone extract

As already mentioned, the acetone extract (section 2.4.1) obtained by hot extraction process was found to undergo extensive polymerization on keeping. Therefore 250 g of the powdered plant material was extracted with acetone at room temperature (27 °C) and the solvent removed at 40 °C under reduced pressure to get the crude acetone extract (6.8 g). 6.3 g of this extract was subjected to column chromatography over silica gel. Elution was started with 5 % EtOAc-Hexane mixture and thereafter the polarity was gradually raised depending on the separation obtained after examining the fractions by TLC. A total of 105 fractions of 80-90 ml each were collected. They were further pooled together according to similarities in TLC into four fractions. The first pool of fractions showed the presence of pinocembrin (**I**) and the diarylheptanoid (**II**). The second pool of fractions (fractions 51-77) weighing 1.53 g which showed a single spot on TLC was kept for crystallization with chloroform-methanol mixture and it yielded 1.4 g of yellow crystals of compound **III** (m.p. 214-215 °C). Compound **III** also gave positive results to Shinoda test and imparted green colour with ferric chloride solution indicating

it to be a flavonoid derivative. The UV spectrum of compound **III** (Figure 2.11) was found to be very informative about its structure as it showed band I absorption at 359 nm. Normally, flavonols absorb in the region 352-385 nm and highly oxygenated flavonols tend to absorb at longer wavelengths. Thus, the UV spectrum indicated compound **III** to be an oxygenated flavonone. Band II (240-285 nm) is normally less affected by the changes in B-ring oxygenation. On the other hand, it is significantly affected by changes in A-ring oxygenation pattern and increases from 250 nm in flavonols with no oxygenation in A ring to 268 nm in 5,7-Dihydroxyflavonol.[65] The absence of hydroxyl groups in either ring is usually evidenced by relatively weak intensity of the relevant band. Compound **III** showed a highly intense band II absorption at 267 nm indicative of a 5,7- Dihydroxyflavonol. To confirm the structure of **III**, other spectroscopic techniques were employed. The IR spectrum showed absorptions arising from –OH and >C=O groups at 3158 cm^{-1} and 1655 cm^{-1} respectively. The ^{1}H NMR spectrum (Figure 2.12), showed two pairs of *ortho* coupled doublets and a triplet together integrating for five protons at δ 8.17-7.51 indicating a substituent free B-ring of a flavonoid. The H-3',4',5' protons occurred upfield (centered at δ 7.47) from the H-2',6' protons (centered at δ 8.17). The 2' and 6' protons appeared downfield due to the deshielding influence of C-ring functions on them. Protons at the 6^{th} and 8^{th} positions appeared separately as doublets (J = 2.0 Hz) at δ 6.19 and δ 6.39 respectively. The H-6 doublet consistently occured upfield than the H-8 doublet. These signals are clearly distinguished from each other because of their widely different paramagnetic induced shifts.[65] The peak corresponding to the 5-OH group was absent which suggested insufficient H-bonding of the 5-OH group with the >C=O group because of the competitive influence of both the 3- and 5-OH for H-bonding. ^{13}C NMR spectrum (Fig. 2.13) showed 15 carbon signals, including the characteristic signal of α, β unsaturated >C=O group at δ 176.7 and the others at δ 165.2,

162.1, 157.7, 145.8, 137.7, 131.9, 130.5, 129.2 (2C), 128.2 (2C), 104.0, 99.1 and δ 94.3. The mass spectrum of compound **III** (Fig. 2.14) gave the molecular ion peak as the base peak at m/z 271 $[M+H]^+$. In the case of flavonols, the molecular ion peak or the $[M+H]^+$ is indeed the base peak and all other peaks will be comparatively small as the RDA process in case of flavonols is minor. The peak at m/z 105 indicated the B^+ ion peak common for flavonols. From all the above spectral details and by comparing with the reported data[65], compound **III** was confirmed as the 5,7-Dihydroxyflavonol commonly known as **Galangin** shown below.



III

This is the first report of the isolation of galangin from *A. galanga* eventhough it has been previously isolated from other *Alpinia* species like *A. officinarum*[26i] and *A. conchigera*.[14d]

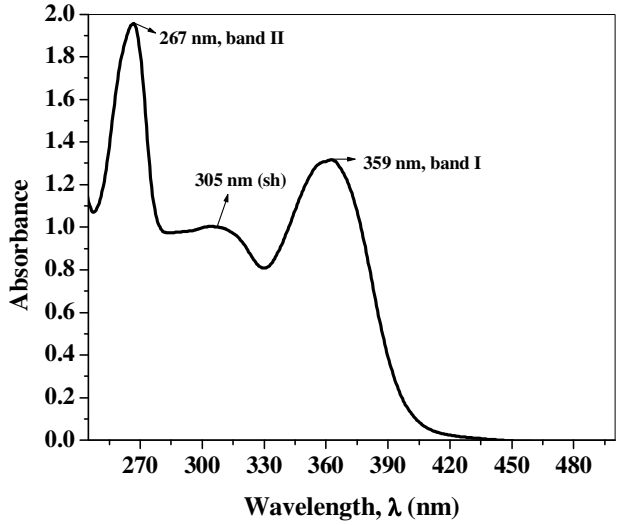


Figure 2.11: UV spectrum of compound **III**

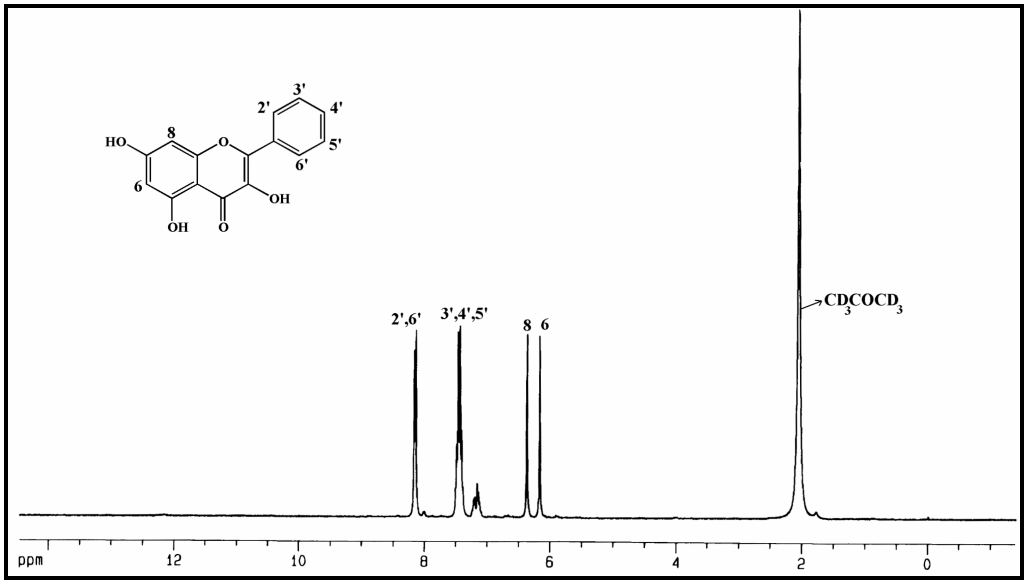


Figure 2.12: ^{1}H NMR Spectrum of compound **III**

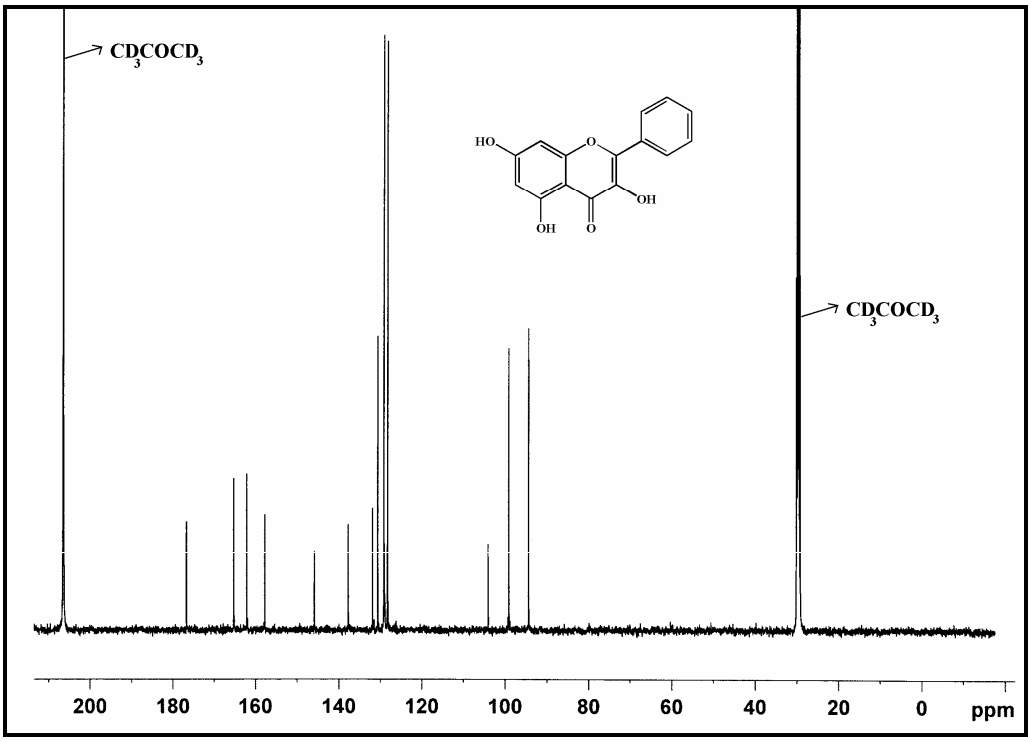


Figure 2.13: ^{13}C NMR Spectrum of compound **III**

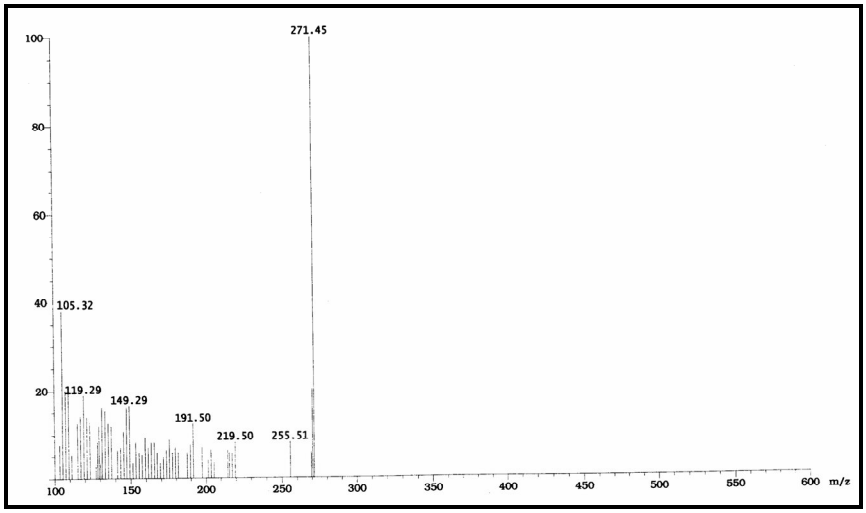
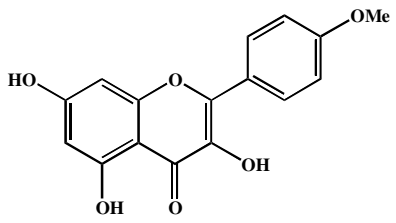


Figure 2.14: Mass Spectrum of compound **III**

The third pool of fractions (78-94; 200 mg) was also found to contain a single compound as indicated by TLC. This was kept for crystallization in chloroform-methanol mixture which upon crystallization, yielded 108 mg of compound **IV** (mp. 198-199 °C) as yellow flakes and was characterized based on its spectral data. As in the case of compound **III**, compound **IV** also reacted positively to Shinoda test and ferric chloride test indicating that **IV** too is a flavonoid derivative. UV spectrum of compound **IV** also (Fig. 2.15) indicated a highly oxygenated flavonol structure with a band I absorption at 367 nm. The band II absorption was intense, at 267 nm, clearly indicating that **IV** also had a structure similar to 5,7-Dihydroxyflavonol. However, band II absorption at 267 nm was accompanied by a shoulder at 253 nm which indicated the presence of an oxygenated 4' substituent.[65] Further evidence of the exact structure of **IV** was made from other spectral studies. In the IR spectrum, absorptions at 3309 cm^{-1} and 1653 cm^{-1} confirmed the presence of –OH and >C=O groups respectively. In the ^{1}H NMR spectrum (Fig. 2.16), the aromatic protons appeared as two pairs of *ortho* coupled doublets centered at δ 8.16 and 7.02 integrating for two protons each. The H-3',5' doublet appeared upfield (centered at δ 7.02, J = 8.9 Hz) from the H-2',6' doublet

(centered at δ 8.16, *J* = 8.9 Hz). For compound **IV**, the 3',5' doublet appeared more upfield than in case of compound **III**. This upfield shift could be attributed to the presence of an oxygenated substituent at the 4' position. This upfield shift arises due to the combined effect of the shielding effect of the oxygen substituent and to the deshielding influence of C-ring functions on H-2' and 6'. Doublets centered at δ 6.43 (*J* = 1.7 Hz) and δ 6.27 (*J* = 1.7 Hz) integrating for one proton each, indicated the H-8 and H-6 protons respectively. The sharp singlet at δ 3.89 integrating for three protons clearly indicated the presence of a methoxy group in the 4' position. In the ^{13}C NMR spectrum (Fig. 2.17), the presence of α, β-unsaturated carbonyl carbon was confirmed from the signal at δ 175.6. The peak at δ 55.2 indicated the $-OCH_3$ carbon. The sixteen carbon signals at δ 175.6, 164.0, 160.8, 160.6, 156.9, 145.4, 137.2, 129.3 (2C), 123.4, 113.8 (2C), 105.4, 98.5, 93.9 and 55.2 indicated the sixteen carbons in compound **IV**. The mass spectrum of compound **IV** (Fig. 2.18) gave the molecular ion peak as the base peak at m/z 300 and all other peaks were comparatively minor as the RDA process in case of flavonols is minor. The $[M-15]^+$ ion peak with a loss of $-CH_3$, i.e., $[M-CH_3]^+$ was observed at m/z 285 and the peak at m/z 135 indicated the B^+ ion peak common for flavonols. From all the spectral data and on comparison of the values with the literature reports,[65] structure of compound **IV** was confirmed as 4'-Methoxy-5,7-dihyroxyflavonol, commonly known as **Kaempferide**. Although kaempferide has been previously isolated from *A. conchigera*[14d] and *A. officinarum*[31], this is the first report of its presence in *A. galanga*.



IV

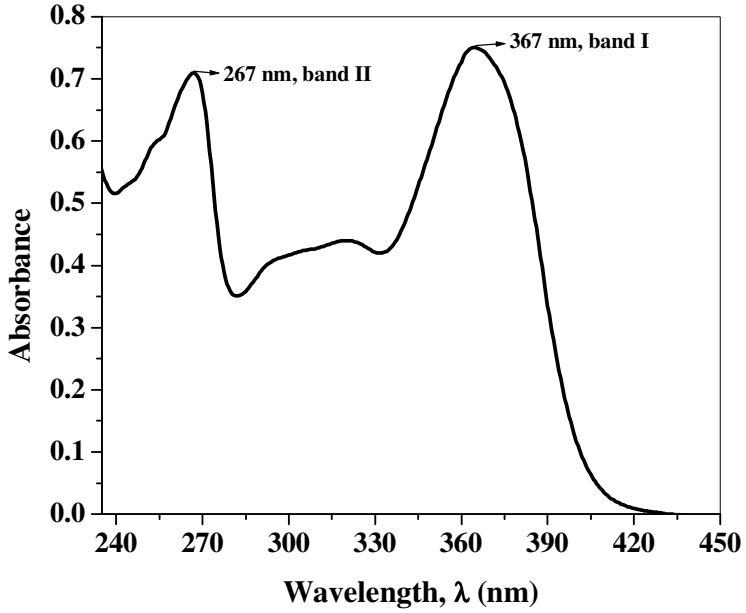


Figure 2.15: UV Spectrum of compound **IV**

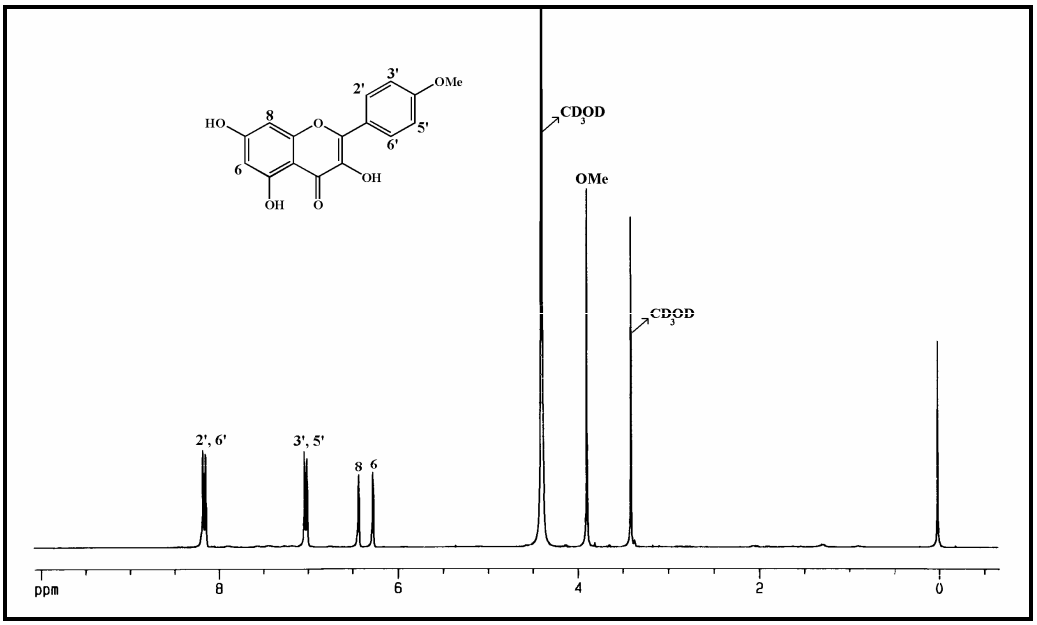


Figure 2.16: ^{1}H NMR Spectrum of compound **IV**

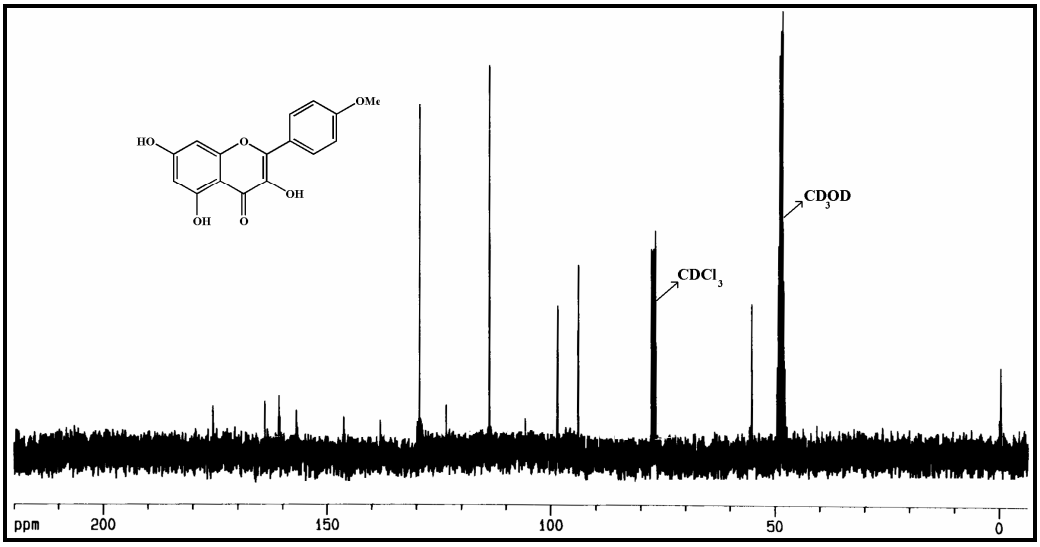


Figure 2.17: ^{13}C NMR Spectrum of compound **IV**

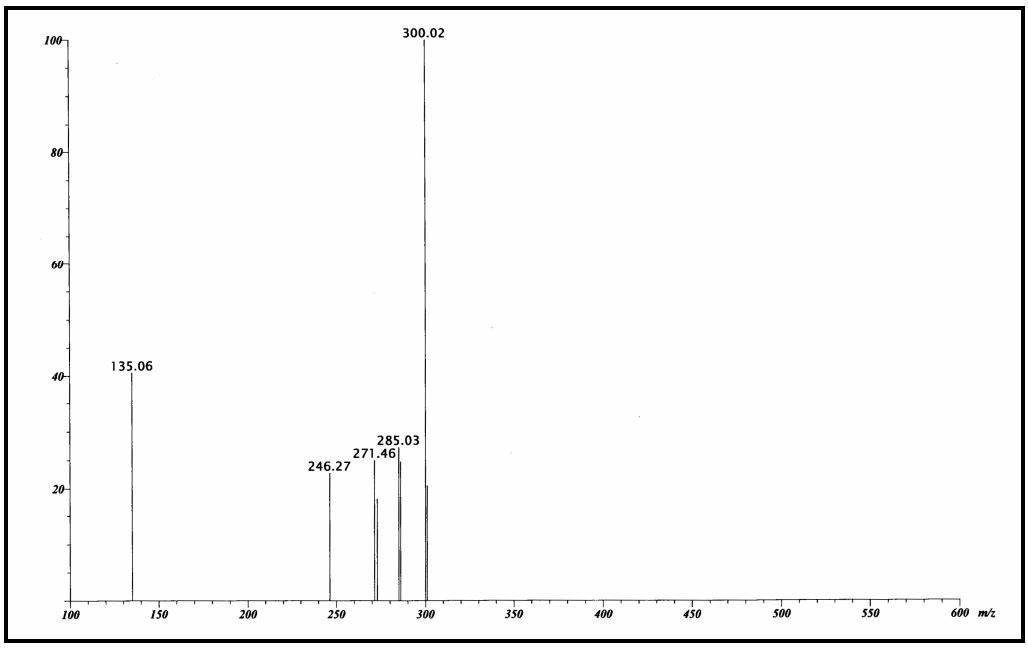


Figure 2.18: Mass Spectrum of compound **IV**

In the present study, we were able to isolate 1.4 g of galangin and 0.108 g of kaempferide from 250 g of plant material (dry weight). i.e., the concentration of galangin and kaempferide in the rhizomes of *Alpinia galanga*

were 5.592 g/kg and 0.432 g/kg of the dry plant material respectively. The extraction when conducted at room temperature using acetone as the solvent minimized any tendency towards loss of material due to polymerization upon heating. Thus, an optimized extraction procedure to obtain maximum quantities of flavonoids galangin and kaempferide from the rhizomes of *Alpinia galanga* using acetone at room temperature was developed.

2.5 Biological activity studies of *Alpinia galanga*

2.5.1 Induction of apoptosis on colon cancer cell lines by pinocembrin

Programmed cell death, known as apoptosis, is an essential cellular homeostasis mechanism that ensures correct development and function of multi-cellular organisms. The pivotal importance of correct execution of apoptosis is apparent from the many human diseases with aberrancies in apoptosis, including cancer. During cancer development, various imbalances can arise in the apoptotic machinery. Consequently, sensitivity towards apoptosis is progressively reduced, which ultimately leads to inappropriate cell survival and malignant progression. However, it has become clear that cancer cells are often reliant on these aberrancies for continued survival. Perhaps counter intuitively, cancer cells can in fact be more prone to apoptosis than normal cells. The apoptosis-prone phenotype of cancer cells is masked and counterbalanced by upregulation of one or more anti-apoptotic mechanisms. Therefore, it is of enormous therapeutic interest to selectively tip the balance of the cellular fate of cancer cells towards apoptosis. Indeed, the rational design of novel agents as well as discovery of novel natural products that can selectively induce apoptosis in cancer cells is a rapidly developing field.

Pinocembrin, isolated from *A. galanga* rhizomes showed remarkable cytotoxicity against colon cancer cell with promising features of apoptotic

pathway, which are the important requirements of an antitumour agent. The cytotoxicity was determined by MTT cell proliferation assay in collaboration with Rajiv Gandhi Centre for Biotechnology, Trivandrum.

For analyzing the cytotoxicity of pinocembrin, different cancer cell lines and two normal cells (lung fibroblasts and umbilical cord-derived endothelial cells) were used. The cells were exposed to 10–200 μM of pinocembrin for 48 h and cell viability was monitored by MTT assay. Pinocembrin failed to induce any significant cell death before 24 h in any of the concentrations tested. Exposure of the cell with 50, 100 and 200 μM of pinocembrin induced marked cytotoxicity after 48 h as evidenced from the MTT assay in a concentration-dependent way (Figure 2.19).

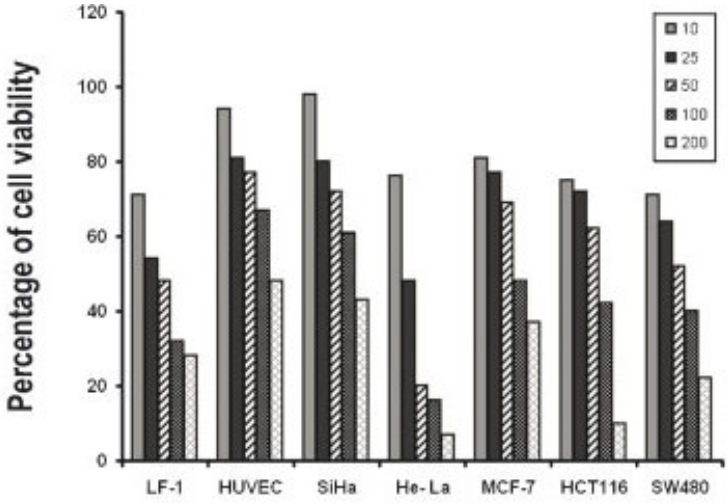


Figure 2.19: Cytotoxicity of pinocembrin (10-200 μM) analysed by MTT assay against a variety of cells

Below 50 μM, pinocembrin failed to induce any significant cell death in most cells except in lung fibroblasts, LF-1 and cervical cancer cell, HeLa as revealed by MTT data. The normal human endothelial cells and the cervical cancer cell line SiHa were relatively resistant to pinocembrin. Cell viabilities were 48 and 43 % respectively in HUVEC and SiHa cells treated with 200 μM of pinocembrin. The colon cancer cell lines SW480 and

HCT116 cells showed comparable cytotoxicity. The percentage viability of cells were 62 and 52 % with 50 μM of pinocembrin, respectively, in SW480 and HCT 116 cells. In breast cancer cell line MCF-7, 37 % cells were viable even in 200 μM concentration of pinocembrin.

Our study shows that pinocembrin isolated from *A. galanga* induces apoptosis in colon cancer cells. This compound showed cytotoxicity against a variety of cancer cells including normal human lung fibroblasts. Quite surprisingly, the normal human endothelial cells and cervical cancer cells SiHa remained relatively resistant to pinocembrin induced cell death. The HeLa cells were highly sensitive to pinocembrin. The reason for differential response is not clear at present. Most probably, these cells may differ in the relative expression status of important signaling intermediates of cell death triggered by this compound.

2.5.2 Antioxidant properties of *Alpinia galanga* and the major compounds isolated from it

As discussed in Chapter 1, the antioxidants are a very important group of biologically active compounds. In order to evaluate the effectiveness of antioxidants in a sample, many different substrates, system compositions and analytical methods are employed in the screening tests. Different free radicals and reactive oxygen species (ROS) are produced in the human body as a result of various biological processes. Each one has its own deleterious effects when produced in the body as already discussed in chapter 1. Therefore, an efficient antioxidant should actively scavenge all these harmful free radicals/ROS. As a result, different methods are necessary to evaluate different antioxidant effects. In the current study, various *in vitro* models for assessing the antioxidant capacity were employed which are discussed in the following sections.

The ethanol extract, acetone extract and the isolated compounds were evaluated for their antioxidant activities. The ethanol extract (14.3 g) was

prepared by the extraction of 100 g of the plant material with ethanol (2L, 24 h) using Soxhlet apparatus. However, this extract was found to be stable. Extraction of 250 g of dried rhizome of *A. galanga* at room temperature using acetone (3 x 1L) gave 6.8 g of the acetone extract of which 0.5 g was used for the antioxidant studies.

2.5.2.1 Total phenolic content of the ethanol (AGE) and acetone extracts (AGA) of *A. galanga*

The preliminary determination of the antioxidative capacity of *A. galanga* rhizomes was carried out by the estimation of the total phenolics using the Folin-Ciocalteau method by expressing the results as equivalents of gallic acid. It is considered as the best method for the total phenolic content determination and is convenient, simple and reproducable. The yellow Folin-Ciocalteau reagent, containing heteropolyphosphotungstates and molybdates, reacts with phenolic compounds in one or two electron reduction reaction and gets converted to a blue species, possibly $(PMoW_{11}O_{40})^{4-}$ whose absorbance is measured at 760 nm.[66] Based on this assay, the total amount of phenolics was 1.93 ± 0.7 g Gallic acid equivalents/100 g of dry rhizomes in AGE and 2.17 ± 0.3 g Gallic acid equivalents/100 g of dry rhizomes in AGA. Thus, it is evident that the concentration of phenolic compounds is higher in the acetone extract compared to the ethanol extract. This may also be due to the fact that ethanol extract contains some starch, glycosides etc.

2.5.2.2 Total flavonoid content

The total flavonoid content was determined by the $AlCl_3$ colorimetric method[67] and is expressed in grams as equivalents of Quercetin. The principle of $AlCl_3$ colorimetric method is that aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of the flavonoids.[68] $AlCl_3$ forms complexes of the type $[Al^{III}(\text{flavonoid-H})_2]^+$ as shown in figure 2.20.

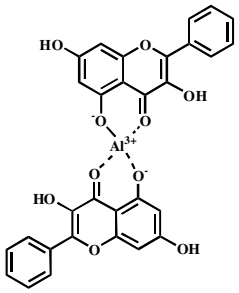


Figure 2.20

The study revealed that AGE contained 3.3 ± 0.9 g Quercetin equivalents/100 g dry rhizomes and AGA contained 2.3 ± 0.4 g Quercetin equivalents/100 g of dry rhizomes. Thus the flavonoid content is higher in the ethanol extract as compared to acetone extract. It is well known that the flavonoids and their glycosides are much polar and are fractionated in the ethanol extract compared to the extracts of solvents of lower polarities.

2.5.2.3 Total antioxidant capacity

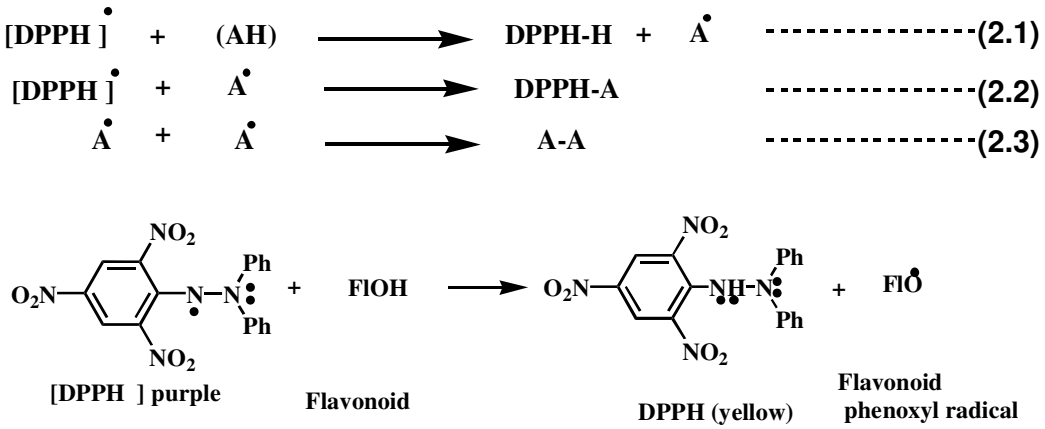
The phosphomolybdenum method of Preito *et al*[69] was used to determine the total antioxidant capacity of the extracts. This method is based upon the reduction of Mo(VI) to Mo(V) by the antioxidant compound and the formation of a green phosphate/Mo(V) complex with an absorption at 695 nm. It is a quantitative method in which, the total antioxidant capacity is expressed as gram equivalents of ascorbic acid. AGE showed total antioxidant capacity of 9.6 ± 1.6 g ascorbic acid equivalents/100 g dry weight of the rhizomes whereas AGA showed a higher total antioxidant capacity of 11.1 ± 1.8 g ascorbic acid equivalents/100g dry weight of the rhizomes indicating the presence of a higher amount of antioxidative compounds in the acetone extract (AGA) than in the ethanol extract (AGE).

2.5.2.4 DPPH$^{\cdot}$ radical scavenging capacity and kinetic studies

The radical scavenging capacity is expressed by the EC_{50} values which can be defined as the concentration of an antioxidant required to decrease the

initial substrate (or free radical) concentration by 50 % (EC_{50}). This is a parameter widely used to measure the antioxidant power.[70] The lower the EC_{50}, higher the antioxidant power.

DPPH· (diphenyl picryl hydrazyl radical) is a stable free radical with absorption maxima in the range of 515-520 nm and is a useful radical for the evaluation of antioxidant capacities.[71] In the DPPH· test, the antioxidants reduce DPPH· to yellow coloured diphenyl picrylhydrazine and the extent of the reaction will depend on the hydrogen donating ability of the antioxidants (Scheme 2.1).[72]



Scheme 2.1

The EC_{50} values were calculated from the percentage of DPPH· remaining after 30 minutes of reaction time. The plot of % DPPH· scavenging capacity against a range of concentrations for each antioxidant gave the EC_{50} values. The extracts and the isolated flavonoids were tested for the DPPH· radical scavenging capacities. Pinocembrin did not show significant antioxidant capacity even at higher concentrations on preliminary investigation by DPPH· assay. Since pinocembrin was obtained only in small quantity and did not scavenge DPPH· effectively, further investigation on the antioxidant capacity of pinocembrin by different methods was not carried out. The extracts, galangin and kaempferide showed radical scavenging capacities and the individual EC_{50} values are given in Table 2.10. Figure 2.21 shows the

plot of concentration of each sample against the % DPPH˙ radical scavenging capacity. As is clear from the figure, the tested compounds showed a concentration dependent scavenging capacity by quenching the DPPH˙ free radicals. The standards used were quercetin and BHA (butylated hydroxy anisole, a synthetic antioxidant used in food industry). However, the samples did not show scavenging capacities as effective as the standards BHA and quercetin. At a concentration of 1000 ppm, kaempferide showed higher activity by scavenging 77 % of the radicals than galangin which could scavenge only 73 % while at lower concentrations, galangin exhibited the highest activity (54 % scavenging capacity at 500 ppm against the 46 % of kaempferide). From the EC_{50} values, the highest antioxidant capacity among the samples analysed was for galangin with an EC_{50} value of 442 ppm. Kaempferide was also good and showed a slightly higher EC_{50} value of 541 ppm. The extracts also showed DPPH˙ radical scavenging with EC_{50} value of 895 and 851 ppm for AGE and AGA respectively indicating AGA to be a slightly better radical scavenger than AGE.

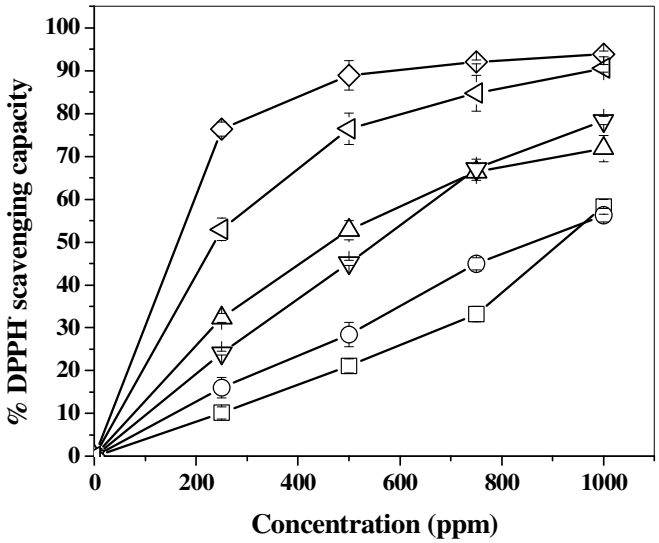
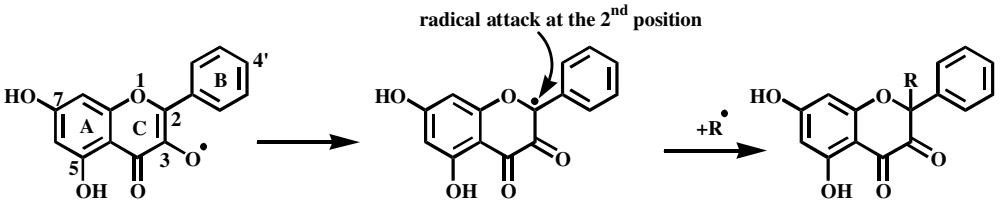


Figure 2.21: DPPH˙ scavenging capacity at different concentrations of (□) AGE, (○) AGA (△) Galangin; (▽) Kaempferide; (◇) Quercetin and (◁) BHA in methanol.

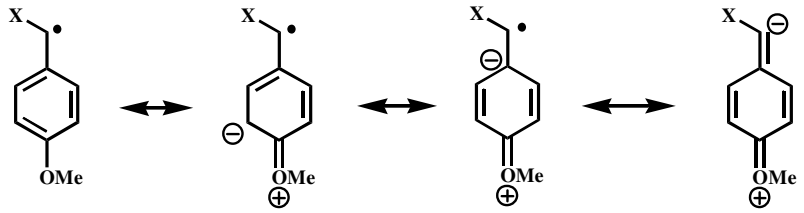
Pinocembrin (5,7-Dihydroxy flavanone) was totally inactive towards the DPPH˙ even at higher concentrations. This inactivity of pinocembrin could be attributed to the absence of –OH group at the third position and the absence of C2-C3 double bond. This corroborates the earlier studies that 5, 7-dihydroxy substituents in the A ring of flavanones do not participate in scavenging free radicals.[73] Galangin showed higher activity than kaempferide because of the presence of both 3-OH group and the C2-C3 double bond. It is reported that flavonoids that lack catechol OH's on ring B, but possessing a 3-OH next to the keto group show a higher radical scavenging capacity.[73] Kaempferide on the other hand, bearing an additional 4'-OMe group in the B ring, showed slightly lower activity than galangin. This can be explained by looking into the mechanism of radical scavenging by flavonols as reported earlier by Amic *et al.*[73] Accordingly, galangin can easily donate 3-hydroxyl hydrogen and form the 3-flavonoid phenoxyl radical. The formed phenol radical undergoes rearrangement leading to radical at position 2. The flavonoid phenoxyl radical is one of the most suitable centres for the unpaired electron. Position 2 is therefore more susceptible to radical attack and radical-radical termination step occurs via addition at position 2 (Scheme 2.2).



Scheme 2.2

In case of kaempferide, there is an electron donating –OMe group at the 4' position of the flavonoid B ring. It has been reported that hydroxyl group in the flavonoid C ring boosts the antioxidant capacity whereas methoxy groups suppresses the antioxidant capacity.[74] An explanation for the

diminished activity due to methoxy group at the 4' position in the B ring has not been put forward earlier. This can be explained by considering the resonance stabilized structure of a *para* methoxy substituted benzyl radical. In the presence of such electron donating groups at the *para* position, the resonance structures shown below are possible (Scheme 2.3). Formation of the last, highly charge separated resonance structure clearly shows that radical formation and stabilization will be retarded.



X = Rest of the ring

Scheme 2.3

This effect is in the order OCH_3 > H.[75] Therefore, the second position in kaempferide is not as prone to radical attack as galangin and radical scavenging through the hydroxyl group at 3rd position in the C ring alone will occur. But in case of galangin since H in the 4' position has much lower inductive effect, the 2nd position in the C ring is readily available for radical attack. This might be the reason for the slightly higher radical scavenging capacity of galangin as compared to kaempferide. The standard quercetin, having a catechol moiety in the flavonoid B ring showed the highest antioxidant capacity. Thus, the order of radical scavenging capacity was found to be galangin > kaempferide > AGA > AGE.

In this study we have shown that *A. galanga* rhizome extract as well as the flavonoids isolated from it showed radical scavenging properties. We have also outlined a kinetic model for the better understanding of the antioxidant behaviour of the flavonoids galangin and kaempferide by free radical

scavenging using DPPH˙ for the first time. Our next objective was therefore to study the kinetic behavior of the free radical scavenging of the flavonoids and the extracts of *A. galanga* rhizomes.

2.5.2.4.1 Kinetic Studies

The method of Espin *et al*[76] was followed for the present kinetic study. As $[DPPH^{\cdot}]_0 << [(AH)_n]_0$ in the reaction medium, DPPH˙ was considered to be depleted from the reaction medium under pseudo first order conditions. The scavenging of DPPH˙ by antioxidants can be represented as in scheme 2.1.[77]

The radical formed gives stable molecules *via* these secondary reactions as shown in equations (2.2) and (2.3).[78] Absorbance decreases as radical is scavenged by antioxidants through the donation of proton to form the reduced form DPPH-H (equation (2.1)). Figure 2.22 gives the spectrophotometric recordings of the disappearance of DPPH˙ in the presence of various antioxidants at different concentrations.

Under pseudo first order reaction conditions, taking into account equation (2.1),

$$\frac{d[DPPH^{\bullet}]}{dt} = k_{obsd}[DPPH^{\bullet}] = k_2[(AH)][DPPH^{\bullet}]$$

The pseudo first order rate constant k_{obsd} obtained from the plot of % $[DPPH^{\cdot}]_{REM}$ against time (t in seconds) (Figure 2.22) was linearly dependent on initial antioxidant concentration ($[AH]_0$) (Figure 2.23). From this plot, the second order rate constants were calculated. The radical scavenging capacity (RSC) of the samples were compared with that of BHA. Kaempferide was the best antioxidant assayed among the flavonoids and extracts obtained from *Alpinia* galanga rhizomes with a k_2 value of 13489 x 10^{-5} $gL^{-1}s^{-1}$. The order of radical scavenging values according to k_2 values was found to be quercetin > BHA > kaempferide > galangin > AGE > AGA (Table 2.9).

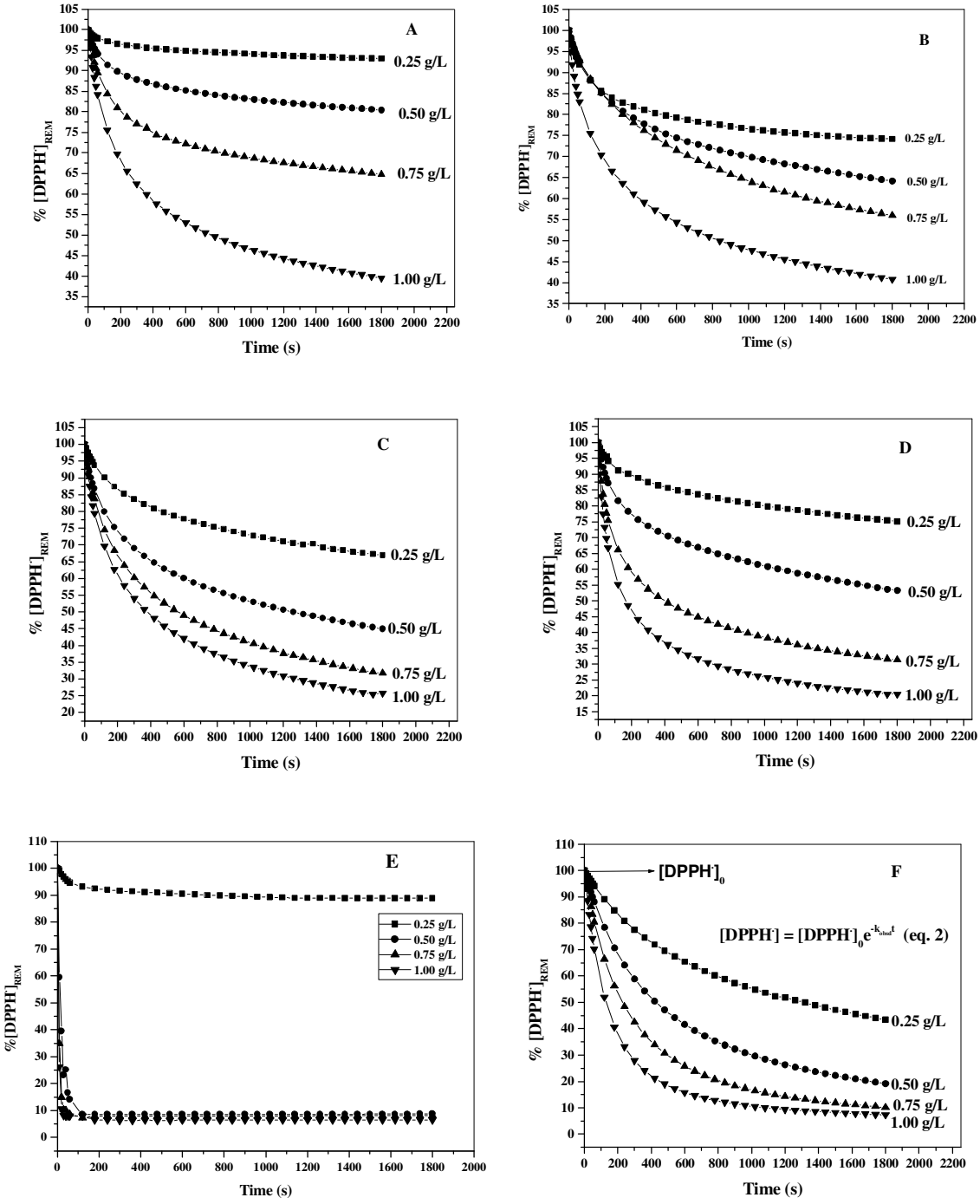


Figure 2.22: Spectrophotometric recordings of the disappearance of DPPH· in the presence of various antioxidants at different concentrations (**A**) AGE (**B**) AGA (**C**) Galangin, (**D**) Kaempferide (**E**) Quercetin and (**F**) BHA.

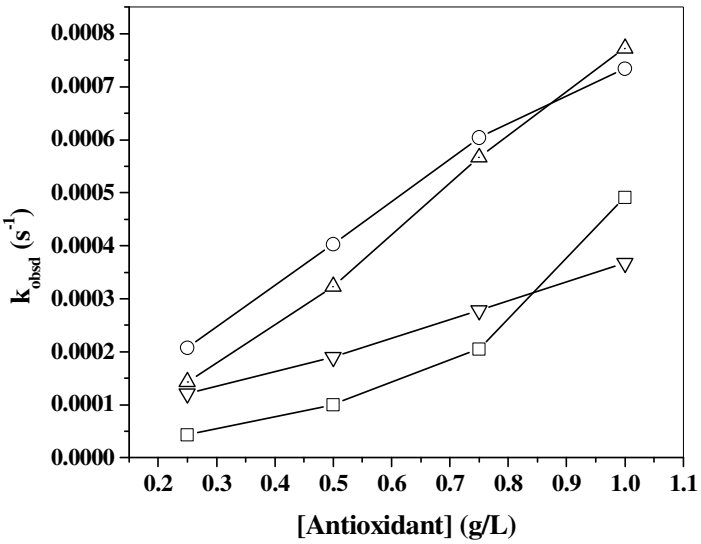


Figure 2.23: Dependence of pseudo-first-order rate constant (k_{obsd}) on the concentration of antioxidants, (□) AGE, (▽) AGA, (○) Galangin, (△) and Kaempferide.

Table 2.9: Kinetic studies of *Alpinia galanga*

No.	Compound	$k_2 \pm$ SD[a]
1	*Alpinia galanga* ethanol extract	57.8963 ± .00023
2	*Alpinia galanga* acetone extract	33.0245 ± .0001
3	Galangin	71.3601 ± .0003
4	Kaempferide	85.3155 ± .00004
5	BHA	125.00 ± .00001
6	Quercetin	13489 ± 0.03783

[a] k_2 (x 10^{-5} $gL^{-1}s^{-1}$), the second order rate constant for the reaction between antioxidants and [DPPH˙], value given as mean ± standard deviation (n = 3)

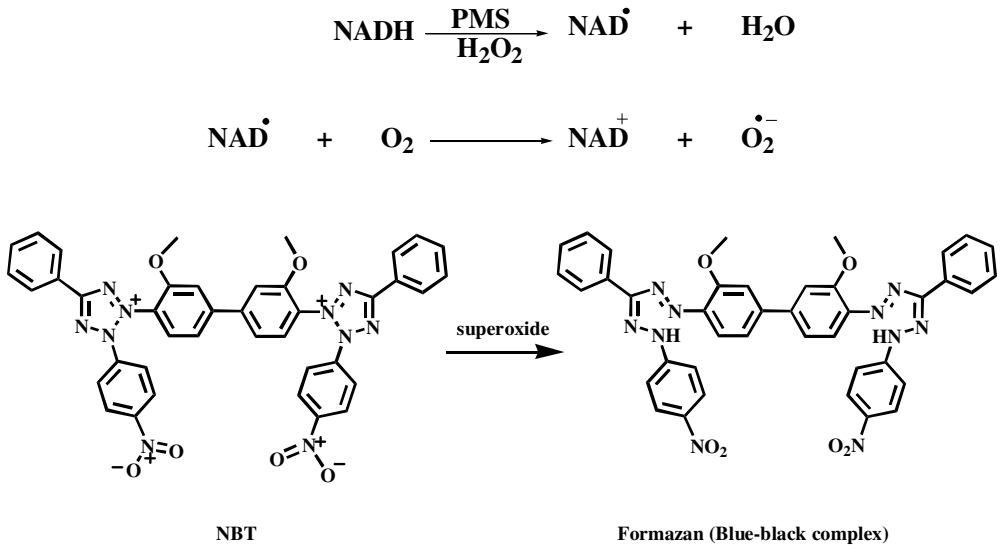
Thus, the values of rate constant of the kinetics of the reaction of galangin and kaempferide towards DPPH˙ radical was contrary to what we had observed according to the EC_{50} values. However, it is known that there is

an optimum concentration for antioxidant capacity and beyond that optimum level they can exhibit pro-oxidant behavior. Here, the pro-oxidant behavior of galangin is more pronounced than that of kaempferide at the tested concentrations. Therefore the rate constant of the reaction of galangin with free radicals was lower than that of kaempferide. It is already reported that some flavonoids can act both as antioxidants and prooxidants, depending on their concentration and free radical source.[79a] The reduced forms of flavonoids act as antioxidants, whereas the oxidized forms such as phenoxyl radicals can have pro-oxidant activities beyond a particular concentration. Here galangin begins to show pro-oxidant character above an optimum concentration. This might be considered as the reason for the decreased k_2 value of galangin compared to kaempferide. Apart from these, recent studies have revealed that the phenolic compounds with multiple hydroxyl groups having higher oxidation-reduction potential react very weakly in the DPPH$^{\cdot}$ assay.[79b]

2.5.2.5 Superoxide radical scavenging capacity

Superoxide radical ($O_2^{\cdot -}$), the one electron reduced form of molecular oxygen, is known to be very harmful to cellular components and also functions as a precursor of more reactive oxygen species.[80] The superoxide radical is produced by a number of enzyme systems in autooxidation reactions and by nonenzymatic electron transfers that univalently reduce molecular oxygen. In cellular oxidation reactions, superoxide radical is normally formed first and its effects can be magnified because it produces other types of cell-damaging free radicals and oxidizing agents, which have been implicated in the initiation of oxidizing reactions associated with aging.[81] Superoxides can result in the formation of H_2O_2 *via* dismutation reaction. The conversion of superoxide and H_2O_2 into more reactive species, such as the hydroxyl radical has been thought to be one of the unfavourable effects caused by superoxide radicals.[82] In the PMS-NADH-NBT (Phenazonium methosulphate - Nicotinamide adenine dinucleotide disodium salt - Nitro blue

tetrazolium chloride) system employed here, the superoxide anion derived from the dissolved oxygen by PMS/NADH coupling reaction reduces NBT to a blue black formazan complex. Any added molecule capable of quenching the generated superoxide radical inhibits the production of formazan complex. The decrease in the absorbance at 560 nm with antioxidants indicates the consumption of the generated superoxide anion in the reaction mixture thereby decreasing NBT reduction (Scheme 2.4).



Scheme 2.4

The assay revealed that the samples demonstrate concentration dependent scavenging capacity in neutralizing the superoxide radicals. Galangin (EC_{50} 903 ppm) and kaempferide (EC_{50} 868 ppm) showed higher superoxide scavenging power than the standard quercetin (EC_{50} 1561 ppm) as shown in figure 2.24 A at lower concentrations. But at higher concentrations, viz., at 1000 ppm, galangin (59 % scavenging power) was slightly more active than kaempferide (57 % scavenging power). At the concentration range of 400-1000 ppm, the extracts did not show any activity. Hence they were analysed at higher concentrations, i.e., at 2000-8000 ppm and the standard

used for comparison at this concentration was α-tocopherol. The extracts showed a concentration dependent scavenging capacity, but lower than the standard. It is clear from figure 2.24 B that at lower concentrations (200-4000 ppm), AGA showed high superoxide scavenging than AGE, but as the concentration increases, AGE was more effective. The EC_{50} values also support the highest scavenging capacity of AGE (7033 ppm) than AGA (10509 ppm). Thus, the superoxide radical scavenging property decreases as kaempferide > galangin > AGE > AGA (Table 2.10). The results clearly indicate that the rhizome extracts and the flavonoids isolated from it are superoxide radical scavengers in a dose dependent manner. In an earlier study on the structure activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers by Cos *et al*[83], the efficiency of galangin as superoxide scavenger has also been proved by employing an enzymatic method.

(**A**) (**B**)

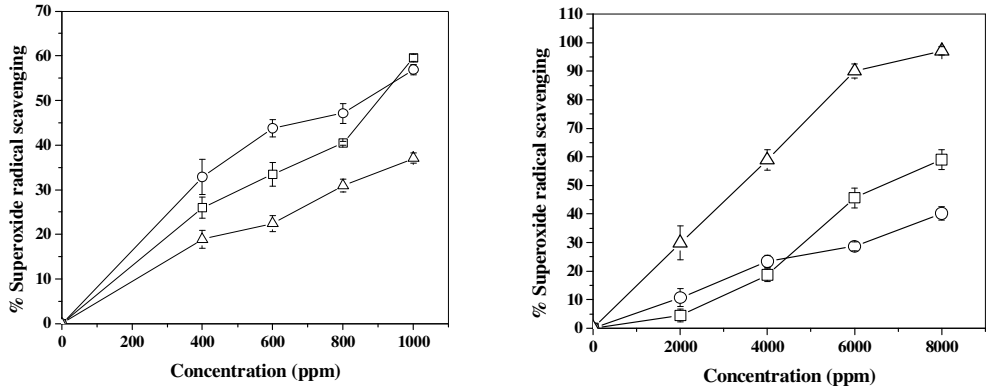
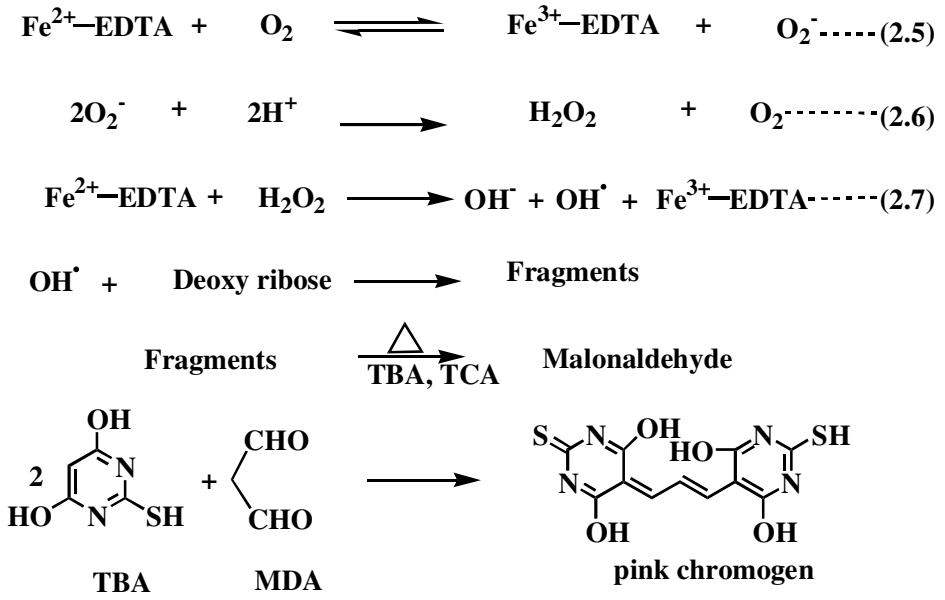


Figure 2.24: The superoxide scavenging capacity of (**A**) (□) Galangin; (○) Kaempferide and (△) Quercetin (**B**) (□) AGE (○) AGA and (△) α-Tocopherol at different concentrations.

2.5.2.6 Hydroxyl radical scavenging capacity

Hydroxyl radical is an extremely reactive oxygen species, capable of modifying almost every molecule in the living cells. This radical has the capacity to cause strand damages in DNA leading to carcinogenesis,

mutagenesis and cytotoxicity. The hydroxyl radical (˙OH) is also capable of quick initiation of lipid peroxidation process by abstracting hydrogen atoms from unsaturated fatty acids.[84] The hydroxyl radical (˙OH) scavenging capacity is measured using the "deoxyribose assay". In this assay, a mixture of ferric chloride and ethylenediamine tetraacetic acid in the presence of ascorbate reacts to form iron(II)-EDTA and oxidized ascorbate. H_2O_2 then reacts with iron(II)-EDTA to generate iron(III)-EDTA and ˙OH in a Fenton reaction system as shown in equation (2.7). Those radicals not scavenged by other components of the reaction mixture attack the sugar deoxyribose and degrade it into a series of fragments, some or all of which react on heating with thiobarbituric acid at acidic pH to give a pink chromogen (Scheme 2.5).



Scheme 2.5

Thus, the scavenging capacity towards hydroxyl radical of a substance added to the reaction mixture is measured on the basis of the inhibition of the degradation of deoxyribose.[85] As is the case with many other free radicals, ˙OH can be neutralized if it is provided with a hydrogen atom. When compounds present in the reaction mixture are capable of scavenging hydroxyl radicals, decrease in absorbance of the mixture at 532 nm is seen

and the colour will change from pink to light yellow at 532 nm. Each of the samples tested here showed high hydroxyl radical scavenging property in a concentration dependent manner (Figure 2.25). The activity was compared with that of the standards BHA and quercetin. Most of the samples showed scavenging capacities more or less equal to that of the standards. Even at a very low concentration of 2.5 ppm, AGE, AGA, galangin and kaempferide showed 83 %, 81 %, 85 % and 84 % hydroxyl radical scavenging whereas the standards BHA and quercetin at the same concentration scavenged 86 % and 79 % of the $OH^{\cdot}$ radicals. The high hydroxyl radical scavenging capacity of galangin and kaempferide could be attributed to the active hydrogen donating ability of hydroxyl substituents present in them. All the tested samples showed more or less the same EC_{50} values ranging from 1.39-1.51 ppm. It is important to mention here that various anti-inflammatory drugs exert some of their beneficial effects by scavenging hydroxyl radicals[85]. Thus the hydroxyl radical scavenging capacities of *A. galanga* extracts as well as the compounds isolated from it become significant as they may act as potent anti-inflammatory agents.

(A) (B) Graph expanded along both the axes

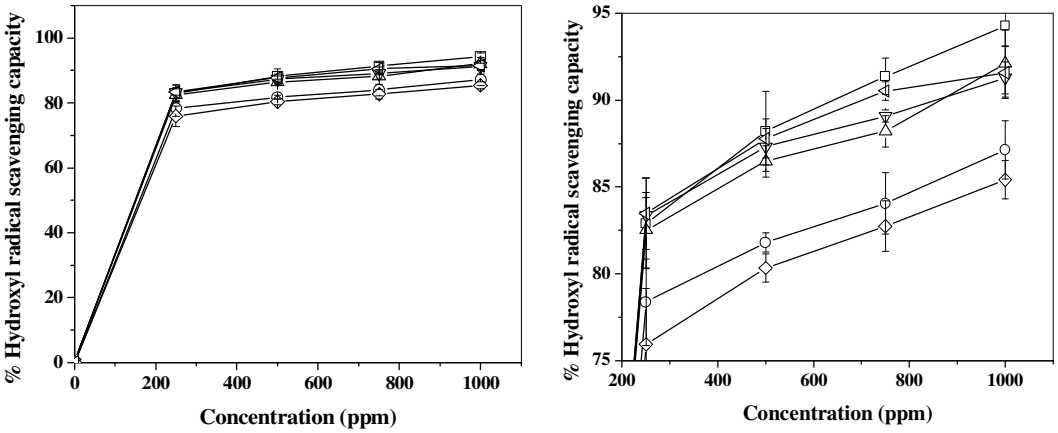


Figure 2.25: The hydroxyl radical scavenging capacity of (□) AGE; (○) AGA; (△) Galangin; (▽) Kaempferide (◇) Quercetin and (◁) BHA at different concentrations.

2.5.2.7 Scavenging of Hydrogen peroxide

Hydrogen peroxide itself is not very active, but sometimes is toxic to cells as it can give rise to hydroxyl radical in the cells.[82] Therefore, removal of hydrogen peroxide is also very important for antioxidative defence in cell or food systems. Polyphenols have been found to protect mammalian and bacterial cells from cytotoxicity induced by hydrogen peroxide.[86] The ability of *A. galanga* extracts, galangin and kaempferide in scavenging hydrogen peroxide was determined according to the method of Ruch *et al*.[87] The plot depicting the scavenging capacity of the extracts, galangin and kaempferide against hydrogen peroxide at various concentrations in comparison to the standard BHA is shown in figure 2.26.

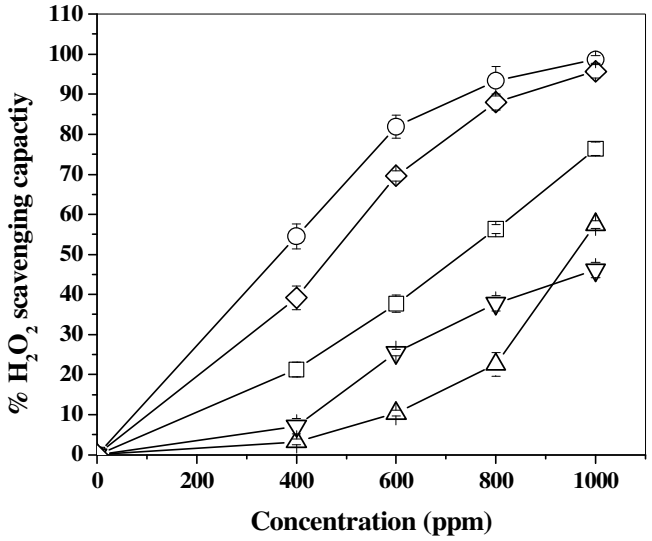


Figure 2.26: The % scavenging of hydrogen peroxide by (□) AGE; (○) AGA; (△) Galangin; (▽) Kaempferide and (◇) BHA at different concentrations.

All the tested compounds were found to destroy hydrogen peroxide in a dose-dependant manner. The EC_{50} values of all the compounds are shown in Table 2.10. The extracts were better scavengers of H_2O_2 than the flavonoids. While galangin and kaempferide could destroy only 58 and 44 % of H_2O_2 at 1000 ppm concentration, AGE destroyed 76 % of H_2O_2 at the same

concentration. At 1000 ppm, AGA could destroy almost 100 % of the H_2O_2 present in the reaction medium. At all the tested concentrations, AGA was found to be superior to other samples and even the standard BHA with an EC_{50} value of 372 ppm (Table 2.10). Thus, the scavenging capacity decreases as AGA > AGE > galangin > kaempferide.

2.5.2.8 Reducing power

The reductive capability was measured from the Fe^{3+}-Fe^{2+} transformation in the presence of the test samples.[88] The reducing property is generally associated with the presence of reductones[89], which have been shown to exert antioxidant action by breaking the free radical chain through donation of a hydrogen atom.[90] Reductones are reported to react with certain precursors of peroxide, thus preventing peroxide formation. The reducing power of the extracts, galangin and kaempferide were found to increase with increasing concentration suggesting that this activity may contribute significantly towards the total antioxidant effect of the samples.

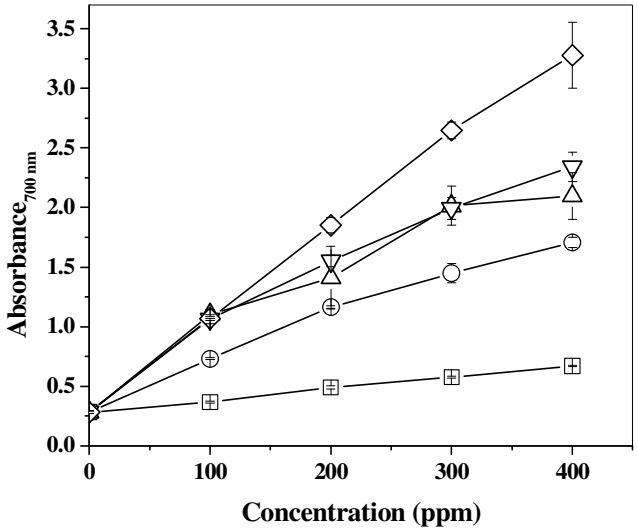


Figure 2.27: Reducing power of (□) AGE; (○) AGA; (△) Galangin; (▽) Kaempferide and (◇) BHA at different concentrations.

Figure 2.27 clearly shows that the extracts as well as galangin and kaempferide can act as electron donors and can react with free radicals and

convert them to more stable products thereby terminating radical chain reactions. Galangin and kaempferide showed more or less equal reducing power, which was higher than that of the extracts. Among the extracts, AGA was found to be a better reducing agent than AGE.

2.5.2.9 Metal chelating activity

The chelating properties were tested against Fe^{2+}. The ability of the extract and compounds to chelate ferrous ions was estimated by the method of Dinis, Madeira and Almeida.[91] It is based upon the principle of complex formation of ferrozine with Fe^{2+}. In presence of other chelating agents, the complex formation will be disrupted with the result that the red colour of the complex will decrease. In this study (Figure 2.28), it was observed that at higher concentrations of the extracts, the formation of the Fe^{2+}-Ferrozine complex was incomplete, indicating that they were able to chelate iron. But the effect was very low compared to the standard EDTA. The flavonoids galangin and kaempferide did not show any metal chelating activity. The inactivity of these flavonoids may be due to the absence of hydroxyl groups B-ring (catechol) of the flavonoid structure which according to the report by van Acker *et al*[92] is important for chelation.

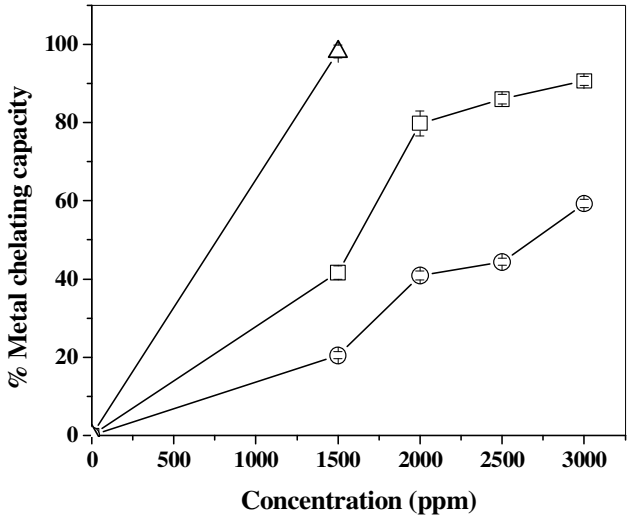


Figure 2.28: Metal chelating activity of (□) AGE; (○) AGA and (△) EDTA at different concentrations.

AGE and AGA showed some chelation of Fe^{2+}, *viz.*, 41 % and 22 % at 500 ppm concentration whereas the standard EDTA showed 90 % chelation at the same concentration. Among the extracts, AGE was better than AGA with 92 % and 59 % Fe^{2+} chelation respectively at 2000 ppm.

2.5.2.10 β-Carotene bleaching method

The antioxidant assay using the discoloration of β-carotene is widely used, because β-carotene is extremely susceptible to free-radical mediated oxidation, resulting from the hydroperoxides formed from linoleic acid. Because of the 11 pairs of double bonds in β-carotene, which are extremely sensitive to oxidation, it is decolorized easily following the initial oxidation of linoleic acid in the medium.[93] The free radical formed by the abstraction of a hydrogen atom from the diallylic methylene group of linoleic acid attacks the highly unsaturated β-carotene models resulting in a decrease in absorption at 470 nm. The presence of different antioxidants can hinder the extent of β-carotene bleaching by neutralizing the linoleate and other free radicals present in the system.[94] Antioxidant capacity of *Alpinia galanga* ethanol (AGE) and acetone extracts (AGA), Galangin and Kaempferide was compared with that of BHA. The antioxidant capacity was evaluated at the final concentration of 500 ppm for the assay. The graphical representation of the result is given in figure 2.29.

The antioxidant capacity of the extract and compounds at 500 ppm concentration are shown in Table 2.10. The antioxidant capacity by β-carotene bleaching method was found to decrease in the order; kaempferide (57.1 %) > *Alpinia galanga* acetone extract (AGA) (55.4 %) > galangin (45.3 %) > *Alpinia galanga* ethanol extract (AGE) (38.8 %). Kaempferide and AGA showed antioxidant capacities higher than that of the standard BHA used in the β-carotene bleaching method.

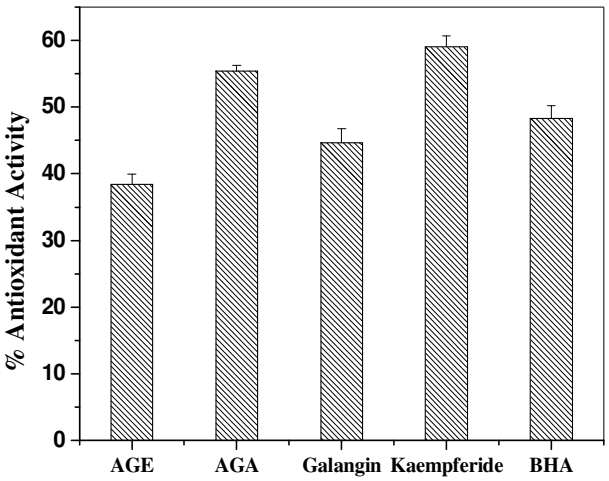


Figure 2.29: Antioxidant capacity of AGE, AGA, galangin, kaempferide and BHA at a concentration of 500 ppm measured by the β-Carotene bleaching method.

2.5.2.11 Inhibition of lipid peroxidation by linoleic acid - thiocyanate method

In biological systems, lipid peroxidation (oxidative degradation of polyunsaturated fatty acid in the cell membranes) generates a number of degradation products that may in turn cause cell damage and cell membrane destruction as shown below.[95] In the present assay, the inhibition of lipid peroxidation is measured using the linoleic acid-thiocyanate method (Scheme 2.6) where hydroperoxide is produced by linoleic acid oxidation in the medium.

$$LH \longrightarrow L^{\bullet} + H^{\bullet}$$

$$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet}$$

$$LOO^{\bullet} + LH \longrightarrow LOOH + L^{\bullet}$$

$$\text{Radicals} \longrightarrow \text{Non radical species}$$

Scheme 2.6

In the presence of hydroperoxide, ferrous chloride and ammonium thiocyanate react to form ferrous thiocyanate which is measured. An antioxidant can inhibit the lipid peroxidation thereby reducing the

hydroperoxide formation. Thus, the ferrous thiocyanate formation will be reduced in case the antioxidant is effective thereby preventing lipid peroxidation.

All the compounds tested here exhibited effective inhibition of lipid peroxidation at a concentration of 1000 ppm. The % of inhibition of lipid peroxidation of the compounds is given in figure 2.30. The effect was compared with that of the standard BHT. The highest inhibition of lipid peroxidation was given by galangin (55 %). Both galangin and AGE showed activities higher than the standard used. The capacity to inhibit lipid peroxidation shown by galangin and the ethanol extract (AGE) was even greater than the standard BHT. This activity is generally higher in bulk polyphenol structures. The % inhibition of lipid peroxidation decreased in the order; galangin (55 %) > AGE (53 %) > kaempferide (49 %) > AGA (46 %).

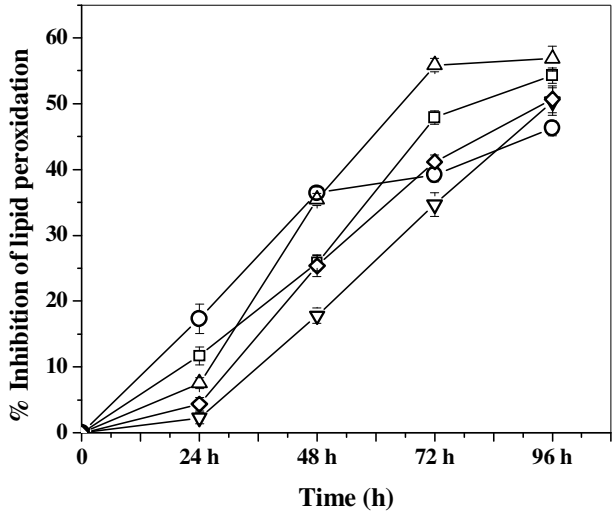
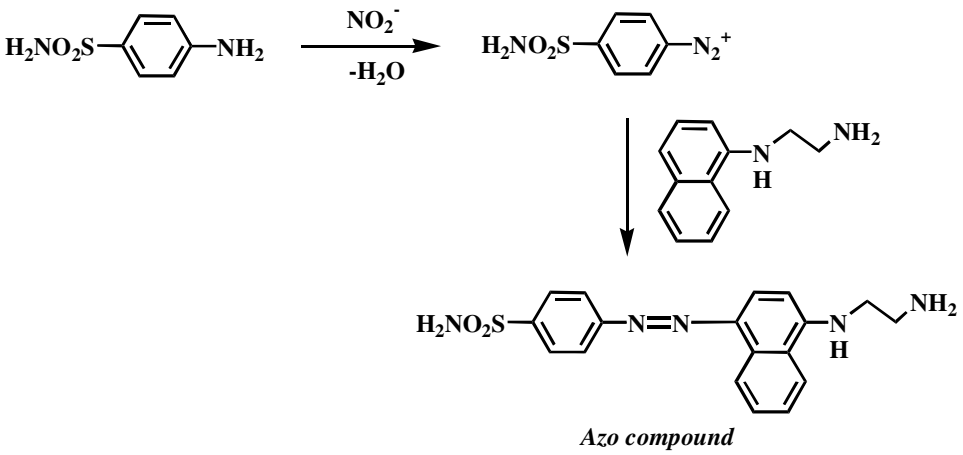


Figure 2.30: The inhibition of lipid peroxidation measured by linoleic acid emulsion-thiocyanate method at a concentration of 1000 ppm by (□) AGE; (○) AGA; (△) Galangin; (▽) Kaempferide and (◇) BHT.

2.5.2.12 Nitric oxide scavenging capacity

Nitric oxide or reactive nitrogen species, formed during the reaction with oxygen or superoxides, such as NO_2, N_2O_4, N_3O_4, NO_3^- and NO_2^- are very reactive. These compounds are responsible for altering the

structural and functional behavior of many cellular components. The nitric oxide scavenging capacity helps to arrest the chain of reactions initiated by excess generation of $NO^{\bullet}$ that are detrimental to human health. Nitric oxide is also implicated for inflammation, cancer and other pathological conditions.[96] The assay is based on the principle that, sodium nitroprusside, in aqueous solution at physiological pH generates nitric oxide ($NO^{\bullet}$) which interacts with O_2 to produce nitrite ions $(NO_2)^-$. The estimation of the nitrite ions is made using Greiss reagent (Sulphanilamide and N-(1-naphthyl)ethylenediamine dihydrochloride (NED) in phosphoric acid). In presence of nitrite ions, diazotisation reaction occurs between sulphanilamide and NED under acidic conditions yielding an azo compound (Scheme 2.7).



Scheme 2.7

Any antioxidant that can scavenge the *in situ* generated nitrite ions results in the inhibition of the production of the azo compound, resulting in a decreased absorbance of the reaction mixture at 546 nm. It is clear from figure 2.31 that the $NO^{\bullet}$ scavenging effects were concentration dependent as the scavenging capacity increased with increase in sample concentration. The $NO^{\bullet}$ scavenging capacity was compared with that of gallic acid. In this assay, AGE and AGA showed very low $NO^{\bullet}$ scavenging effect with high EC_{50}

values (Table 2.10) and scavenged only 20 % and 17 % of $NO^{\bullet}$ at 375 ppm respectively. The nitric oxide scavenging capacity of the flavonoids was found to be higher than that of the extracts, but were low compared to the standard with 45 %, 46 % and 84 % scavenging capacities for galangin, kaempferide and gallic acid respectively. Overall, the nitric oxide scavenging capacity decreases in the order, Galangin ≈ Kaempferide > AGE >AGA.

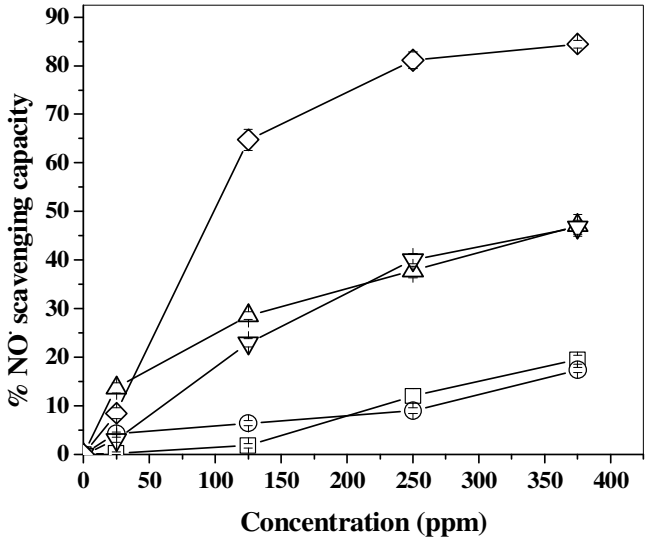


Figure 2.31: Nitric oxide scavenging capacity of (□) AGE; (○) AGA; (△) galangin; (▽) kaempferide and (◇) Gallic acid at different concentrations.

Thus we have shown that the medicinal plant *A. galanga* contains the flavonoids pinocembrin, galangin and kaempferide with galangin being the major compound constituting about 0.6 % of the dry weight of the rhizomes. Pinocembrin isolated from the rhizomes of *A. galanga* triggered bax dependent mitochondrial apoptosis on human colon cancer cell lines. The rhizomes of *A. galanga* possessed antioxidant capacity and its efficiency as an antioxidative agent has been confirmed using various assays. Eventhough the two flavonoids galangin and kaempferide were found to be powerful antioxidants than the crude acetone and ethanol extracts in most of the assays, in H_2O_2 scavenging and Fe^{2+} metal chelation, the extracts were far more active than the flavonoids themselves.

2.6 Experimental

2.6.1 General experimental details

Nuclear magnetic resonance (NMR) spectra were recorded on Bruker DPX 300 MHz NMR spectrometer operated at 300 or 500 MHz for ^{1}H and 75 or 125 MHz for ^{13}C using deuterated chloroform ($CDCl_3$), acetone (CD_3COCD_3) or methanol (CD_3OD) as solvents. Chemical shifts are given in δ scale with TMS as internal reference. Abbreviations used in ^{1}H NMR are: *s* - singlet, *d* - doublet, *dd* - doublet of a doublet, *q* - quartet and *m* - multiplet. Melting points were recorded on Aldrich Meltemp-II apparatus. Mass spectra were recorded using JEOL JMS 600H Mass spectrometer. Analytical thin layer chromatography was performed on silica gel GF_{254} TLC aluminium sheets (Merck). The TLC plates were kept in an air oven for 15 min at 80 °C – 100 °C prior to use. The spots were visualized under UV lamp or in an iodine chamber. The plant materials were dried on RRLT-NC natural convection air drier at 50 °C. Column chromatography was carried out using silica gel (100-200) mesh. Appropriate mixtures of hexane and ethyl acetate were used for elution. The solvents were removed under reduced pressure using Büchi rotary evaporator. All solvents were distilled prior to use. Shinoda test and ferric chloride test were done following the standard procedures. UV Spectra/Absorbances were measured on a Shimadzu UV-1601 UV-vis spectrophotometer using spectroscopic grade methanol as solvent. The IR spectra were recorded on Shimadzu-FTIR spectrometer. The essential oil was analysed on an analytical Shimadzu GC/MS-QP 2010 instrument at 250 °C. GC column: DB-5 (narrow bore, 30 m length, 0.25 mm). The initial temperature was 80 °C for 10 min, then heated at 5 °C/min to 150 °C for 10 min and then heated at 10 °C/min to 300 °C fro 5 min. The carrier gas was helium with a flow rate of 1.18 ml/min. Specific rotations were recorded on Rudolph Research Analytical Autopol I automatic polarimeter.

2.6.2 Chemicals used

Spectroscopic grade methanol, Butylated hydroxy anisole (BHA), Butylated hydroxy toluene (BHT) and gallic acid were bought from E-Merck. DPPH˙, Ferrozine [3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonicacid)-1,2,4-triazine-p,p'-disulfonicacidmonosodium salt hydrate], β-carotene and linoleic acid were purchased from Sigma-Aldrich, Germany. Folin and Ciocalteu's reagent, Ammonium thiocyanate, Tricholoroacetic acid, Chloroform, EDTA (Ethylenediaminetetraaceticacid tri sodium salt), Deoxy ribose, L-ascorbic acid, Nitro blue tetrazolium chloride (NBT), Nicotinamide adenine dinucleotide disodium salt (NADH), Phenazonium methosulphate (PMS) and Quercetin dehydrate were obtained from Sisco Research Laboratories Pvt Ltd, Mumbai. Ferric chloride was obtained from SD Fine Chemicals, India, Hydrogen peroxide from Rankem, Thiobarbituric acid from Fluka, Tocopherol from Lancaster and ammonium molybdate from Nice Chemicals, India. All chemicals used were of analytical grade.

2.6.3 Extraction

The dried rhizomes of *A. galanga* were collected from the medicinal plan garden of Ayurvedic Research Institute, Poojappura, Trivandrum. The rhizomes were powdered using a blender. The hexane extract (20 g) was obtained using successive extraction in a Soxhlet extraction apparatus for 24 h in three lots and then removing the solvents in a rotary evaporator. Also, fresh rhizomes was collected, dried and extracted with acetone at room temperature. The extraction was carried out by immersing the powdered plant material in acetone overnight and then decanting the solvent. Crude acetone extract (6.8 g) was obtained after evaporation of the decanted solvent under reduced pressure at 40 °C in a rotary evaporator. Pure compounds were obtained from the crude extracts using column chromatographic separation.

2.6.4 Chromatographic separation of the extracts

The crude extracts were dissolved in minimum quantity of hexane and then loaded onto a silica gel column. The column was eluted successively with hexane and hexane-ethyl acetate mixtures of increasing polarities (staring from 100 % hexane and ending with 100 % ethyl acetate). The pictorial representation of the isolation procedure of compounds **I** to **IV** is depicted in figures 2.32A and B.

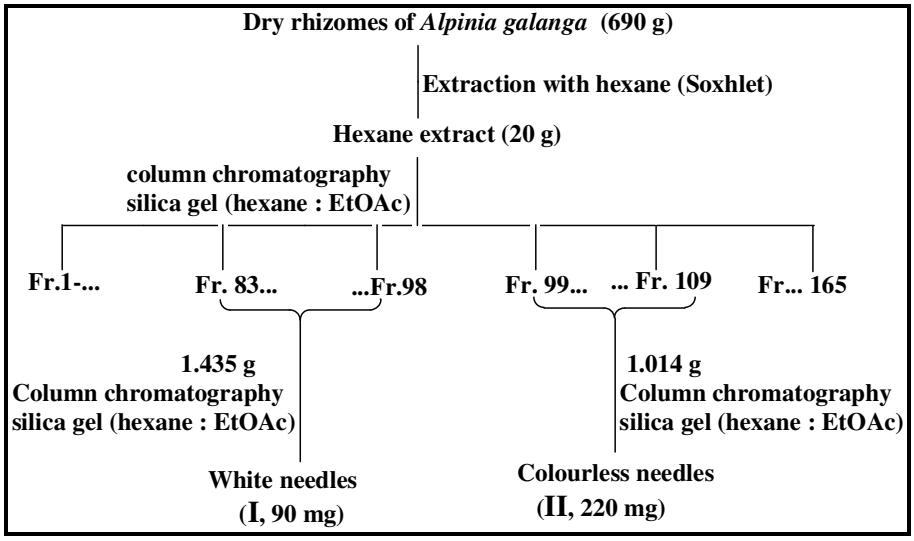


Figure 2.32 A

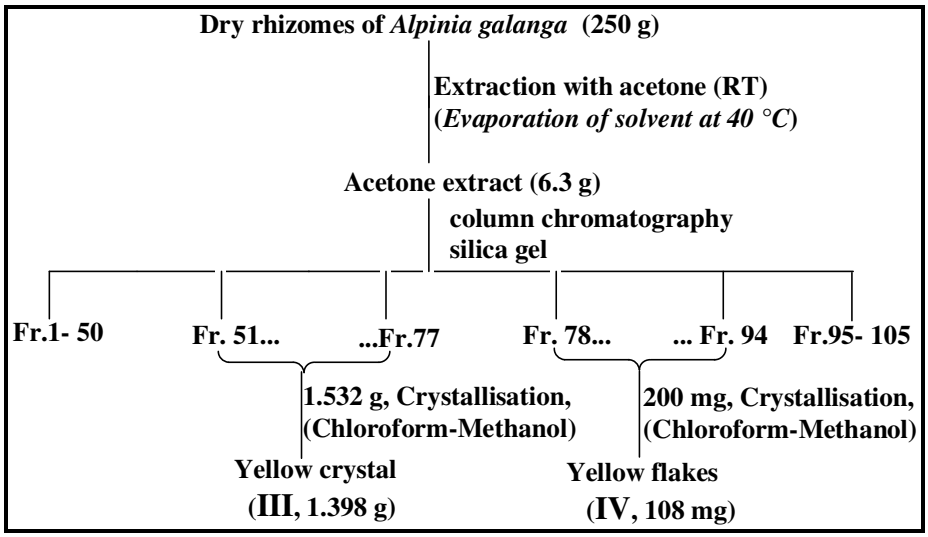
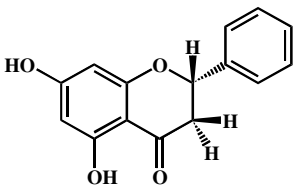


Figure 2.32 B

2.6.4.1 Isolation of compound I

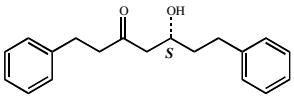
The isolation procedure of compound **I** is represented in figure 2.32A. Compound **I** was obtained on elution with 5% ethyl acetate in hexane. Compound **I** (90 mg) was obtained as off-white needles. UV-vis, IR, ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound, on comparison to literature values, confirmed it to be pinocembrin, the structure of which is shown below.



UV(λ_{max}^{MeOH}nm)	:	326 sh, 289
FT-IR (KBr, ν_{max}/cm^{-1})	:	3428, 3087, 1631, 1583, 1486, 1168, 1089, 827.
^{1}H NMR (300 MHz, $CDCl_3$)	:	δ 12.01 (1H, *s*), 7.45 - 7.37 (5H, *m*), 6.01 (2H, *s*), 5.43 (1H, *dd*, *J* = 12.9, 3.0 Hz), 3.09 (1H, *dd*, *J* = 17.2, 12.9 Hz), 2.83 (1H, *dd*, *J* = 17.2, 3.1 Hz)
^{13}C NMR (75 MHz, $CDCl_3$)	:	δ 195.8, 164.6, 164.3, 163.2, 138.2, 128.9 (3C), 126.1 (2C), 103.2, 96.8, 95.5, 79.2, 43.3
FAB-MS m/z (rel. int.)	:	257.12 $[M+1]^+$ (100), $C_{15}H_{12}O_4$, requires 256.07
m.p. (°C)	:	206-207 °C, lit[21c]. 205-207 °C
$[\alpha]_D^{26}$ (ca 0.965, EtOH)	:	-22.2°, lit.[97] $[\alpha]_D^{20}$ -22.8 °

2.6.4.2 Isolation of compound II

The isolation procedure of compound **II** is as depicted in figure 2.32A. Compound **II** was obtained on elution with 7% ethyl acetate in hexane and on crystallization from DCM-hexane mixture yielded colourless white needles (220 mg). IR, ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and on comparison to the literature values, confirmed it to be the diarylheptanoid, 1,7-diphenyl-5-hydroxy-3-heptanone (dihydroyashabushiket ol) the structure of which is as shown below.

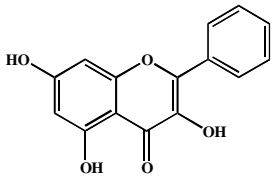


FT-IR (KBr, ν_{max}/cm^{-1})	:	3335, 1723, 1604, 1496, 1453, 1383, 1233, 1078, 744, 703
^{1}H NMR (300 MHz, $CDCl_3$)	:	δ 7.19-7.06 (10H, *m*), 3.96 (1H, *q*, *J* = 6.5 Hz), 2.83 - 2.43 (8H, *m*), 1.76 - 1.56 (2H, *m*)
^{13}C NMR (75 MHz, $CDCl_3$)	:	δ 211.0, 141.7, 140.6, 128.5 (2C), 128.4 (2C), 128.3 (2C), 128.2 (2C), 126.1, 125.8, 66.8, 49.2, 44.9, 38.0, 31.6, 29.4
DEPT-135 (125 MHz, $CDCl_3$) (i) CH, CH_3 (ii) CH_2	:	δ (i) 128.6 (2C), 128.5 (2C), 128.4(2C), 128.3 (2C), 126.2, 125.9, 66.9. (ii) 49.3, 45.0, 38.0, 31.7, 29.5
LRMS m/z (rel. int.)	:	283.15 $[M+1]^+$ (20), $C_{19}H_{22}O_2$, requires 282.16.

m.p. (°C)	:	52-53 °C, lit[21c]. 52-53 °C
$[\alpha]_D^{26}$ (ca 0.862, EtOH)	:	+14.31 °, lit.[98] $[\alpha]_D^{20}$ +14.6°

2.6.4.3 Isolation of compound III

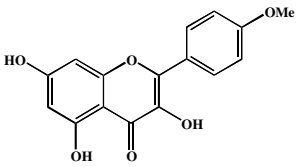
The isolation procedure of compound **III** is also depicted in figure 2.32B. Compound **III** was obtained as yellow crystals (1.4 g). Analysis of the UV-vis, IR, ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and comparison to the literature values, confirmed it to be the 5,7-dihydroxyflavonol, called galangin, the structure of which is shown below.



UV(λ_{max}^{MeOH}nm)	:	359, 305 sh, 267
FT-IR (KBr, ν_{max}/cm^{-1})	:	3556, 3158, 1655, 1608, 1566, 1473, 1315, 849, 772, 704
^{1}H NMR (300 MHz, CD_3CO CD_3)	:	δ 8.17 (2H, *d*, *J* = 6.9 Hz), 7.40-7.51 (3H, *m*), 6.39 (1H, *d*, *J* = 2.0 Hz), 6.19 (1H, *d*, *J* = 2.0 Hz)
^{13}C NMR (75 MHz, CD_3CO CD_3)	:	δ 176.7, 165.2, 162.1, 157.7, 145.8, 137.7, 131.9, 130.5, 129.2 (2C), 128.2 (2C), 104.0, 99.1, 94.3
FAB-MS m/z (rel. int.)	:	271.45 $[M+1]^+$ (100), $C_{15}H_{10}O_5$, requires 270.05
m.p. (°C)	:	m.p. 214-215 °C, lit[26d].214-215 °C

2.6.4.4 Isolation of compound IV

The isolation procedure of compound **IV** is as shown in figure 2.32B. Compound **IV** was obtained as yellow flakes (108 mg). UV-vis, IR, ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and on comparison to the literature values, confirmed it to be the 4'-Methoxy-5,7-dihydroxyflavonol, commonly known as kaempferide, the structure of which is shown below.



UV(λ_{max}^{MeOH}nm)	:	367, 320, 299 sh, 267, 253 sh
FT-IR (KBr, ν_{max}/cm^{-1})	:	3527, 3309, 1653, 1604, 1560, 1510, 1440, 1371, 1307, 1257, 1220, 1176, 833, 759, 713
^{1}H NMR (300 MHz, $CDCl_3$ + CD_3OD)	:	δ 8.16 (2H, *d*, *J* = 8.9 Hz), 7.02 (2H, *d*, *J* = 8.9 Hz), 6.43 (1H, *d*, *J* = 1.7 Hz), 6.27 (1H, *d*, *J* = 1.7 Hz), 3.89 (3H, *s*)
^{13}C NMR (75 MHz, $CDCl_3$ + CD_3OD)	:	δ 175.6, 164.0, 160.8, 160.6, 156.9, 145.4, 137.2, 129.3 (2C), 123.4, 113.8 (2C), 105.4, 98.5, 93.9, 55.2
FAB-MS m/z (rel. int.)	:	300.02 (100), $C_{16}H_{12}O_6$, requires 300.06
m.p. (°C)	:	m.p. 198-199 °C, lit[26d]. 198-199 °C

2.6.5 Cytotoxicity and Apoptosis Measurement

Cytotoxicity was assessed by a modified version of MTT reduction assay.[99] MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]

assay, is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilisation of the cells by the addition of detergent results in the liberation of the crystals, which are solubilised. The number of surviving cells is directly proportional to the level of formazan product created. The color can then be quantified using a simple colorimetric assay. The results can be read on a multi-well scanning spectrophotometer (ELISA reader).

2.6.6 Antioxidant assay procedures

2.6.6.1 Total phenolics: Folin-Ciocalteu's reagent assay

Total phenolic constituents in the ethanol and acetone extracts of *Alpinia galanga* rhizomes was analysed by employing the literature methods involving Folin-Ciocalteu reagent.[100] To 1 ml of the appropriately diluted sample taken in a stoppered test tube, 5 ml of diluted freshly prepared Folin-Ciocalteu reagent (Folin-Ciocalteu reagent : distilled water; 1:10 v/v) was added and the test tube was shaken thoroughly. After 3 min, 4 ml of 7.5 % (w/v) of Na_2CO_3 solution was added and the mixture was allowed to remain at room temperature for 120 min. Absorbance was measured at 760 nm against a reagent blank. Using gallic acid as standard, the total phenolic content is expressed as gallic acid equivalents (mg GAE/100 g dry rhizomes). Data are reported as means ± SD for at least three replications.

2.6.6.2 Total flavonoids: $AlCl_3$ colorimetric assay

Total flavonoid content was determined according to the colorimetric method[67,101] employing quercetin as the standard. To appropriately diluted samples (1 ml), distilled water was added to make up to 5 ml, 0.3 ml of 5 % (w/v) $NaNO_2$ was added to it and placed for 5 min, followed by reaction with 0.3 ml of 10 % (w/v) $AlCl_3$ to form a flavonoid-aluminium complex. At the sixth minute, 2 ml of 1M NaOH was added and the total volume was made to

10 ml with distilled water. The solution was mixed well again and the absorbance was measured against a reagent blank at 510 nm. Blank was prepared by adding all reagents except $AlCl_3$. Distilled water was added in place of $AlCl_3$ in the blank. The total flavonoid content is expressed as quercetin equivalents (mg QE/100 g of dry rhizomes). Data are reported as means ± SD for at least three replications.

2.6.6.3 Total antioxidant capacity:Phosphomolybdenum method

The assay[69,102] is based on the reduction of Mo(VI)-Mo(V) by the extract resulting in the formation of a green phosphate/Mo(V) complex at acidic pH. 0.3 ml of the extract was combined with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and the reaction mixture was incubated at 95 °C for 90 min. After cooling to room temperature, the absorbance of the resulting solution was measured at 695 nm against a blank. Methanol (0.3 ml) in place of extract served as the blank. Ascorbic acid was used as the standard and the total antioxidant capacity is expressed as ascorbic acid equivalents (mg AAE / 100 g of dry rhizomes). Data are reported as means ± SD for at least three replications.

2.6.6.4 DPPH˙ scavenging capacity

DPPH˙ scavenging capacity of the test samples was analysed according to the procedure reported by Brand Williams in 1997.[72] An aliquot of methanol solution containing different concentrations (0.25-1.00 g/l) of samples and the standards were added to 3.9 ml of freshly prepared DPPH˙ (0.025 g/l) reagent in methanol. Absorbance at 515 nm was measured after 30 minutes against a reagent blank. The percentage inhibition of the free radical was calculated as,

$$\% \text{ Inhibition} = [(A_0 - A_1)/A_0] \times 100 \qquad \text{---------- (2.8)}$$

where, A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

2.6.6.5 Kinetic studies

An aliquot of methanol solution containing different concentrations of test samples (0.25-1.00 g/l); BHA, 0.1-1.00 g/l and Quercetin, 0.25-1.00 g/l was added to 3.9 ml of freshly prepared DPPH$^{\cdot}$ (.025 g/l) in methanol separately. The presence of methanol was required to enhance the solubility of the extracts and the compounds to reach pseudo first order assay conditions: i.e., initial radical concentration << antioxidant concentration, ($[DPPH^{\cdot}]_0 << [(AH)_n]_0$). Absorbance at 515 nm was measured at different time intervals for 30 minutes. A total of 180 data points were taken per spectrophotomeric recording of the disappearance of DPPH$^{\cdot}$ in the presence of antioxidant.

The DPPH$^{\cdot}$ concentration ([DPPH$^{\cdot}$]) in the reaction medium was calculated from the following calibration curve determined by linear regression, according to the method of Bondent *et al.*[72]

$$Abs_{515\ nm} = 8.25 \times 10^{-3} + 15.01421[DPPH^{\cdot}] \qquad \text{----- (2.9)}$$

where, [DPPH$^{\cdot}$] is expressed as g litre^{-1}, $r = 0.999$

The percentage of remaining DPPH$^{\cdot}$ (% $DPPH^{\cdot}_{REM}$) at a particular time, t is calculated as follows:

$$\%\ DPPH^{\cdot}_{REM} = [DPPH^{\cdot}]_t/[DPPH^{\cdot}]_0,$$

where, $[DPPH^{\cdot}]_t$ is the radical concentration at any time (t) and $[DPPH^{\cdot}]_0$ is the radical concentration at zero time.

Second order rate constants (k_2) were calculated to determine the radical scavenging capacity of different antioxidants and the standard. DPPH$^{\cdot}$ depleted from the medium under pseudo-first order conditions ($[DPPH^{\cdot}]_0 << [AH]_0$) following the equation,

$$[DPPH^{\cdot}] = [DPPH^{\cdot}]_0\, e^{-k_{obsd}t} \qquad \text{---(2.10)}$$

where [DPPH˙] is the radical concentration at any time (t), $[DPPH^{\cdot}]_0$ is the radical concentration at time zero and k_{obsd} is the pseudo first order rate constant. [DPPH˙] present in the reaction medium was calculated according to equation (2.9).

From equation (2.10),

$\ln [DPPH^{\cdot}] = \ln [DPPH^{\cdot}]_0 - k_{obsd}t$, the pseudo first order rate constant was calculated (Figure 2.22). This constant (k_{obsd}) was linearly dependent on the concentration of each antioxidant and from the slope of this plot, the second order rate (k_2) was determined.[76,103]

Graph preparation and fitting of the experimental data were carried out using Microcal Origin (Version 6.0) program. The mean of three separate determinations of k_2 (triplicate for four different weights of each antioxidant and standard) is shown. Student's t test was applied to calculate the significant difference between values ($P \leq 0.05$). Data are reported as means ± SD for at least three replications.

2.6.6.6 Superoxide radical scavenging capacity

Measurement of superoxide anion scavenging capacity of the samples was based on the method described by Oktay.[104] One ml of Nitroblue tetrazolium (NBT) solution (156µmol/l NBT in 100mmol/l phosphate buffer, pH 7.4), 1 ml NADH solution (468µmol/l in 100 mmol/l phosphate buffer, pH 7.4) and 0.1 ml of sample solutions (different concentration) in methanol were mixed. The reaction was started by adding 100 µl of phenazine methosulphate (PMS) solution (60µmol/l in 100 mmol/l phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 °C for 5 min and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging capacity. The percentage inhibition of superoxide anion generation was calculated from equation (2.8).

% Inhibition = $[(A_0 - A_1)/A_0] \times 100$

where, A_0 is the absorbance of the control and A_1 is the absorbance of the samples.

2.6.6.7 Hydroxyl radical scavenging capacity

This method is based on the principle that the sugar deoxy ribose, on exposure to hydroxyl radicals generated by the Fenton reaction model system, degrades into fragments and generates a pink chromogen up on heating with thiobarbituric acid at low pH.[85] The reaction mixture contained different concentrations of the test samples in a mixture of 3.75 mM deoxyribose, 1 mM H_2O_2, 20 mM potassium phosphate buffer (pH 7.4), 0.1 mM EDTA and 0.1 mM ascorbic acid. It was incubated in a water bath at 37 °C for 60 min. After incubation, 1 ml of 2.8 % trichloroacetic acid was added to it followed by 1 ml of 2 % thiobarbituric acid[105] and boiled for 20 min. The reaction was stopped by keeping the test tubes in an ice water bath for about 10 minutes. A control contained all the reagents except the sample. The absorbance of the resulting solution was measured spectrophotometrically at 532 nm and the % Hydroxyl radical scavenging was calculated from equation (2.8).

2.6.6.8 Scavenging of Hydrogen peroxide

The ability of the test materials to scavenge H_2O_2 was determined according to the method of Ruch *et al.*[87] A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined spectrophotometrically from the absorption at 230 nm with a molar absorptivity of hydrogen peroxide of 81 mol / L cm^{-1}. Different concentrations of the samples in methanol (0.6 ml) were added to 3.4 ml of phosphate buffer containing 20mM H_2O_2. Absorbance of H_2O_2 at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without H_2O_2. The percentage of scavenging of H_2O_2 and also the concentration of H_2O_2 was determined from equation (2.8). The concentration of remaining hydrogen peroxide in the reaction medium was calculated using the equation $A = \varepsilon cl$; $c = A/\varepsilon l$, where A

is the absorbance at 230 nm, ε = 81 mol/L cm^{-1}, c is the concentration of H_2O_2 and l is the length of the cell.

2.6.6.9 Reducing power

Different concentrations of the test samples in 1 ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide ($K_3[Fe(CN)_6]$). The mixture was incubated at 50 °C for 20 min. To the incubated mixture, 2.5 ml of 10 % trichloroacetic acid was added. From it, 2.5 ml was removed and was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1 % $FeCl_3$ was added. The absorbance at 700 nm was measured.[88] Increased absorbance of the reaction mixture indicated increased reducing power which in turn shows the redox property of the sample and hence an increase in the antioxidant capacity.

2.6.6.10 Metal chelating activity

The ability of the extract and compounds to chelate ferrous ions was estimated by the method of Dinis, Madeira and Almeida.[91] The extract in methanol (1 ml) was added to a solution of ferrous chloride (0.1 ml, 20 mM). After 5 min, 0.2 ml, 5 mM ferrozine was added to it and absorbance at 562 nm was taken after 10 min. The prercentage of metal chelation was calculated from equation (2.8).

2.6.6.11 β-Carotene bleaching method

The β-Carotene bleaching method of Hidalgo[106] was used to evaluate the antioxidant capacity the samples. β-Carotene (0.2 mg), linoleic acid (20 mg) and Tween 20 (200 mg) were mixed with 0.5 ml of chloroform. The solvent was removed at 40 °C in a vacuum evaporator and it was diluted with 50 ml of triply distilled, oxygenated water. Aliquots (4 ml) of this emulsion was transferred into test tubes, to which were then added, 0.2 ml of the test samples in methanol. A control contaning 0.2 ml of methanol and 4 ml of the emulsion was also used. The absorbance (470 nm) at the time of addition (t = 0) was taken and the test tubes were incubated at 50 °C in the dark for 60 min

and the absorbance was taken. The antioxidant capacity was calculated knowing the degradation rate of β-Carotene in the sample.

$AA = 100[DR_c - DR_s] / DR_c$;

DR_c = ln (a/b)/60; a = Initial absorbance of control; b = Final absorbance of control.

DR_s = ln (a/b)/60; a = Initial absorbance of sample; b = Final absorbance of sample.

2.6.6.12 Linoleic acid- thiocyanate method

The inhibition of lipid peroxidation was determined according to the ferric thiocyanate method.[107] Test samples in methanol was added to 2.5 ml of linoleic acid emulsion and 2 ml of 0.2 M phosphate buffer (pH 7.0). The reaction mixture was incubated in the dark at 37 °C to accelerate the peroxidation process. The mixture prepared as above without any test sample served as control. Aliquots (0.1 ml) were drawn from the incubation mixture at intervals of 24 h and mixed with 4.7 ml of 75 % of ethanol, 0.1 ml of 30 % ammonium thiocyanate and 0.1 ml of ferrous chloride (20 mM in 3.5 % HCl). BHA was used as the positive control. After mixing for 3 min, the peroxide values were determined by reading the absorbance at 500 nm against a reagent blank each 24 h until 1 day after the absorbance of the control reached a maximum (96 h). The antioxidant capacity (AA) was calculated from equation (2.11).

$AA = 100 - [(OI_{sample}\ t=96\ h / OI_{control}\ t = 96\ h) \times 100]$ ---------- (2.11)

$OI = Absorbance_t / Absorbance_{t=0}$

2.6.6.13 Nitric oxide scavenging capacity

This procedure is based on the method that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent.[108] Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. Different concentrations of the

test samples in methanol (0.2 ml) was mixed with sodium nitroprusside (0.2 ml, 100mM) and made up to 2 ml with phosphate buffer (20 mM, pH 7.4). It was incubated at 25 °C for 150 min. The same reaction mixture without the sample, but an equivalent amount of methanol served as the control. After incubation period, 0.5 ml of Greiss reagent (1% sulphanilamide and 0.1% *N*-(1-naphthyl) ethylenediaminedihydrochloride in 2% phosphoric acid) was added. The absorbance of the chromophore formed was read at 546 nm and the % of nitric oxide scavengers are calculated from equation (2.8).

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Sample	*Scavenging capacity*					*Metal Chelating capacitty*	*Antioxidant capacity by β-Carotene bleaching (500 ppm)*	*Inhibition of Lipid peroxidation, 96 h (1000 ppm)*
	DPPH˙	*Super oxide radical*	*Hydroxyl radical*	*H_2O_2*	*Nitric oxide*			
	EC_{50} (ppm)						**%**	
AGE	**895**	**7033**	**1.51**	**735**	**764**	**1603**	**39**	**53**
AGA	**851**	**10510**	**1.70**	**372**	**862**	**2690**	**55**	**46**
Galangin	**442**	**903**	**1.40**	**961**	**450**	**Nil**	**45**	**55**
Kaempferide	**541**	**868**	**1.41**	**1426**	**447**	**Nil**	**57**	**49**
BHA	**139**	**-**	**1.39**	**472**	**-**	**-**	**48**	**-**
BHT	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**51**
Tocopherol	**-**	**3664**	**-**	**-**	**-**	**-**	**-**	**-**
Gallic acid	**-**	**-**	**-**	**-**	**98.11**		**-**	**-**
EDTA	**-**	**-**	**-**	**-**		**25**	**-**	**-**
Quercetin	**1.67**	**1561**	**2.00**	**-**			**-**	**-**

Table 2.10: Antioxidant capacity of *Alpinia galanga* rhizomes

Chapter 3

Section A

Phytochemical Investigation and Antioxidant Activity Studies on *Alpinia calcarata*

Section B

Comparison of Alpinia *galanga* and *Alpinia calcarata* in terms of chemical constituents and antioxidant activity

Section A

Phytochemical Investigation and Antioxidant Capacity Studies on *Alpinia calcarata*

This chapter deals with the studies on the rhizomes of *Alpinia calcarata*, a Zingiberaceae plant belonging to the genus *Alpinia* which is used extensively in *ayurveda* and is said to have the same medicinal properties as that of *Alpinia galanga*. The study of the chemical constituents of the rhizomes, evaluation of the antioxidant activities by different methods of the crude extracts as well as the individual compounds which have been isolated are discussed in Section A of the present chapter.

3.1 Aim and scope of the present investigation

Figure 3.1: *Alpinia calcarata* plant (Leaves and inflorescence)

A. calcarata (known as *rasana* in Sanskrit and *chittaratha* in Malayalam) is a slender, rhizomatous herb (Figure 3.1), often cultivated in East and South India, Sri Lanka, China and Malaysia. It has white flowers, variegated with red and yellow in pyramidal panicles. The rhizomes are used

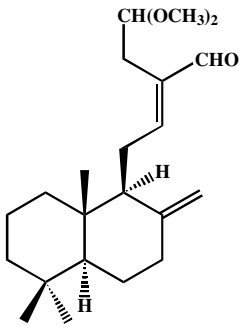
in India, Sri Lanka and China for its medicinal uses such as treatment of bronchitis, cough, respiratory ailments, diabetics, asthma and arthritis.[1] It is an important constituent of the polyherbal formulation, "*Maharasnadi*" recommended by *Ayurvedic* medical practitioners for the treatment of arthritic conditions.[2]

The essential oil composition of *A. calcarata* from various geographical areas have been reported by different groups.[3] The major constituent in the leaf and rhizome oil of *A. calcarata* was found to be 1,8-cineole. The efficacy of the oil against the bruchid, *Callosobruchus maculates* was evaluated by Abeywickrama *et al*.[4] The essential oil was also found to have repellant properties against *Periplanata americana*.[5] The phytochemical investigations of the rhizomes of *A. calcarata* was carried out by Kong *et al*., who found that it contains mainly terpenoids, *viz*., four bis-labdanic diterpenoids, several labdanic diterpenoids, a sesquiterpene-shyobunone and 7-methoxy coumarin (**104**-**118**).[6] The structures of the compounds isolated from the rhizomes of *A. calcarata* is given in chart 3.1. The presence of benzenoids, flavonoids and alkaloids were identified from TLC studies of the leaves of *A. calcarata* grown in India by Merh *et al*.[7] Studies carried out on the extracts of *A. calcarata* have revealed the antibacterial,[8] antifungal[9] and anthelmintic[10] activity in extracts. A recent report from Sri Lanka has confirmed the antinociceptive activity of the rhizomes.[11] It has also been shown that the aqueous extract of the rhizomes of *A. calcarata* increases reproductive potency of male rats.[12] Apart from this, the diterpenes Calcaratarins D (**107**) and E (**108**) were found to have cytotoxic activity against human KB cells *in vitro*.[6b]

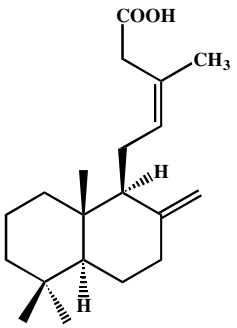
A survey of literature however revealed that no detailed phytochemical investigation of *A. calcarata* rhizome grown and used in South India extensively for medicinal purposes had been carried out. It was especially of interest to us to search for flavonoids in *A. calcarata* as we had already found

several in *A. galanga*. In addition, the study of the antioxidant capacity of the rhizomes and the constituents appeared significant since *A. calcarata* is a herb that is used extensively in *ayurvedic* medicines. Therefore, our aim was to isolate and identify the constituents present in *A. calcarata* rhizomes and to evaluate the antioxidant capacity of the rhizome extract and the isolated compounds.

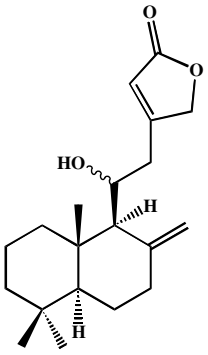
Chart 3.1: Structures of the known phytochemical constituents isolated from the rhizomes of *A. calcarata*



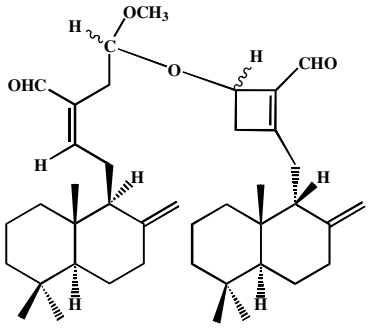
Calcaratarin A
(**104**)



Calcaratarin B
(**105**)

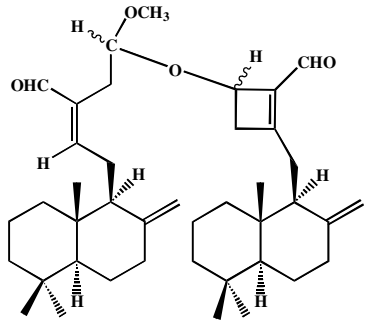


Calcaratarin C
(**106**)

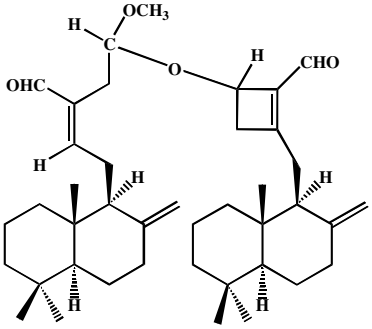


Calcaratarin D
(**107**)

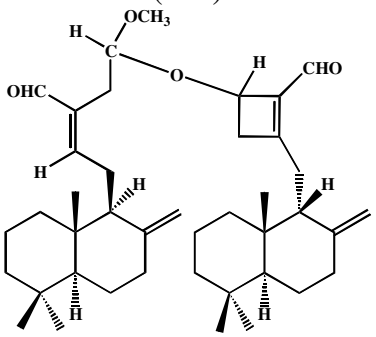
Chart 3.1 continued…



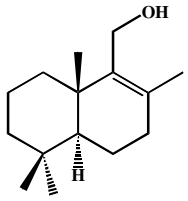
Calcaratarin E
(**108**)



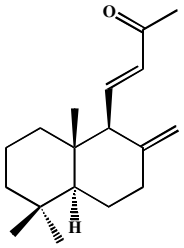
Calcaratarin G
(**109**)



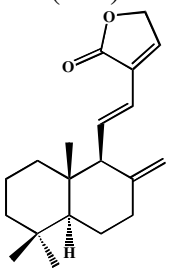
Calcaratarin H
(**110**)



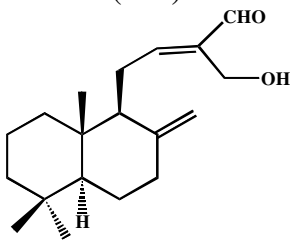
γ-Bicyclohomofarnesol
(**111**)



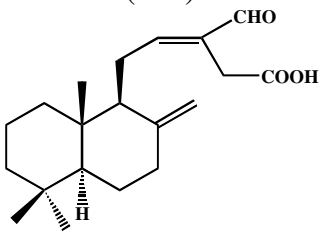
(*E*)-15,16-Bisnorlabda-8(17),11-dien-13-one
(**112**)



Labda-8(17),11,13-trien-15(16)-olide
(**113**)

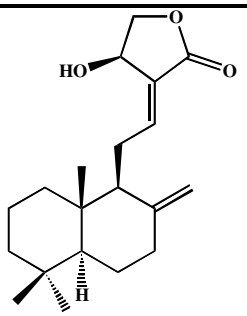


(*E*)-Labda-8(17),12-dien-15-ol-16-al
(**114**)

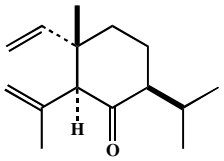


Zerumin A
(**115**)

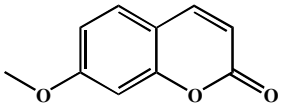
Chart 3.1 continued…



Isocoronarin D
(**116**)



Shyobunone
(**117**)



7-Methoxycoumarin
(**118**)

3.2 Essential oil composition of *A. calcarata* rhizomes

Dried rhizomes of *A. calcarata* (100 g) was coarsely powdered and was subjected to hydrodistillation in a Clevenger type apparatus. The oil obtained (0.2 %) was analyzed on an analytical Shimadzu QP-2010 GC/MS. Most of the constituents were identified by comparison of their mass spectra with those from the library of standards. The major constituents present in the rhizome essential oil are given below in Table3.1.

Table 3.1: Essential oil constituents of the rhizomes of *A. calcarata*

No	Compound	Percentage (%)
1	α-Pinene	3.46
2	Camphene	4.87
3	β-Pinene	6.75
4	β-Myrcene	0.47
5	**Eucalyptol**	**12.78**
6	γ-Terpinene	0.93
7	α-Terpinene	0.36
8	Fenchyl alcohol	1.07
9	Camphor	0.34

10	Camphene hydrate	4.06
11	*endo* Borneol	0.34
12	Pinocamphone	1.00
13	Terpinene-4-ol	0.27
14	3-Cyclohexene-1-methanol	0.43
15	α-Terpineol	1.63
16	**Fenchyl acetate**	**20.25**
17	Bornyl acetate	1.34
18	Aromadendrene	1.58
19	Cadinene	0.95
20	Methyl cinnamate	5.23
21	Sesquithujene	1.18
22	Germacrene D	0.68
23	Bicyclogermacrene	0.32
24	α-Bergamotene	0.63
25	Caryophyllene	2.68
26	γ-Muurolene	0.34
27	α-Humulene	0.58
28	β-Farnesene	0.36
29	α-Cubebene	1.23
30	γ-Selinene	0.43
31	Valencene	1.21
32	β-Chamigrene	1.63
33	β-Bisabolene	0.69
34	Selinene	1.22
35	Sesquiphellandrene	1.51
36	Elemol	1.23
37	Caryophyllene oxide	6.32
38	Azulenol	1.58
39	Eudesmol	1.26
40	α-Eudesmol	0.48
41	Selin-11-en-4-alpha-ol	0.47
42	Hedycaryol	0.69

It was found that the major constituents present in the rhizome essential oil studied here were fenchyl acetate and eucalyptol while 1,8-cineole was the major constituent of the rhizome oil reported earlier by several groups.[3]

3.3 Extraction, Isolation and Characterization of compounds from the hexane extract of *A. calcarata* rhizomes

3.3.1 Plant material and extraction

The rhizomes of *A. calcarata* were collected from National Institute for Interdisciplinary Science and Technology (NIIST) medicinal plant garden. A voucher specimen (TBGT 20271) is deposited in the Tropical Botanical Garden and Research Institute (TBGRI) Herbarium, Palode, Kerala, India. Fresh rhizomes (1.5 kg) collected from NIIST campus was washed, cut into small pieces and dried in an oven at 50 °C. The powdered rhizome (350 g) was extracted three times using dichloromethane (2l each) at room temperature (27 °C). The crude extract was combined and the solvent evaporated at 40 °C to yield 16.7 g of dichloromethane extract.

3.3.2 Analysis of the dichloromethane extract and isolation of the major component

This dichloromethane extract was subjected to column chromatography on silica gel (100-200 mesh) employing hexane-ethyl acetate mixtures of increasing polarities (starting with pure hexane and ending with pure ethyl acetate) as eluent to yield 157 fractions. Based on TLC's, the fractions were finally pooled together into five fractions.

The first (1-25; 660 mg), second (26-30; 175 mg) and third fraction (31-40; 397 mg) pools obtained on elution with pure hexane was found to contain many components of very low polarity, very similar to the essential oil. Therefore, these three fractions were analyzed by GC-MS on an analytical Schimadzu QP-2010 GC-MS instrument. The major compounds present in the first, second and third fractions are given in Tables 3.2, 3.3 and 3.4 respectively. The major compound present in the first fraction pool was carotol (57.74 %). Second pool of fractions gave major peak corresponding to the molecular mass 162 which corresponds to methyl cinnamate. However,

another compound with molecular mass 159 corresponding to 1-benzyl-3-pyrroline was also present. Eventhough it matches with the standard compound from the library search (Wiley library), we cannot confirm it. The third pool of fractions showed a major constituent (83% of the total peak area) with molecular mass 302. This was identified as (*E*)-labda-8(17),12-diene-15,16-dial after the structural identification of the same compound from the fourth pool and reconfirmation with pure compound under the GC-MS conditions.

Table 3.2: Constituents analysed by GC-MS from the fractions 1-25

No.	Compound	Percentage (%)
1	Camphor	2.03
2	**Fenchyl acetate**	**20.21**
3	Bornyl acetate	0.93
4	Terpenyl acetate	0.94
5	β-Farnesene	0.36
6	Gynolutone	1.07
7	Limoneneoxide	0.60
8	2-Butanone,4-(2,6,6-trimethyl-2-cyclohexene-1-ylidene)	0.34
9	Caryophyllene oxide	2.68
10	**Carotol**	**57.74**
11	Thujopsene	0.39
12	Humulene epoxide	0.58
13	Daucol	0.21
14	Longiborneol	0.13
15	3-Cyclohexene-1-carboxaldehyde-1,3,4-timethyl	5.62
16	Longifolol	0.36
17	Ethyl heptanoate	0.27
18	Germacrene D-4-ol	0.32
19	Kauran-18-al	1.00
20	4,8,13-Cyclotetradecatriene-1,3-diol	1.07
21	4-Amino-4'-nitrostilbene	1.59
22	Isolongifolol	0.63
23	1,2-Benzenedicarboxylic acid	0.43
24	Farnesol	0.17

Table 3.3: Constituents analysed by GC-MS from the fractions 26-30

No	Compound	Percentage (%)
1	Camphor	0.80
2	**Methyl cinnamate**	**20.84**
3	Caryophyllene oxide	1.39
4	Selina-3,7(11)-diene	0.27
5	Cubenol	5.31
6	Torreyol	0.92
7	Elemol	0.98
8	Sesquilavundulol	1.22
9	Germacrene D-4-ol	0.68
10	Cedrol	0.15
11	Nopol	0.33
12	**1-Benzyl-3-pyrroline**	**13.42**
13	Kauran-8-ol	8.92
14	Longipinanol	0.22
15	4,6-Heptadien-3-one	0.84
16	Catalponol	0.25
17	Unidentified constituents	43.22

Table 3.4: Constituents analysed by GC-MS from the fractions 31-40

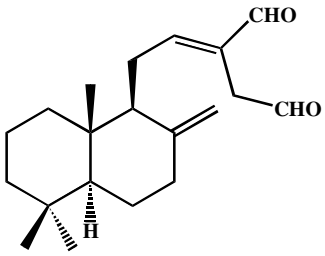
No.	Compound	Percentage (%)
1	Germacrene D -4-ol	0.65
2	Selina-11-en-4-alpha-ol	0.68
3	Eudesmol	4.11
4	Sesquilavundulol	1.22
5	Sesquilavundulol acetate	0.13
6	Longiborneol	0.21
7	Bergamatol	0.06
8	Longifolol	0.18
9	Caryophyllene	0.14
10	2-Phenanthrene amine	0.43
11	Thujopsene	0.39
12	Manool	0.12
13	Thujapsanone	0.34
14	7-Phenyl-4-*trans*-heptenone	5.96

Table 3.4 continued…

15	**(*E*)-labda-8(17),12-diene-15,16-dial**	**83**
16	Sandaracopimaradiene	0.20
17	Caryophyllene oxide	0.16
18	4,6-Heptadiene-3-one	0.51
19	Thujopsadiene	0.22

The fourth fraction (41-61; 1.6 g) upon flash column chromatography using prepacked column employing hexane as eluent yielded 200 mg of compound **V** as an yellow oil in pure form. TLC showed it to be UV active. The IR spectrum of compound **V** showed strong absorptions at 1728 and 1683 cm^{-1} suggesting the presence of both carbonyl and α,β-unsaturated carbonyl group. Absorptions at 2931, 1644 and 889 cm^{-1} indicated the presence of an exomethylene group. The ^{1}H NMR spectrum (Figure 3.2) indicated the presence of two aldehydic groups from the singlets present at δ 9.64 and 9.41. Another signal appearing as a triplet centered at δ 6.77 could be attributed to olefinic proton. Two doublets each integrating for one proton at δ 4.86 and 4.37 with a *J* value of 1.0 Hz indicated the presence of exomethylene protons. The presence of three quaternary methyl groups could be surmised from the singlets integrating for three protons each at δ 0.89, 0.82 and 0.73. The ^{13}C NMR spectrum (Figure 3.3) also supported the presence of two aldehydic groups from the signals at δ 197.3 and 193.5 and the signals for the exocyclic olefinic group through the signals at δ 148.0 and 107.8. The rapidly gleaned information from the ^{13}C NMR spectrum was the presence of twenty carbons indicating the compound to be a diterpene. The mass spectrum gave base peak at m/z 302. Further, the mass spectrum also gave a characteristic base peak at m/z 137 supportive of the labdane type skeleton. From all the above spectral details, compound **V** was deduced as (*E*)-labda-8(17),12-diene-15,16-dial shown below.[13] This is a cytotoxic, antifungal diterpene, first reported from *A. speciosa*[13] and later from *A. galanga*,[14] *A. chinensis*,[15] A. *katsumadai*[16] and *A.*

malaccensis.[17] However, this is the first report of its presence in the rhizomes of *A. calcarata*.



V

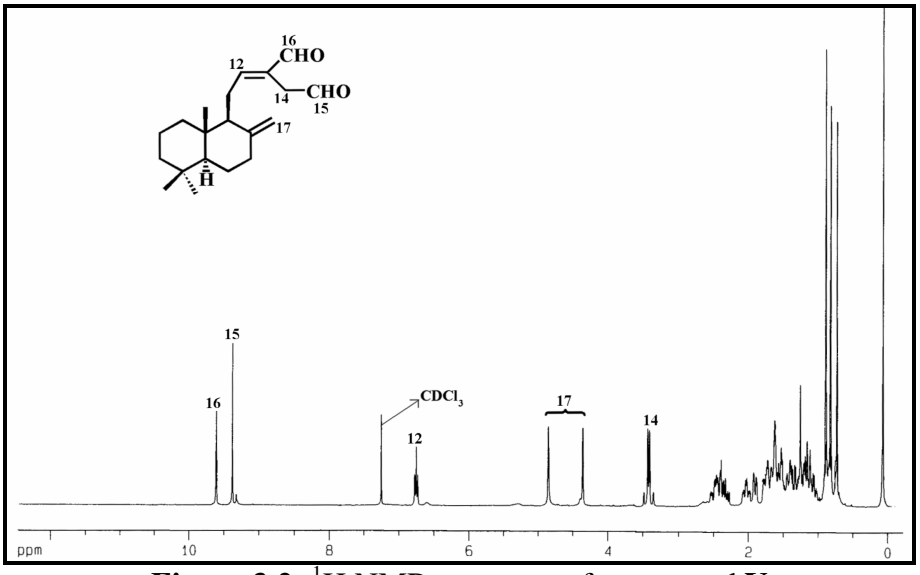


Figure 3.2: ^{1}H NMR spectrum of compound **V**

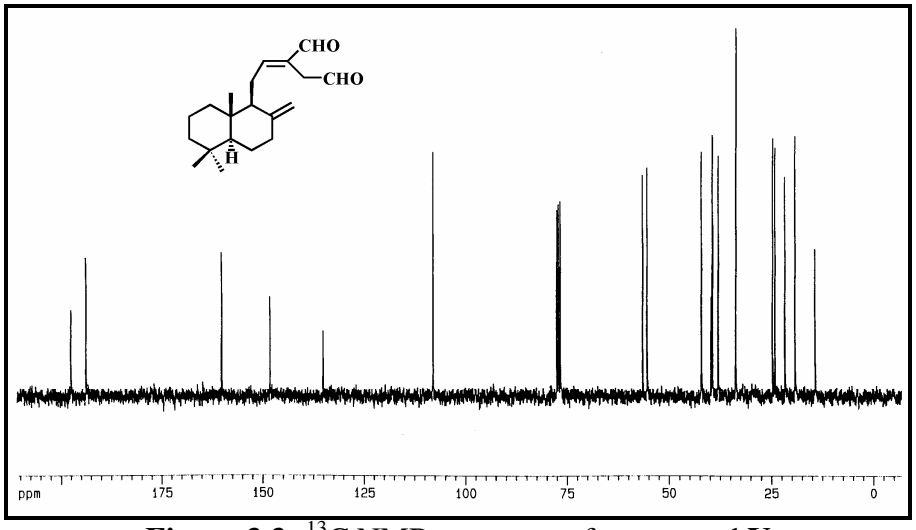


Figure 3.3: ^{13}C NMR spectrum of compound **V**

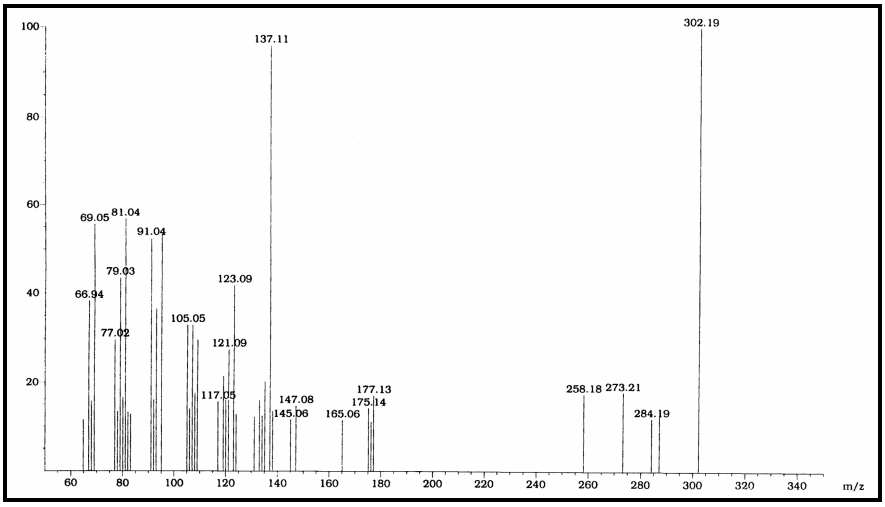


Figure 3.4: Mass spectrum of compound **V**

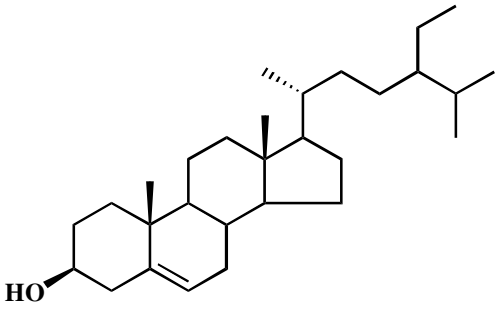
The fifth and sixth fractions were found to contain many polar constituents upon examination by TLC from which components could not be isolated in pure form. In order to isolate and characterize the polar constituents in the extract of *A. calcarata* rhizomes, a second extraction of the rhizome was set up on a large scale to get more of the polar compounds.

3.3.3 Isolation of compounds from the acetone extract

Fresh rhizomes of *A. calcarata* (4 kg) were collected, washed, dried and powdered. The air dried powdered rhizomes (1.4 kg) of *A. calcarata* were extracted with acetone at room temperature. The solvent on evaporation under reduced pressure yielded 72.5 g of the extract. Since several less polar compounds have already been isolated from *A. calcarata*,[6] and our previous extraction has revealed the presence of several less polar constituents, the present study was aimed at the more polar constituents. Therefore, the acetone extract was washed repeatedly with hexane (3 x 200 ml) to remove the hexane soluble less polar part. The hexane soluble part on solvent evaporation under reduced pressure yielded 25.2 g of the hexane extract. The hexane extract on evaluation by TLC revealed the presence of dialdehyde (**V**) as the major component along with other less polar compounds. The residue (defatted extract) was dried, weighed (47.3 g) and subjected to further analysis. From

this 500 mg of the extract was kept aside for antioxidant studies. Thus, 46.8 g of the residue was then subjected to careful column chromatography using silica gel (500 g, 100-200 mesh) starting with hexane as eluent and thereafter gradually raising the polarities depending on the separation obtained after examining the fractions by TLC. A total of 370 fractions of 80-90 ml each were collected. They were further pooled together according to similarities in TLC into fifteen fraction pools.

The fourth fraction pool (64-105; 2.09 g) on evaluation showed two major spots in TLC. This mixture was very carefully separated by column chromatography with hexane:ethylacetate (99:1 – 97:3) as the eluent, afforded forty fractions which were again pooled further into four fraction pools *viz.*, 4A, 4B, 4C and 4D based on TLC. Fraction 4C (281 mg) was further purified by flash column chromatography, eluted with 1% ethyl acetate-hexane mixture which gave 70 mg of white crystalline compound **VI** (m.p: 136 – 137 °C). The IR, ^{1}H NMR (Figure 3.5) and ^{13}C NMR (Figure 3.6) spectral details of this compound matched with that of β-sitosterol. Hence compound **VI** is assigned the structure of β-sitosterol as given below. The compound was checked by co-spot with standard also. β-Sitosterol is being isolated for the first time from *A. calcarata* even though it has been previously isolated from many *Alpinia* species[18] and is a common secondary metabolite found in most plants.



VI

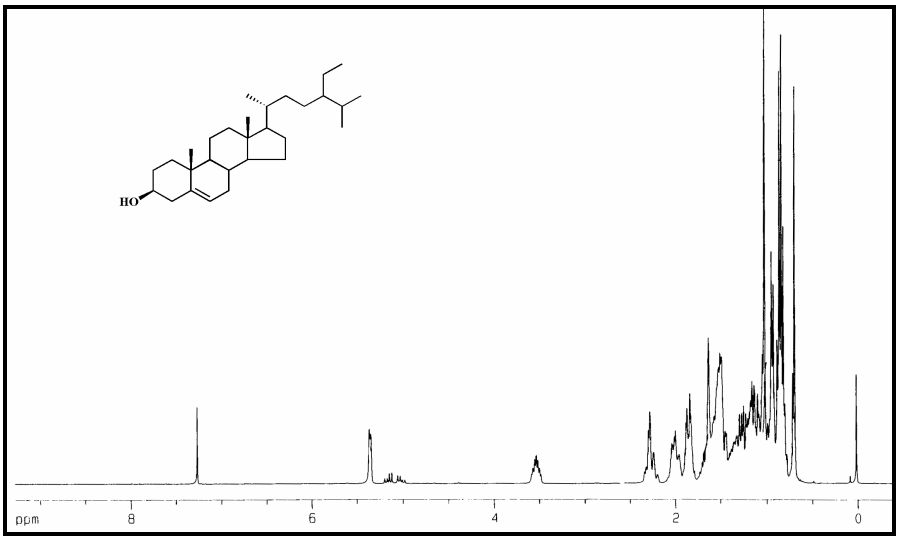


Figure 3.5: ^{1}H NMR spectrum of compound **VI**

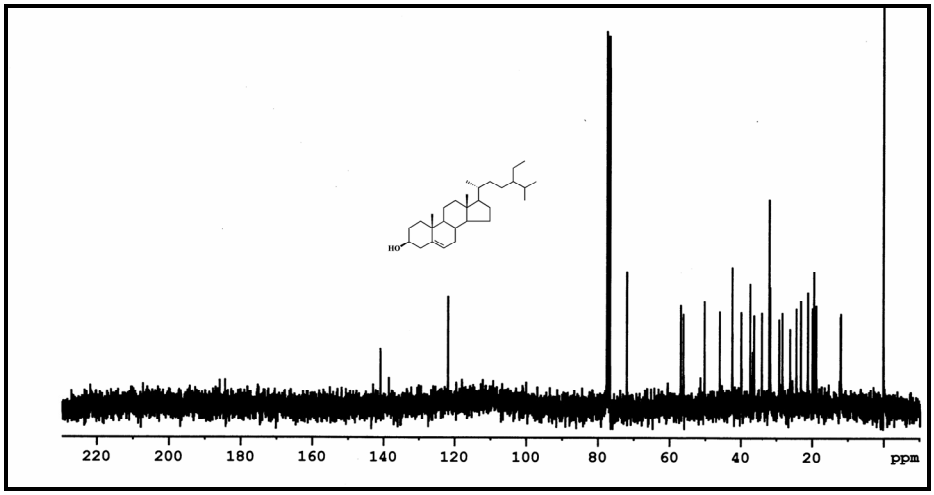


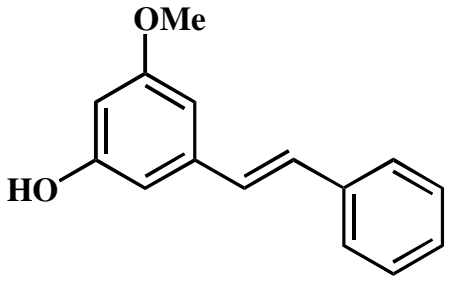
Figure 3.6: ^{13}C NMR spectrum of compound **VI**

Fraction 4D (134 mg) was found to contain a major spot on examination by TLC. It was further purified using flash column chromatography employing 3% ethyl acetate-hexane mixture as eluent to give 16 mg of pure compound **VII** as light yellow thick liquid. The IR spectrum

indicated the presence of hydroxyl group in compound **VII** from the absorption at 3392 cm^{-1}.

The ^{1}H NMR spectrum of compound **VII** (Figure 3.7) indicated the presence of an –OMe group which gave a singlet at δ 3.82 integrating for three protons. A doublet of a doublet for two protons between δ 7.09 and 6.96 with a *J* value of 16.3 Hz could be attributed to *trans* olefinic protons. The ^{1}H NMR spectrum also indicated the presence of two aromatic groups, one unsubstituted (five aromatic protons, δ 7.51-7.24) and the other a 3,5 disubstituted one (three aromatic protons, δ 6.65, 6.61 and 6.34), thus suggesting that compound **VII** could be a *trans* stilbene with substitution in one of the aromatic rings. The ^{13}C NMR spectrum (Figure 3.8) also confirmed the presence of an –OMe group (δ 55.4), olefinic (δ 129.4 and 128.3) and the aromatic carbons.

The mass spectrum (Figure 3.9) gave the base peak at m/z 226. From all the above spectral data and by comparing the values with those in the literature, the structure of compound **VII** was confirmed as that of 3-methoxy-5-hydroxy stilbene which is isolated from *A. calcarata* for the first time and has been previously isolated from *A. katsumadai*.[16] The structure of compound **VII** is shown below.



VII

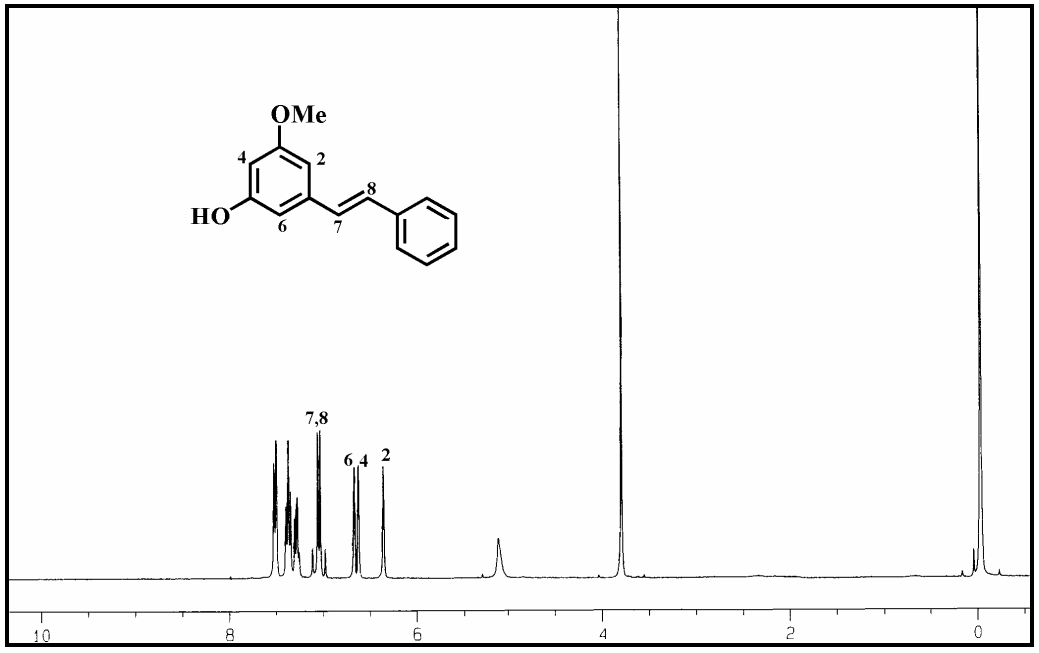


Figure 3.7: ^{1}H NMR spectrum of compound **VII**

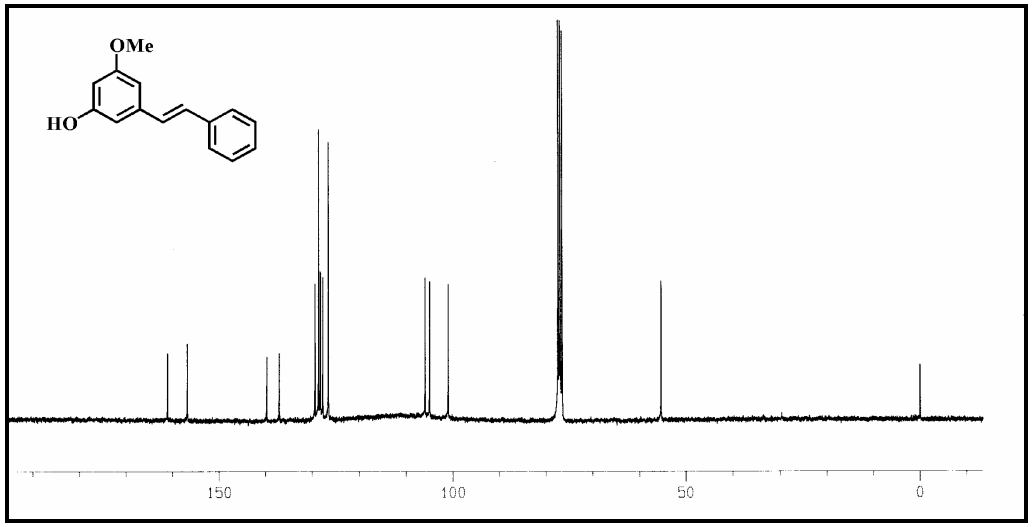


Figure 3.8: ^{13}C NMR spectrum of compound **VII**

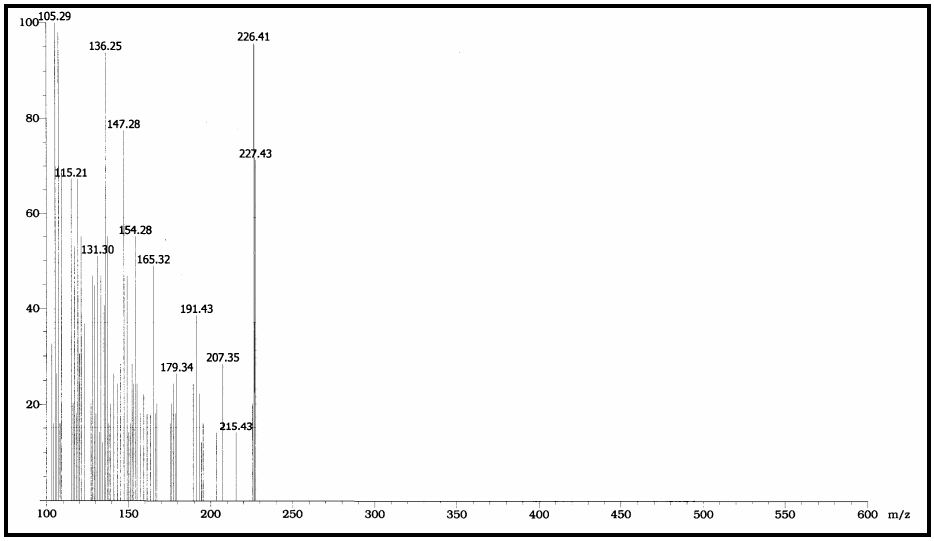
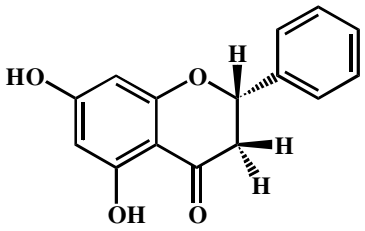


Figure 3.9: Mass spectrum of compound **VII**

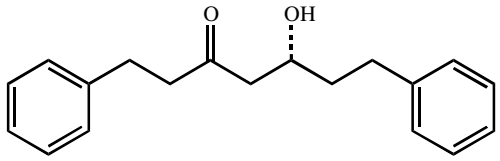
The fifth fraction pool (106-113; 2.4 g) upon TLC showed the presence of a major UV active component. This fraction on further column chromatographic separation with 95:5 hexane:ethyl acetate as the eluent, afforded compound **VIII** as off white needles (951 mg; m.p: 206-207 °C). It was analyzed by various spectroscopic techniques and by co-TLC, and found that compound **VIII** was pinocembrin, previously isolated by us from *A. galanga* (same as compound **I**) and whose spectral details are discussed in chapter 2 of this thesis.



VIII

The sixth pool of fractions (114-149; 3.1 g) was rechromatographed on silica gel using hexane-ethyl acetate mixtures (95:5-80:20) as the eluent which afforded compounds **IX**, **X**, **XI** and **XII** in their pure form. Compound **IX** (48 mg, m.p: 52-53 °C) which was obtained as colorless needles upon

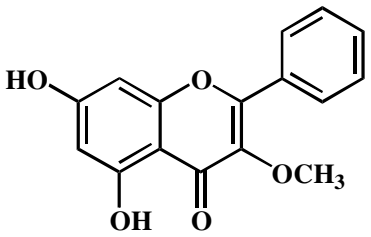
spectral analysis and identification by TLC, was found to be 1,7-Diphenyl-5-hydroxy-3-heptanone called also as dihydroyashabushiketol (same as compound **II**, shown in Chapter II) earlier obtained from *A. galanga*.



IX

Compound **X** was obtained as light yellow amorphous solid (224.3 mg; 288-291 °C). The structure of compound **X** was assigned based on spectroscopic evidences such as UV-vis, ^{1}H NMR, ^{13}C NMR and mass spectral details. Alcoholic solution of compound **X** gave positive results towards Shinoda test and imparted green coloration towards ferric chloride solution indicating **X** to be a flavonoid derivative. The UV spectrum of compound **X** (Figure 3.10) showed absorption maximum at 266 nm (band II) and two shoulders at 312 and 340 nm. The IR spectrum showed peaks corresponding to >C=O group at 1647 cm^{-1} and hydroxyl groups at 3155 cm^{-1}. The ^{1}H NMR spectrum (Figure 3.11) indicated the presence of an –OMe group from the sharp singlet integrating for three protons at δ 3.83. The five aromatic protons due to the free rotation of the B-ring of the flavonoid derivative appeared as two pairs of *ortho* coupled doublets integrating for two and three protons in the range δ 8.07-7.52 downfield to the A-ring protons indicating a substituent free B-ring. Two upfield doublets (J = 1.9 Hz) at δ 6.43 and δ 6.29 respectively indicated the A-ring protons of a 5,7-Dihydroxy flavonol. The peak for the 5-OH group was observed at δ 12.16 indicating a substitution on the 3-OH group. The ^{13}C NMR spectrum (Figure 3.12) also confirmed the presence of an –OMe group from the signal at δ 60.2. Furthermore, it showed 16 carbon signals, including the characteristic signal of >C=O group at δ 178.5. The mass spectrum of compound **X** (Figure 3.13) gave the base peak at m/z 285 which is the $[M+1]^{+}$ peak. Further the peaks at

$[M\text{-}CH_3]^+$ at m/z 270 and the peak at m/z 105 (B^+) confirmed a flavonol structure. From all the above spectral data, compound **X** was assigned to be the 3-methyl ether of galangin which was further confirmed from the UV-vis spectra reported for galangin 3-methyl ether (266, 312sh, 340sh) isolated earlier from *A. officinarum*.[19] This is the first report of its presence in *A. calcarata*. The structure of compound **X**, galangin-3-methyl ether is as shown.



X

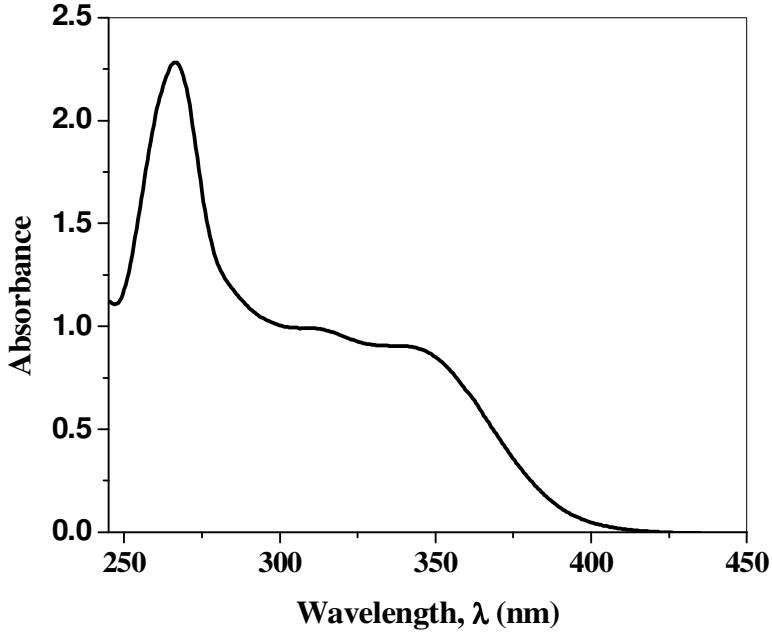


Figure 3.10: UV spectrum of compound X

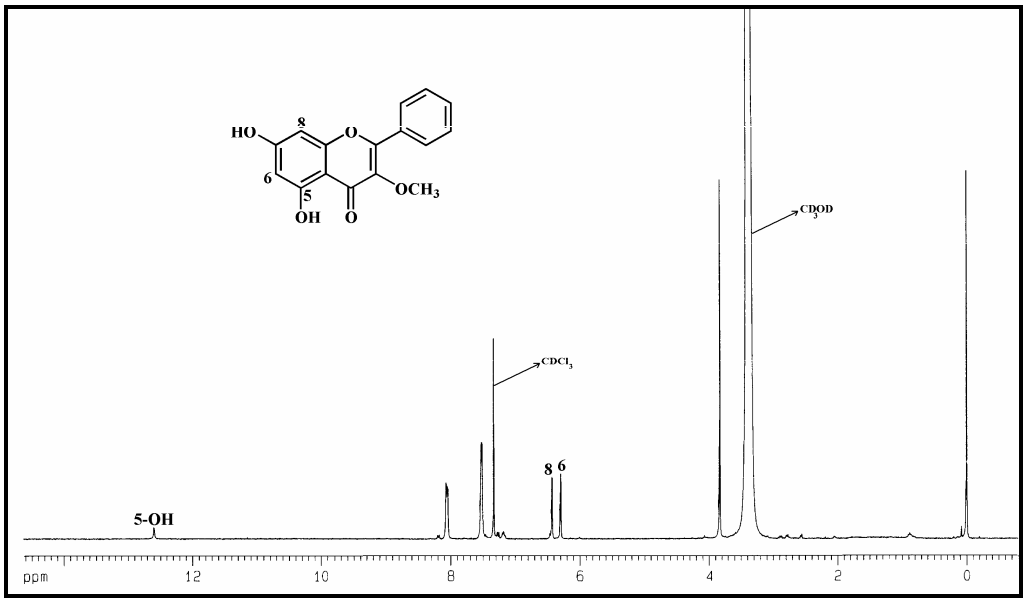


Figure 3.11: ^{1}H NMR spectrum of compound **X**

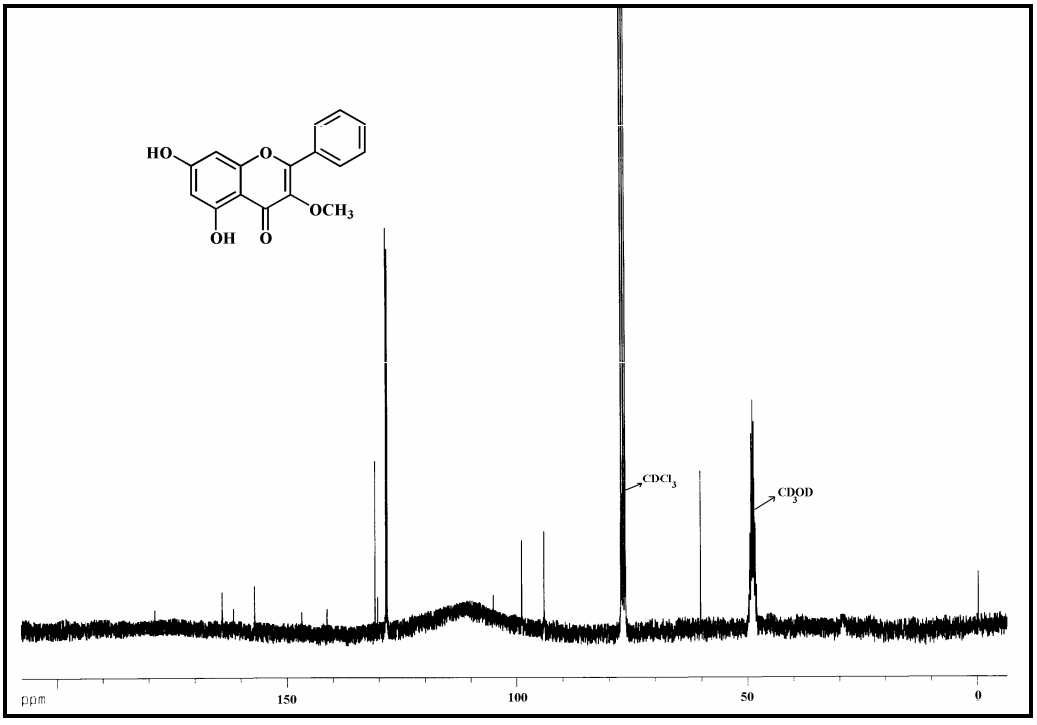


Figure 3.12: ^{13}C NMR spectrum of compound **X**

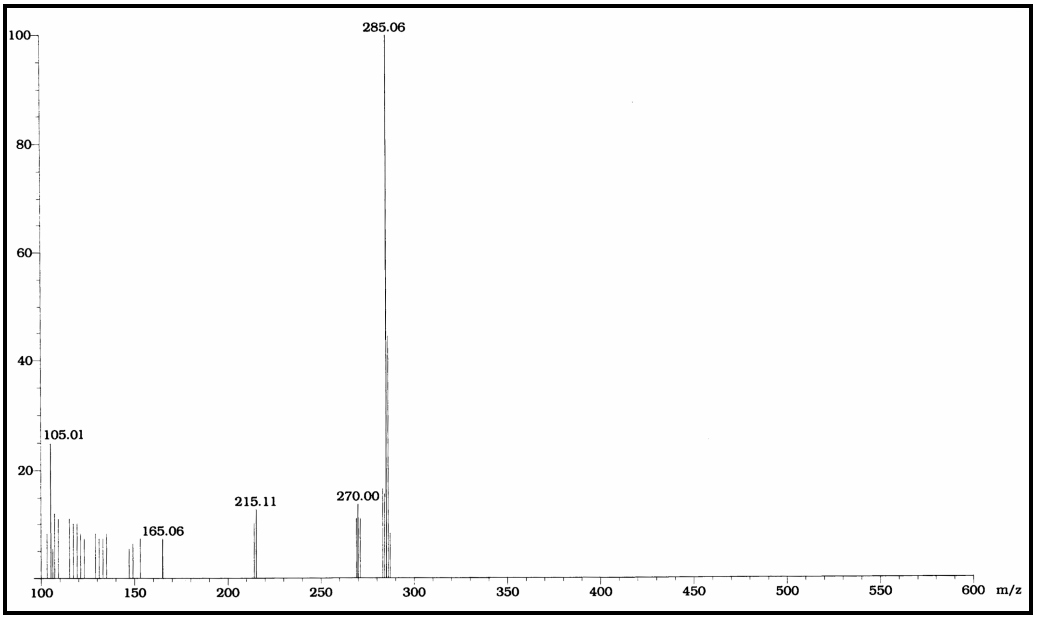
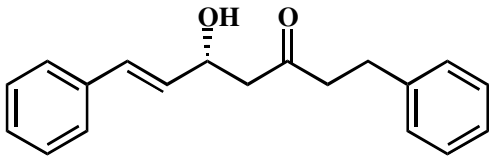


Figure 3.13: Mass spectrum of compound **X**

Compound **XI** was obtained as yellow needles (32 mg; m. p: 58-60 °C). The IR spectrum of compound **XI** indicated the presence of a saturated ketone from the absorbance at 1705 cm^{-1}. The ^{1}H NMR spectrum of compound **XI** (Figure 3.14) indicated the presence of two phenyl groups from the multiplet integrating for ten protons between δ 7.38-7.16. The presence of a pair of *trans* olefinic protons could be surmised from the two signals at δ 6.62 and 6.18 integrating for one proton each with a *J* value of 15.9 Hz. The signal at δ 6.62 was due to the olefinic proton near to the phenyl group and the signal at δ 6.18 arised due to the olefinic proton near the hydroxyl group. This signal appeared as a doublet of a doublet as it was coupled with the proton of the hydroxylated methine which appeared at δ 4.75. This methine signal appeared as a multiplet due to its coupling with the nearby four protons. Two triplets integrating for two protons each (*J* = 7.2 Hz) at δ 2.92 and 2.80 could be attributed to two methylene groups. The ^{13}C NMR spectrum (Figure 3.15 A) indicated the presence of three methylenic carbons

from the signals at δ 29.4, 45.1 and 49.3. The signal at δ 68.5 could be attributed to the hydroxylated methine and the carbonyl carbon could be confirmed from the signal at δ 210.1. The olefinic carbons gave signals at δ 130.4 and 130.1. The DEPT-135 NMR spectrum (Figure 3.15 B) gave signals for three -CH_2- groups in the aliphatic region. In the mass spectrum (Figure 3.16), the molecular ion peak at 280.9 further confirmed that compound **XI** is the diarylheptanoid, 1,7-diphenyl-5-hydroxy-6-heptene-3-one as shown below.



XI

Compound **XI**, 1,7-diphenyl-5-hydroxy-6-heptene-3-one was earlier isolated from *A. katsumadai*,[20] *A. rafflesiana*[21] and also from *A. nutans*.[22]

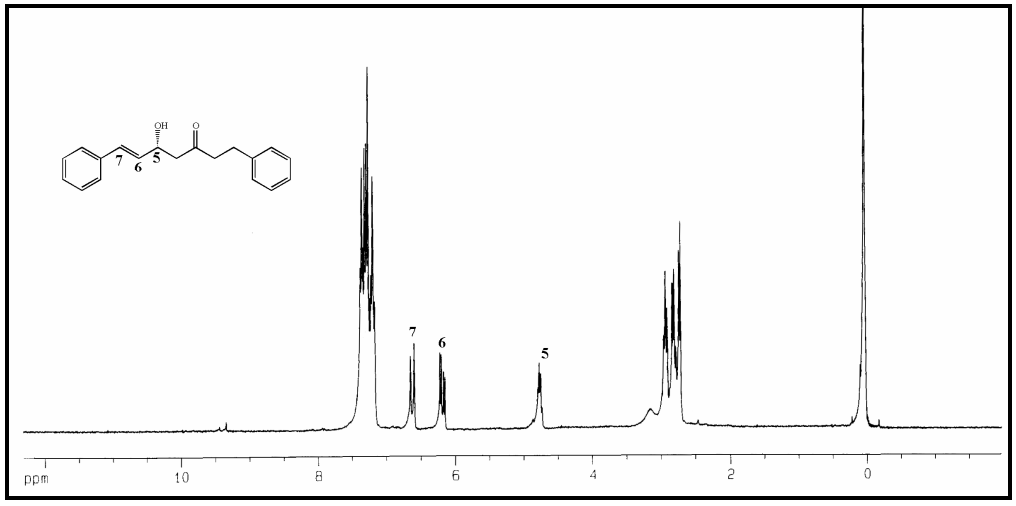


Figure 3.14: ^{1}H NMR spectrum of compound **XI**

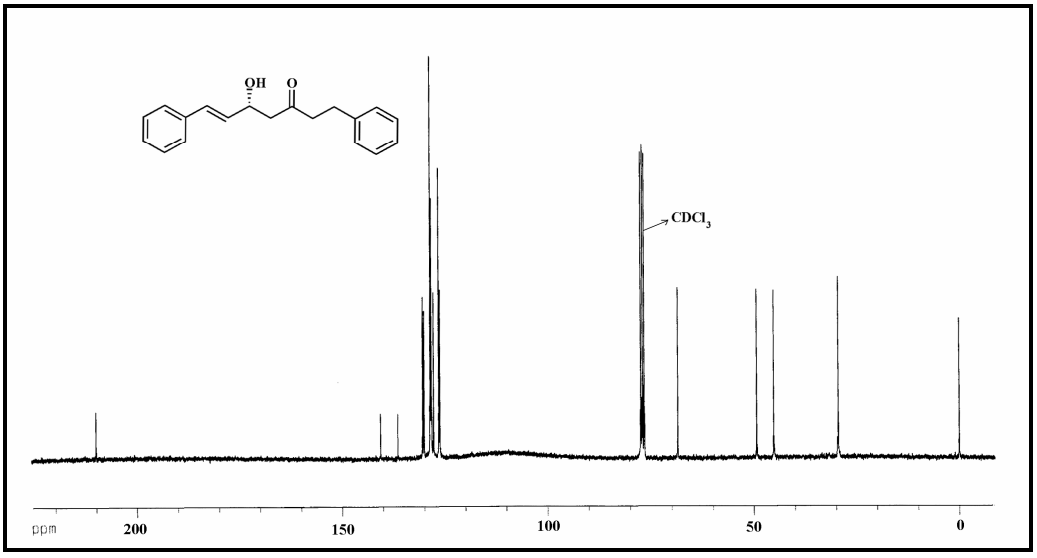


Figure 3.15 A: ^{13}C NMR spectrum of compound **XI**

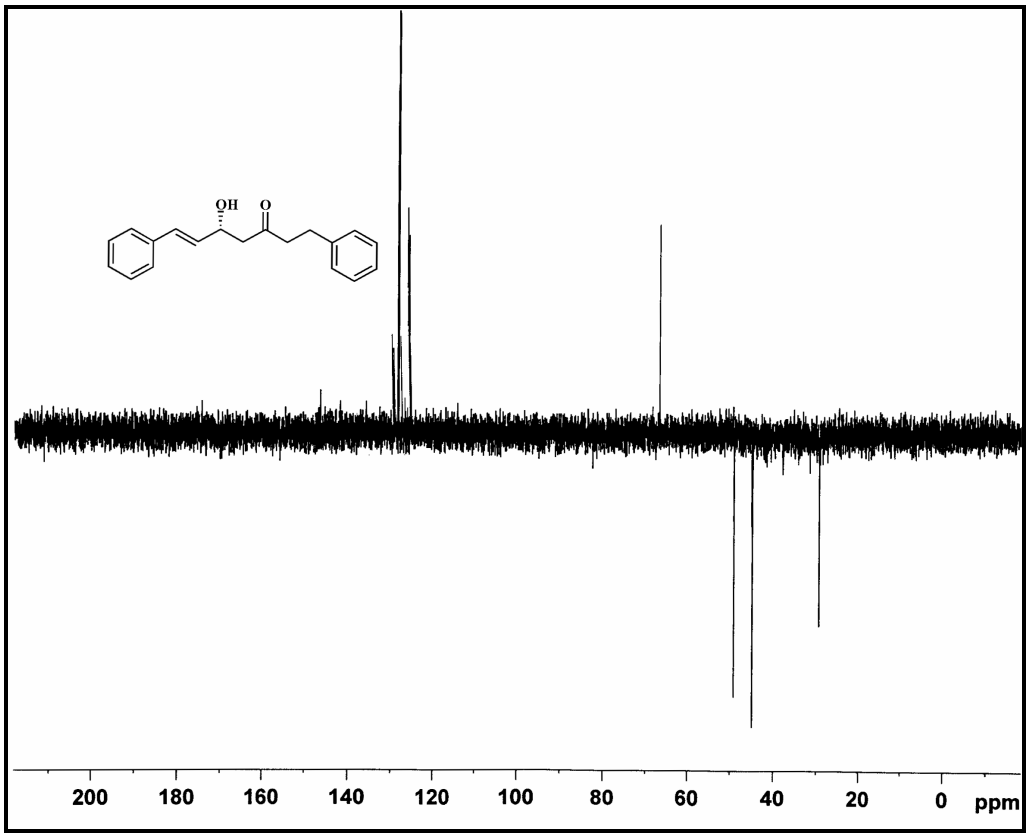


Figure 3.15 B: DEPT-135 NMR spectrum of compound **XI**

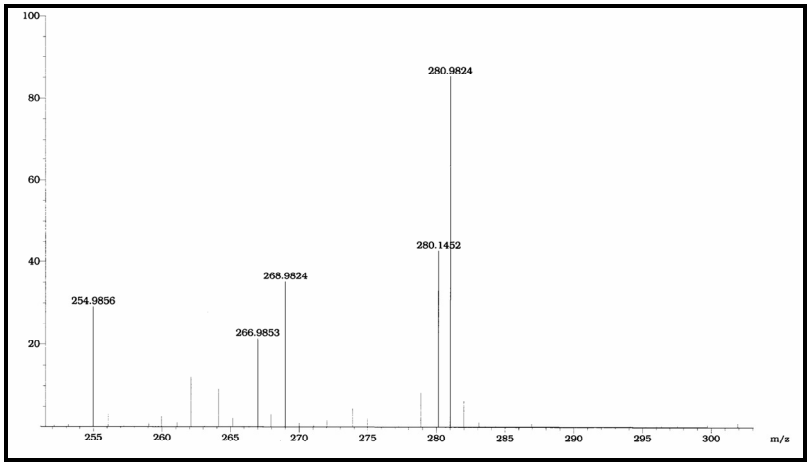
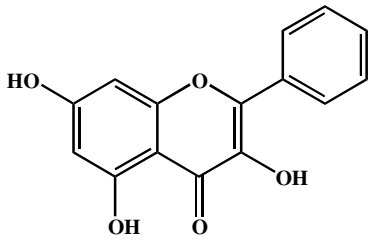


Figure 3.16: Mass spectrum of compound **XI**

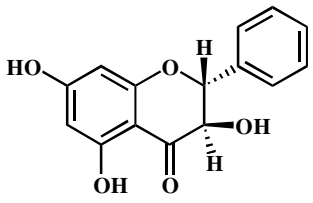
Compound **XII** was obtained as yellow crystals (30.5 mg; m.p. 214-215 °C). Upon detailed spectral analysis and on analysis by TLC, compound **XII** was found to be the 5,7-Dihydroxy flavanone commonly known as galangin (same as compound **III**, chapter 2) isolated for the first time from *A. calcarata*, eventhough other *Alpinia* species such as *A. officinarum*[23] and *A. conchigera*[24] were found to contain galangin as one of the constituents.



XII

The seventh pool of fractions (150-190; 941.6 mg) upon column chromatographic separation with hexane-ethyl acetate (90:10-70:30) as eluent yielded 42.9 mg of pure compound **XIII** (colourless needles crystallised from DCM-hexane mixture, m.p: 173.5-174.5 °C) which was identified to be a flavonoid derivative from the positive results on colour reactions (Shinoda and ferric chloride tests) and from the UV spectrum (Figure 3.17). The UV spectrum showed a low intensity band I absorption at 330 nm and band II at 290 nm. IR spectrum showed –OH absorption at 3566 cm^{-1} and >C=O

absorption at 1610 cm^{-1}. In the ^{1}H NMR spectrum (Figure 3.18) the doublets at δ 5.16 and 4.64 integrating for one proton each with a *J* value of 11.2 Hz indicated the protons at C-2 and C-3 positions respectively of a dihydroflavonol that are *trans* diaxially related to each other. The H-2 signal at δ 5.16 is well separated downfield from the H-3 signal which is at δ 4.64. The *ortho* coupled doublets for five protons between δ 7.59 and 7.37 indicated the unsubstituted B-ring aromatic protons. The protons at the 6^{th} and 8^{th} positions appeared separately as doublets (*J* = 2.0 Hz) at δ 5.95 and δ 5.99 respectively indicating that compound **XIII** is a 5,7-dihydroxydihydroflavonol. The peak for the 5-OH group at δ 11.7 indicated that the 3-OH group is in a different plane, resulting in effective hydrogen bonding between the carbonyl group and the 5-OH group. Further confirmation of the structure was gathered from the ^{13}C NMR spectrum (Figure 3.19) which gave signals at δ 84.6 and 73.4 for the C-2 and C-3 carbons respectively. It showed 15 carbon signals, including the characteristic signal of >C=O group at δ 198.1 apart from the other characteristic signals. In the mass spectrum, (Figure 3.20), **XIII** typically fragmented as a dihydroflavanol by the Retro Diels Alder (RDA) reaction to give m/z peaks that correspond to $[M+H]^{+}$ and the RDA product at 273 and 153 respectively. Based on all the spectral data and comparing the values with those reported in the literature[25], the structure of compound **XIII** was confirmed as the 5,7-Dihydroxydihydroflavanol commonly known as pinobanksin, previously isolated from *A. officinarum.*[25] The structure of pinobanksin is as shown below.



XIII

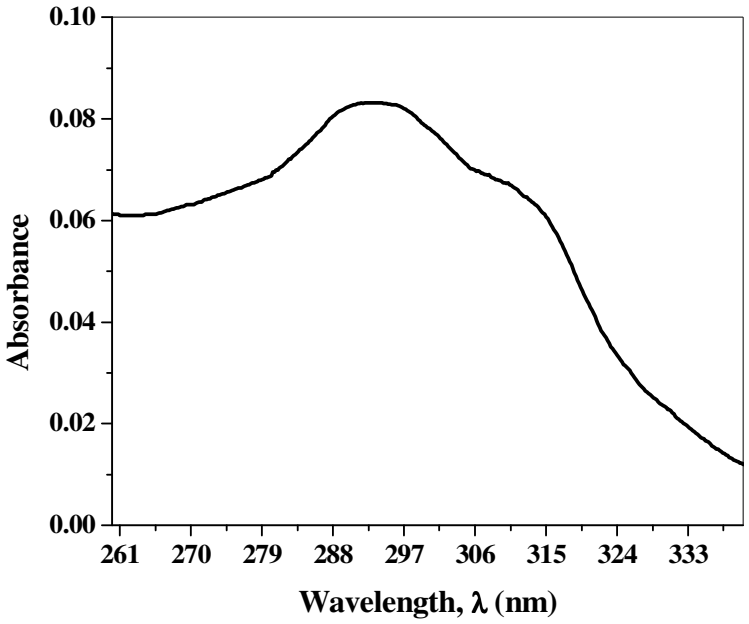


Figure 3.17: UV spectrum of compound XIII

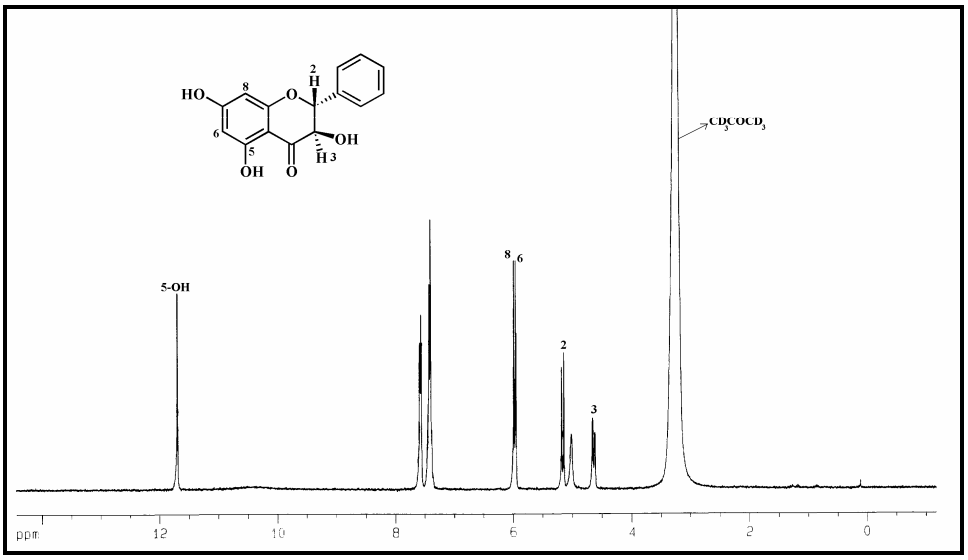


Figure 3.18: ^{1}H NMR spectrum of compound XIII

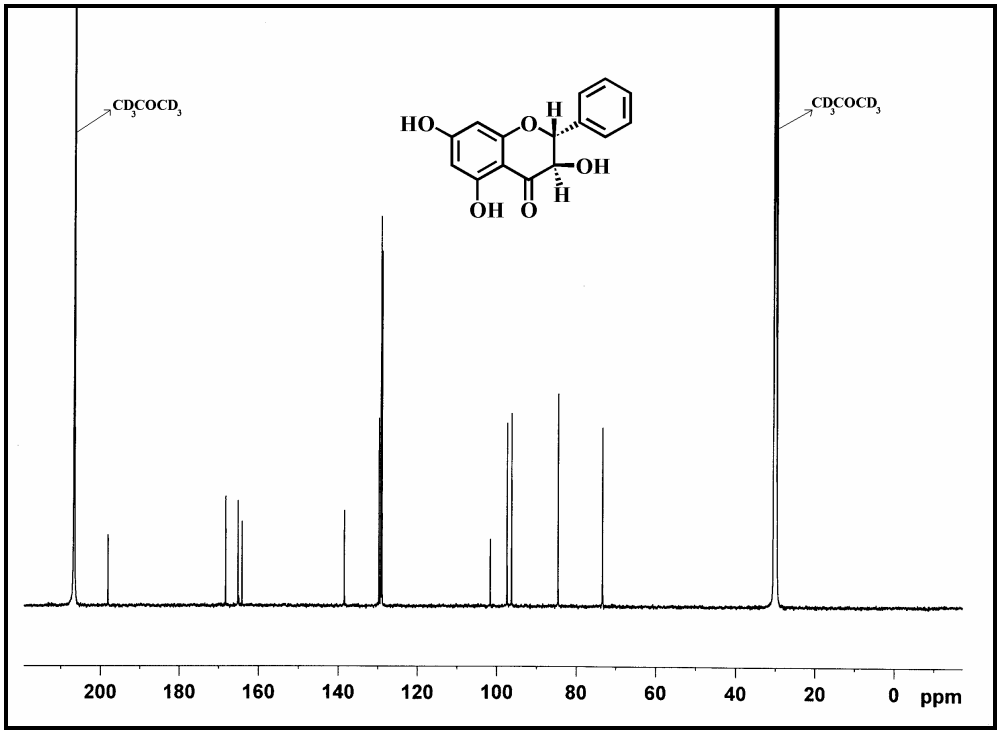


Figure 3.19: ^{13}C NMR spectrum of compound XIII

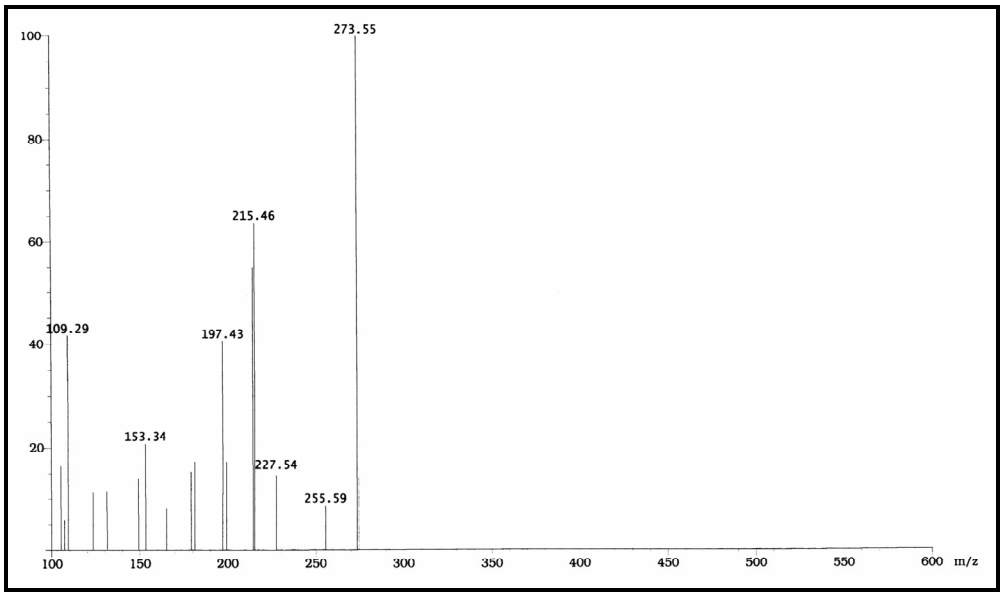
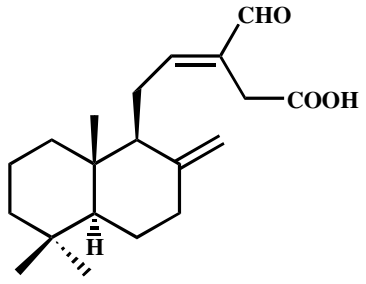


Figure 3.20: Mass spectrum of compound XIII

The eighth fraction pool (191-235; 1.3 g) upon column chromatography on silica gel employing hexane-ethyl acetate mixtures of increasing polarity (70:30 – 50:50) as eluent yielded 145 mg of compound **XIV** as an yellow oil. The IR spectrum of compound **XIV** showed the presence of an α,β-unsaturated aldehyde (1688 cm^{-1}) and a carboxylic acid group (1695 cm^{-1}). Absorptions at 3080, 1640 and 890 cm^{-1} indicated the presence of exomethylene group. The ^{1}H NMR spectrum (Figure 3.21) was characteristic of a labdane type diterpene. The three quaternary methyl group singlets integrating for three protons each appeared at δ 0.75, 0.83 and 0.89. The exomethylene group could be surmised from the doublets for one proton each centered at δ 4.86 and 4.39 each with a *J* value of 1 Hz. The ^{1}H NMR spectrum also showed the signals due to an olefinic proton at δ 6.70 and an allylic methylene group adjacent to a carboxylic acid as a doublet of doublet centered at δ 3.38. The signal at δ 9.38 could be attributed to the aldehydic group. The ^{13}C NMR (Figure 3.22) spectral data except for the carbonyl signal at δ 175.2 was very similar to that of compound **V**, the (*E*)-labda-8(17),12-diene-15,16-dial isolated from the first fractions. The mass spectrum (Figure 3.23) gave the molecular ion peak as the base peak at m/z 318. From all the spectral data and by comparing with the literature values, compound **XIV** was assigned the structure of the labdane diterpene (*E*)-labda-8(17),12-diene-15-ol,16-al, commonly known as Zerumin A whose structure is as shown and has been earlier reported from this species by Kong *et al.*[6a]



XIV

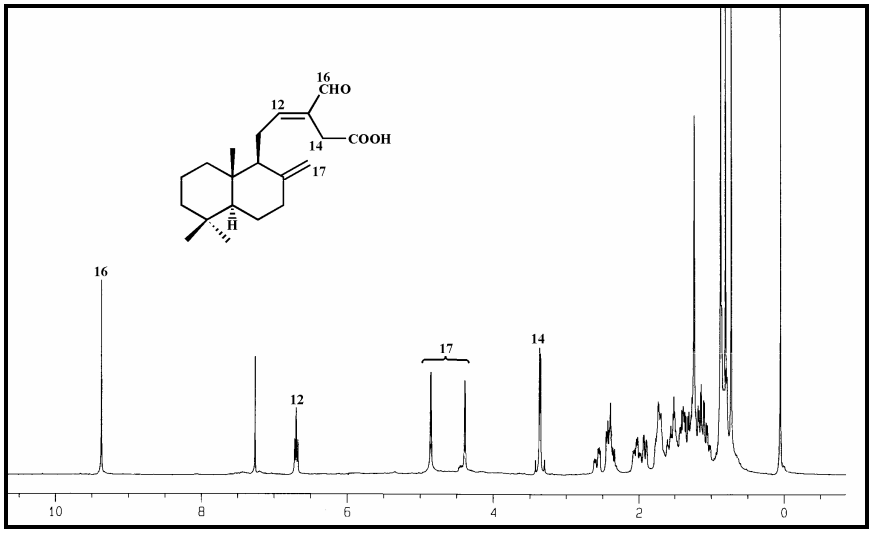


Figure 3.21: ^{1}H NMR spectrum of compound **XIV**

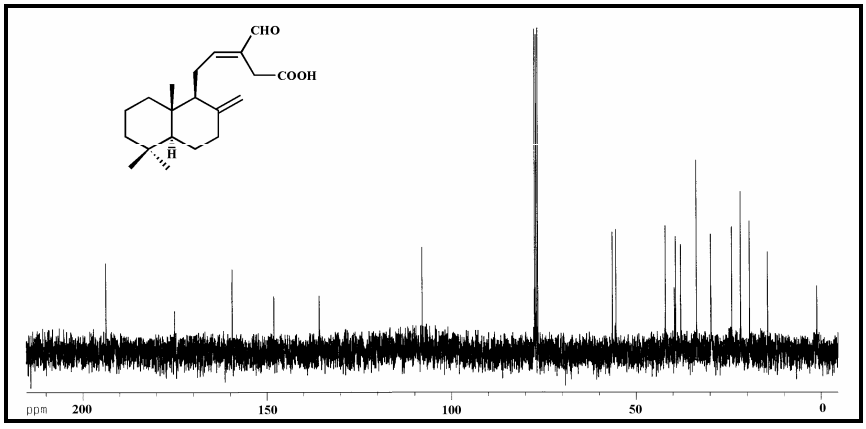


Figure 3.22: ^{13}C NMR spectrum of compound **XIV**

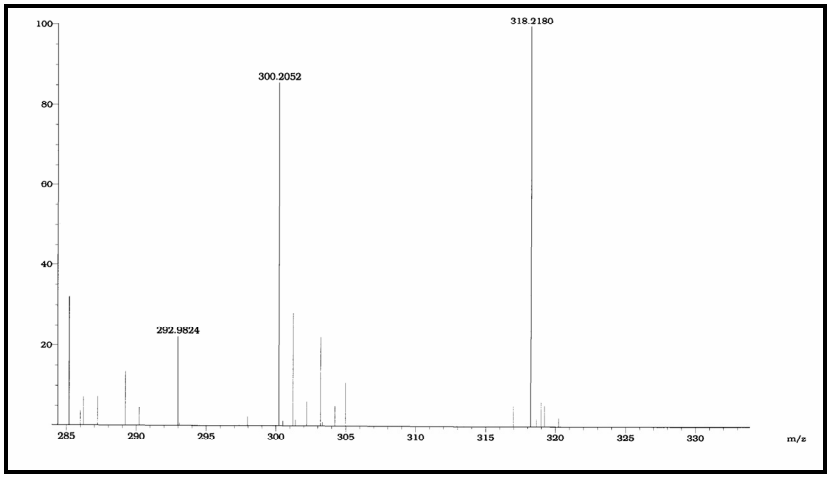


Figure 3.23: Mass spectrum of compound **XIV**

Among the compounds **V**-**XIV** isolated by us, only Zerumin A has been previously isolated from the rhizomes of *A. calcarata*. It is of interest to note that major compound isolated from the rhizomes of *A. calcarata* is the 5,7-dihydroxyflavanone-pinocembrin, whose role as an apoptosis inducer in human colon cancer cells has been discovered and is discussed in Chapter 2.

3.4 ANTIOXIDANT PROPERTIES OF *ALPINIA CALCARATA* AND THE MAJOR COMPOUNDS ISOLATED FROM IT

The defatted acetone extract, 500 mg (obtained as per procedure section 3.3.3) termed as ACA was used for the antioxidant study. Similarly 50 g of the dry rhizomes was extracted with 200 ml ethanol for 72 h at 27 °C. The solvent was removed in vacuum at 50 °C to give dark gummy residue of total ethanol extract (7.2 g; termed as ACE). Both ACA and ACE were used for antioxidant evaluation using different assays along with the pure flavonoids and *trans* stilbene isolated from the rhizomes of *A. calcarata*. Among the flavonoids, galangin was not tested here, as its antioxidant potential is already discussed in chapter 2.

3.4.1 Total antioxidant capacity, total flavonoid content and total phenolic content of ACA and ACE

The total antioxidant capacity (TAC) of the defatted acetone and ethanol extracts expressed in Ascorbic acid equivalents (*AAE*) are presented in Table 3.5. *AAE* varied from 3.5 ± 1.3 g *AAE*/100 g dry rhizomes in the ethanol extract to 9.2 ± 1.8 g *AAE*/100 g dry rhizomes in the defatted acetone extract. Comparing both the extracts, the defatted acetone extract showed higher *AAE* than the total ethanol extract. The total flavonoid content and the total phenolic content in both the extracts were determined and are also shown in Table 3.5. The total flavonoid content is expressed as quercetin equivalents

(*QE*) whereas the total phenolic content is expressed as gallic acid equivalents (*GAE*) per 100 g of the dry rhizomes.

Phenolic compounds like flavonoids protect human body from harmful free radicals, whose formation is associated with the normal natural metabolism in aerobic cells.[26a] The antiradical activity of phenolics and flavonoids depend mainly on their chemical structure.[26b] Natural polyphenols, depending on the structural characteristics are capable of destroying free radicals, chelate metal catalysts; activate the enzymes responsible for antioxidant capacity etc.[26c] Here, the total phenolic content of the extracts studied varied from 0.5 ± 0.03 g *GAE*/100 g dry rhizomes for the total ethanol extract to 1.9 ± 0.7 g *GAE*/ 100 g dry rhizomes for the defatted acetone extract. This indicated that most of the phenolics in the rhizomes of *A. calcarata* were extracted with acetone and could be concentrated by the defatting process. The total phenolic content in both the extracts (*viz*., total ethanol extract, ACE and the defatted acetone extract, ACA) were higher than their corresponding total flavonoid content. The total flavonoid content of both the extracts ranged from 0.26 ± 0.05 g *QE* to 0.83 ± 0.04 g *QE* per 100 g dry rhizomes respectively. The flavonoid content was also higher in the defatted acetone extract (ACA) than the total ethanol extract (ACE). Therefore it is obvious from Table 3.5 that the antioxidant phytoconstituents were higher in the defatted acetone extract (ACA). This higher phenolic and flavonoid content in the defatted acetone extract point to the possibility that these phenolics and flavonoids have contributed much to the higher total antioxidant capacity. The decreased activity of the total ethanol extract may be because of the lower percentage composition of phenolics and flavonoids in it as the extract contained the essential oil, terpenes and some starch too.

Table 3.5: Total antioxidant capacity, total flavonoid content and total phenolic content of *A. calcarata* rhizomes

Sample	TAC[a] ± SD[d]	TFC[b] ± SD[d]	TPC[c] ± SD[d]
ACE	3.5 ± 1.3	0.26 ±0.05	0.5 ± 0.03
ACA	9.2 ± 1.8	0.83 ± 0.04	1.9 ± 0.7

[a]Total antioxidant capacity expressed in grams of Ascorbic acid equivalents/100 g of dry rhizomes
[b]Total flavonoid content expressed in grams of Quercetin equivalents/100 g of dry rhizomes
[c]Total phenolic content expressed in grams of Gallic acid equivalents/100 g of dry rhizomes
[d]Values given as mean ± standard deviation (n = 3)

3.4.2 DPPH$^{\cdot}$ radical scavenging capacity

ACE, ACA and the flavonoids galangin-3-methylether and pinobanksin were initially tested for antioxidant capacity using the diphenyl picryl hydrazyl (DPPH$^{\cdot}$) radical[27] and the results show moderate radical scavenging capacity for the extracts and the isolates. Among the extracts, at a concentration of 1000 ppm, the defatted acetone extract, ACA (65 %) showed slightly higher scavenging property than the ethanol extract, ACE (62 %). The flavonoids galangin-3-methyl ether and pinobanksin did not show any DPPH$^{\cdot}$ radical scavenging capacity. The EC_{50} values are depicted in Table 3.6. The plot of the DPPH$^{\cdot}$ radical scavenging capacity of all the extracts and isolated compounds at different concentration is presented in figure 3.24. The high free radical scavenging capacity of the defatted acetone extract accords with the fact that antioxidant phytoconstituent concentration in it was higher than in the total ethanol extract. Furthermore, there is also agreement between total phenolic content, total flavonoid content and the free radical scavenging ability of the defatted acetone extract. These results agree with earlier reports where correlations were established between total phenolic content, total flavonoid content and observed antioxidant activities.[28] Antioxidant capacity of flavonoids have been studied in detail and structure-activity relationships have been proposed to explain the results.[29] It is generally accepted that the presence of hydroxyl groups in the B ring of flavonoids as in quercetin greatly

enhances the antioxidant capacity which clearly explains its highest free radical scavenging capacity. Pinocembrin, galangin-3-methyl ether and pinobanksin eventhough have two hydroxyls in A ring, did not possess radical scavenging capacity showing the inactivity of the A ring hydroxyls to participate or aid in the radical scavenging process. Also, pinocembrin and pinobanksin lack conjugation between C2-C3. It is generally accepted that flavonoids lacking conjugation between C2-C3 or methoxylation of the hydroxyl groups present in them significantly reduce the antioxidant capacity of the molecule.[30] Summarizing the results obtained, the radical scavenging capacity was ACA > ACE, with pinocembrin, galangin-3-methyl ether and pinobanksin possessing no DPPH radical scavenging capacity.

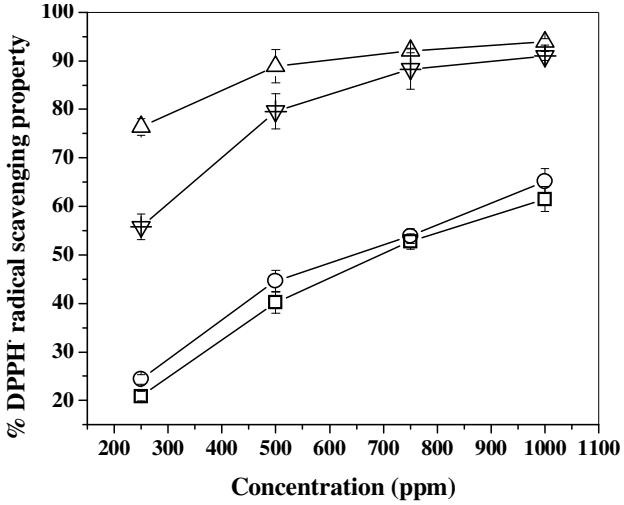


Figure 3.24: DPPH˙ radical scavenging capacities at different concentrations of (○) ACA; (□) ACE; (△) Quercetin; (▽) BHA

3.4.3 Superoxide radical scavenging capacity

In the present investigation, the extracts and the flavonoids were also tested for their superoxide scavenging properties. The flavonoids galangin-3-methyl ether and pinobanksin did not show any activity against the superoxide radical. Moreover, the superoxide scavenging power of the extracts were much lower compared to the standards. Among the extracts, ACA showed a higher scavenging power for the superoxide radical with 67 %

scavenging capacity at a concentration of 8000 ppm. ACE showed only 39 % superoxide scavenging capacity at this concentration, whereas the standard tocopherol scavenged 97 % of the superoxide radicals produced. The EC_{50} values are given in Table 3.6. Here also, the defatted acetone extract was more effective than the total ethanol extract showing a correlation between the total phenolic content and the antioxidant capacity.

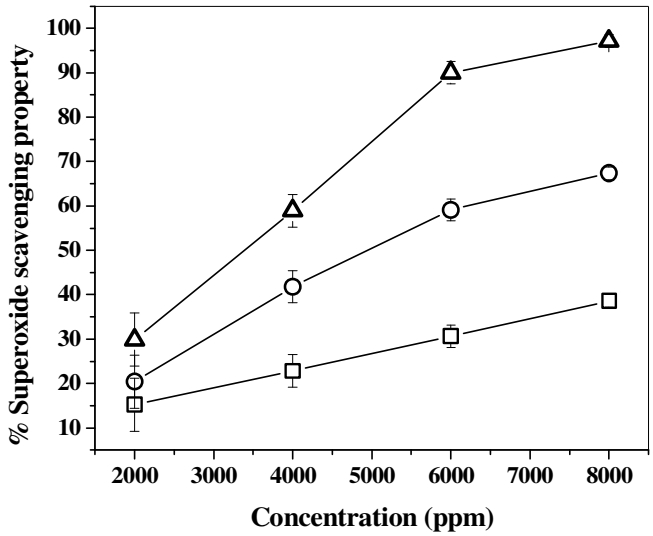


Figure 3.25: Superoxide scavenging capacities at different concentrations of (○) ACA; (□) ACE and (△) Tocopherol

3.4.4 Hydroxyl radical scavenging capacity

The hydroxyl radical scavenging capacity of the extracts and flavonoids are depicted in figure 3.26. Both the extracts as well as the flavonoids tested showed excellent hydroxyl radical scavenging capacity. At a concentration of 25 ppm, galangin-3-methyl ether showed the best hydroxyl radical scavenging (89 %) whereas quercetin showed slightly lower activity (85 %) at the same concentration. ACA showed 87 % effect at the same concentration whereas ACE showed 86.5 % scavenging power. Interestingly, the flavonoids pinocembrin (84 %) and pinobanksin (86 %) as well as the *trans* stilbene (85 %) showed an efficient hydroxyl radical scavenging ability at 25 ppm. The EC_{50} values are given in Table 3.6. The hydroxyl radical

scavenging capacity decreases in the order galangin-3-methyl ether > ACA > ACE > Pinobanksin > stilbene > pinocembrin. The EC_{50} values are given in Table 3.6. It is important to mention here that various anti-inflammatory drugs exert some of their beneficial effects by scavenging hydroxyl radicals.[31] Thus the hydroxyl radical scavenging capacities of *A. calcarata* extracts as well as the compounds isolated becomes significant as they may act as potent anti-inflammatory agents.

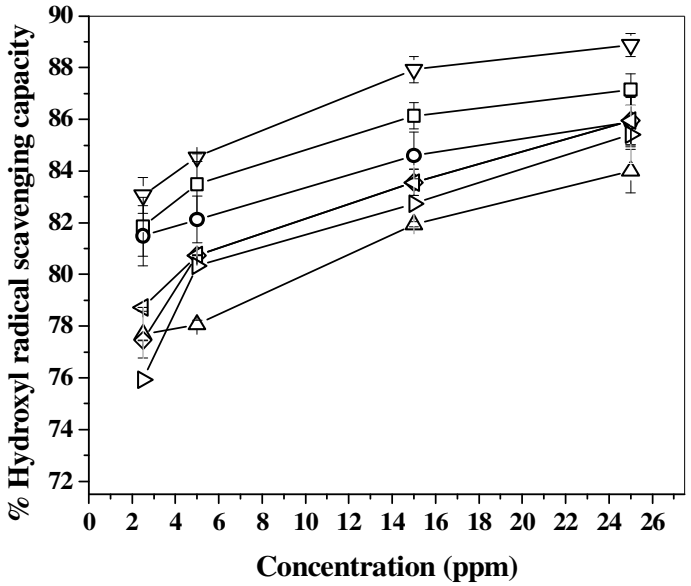


Figure 3.26: % Hydroxyl radical scavenging power of (□) ACA; (○) ACE; (△) Pinocembrin; (▽) Galangin-3-methyl ether; (◇) Pinobanksin; (◁) Stilbene and (▷) Quercetin at different concentrations

3.4.5 Scavenging of Hydrogen peroxide

Figure 3.27 depicts the H_2O_2 scavenging ability of the extracts and the isolated flavonoids. Among the compounds tested, the *trans* stilbene showed the highest H_2O_2 scavenging of 99 % and pinocembrin showed 59 % scavenging capacity at 400 ppm while the standard BHA used here showed only 39 % scavenging of H_2O_2 at this concentration. Both pinobanksin and galangin-3-methyl ether also showed promising scavenging activities (48 % and 45 % respectively) at 400 ppm next to pinocembrin. However the

extracts showed very negligible activity at this concentration. Thus the H_2O_2 scavenging ability of the test materials decrease in the order stilbene > pinocembrin > pinobanksin > galangin-3-methyl ether with the extracts with exceptionally lower activities. The concentration of remaining hydrogen peroxide in the reaction medium was calculated using the equation and as is clear from the scavenging capacity, the concentration of H_2O_2 was only 0.11mM and 7 mM in the reaction mixtures after the reaction with stilbene and pinocembrin respectively.

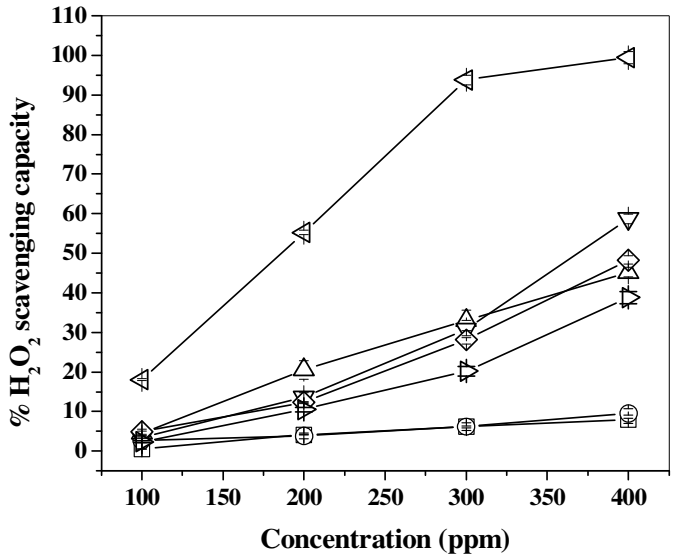


Figure 3.27: Hydrogen peroxide scavenging capacities at different concentrations of (○) ACA; (□) ACE; (△) Galangin-3-methyl ether; (▽) Pinocembrin; (◇) Pinobanksin; (◁) Stilbene and (▷) BHA.

3.4.6 Reducing power

The reducing powers of ACE, ACA, pinocembrin, galangin-3-methyl ether, pinobanksin and stilbene were assessed based on their ability to reduce Fe (III) to Fe (II) and the results are presented in figure 3.28. An increased absorbance indicates a higher reducing power. Thus the maximum absorbance at 40 ppm was shown by stilbene showing the highest reducing property. All the compounds evaluated showed reducing capacities indicating that they can

act as electron donors and can react with free radicals to convert them to more stable products and thereby terminate radical chain reactions.

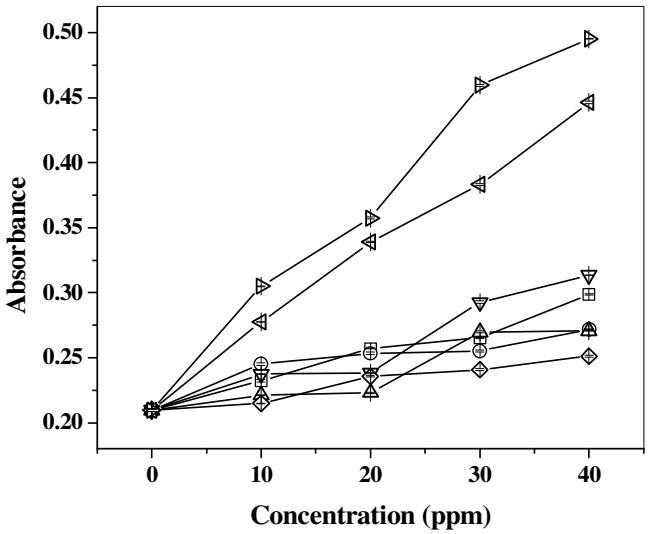


Figure 3.28: Reducing power at different concentrations of (○) ACA; (□) ACE; (△) Galangin-3-methyl ether; (▽) Pinocembrin; (◇) Pinobanksin; (◁) Stilbene and (▷) BHA

Table 3.6: EC_{50} values

Sample	DPPH˙	˙OH	H_2O_2
	Scavenging capacity EC_{50}		
Ethanol extract (ACE)	690	1.6	> 1000
Defatted acetone extract (ACA)	650	1.4	> 1000
Pinocembrin	>1000	2.1	367
Galangin-3-methyl ether	>1000	1.2	437
Pinobanksin	>1000	1.8	407
Stilbene	>1000	1.9	186

Thus *A. calcarata* rhizomes contains a variety of powerful antioxidative compounds have been isolated for the first time and evaluated for their biological activity. The extracts and the isolated flavonoids were efficient in scavenging different free radicals and reactive oxygen species. The *trans* stilbene, 3-methoxy-5-hydroxy stilbene (compound **VII**) was very

effective in scavenging hydrogen peroxide. Even though *A. calcarata* and some of the compounds isolated showed efficient activities from the above mentioned tests, for another set of tests such as chelating power, nitric oxide scavenging, antioxidant capacity by β-carotene bleaching assay and for the inhibition of lipid peroxidation assay the extracts and the pure compounds did not show any positive results. It should be emphasized that this study was focused on the chemical aspects of using *A. calcarata* rhizome extract as a substitute to *A. galanga* rhizomes in herbal formulations. The results obtained indicated that there are differences in the chemical composition of *A. calcarata* and *A. galanga* as the latter is a much more efficient antioxidant as concluded from the present study.

A comparison of *A. galanga* and *A. calcarata* in terms of chemical constituents and antioxidant capacity is discussed in section B of this chapter.

3.5 Experimental

General experimental procedures and chemicals used are as reported in chapter 2 of this thesis. Flash chromatography was done on a Büchi Sepacore flash chromatogram using prepacked cartridges. Drying of the plant material was carried out in RRLT-NC drier developed inhouse.

3.5.1 Extraction

The extraction was carried out by immersing the powdered plant material in acetone/ethanol overnight and then decanting the solvent. Crude extracts were obtained after evaporation of the decanted solvent under reduced pressure at ambient temperatures in a rotary evaporator. Pure compounds were obtained from the crude extracts using column chromatographic separation.

3.5.2 Chromatographic separation of the extracts

The crude extracts were dissolved in minimum quantity of hexane and then loaded onto a silica gel column. The column was eluted successively with hexane and hexane-ethyl acetate mixtures of increasing polarities

(staring from 100 % hexane and ending with 100 % ethyl acetate). The pictorial representation of the isolation procedure of compounds **V** to **XIV** is depicted in figures 3.29A and 3.29 B.

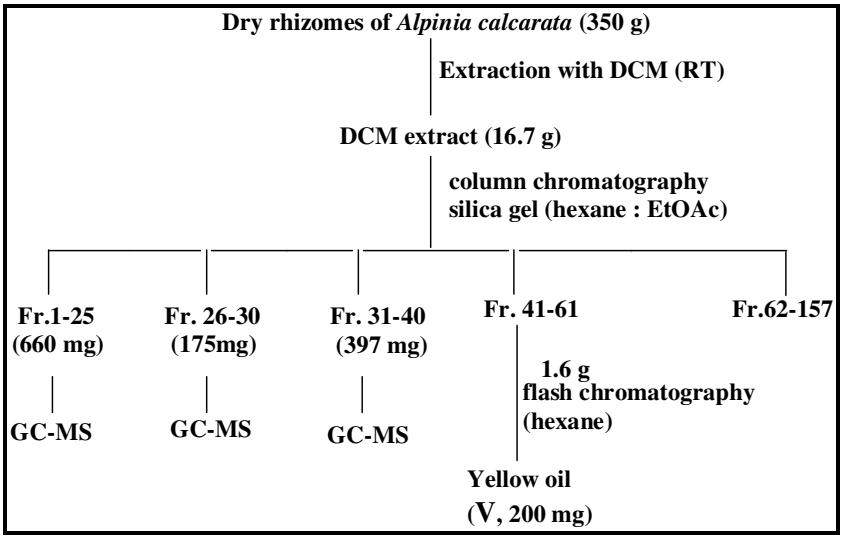


Figure. 3.29 A: Pictorial representation of the isolation of compound **V**

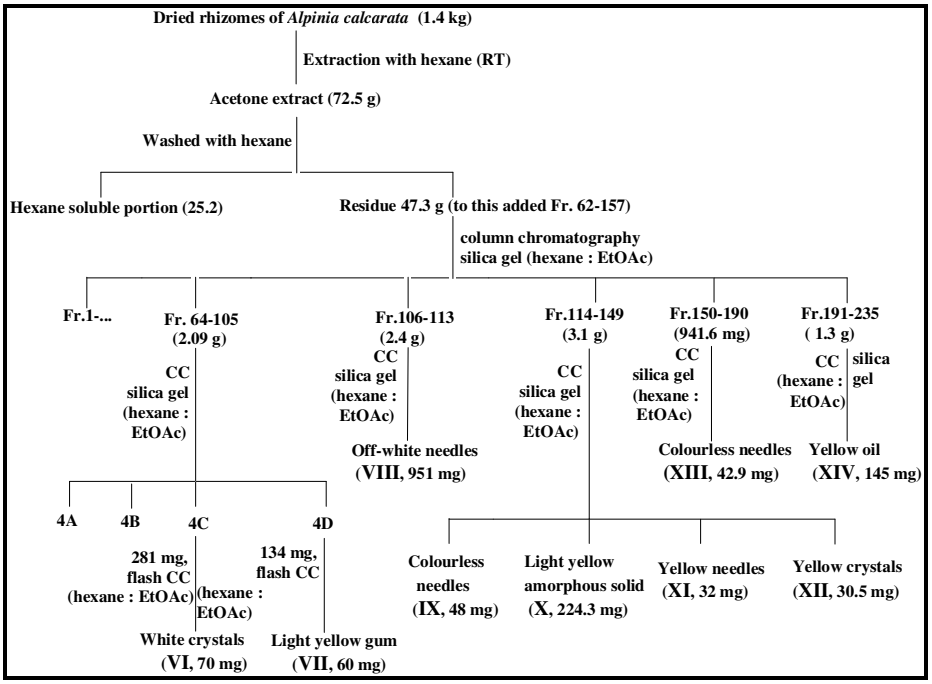
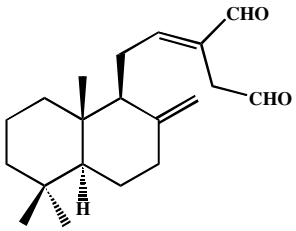


Figure. 3.29 B: Pictorial representation of the isolation of compounds **VI-XIV**

3.5.2.1 Isolation of compound V

The isolation procedure of compound **V** is represented in figure 3.29A. It was obtained on elution with pure hexane in a flash chromatogram. Compound **V** (450 mg) was obtained as an yellow oil. IR, ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound, on comparison to literature values, confirmed it to be (*E*)-labda-8(17),12-diene-15,16-dial, the structure of which is assigned as shown.

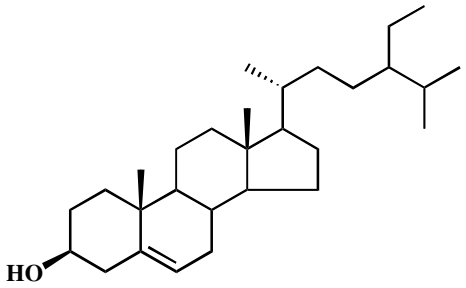


FT-IR (neat, ν_{max}/cm^{-1})	:	2931, 2870, 2845, 1728, 1683, 1644, 889.
^{1}H NMR (300 MHz, $CDCl_3$)	:	δ 9.64 (1H, *s*), 9.41 (1H, *s*), 6.77 (1H, *t*, $J_1 = J_2 = 6.5$ Hz), 4.86 (1H, *d*, $J = 1.0$ Hz), 4.37 (1H, *d*, $J = 1.0$ Hz), 3.43 (2H, *dd*, $J = 16.8, 6.5$ Hz), 2.54-2.39 (3H, *m*), 1.93-1.11 (11H, *m*), 0.89 (3H, *s*), 0.82 (3H, *s*), 0.73 (3H, *s*)
^{13}C NMR (75 MHz, $CDCl_3$)	:	δ 197.3, 193.5, 148.0, 159.9, 134.8, 107.8, 56.4, 55.3, 41.9, 39.6, 39.3, 39.2, 37.8, 33.5, 29.6, 24.6, 24.1, 21.7, 19.2, 14.4
FAB-MS m/z (rel. int.)	:	302.19 $[M]^+$ (100), $C_{20}H_{30}O_2$, requires 302.22

3.5.2.2 Isolation of compound VI

Figure 3.29B depicts the isolation procedure of compound **VI**. It was obtained on column chromatographic separation of the fraction with 1 % Ethyl acetate-hexane mixture to give 70 mg of white crystalline compound

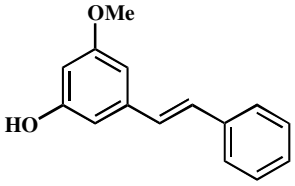
VI. The melting point determination gave a value of 136-137 °C. From various spectroscopic data, compound **VI** was confirmed as β -sitosterol whose structure is as shown.



VI

3.5.2.3 Isolation of compound VII

Compound **VII** was obtained as a light yellow thick liquid (16 mg) and the isolation procedure is as shown in figure 3.29B. The structure of **VII** is assigned from various spectroscopic techniques and by comparing the values with that of the reported one. Following those, the compound **VII** was found to be 3-methoxy-5-hydroxy stilbene and the structure is as shown below.



FT-IR (neat, ν_{max}/cm^{-1})	:	3392, 2923, 1604, 1593, 1495, 1454, 1347, 1262, 1194, 1148, 1058, 959, 692
^1H NMR (300 MHz, $CDCl_3$)	:	δ 7.51-7.24 (5H, *m*), 7.09-6.96 (2H, *dd*, *J* = 16.3, 7.2 Hz), 6.65 (1H, *s*), 6.61 (1H, *s*), 6.34 (1H, *s*), 3.82 (3H, *s*)
^{13}C NMR (75 MHz, $CDCl_3$)	:	δ 161.1, 156.8, 139.7, 137.0, 129.4, 128.7 (2C), 128.3, 127.8, 126.6 (2C), 106.0, 105.0, 101.0, 55.4
FAB-MS m/z (rel. int.)	:	226.41 $[M]^+$ (100), $C_{15}H_{14}O_2$, requires 226.10

3.5.2.4 Isolation of compound VIII

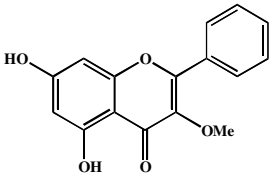
Compound **VIII** was isolated from the rhizomes of *A. calcarata* as represented in figure 3.29B. Compound **VIII** was obtained as off-white needles (951 mg) by eluting the column with 5% ethyl acetate in hexane mixture. On evaluation of the structure of **VIII** and on comparison with TLC, compound **VIII** was confirmed as the 5,7-Dihydroxyflavanone, Pinocembrin which was isolated earlier from *A. galanga* by us (compound **I**) and is discussed in detail in chapter 2.

3.5.2.5 Isolation of compound IX

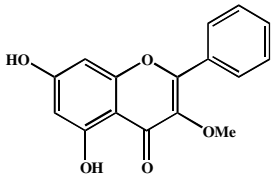
Figure 3.29B represents the isolation procedure for compound **IX**. It is obtained as colourless needles (48 mg). Detailed investigation of various spectroscopic data of compound **IX** revealed that it was the diarylheptanoid, 1,7-Diphenyl-5-hydroxy-3-heptanone (dihydroyashabushiketol). Further evidence was made by comparing the TLC with that of compound **II** isolated from *A. galanga*. The structural assignment and detailed spectral data are discussed in chapter 2.

3.5.2.6 Isolation of compound X

The isolation of compound **X** is as summarized in figure 3.29B. It is obtained as light yellow amorphous solid (224.3 mg). UV-vis, IR, ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound and on comparison to the literature values, confirmed it to be galangin-3-methyl ether whose structure is shown below.

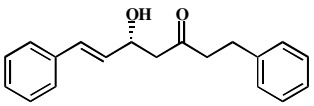


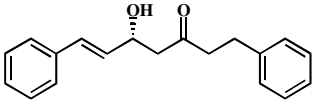
UV(λ_{max}^{MeOH}nm)	:	340 sh, 312 sh, 266
FT-IR (KBr, ν_{max}/cm^{-1})	:	3155, 2551, 1647, 1608, 1570, 1496, 1363, 1305, 1222, 1161, 1018, 711.
^{1}H NMR (300 MHz, CD_3OD +	:	δ 12.16 (1H, *s*), 8.07-8.04 (2H, *m*), 7.52 (3H, *t*, $J_1 = J_2$

	$CDCl_3$)	= 3.2 Hz), 6.43 (1H, *d*, *J* = 1.9 Hz), 6.29 (1H, *d*, *J* = 1.9 Hz), 3.83 (3H, *s*)
	^{13}C NMR (75 MHz, CD_3OD + $CDCl_3$)	: δ 178.5, 164.1, 160.1, 157.0, 147.0, 141.9, 130.8, 130.3, 128.5 (2C), 128.2 (2C), 105.2, 98.9, 94.1, 60.2
	FAB-MS m/z (rel. int.)	: 285.06 $[M+H]^+$ (100), $C_{16}H_{12}O_5$, requires 284.07
	m.p. (°C)	: m.p. 288-291 °C, lit[19].288-299 °C

3.5.2.7 Isolation of compound XI

Compound **XI** was obtained as yellow needles (32 mg) and figure 3.29B summarized the isolation procedure. IR, ^1H NMR, ^{13}C NMR and mass spectral studies of this compound and on comparison to the literature values, confirmed it to be the diarylheptanoid-1,7-diphenyl-5-hydroxy-6-heptene-3-one whose structure is shown below.

	FT-IR (KBr, ν_{max}/cm^{-1})	: 3574, 1705, 1603, 1467, 1425, 1387, 1288, 1263, 1169, 1082, 780, 719, 710
	^1H NMR (300 MHz, $CDCl_3$)	: δ 7.38-7.16 (10H, *m*), 6.62 (1H, *d*, *J* = 15.9 Hz), 6.18 (1H, *dd*, *J* = 15.9, 6.1 Hz), 4.75 (1H, *m*), 2.92 (2H, *t*, *J* = 7.1 Hz), 2.80 (2H, *t*, *J* = 6.9 Hz), 2.71 (2H, *d*, *J* = 6.3 Hz)



^{13}C NMR (75 MHz, $CDCl_3$)	:	δ 210.1, 140.6, 136.4, 130.4, 130.1, 128.5 (4C), 128.4 (2C), 127.7, 126.5 (2C), 126.2, 68.5, 49.3, 45.1, 29.4
DEPT-135 NMR (125 MHz, $CDCl_3$)	:	(i) 130.4, 130.1, 128.9, 128.6, 128.5, 128.4, 128.3, 127.8, 127.3, 126.5, 126.3, 66.9
(i) CH		
(ii) CH_2		(ii) 49.3, 45.0, 30.1
HRMS m/z (rel. int.)	:	280.9824 $[M]^+$ (90), $C_{19}H_{20}O_2$, requires 280.1463.
m.p. (°C)	:	58 - 60 °C, lit[20]. 59.5-60.5 °C
$[\alpha]_D^{26}$ (ca, 0.528, $CHCl_3$)	:	+17.2 °, lit[20]. $[\alpha]_D^{20}$ +17.8 °

3.5.2.8 Isolation of compound XII

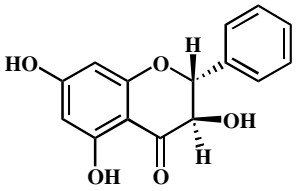
Compound **XII** was obtained as yellow crystals (30.5 mg) and the isolation procedure is depicted in figure 3.29B. Spectral analysis in detail confirmed the compound **XII** is similar to compound **III** obtained earlier from *A. galanga* as discussed in chapter2.

3.5.2.9 Isolation of compound XIII

Compound **XIII** was obtained as colorless needles (42.9 mg) on column chromatographic separation of the fraction as depicted in figure 3.29B. UV-vis, IR, ^{1}H NMR, ^{13}C NMR and mass spectral studies of this compound, on comparison to literature values, confirmed it to be Pinobanksin, the structure of which is assigned as shown.

UV(λ_{max}^{MeOH}nm)	:	330 sh, 290
FT-IR (KBr,	:	3566, 3429, 1618, 1469,

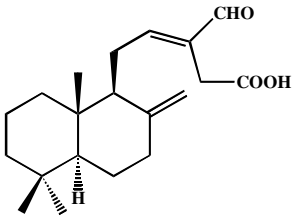
ν_{max}/cm^{-1})		1371, 1469, 1371, 1278, 1168, 1136, 1082, 999, 835, 763, 698
^1H NMR (300 MHz, CD_3COCD_3)	:	δ 11.7 (1H, *s*), 7.59 (2H, *dd*, *J* = 7.5, 1.5 Hz), 7.46 - 7.37 (3H, *m*), 5.99 (1H, *d*, *J* = 2.0 Hz), 5.95 (1H, *d*, *J* = 2.0 Hz), 5.16 (1H, *d*, *J* = 11.2 Hz), 4.64 (1H, *d*, *J* = 11.2 Hz)
^{13}C NMR (75 MHz, CD_3COCD_3)	:	δ 198.1, 168.3, 165.2, 164.2, 138.5, 129.8, 129.3 (2C), 129.0 (2C), 101.7, 97.4, 96.3, 84.6, 73.4
FAB-MS m/z (rel. int.)	:	273.55 $[M+1]^+$ (100), $C_{15}H_{12}O_5$, requires 272.07
m.p. (°C)	:	173.5-174.5 °C, lit[32]. 171-174 °C
$[\alpha]_D^{26}$ (ca,1.000, $CHCl_3$)	:	+6.3°, lit[32]. $[\alpha]_D$ +6.1°



3.5.2.10 Isolation of compound XIV

Compound **XIV** was obtained as an yellow oil (145 mg). It was obtained from the last fraction as depicted in figure 3.29B. Detailed analysis by IR, ^1H NMR, ^{13}C NMR and mass spectral studies and by comparing the values from the literature, this compound was confirmed as Zerumin A whose structure is shown below.

FT-IR (neat, ν_{max}/cm^{-1})	:	3080, 1695, 1682 1640, 890



^{1}H NMR (300 MHz, $CDCl_3$)	:	δ 9.38 (1H, *s*), 6.70 (1H, *t*, $J_1 = J_2 = 6.5$ Hz), 4.86 (1H, *d*, $J = 1.0$ Hz), 4.39 (1H, *d*, $J = 1.0$ Hz), 3.38 (2H, *dd*, $J = 16.6, 6.0$ Hz), 2.56-2.40 (3H, *m*), 1.75-1.12 (11H, *m*), 0.89 (3H, *s*), 0.83 (3H, *s*), 0.75 (3H, *s*)
^{13}C NMR (75 MHz, $CDCl_3$)	:	δ 193.5, 175.2, 159.3, 147.8, 135.6, 107.8, 56.2, 55.2, 41.9, 39.4, 39.1, 37.7, 33.5 (2C), 29.4, 24.5, 24.0, 21.6, 19.2, 14.3
HRMS m/z (rel. int.)	:	318.2180 $[M]^+$ (100), $C_{20}H_{30}O_3$, requires 318.2195

Section B

Comparison of *A. galanga* and *A. calcarata* in terms of chemical constituents and antioxidant capacity

Alpinia galanga and *Alpinia calcarata* are two species of the genus *Alpinia* that is widely employed in traditional medicines practiced in India and Sri Lanka. Eventhough the medicinal properties of *A. galanga* are well studied, the properties of *A. calcarata* are not well documented. In Wealth of India, it is mentioned that *A. calcarata* has the same medicinal properties as that of *A. galanga*. Our studies on the phytochemical constituents and biological activity of *A. galanga* and *A. calcarata* have pointed out differences between both the species in terms of constituents as well as the biological activity. In this section, we discuss briefly the comparison between *A. galanga* and *A. calcarata*.

3.6 Comparison in terms of phytoconstituents

Literature reports indicate the presence of phenyl propanoids such as chavicol acetate, acetoxy cineoles and a few phenolic compounds from the rhizomes of *A. galanga* whereas only diterpenes have been isolated so far from *A. calcarata* rhizomes. In our present study, we obtained three flavonoids and an aryl heptanoid from *A. galanga* (Table 3.7). Four flavonoids, two arylheptanoids, a *trans* stilbene and two diterpenes along with β-sitosterol were isolated from *A. calcarata*. Table 3.7 depicts the percentage concentration of compounds isolated by us from *A. galanga* and *A. calcarata*. From Table 3.7, it is clear that there are marked differences in the constituents of the rhizomes of both the species. Galangin was the major constituent

(highest yield) in *A. galanga* rhizomes, whereas pinocembrin was the major compound in *A. calcarata* rhizomes. Pinocembrin, galangin and 1,7-diphenyl-5-hydroxy-3-heptanone are the common constituents of both the rhizomes, but shows variation in the percentage composition in the two rhizomes as shown in figure 3.30. *A. galanga* is a rich source of the antioxidative flavonoid galangin whereas, from *A. calcarata*, considerable amounts of pinocembrin, which is a powerful inducer of apoptosis in colon cancer cells has been isolated. Both the rhizomes vary in their chemical constituents and hence there will be variations in their biological activities.

Table 3.7: Compounds isolated in the present study from *A. galanga* and *A. calcatara* rhizomes

Specie	Compounds isolated	Weight[a] (mg)	%
Alpinia galanga	Pinocembrin (**I**)	13	0.013
	1,7-Diphenyl-5-hydroxy-3-heptanone (**II**)	32	0.032
	Galangin (**III**)	560	0.560
	Kaempferide (**IV**)	43	0.043
Alpinia calcarata	(*E*)-Labda-8(17),12-diene-15,16-dial (**V**)	61	0.061
	β-Sitosterol (**VI**)	5	0.005
	3-methoxy-5-hydroxy stilbene (**VII**)	4	0.004
	Pinocembrin (**I/VIII**)	70	0.070
	1,7-Diphenyl-5-hydroxy-3-heptanone (**II/IX**)	3	0.003
	Galangin-3-methyl ether (**X**)	22	0.022
	1,7-diphenyl-5-hydroxy-6-heptene-3-one (**XI**)	2	0.002
	Galangin (**III/XII**)	2	0.002
	Pinobanksin (**XIII**)	3	0.003
	Zerumin A (**XIV**)	11	0.011

[a] in 100g of the rhizomes (dry)

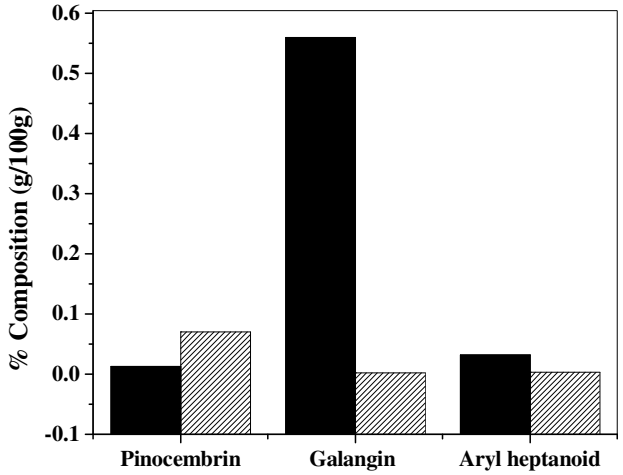


Figure 3.30: % Composition of the common constituents of (■) *A. galanga* and (▨) *A. calcarata*

As a result, the comparison between the antioxidant profiles of both the ethanol and acetone extracts of *A. galanga* (AGE and AGA respectively) and *A. calcarata* (ACE and ACA) has been made and is discussed below.

3.7 Comparison of the antioxidant capacities of *A. galanga* and *A. calcarata*

3.7.1 Total phenolic content, total flavonoid content and total antioxidant capacity

The total phenolic content, total flavonoid content and total antioxidant capacity of the ethanol extracts of both *A. galanga* (AGE) and *A. calcarata* (ACE) were compared and is presented in figure 3.31. From the figure, it is clear that the total phenolic content, total flavonoid content and the total antioxidant capacity of *A. galanga* is higher than *A. calcarata*, thus confirming the presence of higher amounts of antioxidative compounds in *A. galanga*.

In a similar way, the acetone extract of *A. galanga* (AGA) and the defatted acetone extract of *A. calcarata* (ACA) were compared and is represented in figure 3.32. The total phenolic and flavonoid contents of the

extracts of both showed almost similar results, with *A. galanga* having slightly higher values. The total antioxidant capacity of the acetone extract *A. galanga* rhizomes was much higher than the defatted acetone extract of *A. calcarata* rhizome.

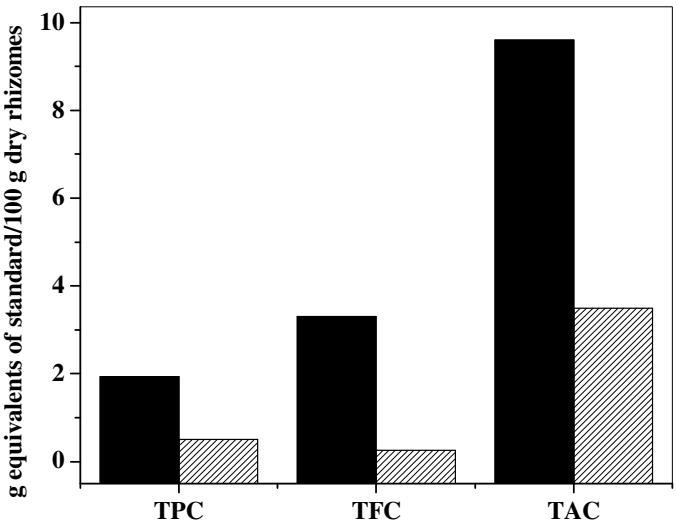


Figure 3.31: The total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant capacity (TAC) of the ethanol extract of (■) *A. galanga* AGE and (▨) *A. calcarata* ACE

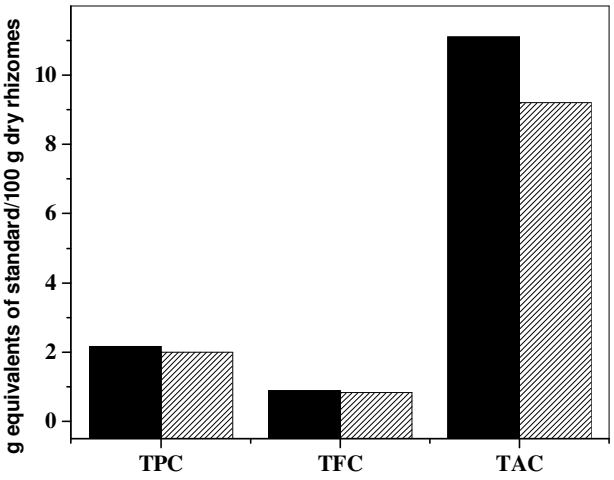


Figure 3.32: The total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant capacity (TAC) of the acetone extract of (■) *A. galanga* (AGA) and (□) *A. calcarata* (ACA)

Thus, from the preliminary analyses, *A. galanga* was found to possess higher antioxidant capacity than *A. calcarata* which was further proved by the individual antioxidant measurements using different assays. Even though the ethanol extract of *A. galanga* showed higher antioxidant capacity, the acetone extract of *A. galanga* showed only a little variation in the antioxidant capacity in comparison with defatted acetone extract of *A. calcarata*.

3.7.2 DPPH˙ radical scavenging capacity

The DPPH˙ radical scavenging capacities of both the extracts of *A. galanga* and *A. calcarata* were compared and is represented in Figure 3.33.

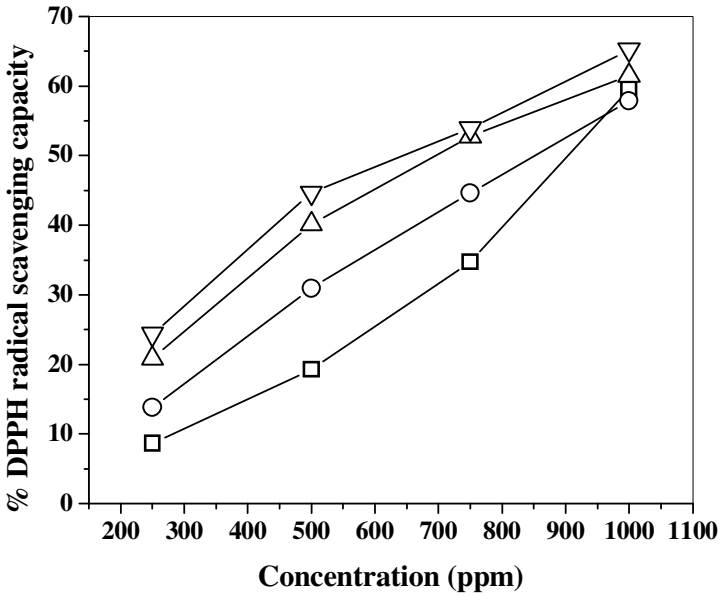


Figure 3.33: DPPH radical scavenging capacities at different concentrations of (□) AGE; (○) AGA; (△) ACE; (▽) ACA

The highest free radical scavenging capacity was shown by *A. calcarata* acetone extract when measured by the DPPH˙ method and decreases in the order *A. calcarata* defatted acetone extract (ACA) > *A. calcarata* ethanol extract (ACE) > *A. galanga* acetone extract (AGA) > *A. galanga* ethanol extract (AGE). Eventhough *A. calcarata* acetone extract showed higher radical scavenging capacity, the major compound isolated from it viz., pinocembrin exhibited very low activity. On the other hand, the

major compound isolated from *A. galanga* viz., galangin showed very high DPPH˙scavenging capacity. Thus, the higher free radical scavenging capacity of *A. calcarata* might be due to the synergistic effect of all the compounds present in the extract.

3.7.3 Superoxide radical scavenging capacity

Figure 3.34 summarizes the superoxide radical scavenging capacities of *A. galanga* and *A.calcarata* extracts.

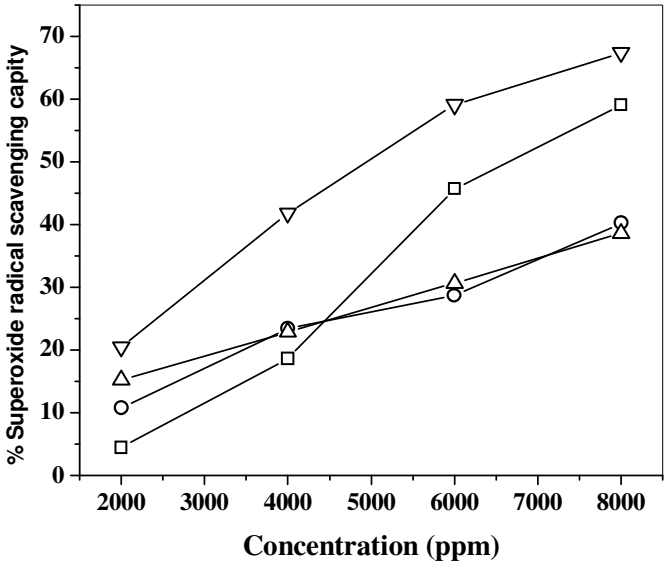


Figure 3.34: Superoxide radical scavenging capacities at different concentrations of (□) EE; (○) AE; (△) ACE; (▽) ACA

It is clear from the figure that the defatted acetone extract of *A. calcarata* was most efficient in scavenging the superoxide radical among all the extracts studied with the ability of scavenging 67 % of the superoxide radical produced at a concentration of 8000 ppm. *A. galanga* acetone extract showed the next highest activity by scavenging 59 % of the radicals produced at the same concentration. *A. calcarata* ethanol extract showed slightly higher activity at a lower concentration (15 % at 2000 ppm) than *A. galanga* (AGE 4 % and AGA 11 % at 2000 ppm), but showed a decreased activity at higher concentrations. Thus the superoxide radical scavenging capacities of the extract decreased in the order ACA > AGE > AGA > ACE.

3.7.4 Hydroxyl radical scavenging capacity

A. galanga and *A. calcarata* extracts were found to be highly efficient in the scavenging of the hydroxyl radicals. Of these, *A. galanga* ethanol extract (AGE) was most effective among all four extracts with scavenging of 93 % of the hydroxyl radicals at a concentration of 25 ppm. Figure 3.35 summarizes the hydroxyl radical scavenging capacity at different concentrations of the extracts. Other extracts also showed good hydroxyl radical scavenging activities with AGA, ACE and ACA showing 88 %, 86 % and 87 % hydroxyl radical scavenging capacity respectively at 25 ppm concentration. Thus, the activity decreases in the order AGE > AGA > ACA > ACE.

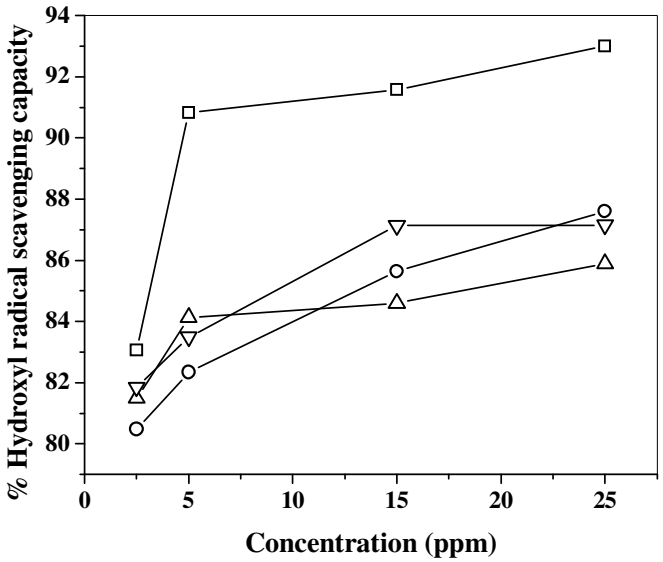


Figure 3.35: Hydroxyl radical scavenging capacities at different concentrations of (□) AGE; (○) AGA; (△) ACE; (▽) ACA

3.7.5 Hydrogen peroxide scavenging capacity

Hydrogen peroxide scavenging capacity was observed for *A. galanga* acetone extract whereas *A. calcarata* did not scavenge hydrogen peroxide even at higher concentrations. At 400 ppm, *A. galanga* ethanol and acetone extracts showed 25 % and 55 % H_2O_2 scavenging capacities respectively

whereas *A. calcarata* extracts showed only 8 % and 9 % scavenging capacities respectively for the ethanol extract and the defatted acetone extract (Figure 3.36).

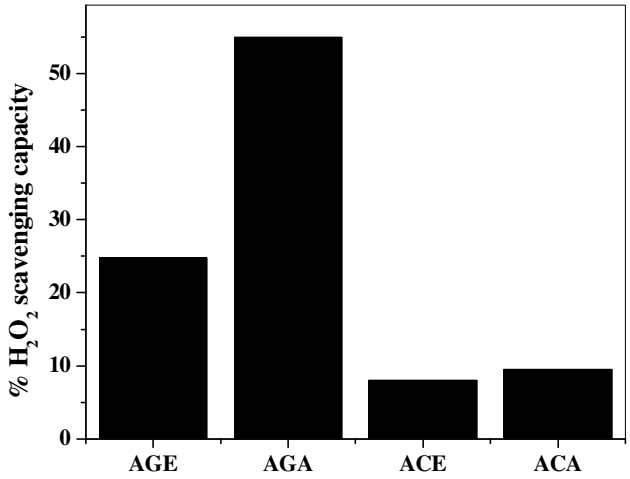


Figure 3.36: Hydrogen peroxide scavenging capacity of different extracts at a concentration of 400 ppm

A. calcarata extracts did not show any (i) metal chelating activity, (ii) β -carotene bleaching, (iii) inhibition of lipid peroxidation and (iv) nitric oxide scavenging capacity. On the other hand, *A. galanga* extracts showed activity in all the methods except in metal chelating. However in $DPPH^{\cdot}$ scavenging, both the extracts of *A. calcarata* showed higher activity than *A. galanga*. In the antioxidant assay by superoxide radical scavenging, *A. calcarata* defatted acetone extract exhibited a higher potential than *A. galanga*. In all the other assays the antioxidant capacity of *A. galanga* dominated over that of *A. calcarata*. Summarized from all the results, *A. galanga* could be considered as an efficient antioxidant than *A. calcarata*. Also, the antioxidative flavonoid galangin was obtained in larger amounts from *A. galanga* (0.56 %) whereas in *A. calcarata* it was obtained only in very smaller amounts (0.002 %). On the other hand, pinocembrin, an apoptosis inducer in human colon cancer cells, was obtained in larger

amounts from the rhizomes of *A. calcarata* (0.07 %) than from the rhizomes of *A. galanga* (0.01 %).

From all the above observations viz., phytochemical and activity guided assays, it was observed that there is considerable difference between the two species of the genus *Alpinia*, viz., *A. calcarata* and *A. galanga*.

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Chapter 4

Phytochemical Investigation of *Kaempferia pulchra*

This chapter deals with the isolation and characterization of chemical constituents from *Kaempferia pulchra* (syn. *Kaempferia elegans*), a member of the genus *Kaempferia* belonging to Zingiberaceae family.

4.1 Introduction - *Kaempferia*

Kaempferia is a genus of rhizomatous herbs distributed in the tropics and subtropics of Asia and Africa.[1] These tuberous and fleshy rooted plants are named after Engelbert Kaempfer (1651-1716), a German physician who wrote a book on Japanese medicinal plants. The species under this genus are herbaceous perennials, mostly grown as ornamental plants because of their beautiful flowers and foliage. Leaves are elliptic or ovate-lanceolate and acuminate. Flowers appear in terminal scapes. Corolla tube is elongated, exerted and fragrant. Most species naturally go dormant for a portion of the year.[2] Phytochemistry and biological activities of the commonly studied species in this genus is discussed briefly below.

K. angustifolia is indigeneous to south-east Asia. In India, it is found at the foot of the eastern Himalayas and in North Bengal. It is used to treat abdominal illness, including dysentery and diarrhoea.[3] A study on the essential oil of *K. angustifolia* revealed that *n*-pentadecane and camphene respectively were the major constituents of the rhizomes and lateral parts.[4] A study by Williams and Harborne has confirmed the presence of the flavonoids in *K. angustifolia*.[5] A Thai species of *Kaempferia* (Thai name: *krachaikao*) has yielded cyclohexane diepoxides and zeylenol (**357**) from the rhizomes.[6] Following that report, *K. angustilfolia* rhizomes were also evaluated in detail.

As a result, several cyclohexane diepoxides and other oxygenated cyclohexane derivatives have been isolated from *K. angustifolia* rhizomes (Table 4.1, chart 4.1).[7]

K. galanga is an important medicinal plant in Indian traditional medicines and is a glabrous, perennial, aromatic herb with very fragrant underground parts. The rhizomes possess a wide range of medicinal properties as described in P. S. Varier's book on Indian medicinal plants.[3] The root stocks are bitter, thermogenic, vulnerary, antihelmintic, febrifuge and stimulant. They are said to be good for dyspepsia, leprosy, skin diseases, rheumatism, asthma, cough, bronchitis, wounds, ulcers, helminthiasis, fever, malarial fever, spleenopathy, cephalalgia, inflammatory tumors, nasal obstruction, halitosis, strangury and hemorrhoids. The leaves are used for pharyngodynia, ophthalmia, swellings, fever and rheumatism.[3] In Chinese medicine, the rhizomes of *K. galanga* has been used as an aromatic stomachic and as an incense.[8] The rhizomes showed sedative,[9] wound healing,[10] nematicidal,[11] larvicidal[12] and pharmacological[13] activities. Various compounds including cinnamic acid derivatives, terpenes and hydrocarbons, camphene, *l*-Δ^3-carene, borneol, *para*-methoxystyrene and pentadecane have been isolated from the rhizomes.[14] Ethyl cinnamate, *para* methoxy cinnamic acid as well as ethyl *para* methoxy cinnamate were the major constituents of the rhizomes of *K. galanga*.[15] In 1987, Kiuchi *et al* isolated a new monoterpene ketone **367** (chart 4.1) from the rhizomes.[16] *para* Methoxy cinnamic ester from *K. galanga* serves as a monamine oxidase inhibitor and thus, could be used as a candidate in drugs for treating depression.[17] Ethyl cinnamate isolated from the rhizomes exhibited vasorelaxant effect.[18] *K. galanga* extract was also found to have cytotoxic activity.[19] In another study, *para* methoxy cinnamate isolated from *K. galanga* was found to have lethal effects on mosquito larvae.[20] Also, ethylcinnamate as well as *para* methoxy cinnamate exhibited pesticidal activity.[21] The essential oil of *K. galanga*

showed antibacterial (*E. coli* and *S. aureus*)[22] and antifungal (five dermatophytes)[23] activity. The leaves showed antinociceptive and anti-inflammatory activities.[24]

K. marginata is a Thai medicinal plant. The root of this plant is used in the treatment of allergy, fever and swollen leg. Oxygenated pimarane diterpenes exhibiting antimalarial, antituberculous and antifungal activities and other compounds have been isolated from the whole plant extract of *K. marginata* (Table 4.1, chart 4.1).[25]

K. pandurata, another Thai medicinal plant, has been used traditionally in food and also for medicinal purposes like the treatment of stomachic disorders. It is also known for its insecticidal, antimutagenic, antitumor, anti-inflammatory and antibacterial activites.[26] Monoterpenoids, flavanones, chalcones and unusual dihydrochalcone derivatives which show biological activities have been isolated from the rhizomes of *K. pandurata*.[27] Panduratin A **384** (chart 4.1) isolated from *K. pandurata* possess various biological activities including anti-inflammatory and anticancer activities.[28]

K. parviflora is used among local people in the northeast of Thailand for the treatment of colic disorder, peptic and duodenal ulcers. It is known as a health promoting herb and a tonic made from its rhizomes is believed to relieve impotance symptoms. Several bioactive flavonoids and phenolic glycosides were isolated from the rhizomes of *K. parviflora* and the rhizomes itself show a wide array of biological activities.[29]

K. pulchra (syn. *K. elegans*) is an ornamental plant distributed in many parts of Thailand. It is also found in Indochina, Malaysia, Indonesia and South India. *K. pulchra* is a less studied plant of the genus *Kaempferia* and will be discussed in detail in the next section of this chapter.

K. rotunda is a plant indigenous to Southeast Asia. This is a handsome aromatic herb with tuberous rhizomes, distributed throughout India and cultivated for medicinal and ornamental purposes.[1] The rhizomes and young

leaves are used as a flavoring agent. It is also used as an ingredient in cosmetic products. The rhizomes of *K. rotunda* possess a wide range of medicinal uses such as in gastropathy and in the treatment of inflammations, wounds, ulcers, blood clots, tumors etc as described in P. S. Varier's book on Indian medicinal plants.[3] On steam distillation, the rhizomes yielded a light yellow volatile oil with an unpleasant odour. The oil contains mainly cineol and methyl chavicol. The tubers of the plant are widely used for local application to reduce tumors, swellings and for healing wounds. They are also considered stomachic and is administered for gastric complaints. They help in the removal of blood clots and other purpulent matter in the body. The juice of the tubers is given in dropsical affections of hand and feet and of effusions in joints, it is also considered efficacious in resolving phlegm. The juice however causes salivation and vomiting. The herb is used in oilments for healing wounds.[1] A number of cyclohexane oxide derivatives especially crotepoxide have been isolated from *K. rotunda*.[30,32] Benzyl benzoate and crotepoxide isolated from the rhizomes exhibited insecticidal activity.[31] The plant has also been attributed to contain flavonoids, chalcones, quercetin, flavonols, β-sitosterol, stigmasterol, syringic acid, protocatechuic acid and some hydrocarbons.[1,30a] There has also been a report on the antioxidant property of *K. rotunda* evaluated by the lipid peroxidation assay.[33] Chalcone **361** isolated from the chloroform extract of *K. rotunda* also exhibited antioxidant activity by scavenging free radicals.[34] Studies have shown that benzyl benzoate was the major constituent of the essential oil of the rhizomes whereas *n*-pentadecane prevailed in the lateral parts.[4, 35]

The main compounds isolated from different species of *Kaempferia* are given in Table 4.1. Their structures are given in Chart 4.1.

Table 4.1: Known phytochemical constituents isolated from various *Kaempferia* species

Plant	**Compound**	**No.**
Kaempferia sp. (Thai name: krachaikao)	Crotepoxide	355
	Bosenboxide	356
	(+)-Zeylenol	357
	(-)–(1*R*,2*S*,3*R*,4*S*)-2-Benzoyloxymethyl cyclohex-5-ene-1,2,3,4-tetrol-1,4-dibenzoate	358
	(1*R*,2*S*,3*R*,4*S*)-2-hydroxymethylcyclohex-5-ene-1,2,3,4-tetrol-1,4-dibenzoate	359
K. angustifolia	(-)–(3*S*,4*R*,5*S*,7*S*)-5-Benzoyloxymethyl-6-oxadicyclo[4.1.0]hept-1-ene-3,4-diol-3-benzoate [(-)-*pipoxide*]	360
	(-)–(1*R*,2*S*,3*R*,4*S*)-2-Benzoyloxymethyl cyclohex-5-ene-1,2,3,4-tetrol-1,4-dibenzoate	358
	(1*R*,2*S*,3*R*,4*S*)-2-hydroxymethylcyclohex-5-ene-1,2,3,4-tetrol-1,4-dibenzoate	359
	Crotepoxide	355
	Bosenboxide	356
	(+)-Zeylenol	357
	2’-Hydroxy-4,4’6’-trimethoxychalcone	361
	Quercetin	362
K. galanga	*p*-Methoxyethylcinnamate	363
	Ethylcinnamate	364
	p-Methoxycinnamic acid	365
	Cinnamaldehyde	366

	Table 4.1 continued...	
	3-Caren-5-one	367
K. marginata	(1*R*,2*S*,5*S*,7*S*,9*R*,10*S*,13*R*)-1,2,7-trihydroxy pimara-8(14),15-diene	368
	(1*R*,2*S*,5*S*,*9S*,10*S*,11*R*,13*R*)-1,2,11-trihydr-oxypimara-8(14),15-diene	369
	(1*S*,5*S*,7*R*,9*R*,10*S*,11*R*,13*R*)-1,7,11-trihydr-oxypimara-8(14),15-diene	370
	(1*S*,5*S*,9*S*,10*S*,11*R*,13*R*)-1,11-dihydroxy pimara-8(14),15-diene	371
	(5*S*,6*R*,9*S*,10*S*,13*R*)-6-hydroxypimara-8(14),15-diene-1-one	372
	(1*R*,2*S*,5*S*,7*S*,9*R*,10*S*,13*R*)-1,2-dihydroxy pimara-8(14),15-diene-7-one	373
	Sandaracopimaradiene	374
	Sandaracopimaradien-1α-ol	375
	2α-Acetoxysandaracopimaradien-1α-ol	376
	Sandaracopimaradien-1α,2α-diol	377
K. pandurata	Geranial	378
	Neral	379
	Pinostrobin	380
	Alpinetin	381
	Pinocembrin	382
	Cardamonin	383
	Panduratin A	384
	Isopanduratin A	385
	Compound B	386
	4-Hydroxypanduratin A	387

	Table 4.1 continued…	
K. parviflora	5-Hydroxy-3,7-dimethoxyflavone	388
	5-Hydroxy-7-methoxyflavone	389
	5-Hydroxy-3,7,4'-trimethoxyflavone	390
	5-Hydroxy-7,4'-dimethoxyflavone	391
	5-Hydroxy-3,7,3',4'-tetramethoxyflavone	392
	3,5,7-Trimethoxyflavone	393
	3,5,7,4'-Tetramethoxyflavone	394
	5,7,4'-Trimethoxyflavone	395
	5,7,3',4'-Tetramethoxyflavone	396
	5,7-Dimethoxyflavone	397
	3,5,7,3',4'-Pentamethoxyflavone	398
	rel-(5a*S*,10b*S*)-5a,10b-Dihydro-1,3,5a,9-tetrahydroxy-8-methoxy-6*H*-benz[b]indeno [1,2-*d*]furan-6-one 5a-O-[a-L-rhamnopyranosyl-(1 ⟶ 6)-β-D-glucopyranoside]	399
	rel-(5a*S*,10b*R*)-5a,10b-Dihydro-1,3,5a,9-tetrahydroxy-8-methoxy-6*H*-benz[b]indeno [1,2-*d*]furan-6-one 5a-O-[a-L-rhamnopyranosyl-(1⟶6)-β-D-glucopyranoside]	400
	(2*R*,3*S*,4*S*)-3-O-[α-L-rhamnopyranosyl-(1⟶6)-β-D-glucopyranosyl]-3'-*O*-methyl-*ent*-epicatechin-(2α⟶O⟶3,4α⟶4)-(5a*S*,10b*S*)-5a,10b-dihydro-1,3,5a,9-tetrahydroxy-8-methoxy-6*H*-benz[b]indeno[1,2-*d*]furan-6-one-5a-*O*-[α-L-rhamnopyranosyl-(1⟶6)-β-D-glucopyranoside]	401
	5,3'-Dihydroxy-3,7,4'-trimethoxyflavone	402

	Table 4.1 continued…	
	β– Sitosteryl myristate	403
	(*E*)-2'-Hydroxy-4',6'-Dimethoxychalcone	404
	(1*E*,6*E*)-1,7,diphenyl-1,6-heptadiene-3,5-dione	405
	5-Hydroxy-7,3',4'-trimethoxyflavone	406
	(2*S*)-5,7-Dimethoxyflavanone	407
	4'-Hydroxy-5,7-dimethoxyflavone	408
K. species (Thai name: chung - ngang)	Sandaracopimaradiene-9α-ol-1-one	409
	Sandaracopimaradien-1α,9α-diol	410
	6β-Acetoxysandaracopimaradien-9α-ol-1-one	411
	Sandaracopimaradien-6β,9α-diol-1-one	412
	6β-Acetoxysandaracopimaradien-1α,9α-diol	413
	Sandaracopimaradien-1α,6β,9α-triol	414
K. pulchra (syn. *K. elegans*)	2α-Acetoxysandaracopimaradien-1α-ol	376
	Sandaracopimaradien-1α,2α-diol	377
	p-Methoxyethylcinnamate	363
K. rotunda	Benzylbenzoate	415
	Crotepoxide	355
	(-)–(1*R*,2*R*,4*R*,5*S*,6*R*,7*R*)-4-Benzoyloxymethyl-3,8-dioxatricyclo[5.1.0.$0^{2,4}$]octane-5,6-diol-6-acetate	416
	(+)–(1*R*,2*R*,4*R*,5*S*,6*R*,7*R*)-4-Benzoyloxymethyl-3,8-dioxatricyclo[5.1.0.$0^{2,4}$]octane-5,6-diol-5-acetate	417

	Table 4.1 continued…	
	(-)–(1*R*,2*R*,4*R*,5*S*,6*R*,7*R*)-4-Benzoyloxymethyl-3,8-dioxatricyclo[5.1.0.$0^{2,4}$]octane-5,6-diol-6-benzoate	418
	(-)-Zeylenol	419
	(-)-6-Acetylzeylenol	420
	(-)-Rotepoxide A	421
	(-)-Rotepoxide B	422
	2-Acetylrotepoxide A	423
	2-Acetylrotepoxide B	424
	Rotundol	425
	2-(Benzoyloxymethyl)phenyl(3,6-di-*O*-acetyl)-β-glucopyranoside	426
	2'-Hydroxy-4,4'6'-trimethoxychalcone	361

Chart 4.1: Structures of the known phytochemical constituents isolated from various *Kaempferia* species

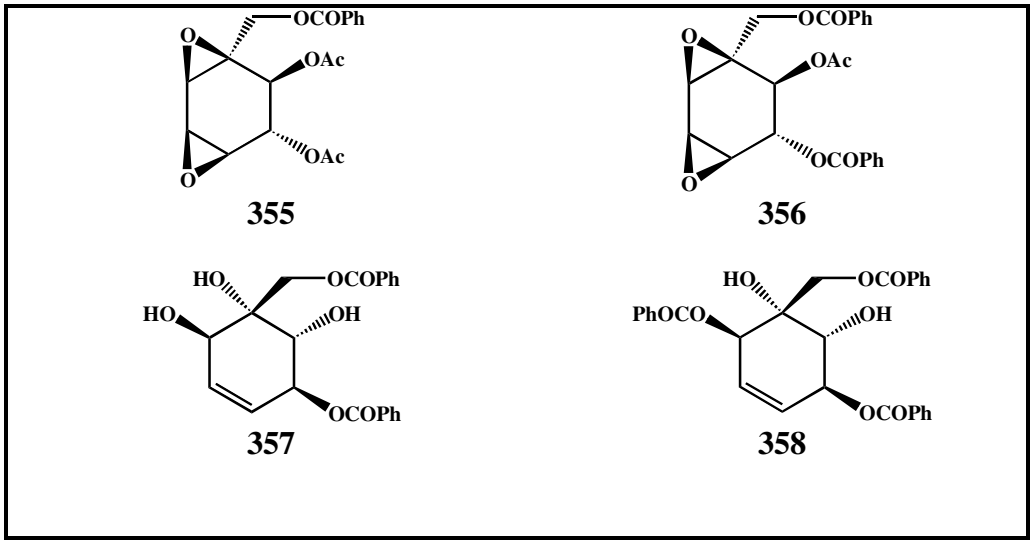
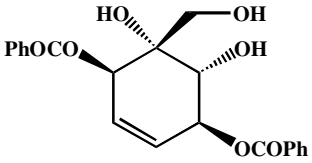


Chart 4.1 continued…



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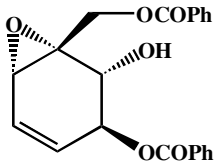

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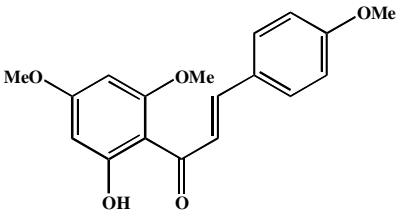

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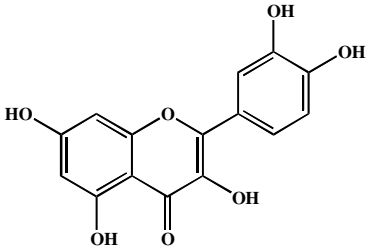

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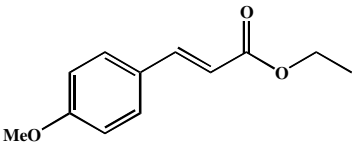

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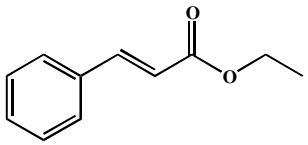

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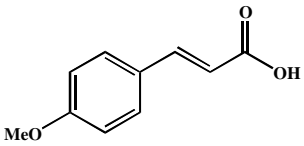

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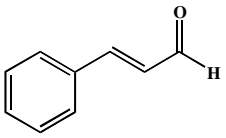

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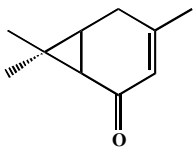

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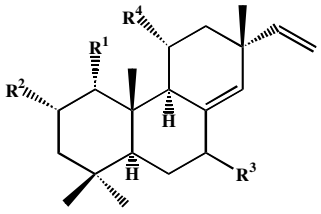
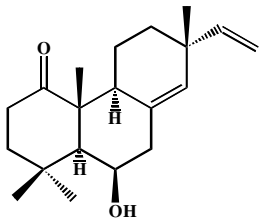


368; $R^1 = R^2 = OH$, $R^3 = \beta\text{-OH}$, $R^4 = H$
369; $R^1 = R^2 = R^4 = OH$, $R^3 = H$
370; $R^1 = R^4 = OH$, $R^3 = \alpha\text{-OH}$, $R^2 = H$
371; $R^1 = R^4 = OH$, $R^2 = R^3 = H$
373; $R^1 = R^2 = OH$, $R^3 = O$, $R^4 = H$

Chart 4.1 continued…



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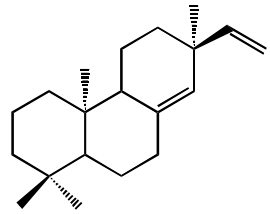

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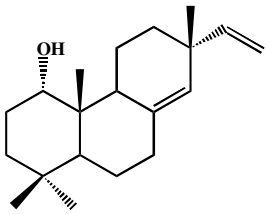

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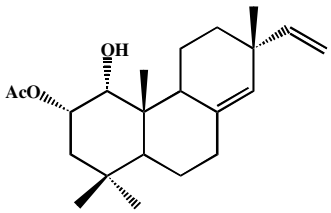

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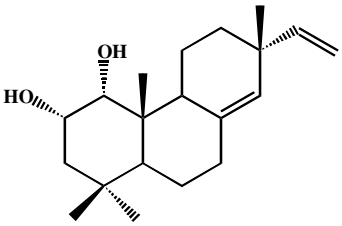

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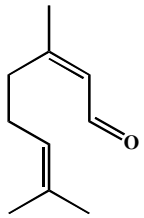

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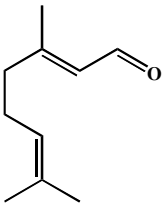

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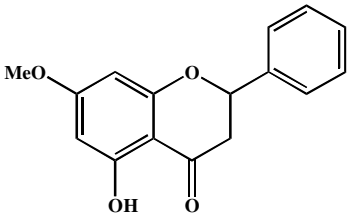

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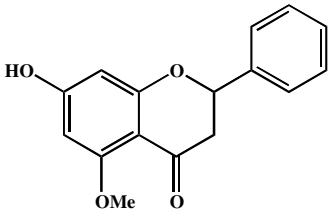

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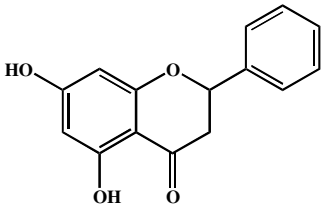

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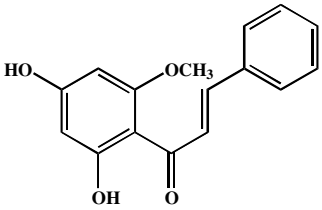

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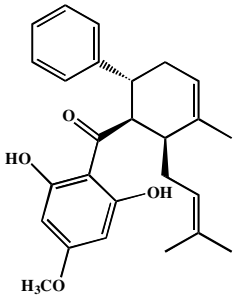
Chart 4.1 continued…



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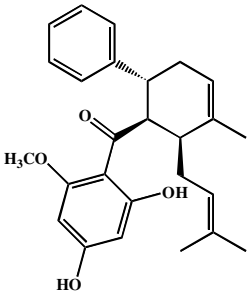

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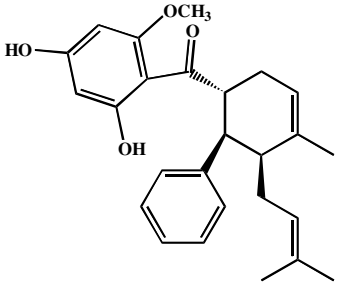

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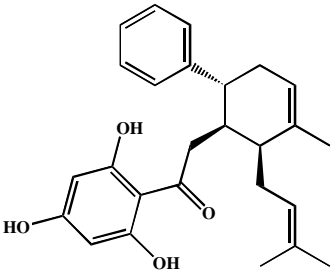

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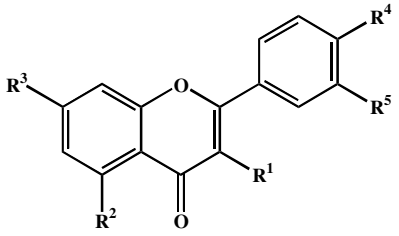


388; $R^1 = R^3 = OMe, R^2 = OH, R^4 = R^5 = H$

389; $R^1 = R^4 = R^5 = H, R^2 = OH, R^3 = OMe$

390; $R^1 = R^3 = R^4 = OMe, R^2 = OH$

391; $R^1 = H, R^2 = OH, R^3 = R^4 = OMe$

392; $R^1 = R^3 = R^4 = R^5 = OMe; R^2 = OH$

393; $R^1 = R^2 = R^3 = OMe, R^4 = R^5 = H$

394; $R^1 = R^2 = R^3 = R^4 = OMe, R^5 = H$

395; $R^2 = R^3 = R^4 = OMe, R^1 = R^5 = H$

396; $R^2 = R^3 = R^4 = R^5 = OMe, R^1 = H$

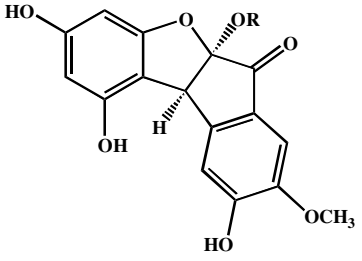
397; $R^2 = R^3 = OMe, R^1 = R^4 = R^5 = H$

398; $R^1 = R^2 = R^3 = R^4 = R^5 = OMe$

402; $R^2 = R^5 = OH, R^1 = R^3 = R^4 = OMe$

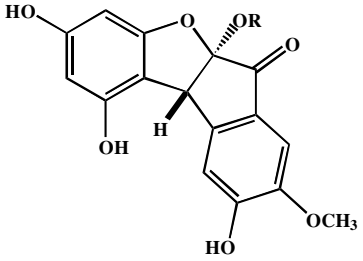
406; $R^2 = OH, R^3 = R^4 = R^5 = OMe, R^1 = H$

408; $R^2 = R^3 = OMe, R^4 = OH, R^1 = H$



R = α-L-Rha-(1→6)-β-D-Glc-

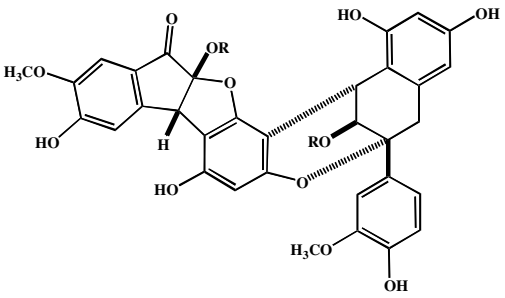
399



R = α-L-Rha-(1→6)-β-D-Glc-

400

Chart 4.1 continued…



R = α-L-Rha-(1→6)-β-D-Glc

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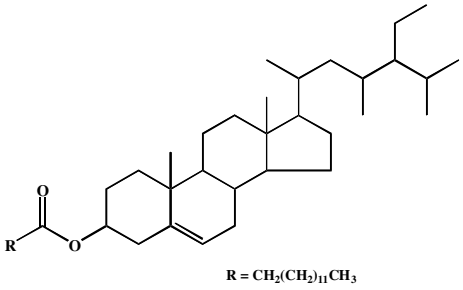

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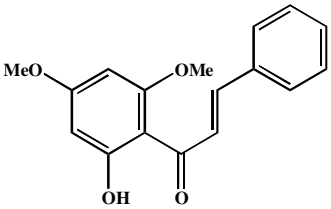

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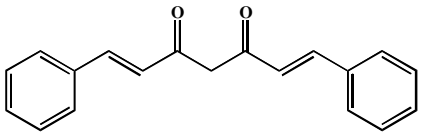

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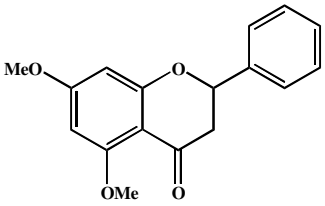

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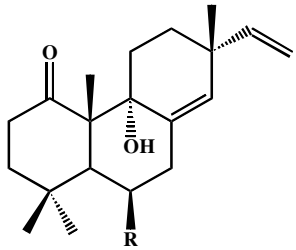
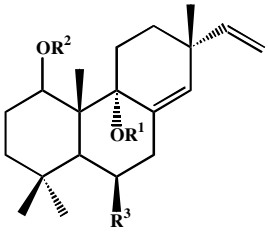


409; R = H

411; R = OAc

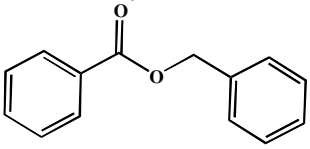
412; R = OH



410; $R^1 = R^2 = R^3 = H$

413; $R^1 = R^2 = R^3 = OAc$

414; $R^1 = R^2 = R^3 = OH$



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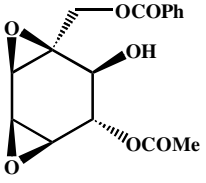

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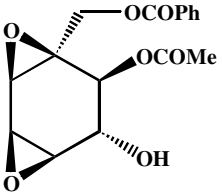

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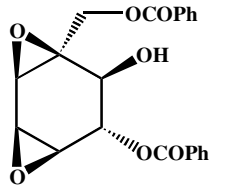

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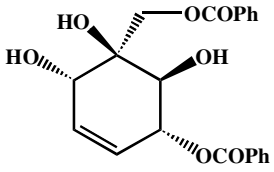

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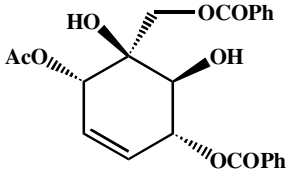

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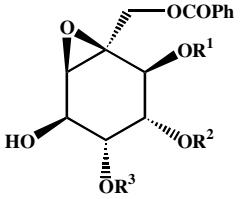
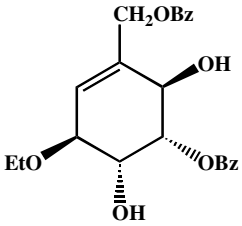


421; $R^1 = R^3 = H, R^2 = Bz$

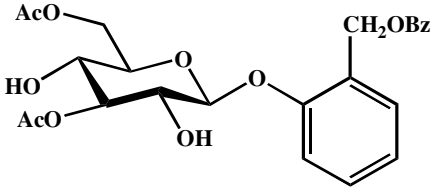
422; $R^1 = R^2 = H, R^3 = Bz$

423; $R^1 = Ac, R^2 = Bz, R^3 = H$

424; $R^1 = Ac, R^2 = H, R^3 = Bz$



425



426

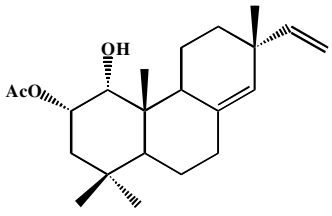
4.2 Aim and scope of the present investigation

Figure 4.1: Photograph of *Kaempferia pulchra* plant with flowers

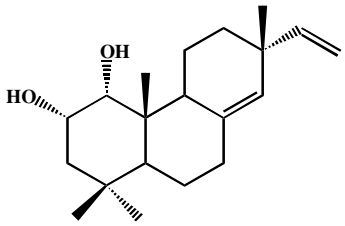
Kaempferia pulchra is a small plant with very colourful leaves. The leaves are oval in shape and have an odd pattern to them. The flowers are purple in colour with a white centre which is formed in the centre of the leaves. The plants make excellent ground covers and are easy to grow. It is grown as a garden plant in Kerala and the rhizomes look very similar to that of *K. rotunda*.

The presence of flavonoids in the leaves of *K. pulchra* was reported earlier.[5] Studies on the rhizomes of a thai species of *Kaempferia* (Thai name: chung-ngang) led to the isolation of six diterpenes of the sandaracopimaradiene series.[36] Following that, Tuchinda *et al*[37] reported the isolation of pimarane diterpenes **376** and **377** from *K. pulchra*. The isolation of *para* methoxy ethyl cinnamate (**363**), the compound common for the genus *Kaempferia* has also been isolated from the rhizomes. Chart 4.2 shows the structures of the isolated compounds from the rhizomes of *K. pulchra*. Topical anti-inflammatory activity of the two pimarane diterpenes from *K. pulchra* has also been studied.[38]

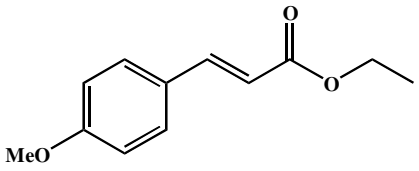
Chart 4.2: Known compounds isolated from the rhizomes of *K. pulchra*



2α-Acetoxysandaracopimaradien-1α-ol
(**376**)



Sandaracopimaradien-1α,2α-diol
(**377**)



para-Methoxyethylcinnamate
363

Due to the lack of published literature further on the constituents of this plant as well as because it is used as a substitute for *Kaempferia rotunda* in traditional systems of medicine, we selected *Kaempferia pulchra* for our present study.

4.3 Extraction, Isolation and Characterization of compounds from the acetone extract of *K. pulchra* rhizomes

4.3.1 Plant material and extraction

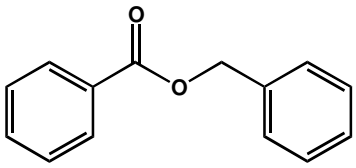
The fresh rhizomes of *Kaempferia pulchra* were collected from a certified medicinal plant grower. A voucher specimen (TBGT 20270) has been deposited in the Tropical Botanical Garden and Research Institute (TBGRI) Herbarium, Palode, Kerala, India. Fresh rhizomes (2 kg) were washed, cut into small pieces and dried in an oven at 50 °C. The powdered rhizome (360 g) was extracted using acetone (1.5 l x 3) at room temperature (27 °C). Extract was collected and the solvent was evaporated to yield 9.2 g of acetone extract. 200 mg of the extract was kept aside for antioxidant studies. Similarly ethanol extract (200 mg, kept for antioxidant studies) was obtained by extracting 100 g of the dried powdered rhizomes by room temperature extraction and then evaporating the solvent in a rotary evaporator at 50 °C.

4.3.2 Isolation of compounds from the acetone extract

The acetone extract (9 g) was then subjected to careful column chromatography using silica gel (300 g, 100-200 mesh) starting with hexane as eluant and thereafter gradually raising the polarities depending on the separation obtained after examining the fractions by TLC. A total of 150 fractions of 80-90 ml each were collected. They were further pooled together according to similarities in TLC into six fraction pools.

The second fraction pool (22-40; 1.02 g) was purified further by flash chromatography using 1 % ethyl acetate in hexane as eluant which yielded

compound **XV** (300mg) as light yellow oil. It was analyzed by various spectroscopic techniques. The IR spectrum gave the indication of an ester group from the absorption at 1726 cm^{-1}. In the ^{1}H NMR spectrum (Figure 4.2), signals between δ 8.07-7.28 integrating for ten protons indicated the presence of two aromatic rings. A sharp singlet at δ 5.32 integrating for two protons indicated a methylene group. The presence of an ester carbonyl group and the methylene carbon was further confirmed from the respective signals at δ 166.1 and 66.4 in the ^{13}C NMR spectrum (Figure 4.3). From these spectral data and by comparison with that of an authentic sample, compound **XV** was confirmed as benzyl benzoate, whose structure is as shown.



XV

Benzyl benzoate has been previously isolated as an insecticidal component from the rhizomes of *K. rotunda*.[31] However, this is the first report of the presence of benzyl benzoate in *K. pulchra*.

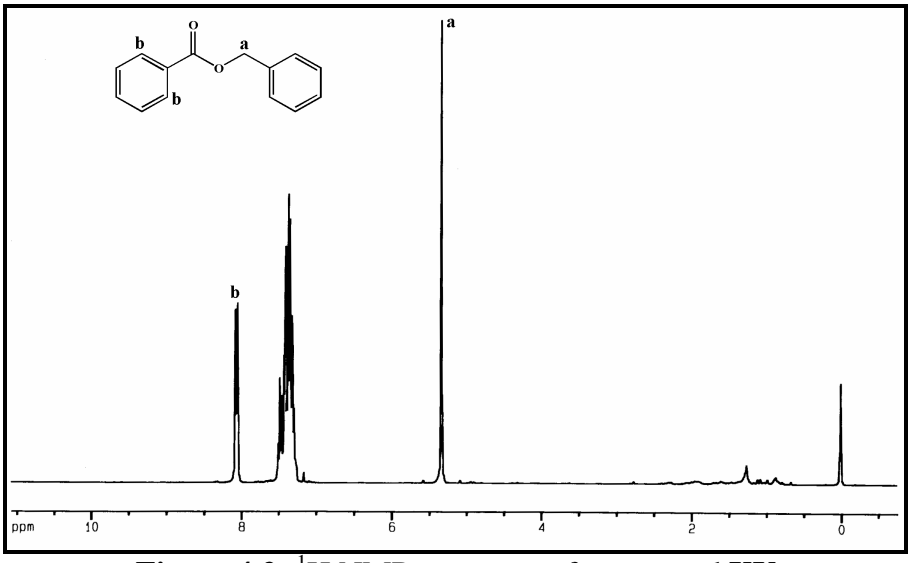


Figure 4.2: ^{1}H NMR spectrum of compound **XV**

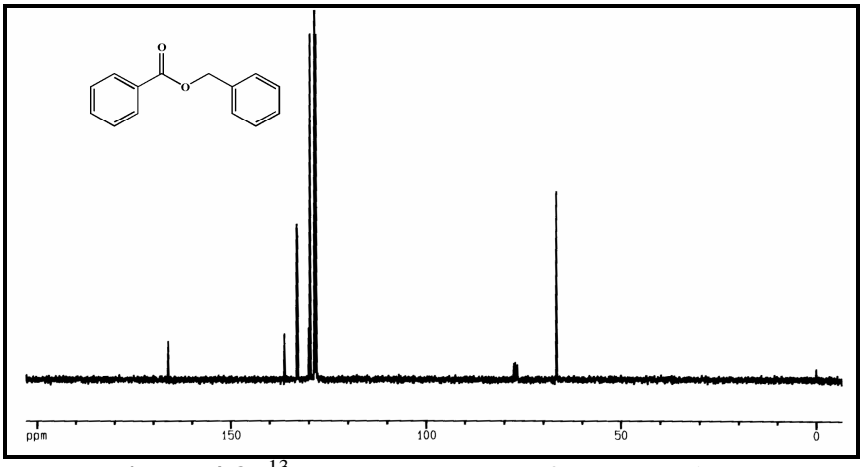
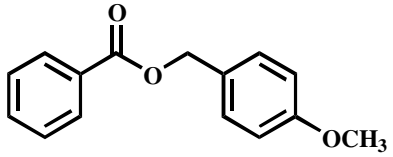


Figure 4.3: ^{13}C NMR spectrum of compound **XV**

The remaining fractions after obtaining compound **XV** were mixed together (400 mg) and was purified by recycling HPLC using chloroform as the solvent which yielded compound **XVI** (yellow oil, 5 mg) and **XVII** (white powder, 8 mg, m.p. 88-90 °C) in pure form. Both the compounds were characterized using various chromatographic techniques.

Compound **XVI** appeared just below benzyl benzoate in TLC. The IR spectrum gave absorptions at 3749 cm^{-1} indicating the presence hydroxyl group and at 1721 cm^{-1} indicating an ester group. In the ^{1}H NMR spectrum (Figure 4.4) the presence of a sharp singlet integrating for three protons at δ 3.86 confirmed the presence of an –OMe group. Nine aromatic protons were present as indicated by the signals between δ 8.10-6.91. A signal for two protons at δ 5.42 indicated the methylene proton adjacent to an ester group. The ^{13}C NMR spectrum (Figure 4.5) also confirmed the presence of –OMe group from the signal at δ 55.5. From all the spectroscopic data and further comparison with the authentic sample, compound **XVI** was confirmed to be *para* methoxy benzyl benzoate, whose structure is as shown below.



XVI

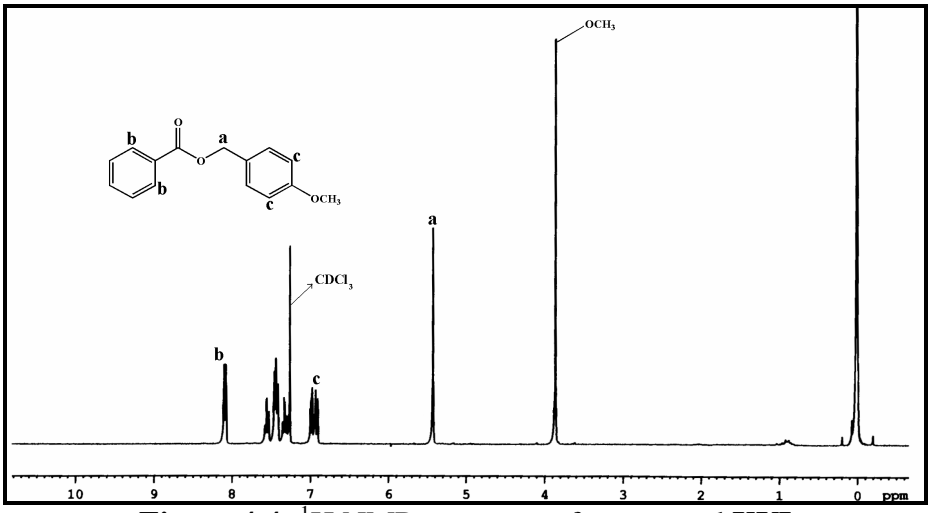


Figure 4.4: ^{1}H NMR spectrum of compound **XVI**

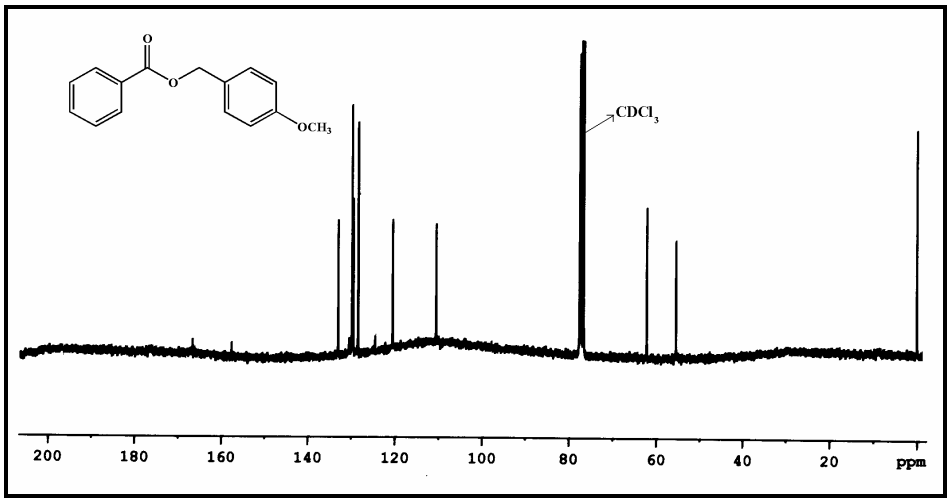
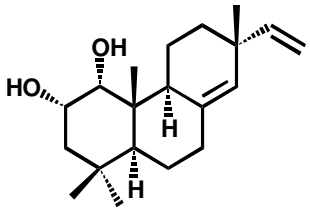


Figure 4.5: ^{13}C NMR spectrum of compound **XVI**

Compound **XVII** obtained as a white powder (8 mg, m.p. 88-90 °C) was analyzed using various spectroscopic data. The presence of hydroxyl groups were surmised from the IR absorption at 3375 cm^{-1}. The ^{1}H NMR spectrum (Figure 4.6) indicated the presence of four methyl groups from the

signals at δ 0.79, 0.87, 0.92 and 1.06. The protons attached to the hydroxyl carbons appeared at δ 3.85 and 4.22 and in the ^{13}C NMR spectrum (Figure 4.7) signals at δ 73.4 and 65.3 indicated the hydroxylated carbons. The singlet at δ 5.53 in the ^{1}H NMR spectrum indicated an olefinic proton perhaps at a ring junction. A doublet of a doublet and another doublet with fine structure for two protons between δ 4.99-4.92 indicated terminal olefinic protons. Presence of a vinylic proton was surmised from the doublet of a doublet at δ 5.79. In the ^{13}C NMR spectrum, the signals at δ 139.6 and 133.9 indicated the cyclic olefinic carbons. The terminal olefinic carbons appeared at δ 148.3 and 110.6. The mass spectrum (Figure 4.8) also gave the molecular ion peak at m/z 304. From all these spectral values and by comparing with that of the reported one, compound **XVII** was confirmed as sandaracopimaradien-1α,2 α-diol which was isolated from *K. pulchra* by Tuchinda *et al*,[37] whose structure is as shown below.



XVII

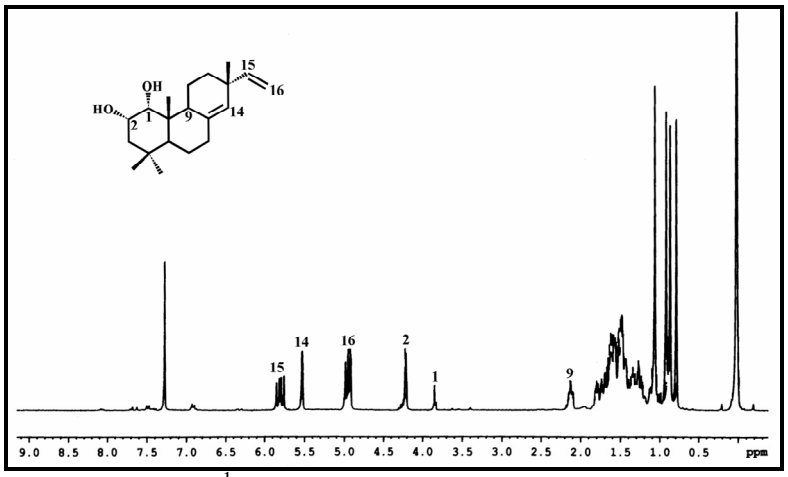


Figure 4.6: ^{1}H NMR spectrum of compound **XVII**

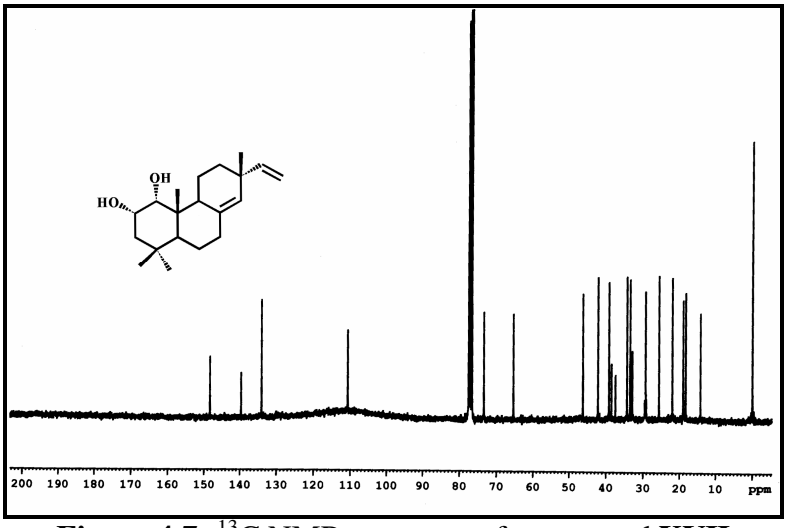


Figure 4.7: ^{13}C NMR spectrum of compound **XVII**

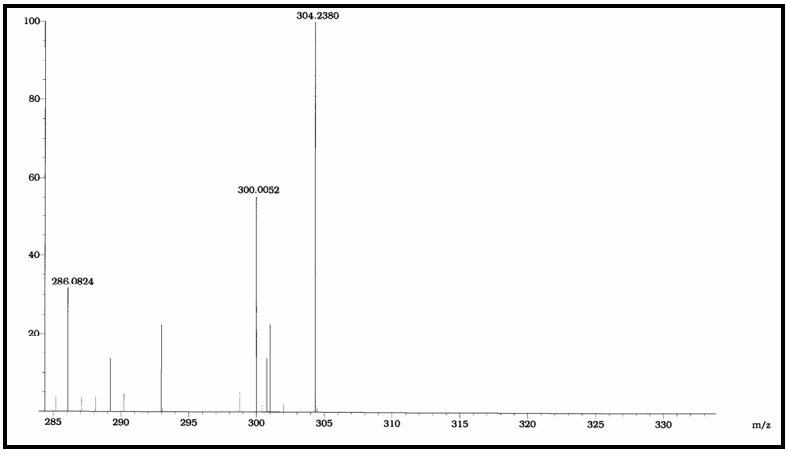
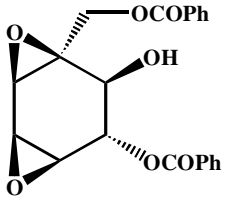


Figure 4.8: Mass spectrum of compound **XVII**

The fourth fraction pool (64-100; 700 mg) upon purification by silica gel column chromatography using hexane-EtOAc mixture (95:5) as eluent provided 71 mg of compound **XVIII** (m.p. 178-179 °C) obtained as light yellow needle like crystals after crystallizing from hexane-dichloromethane mixture. The compound was characterized after various spectral studies. The IR spectrum showed absorption at 3461cm^{-1} indicating the presence of –OH group. The absorption at 1712 cm^{-1} showed the presence of an ester >C=O group. Absorptions at 922 and 851 cm^{-1} were indicative of epoxy ring in the molecule. The ^{1}H NMR spectrum (Figure 4.9) showed a multiplet between δ

8.01 - 7.37 integrating for 10 protons indicating the presence of two aromatic rings. Two doublets centered at δ 4.62 and 4.48 integrating for one proton each coupled to each other with a *J* value of 12.3 Hz indicated the presence of methylene protons next to a polar functionality. The ^{13}C NMR spectrum (Figure 4.10) showed two peaks at δ 165.6 and 166.2 indicating the carbon atoms of ester carbonyl groups. The peaks at δ 133.8, 133.7, 130.0 (4C), 129.3, 129.1, 128.8 (2C) and 128.7 (2C) indicated the aromatic carbon atoms. Further the mass spectrum gave a molecular ion peak at m/z 383 for [M+1] (Figure 4.11). From all these data as well as by comparison with the spectral details reported in the literature[30b], the structure of **XVIII** was confirmed as **4-Benzoyloxymethyl-3,8-dioxatricyclo[5.1.0.0^{2,4}]octane-5,6-diol-6-benzoate** as shown below. This compound was isolated earlier from *K. rotunda*.[30b] However, this is the first report of its presence in *K. pulchra*.



XVIII

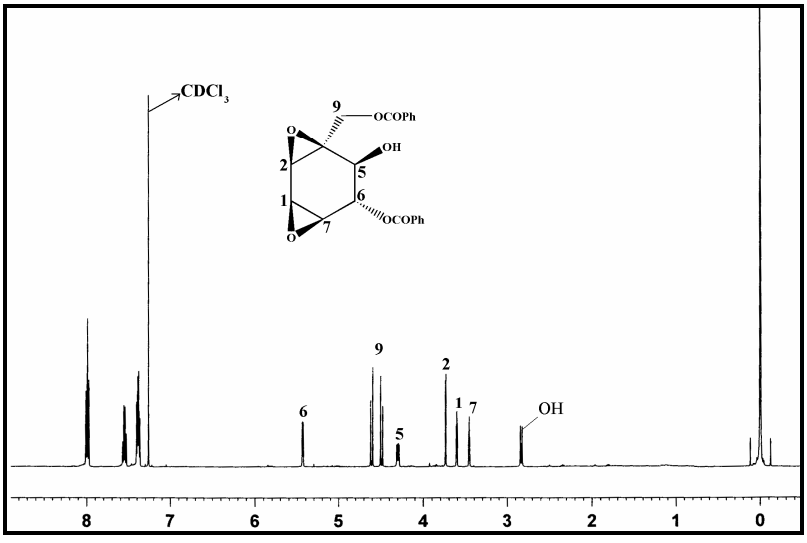


Figure 4.9: ^{1}H NMR spectrum of compound **XVIII**

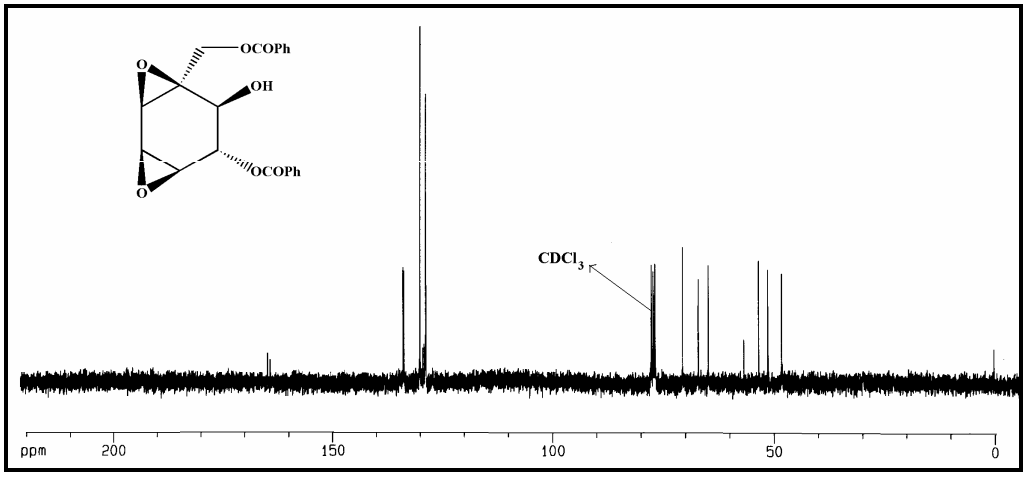


Figure 4.10: ^{13}C NMR spectrum of compound **XVIII**

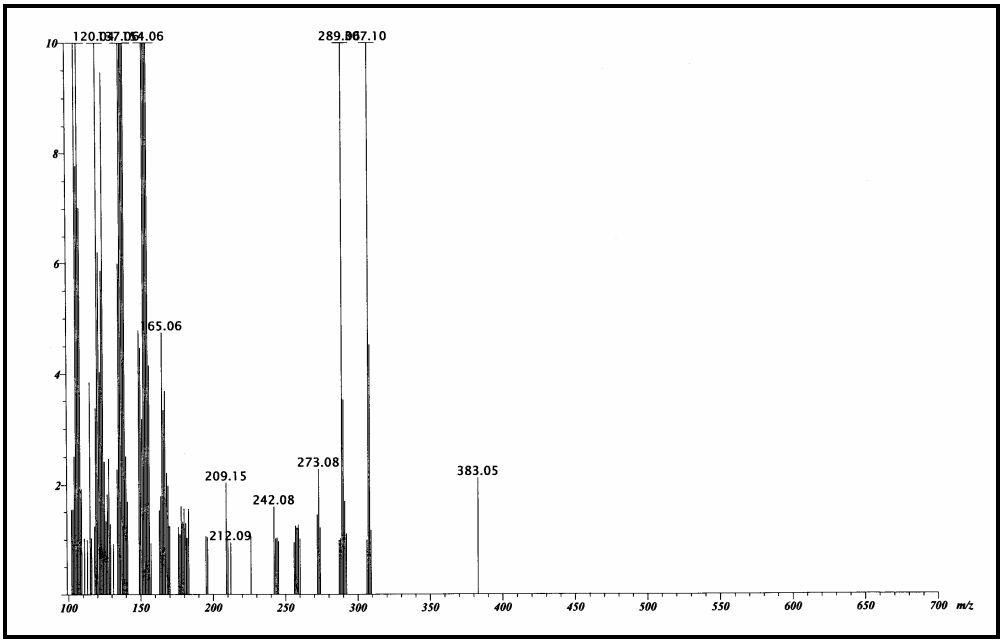
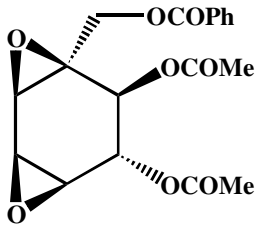


Figure 4.11: Mass spectrum of compound **XVIII**

The fifth fraction pool (101-144; 1.4 g), on column chromatographic purification by silica gel using hexane-EtOAc mixtures (90:10 – 60:40) yielded three compounds **XIX**, **XX** and **XXI** in pure form. Compound **XIX** was crystallized from hexane-dichloromethane mixture as white crystals (1.2 g; m.p. 152-153 °C). It was analyzed based on various spectroscopic data. The

IR spectrum showed the presence of two types of carbonyl groups present in the molecule based on two >C=O stretching vibrations; one was at 1766 cm^{-1} and other at 1726 cm^{-1} suggestive of two ester groups. The absorptions at 904 cm^{-1} and 863 cm^{-1} suggested the presence of epoxy ring. In the ^{1}H NMR spectrum (Figure 4.12), two singlets at δ 2.03 and 2.12 integrating for 3 protons each indicated the presence of two acetate groups. The signals from δ 8.04 to 7.44 integrating for 5 protons indicated the presence of an aromatic group. Two doublets centered at δ 4.99 and 5.71 with *J* value of 9.0 Hz, integrating for one proton each, indicated the presence of two adjacent deshielded protons perhaps adjacent to oxygen functionality. Doublets centered at δ 4.24 and 4.57 which were coupled to each other indicated the presence of CH_2 protons, once again adjacent to a polar group. The peaks centered at δ 3.67, 3.46, 3.11 indicated the presence of protons attached to the strained carbons bearing epoxy ring. The ^{13}C NMR spectrum (Figure 4.13) showed 18 carbon peaks in the compound. The peaks at δ 133.6, 129.8 (2C), 129.2 and 128.6 (2C) confirmed the presence of a benzene ring. The signals at δ 170.1, 169.8 and 165.5 indicated the presence of three ester carbonyl groups and the peaks at δ 20.7 and 20.6 suggested the presence of two acetoxy methyl groups. The mass spectrum (Figure 4.14) gave the molecular ion peak at m/z 362 for the $[M]^{+}$ ion. From all the spectral data and from the literature reports, compound **XIX** was confirmed as the cyclohexane diepoxide **4-Benzoyloxymethyl-3,8-dioxatricyclo[5.1.0.$0^{2,4}$]octane-5,6-dioldiacetate**, commonly known as **crotepoxide**. The structure of crotepoxide is as shown below.



XIX

Crotepoxide has been isolated earlier from *K. rotunda*[30b] as well as from *Bosenbergia* species of the Zingiberaceae family. It is also an important constituent of *Piper attenuatum*.[30c] Crotepoxide was found to possess insecticidal activity[31] and tumor inhibiting property which makes its isolation from *K. pulchra* significant.

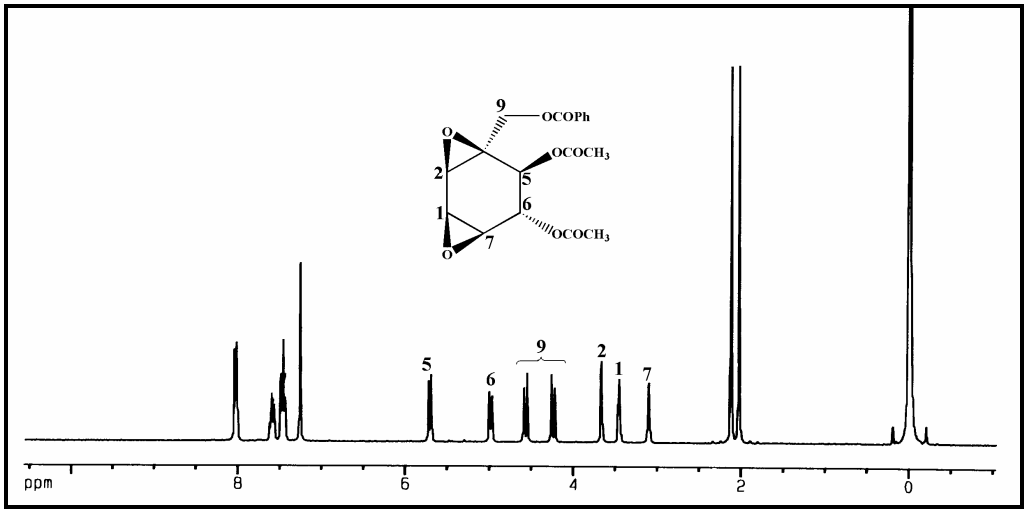


Figure 4.12: ^{1}H NMR spectrum of compound **XIX**

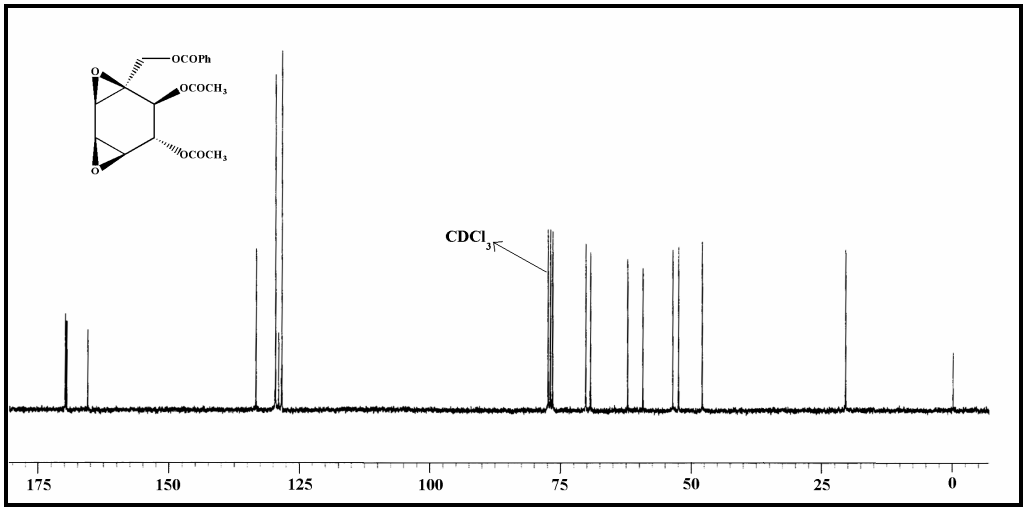


Figure 4.13: ^{13}C NMR spectrum of compound **XIX**

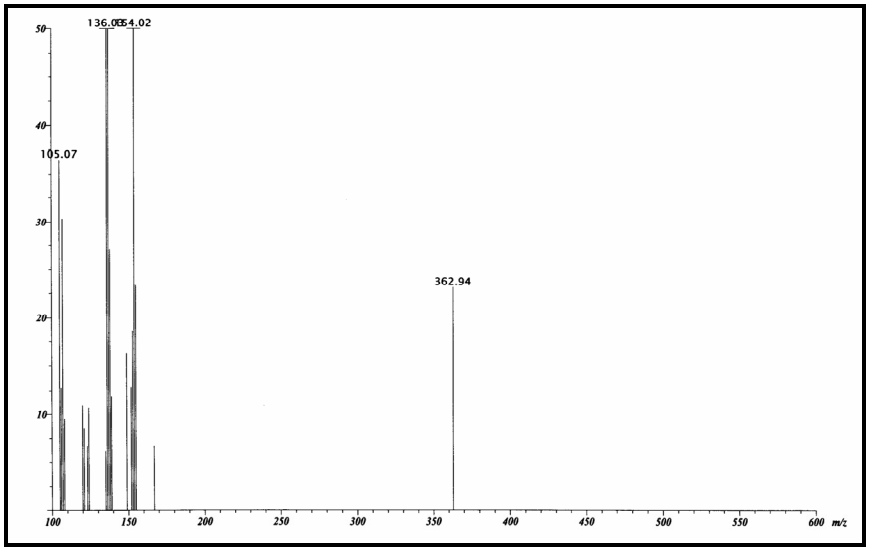
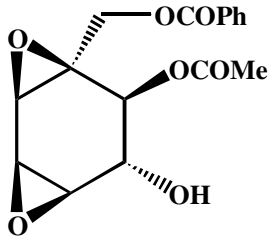


Figure 4.14: Mass spectrum of compound **XIX**

Compound **XX** crystallized from hexane-dichloromethane mixture was obtained as yellow crystals (23 mg, m.p. 136-137 °C). The structure of this compound was also deduced by studies of IR, ^{1}H NMR and ^{13}C NMR spectral data. The absorption at 3294 cm^{-1} in the IR spectrum indicated the presence of -OH functionality, while absorptions at 1748 cm^{-1} and 1722 cm^{-1} showed two ester carbonyl groups. The peaks at 908 cm^{-1} and 852 cm^{-1} indicated the absorptions due to epoxy ring. The ^{1}H NMR spectrum (Figure 4.15) gave insight to further structural details of this compound. A sharp singlet integrating for three protons at δ 2.19 indicated the presence of an acetate group and the signals from δ 8.03 to 7.44 integrating for five protons indicated the presence of an aromatic ring. One proton doublet centered at δ 5.46 indicated the presence of a proton attached to the carbon bearing the acetate group. Two doublets centered at δ 4.60 and 4.24 with *J* value of 12.1 Hz, integrating for one proton each indicated the presence of a deshielded methylene group. The peaks centered at δ 3.63, 3.18 and 2.70 indicated the presence of protons attached to the strained carbon atom bearing the epoxy ring. The ^{13}C NMR spectrum (Figure 4.16) showed 14 carbon signals. Peaks

at δ 171.5 and 165.9 confirmed the presence of two ester functionalites. The signals at δ 133.5 129.7 (2C), 129.1 and 128.5 (2C) indicated the presence of aromatic carbon atoms. The peak at δ 20.8 indicated the presence of methyl group of the acetate functionality. The mass spectrum (Figure 4.17) gave molecular ion peak at m/z 321 $[M+H]^+$. From all these data and on comparison with data of compounds isolated from other *Kaempferia* species[30b], the structure of compound **XX** was confirmed as **4-Benzoyloxymethyl-3,8-dioxatricyclooctane-5,6-diol-5-acetate** which was earlier isolated from *K. rotunda*, as shown below.



XX

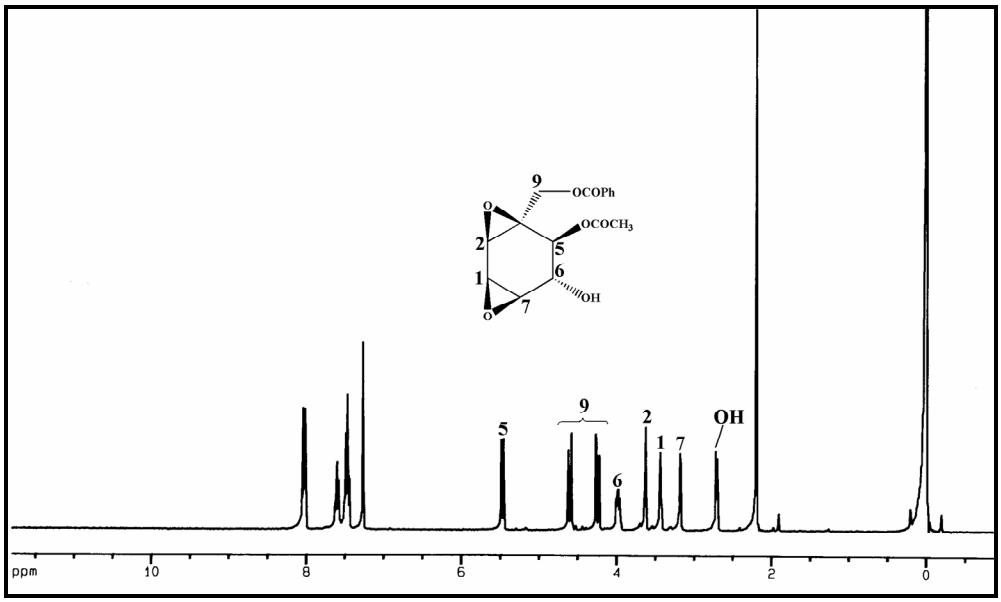


Figure 4.15: ^{1}H NMR spectrum of compound **XX**

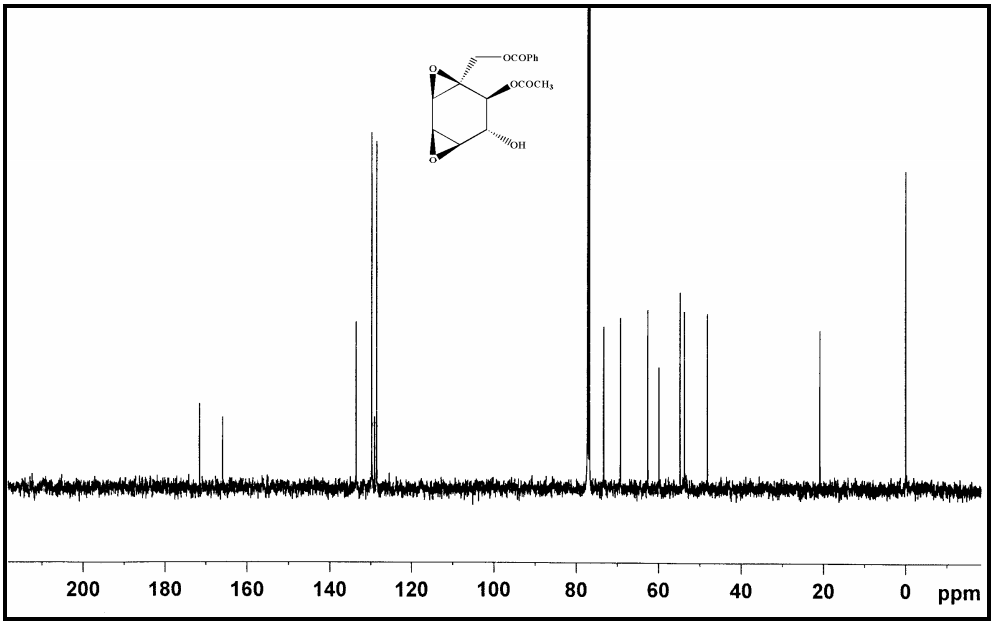


Figure 4.16: ^{13}C NMR spectrum of compound **XX**

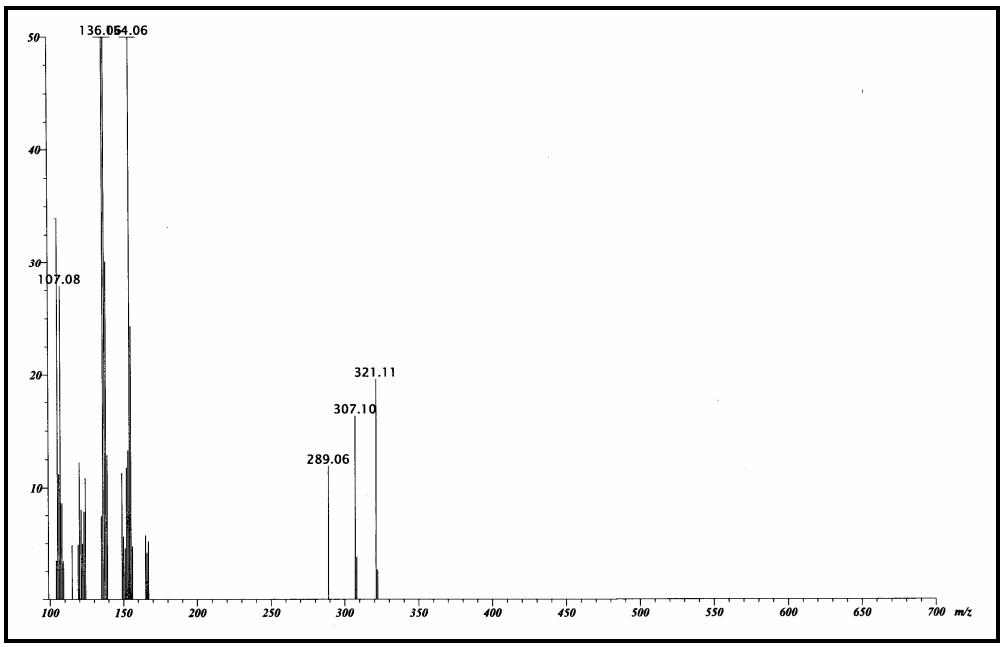
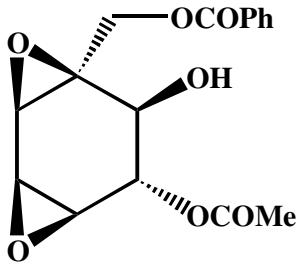


Figure 4.17: Mass spectrum of compound **XX**

Compound **XXI** was crystallized from hexane-dichloromethane as colourless needles (12 mg; m.p. 142-143 °C). The structure of **XXI** was

confirmed after various spectral analyses. The IR spectrum showed absorption at 3495 cm^{-1} indicating the presence of –OH group. The absorptions at 1748 and 1715 cm^{-1} indicated the presence of two ester groups. The absorptions at 913cm^{-1} and 863 cm^{-1} indicated epoxy group absorptions. The ^{1}H NMR spectrum (Figure 4.18) showed a sharp, three proton singlet at δ 1.89 indicating the presence of an acetate group and the signals ranging from δ 8.07 to 7.44 integrating for five protons indicated the presence of an aromatic ring. Doublet of a doublet at δ 5.17 for one proton indicated the presence of the proton attached to the carbon bearing the acetate group. The two doublets centered at δ 4.54 and 4.41 each integrating for one proton with the same coupling constant (J = 12.2 Hz) indicated the presence of a deshielded -CH_2- group. The peaks centered at δ 3.54, 3.31 and 2.72 indicated the presence of protons attached to the strained carbon atom bearing the epoxy ring. The ^{13}C NMR spectrum (Figure 4.19) showed 14 carbon signals. Peaks at δ 169.9 and 165.6, confirmed the presence of ester functionalities. The signals at δ 133.6, 129.8 (2C), 129.3 and 128.6 (2C) indicated the presence of aromatic carbon atoms. Signal at δ 20.5 indicated the methyl group of the acetate functionality. The mass spectrum (Figure 4.20) gave molecular ion peak at m/z 321 for the $[M+1]^{+}$ ion. From these data and on comparison with data of compounds isolated from *K. rotunda*, the structure of compound **XXI** was confirmed as **4-Benzoyloxymethyl-3,8-dioxatricyclooctane-5,6-diol-6-acetate** as shown below.



XXI

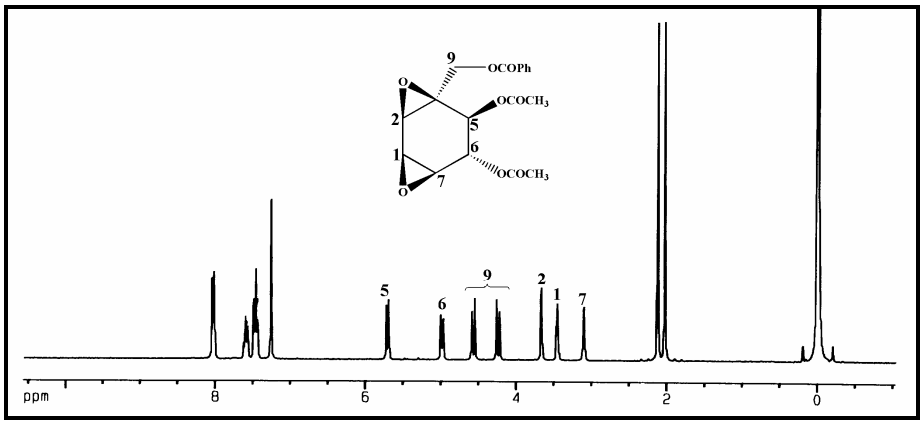


Figure 4.18: ^{1}H NMR spectrum of compound **XXI**

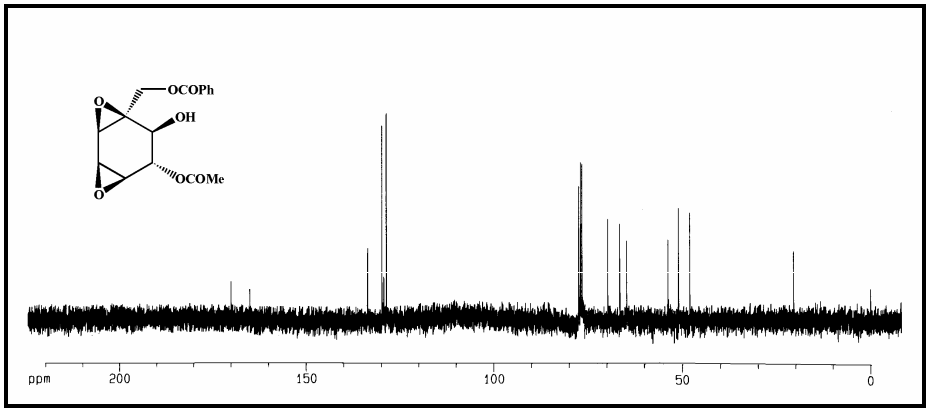


Figure 4.19: ^{13}C NMR spectrum of compound **XXI**

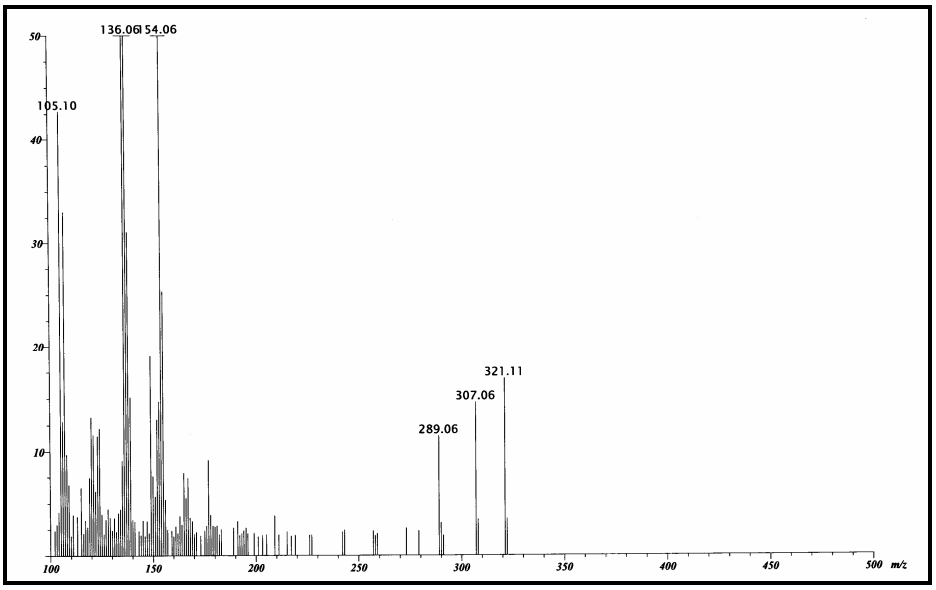
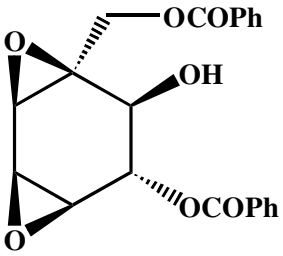


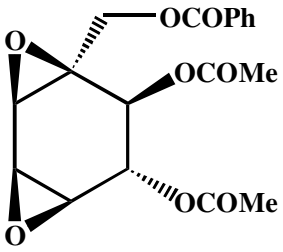
Figure 4.20: Mass spectrum of compound **XXI**

4.3.3 Biological activity

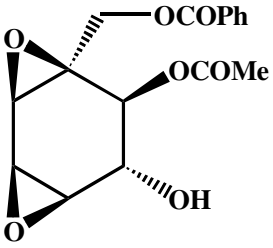
The acetone and ethanol extracts as well as the pure compounds isolated from *K. pulchra* were tested for antioxidant activity by various *in vitro* methods. However, significant antioxidant activity was not observed. The isolated compounds were also tested for anticancer activity in collaboration with Rajiv Gandhi centre for Biotechnology (RGCB). Preliminary experiments on the cytotoxicity/anticancer activity of the pure compounds viz., **XVIII**, crotepoxide (**XIX**), **XX** and **XXI** gave positive results, i.e., the compounds **XX** and **XIX** showed anticancer activity on MCF-7 breast cancer cell lines, with compound **XX** showing the highest activity. The cell viability on treatment with **XX** was lowest (19% cell viability at 50 μM of the sample) as shown in figure 4.21. Compound **XXI** and **XVIII** did not show remarkable anticancer activity. Further work is in progress at RGCB inorder to confirm the *in vitro* anticancer potential of the compounds isolated from *K. pulchra*.



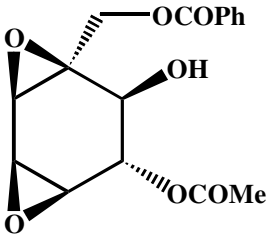
XVIII



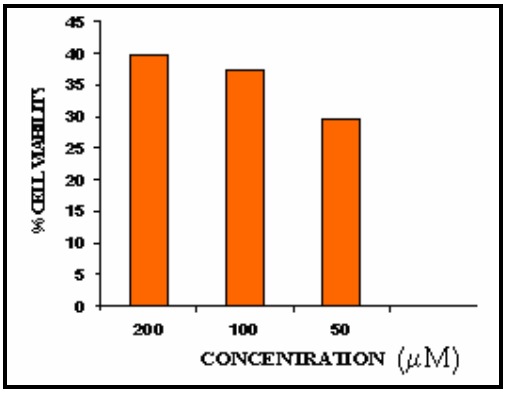
XIX

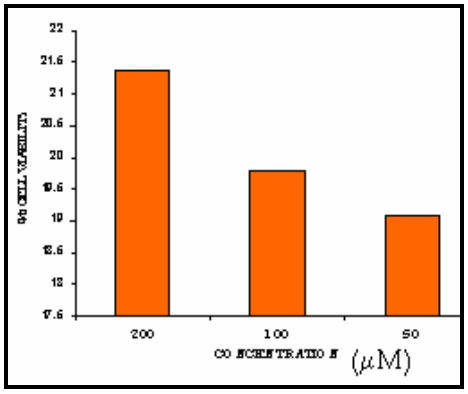


XX



XXI





Crotepoxide (**XIX**) Compound **XX**

Figure 4.21: % Cell viability of compounds **XIX** and **XX** against breast cancer cell lines

4.4 Experimental

General experimental procedures for the isolation of compounds are as reported in Chapter 2 of this thesis.

4.4.1 Extraction

The extraction was carried out by immersing the powdered plant material in acetone overnight and then decanting the solvent. Crude extracts were obtained after evaporation of the decanted solvent under reduced pressure at ambient temperatures in a rotary evaporator. Pure compounds were obtained from the crude extracts using column chromatographic separation.

4.4.2 Chromatographic separation of the extracts

The crude extracts were dissolved in minimum quantity of hexane and then loaded onto a silica gel column. The column was eluted successively with hexane and hexane-ethyl acetate mixtures of increasing polarities (staring from 100 % hexane and ending with 100 % ethyl acetate). The isolation procedure of compounds **XV** to **XXI** is depicted in figure 4.22. Purification by recycling HPLC was carried out on a LC-9101 Recycling

preparative HPLC instrument (Japan Analytical Industry Co., Ltd) using spectroscopic grade chloroform as the solvent.

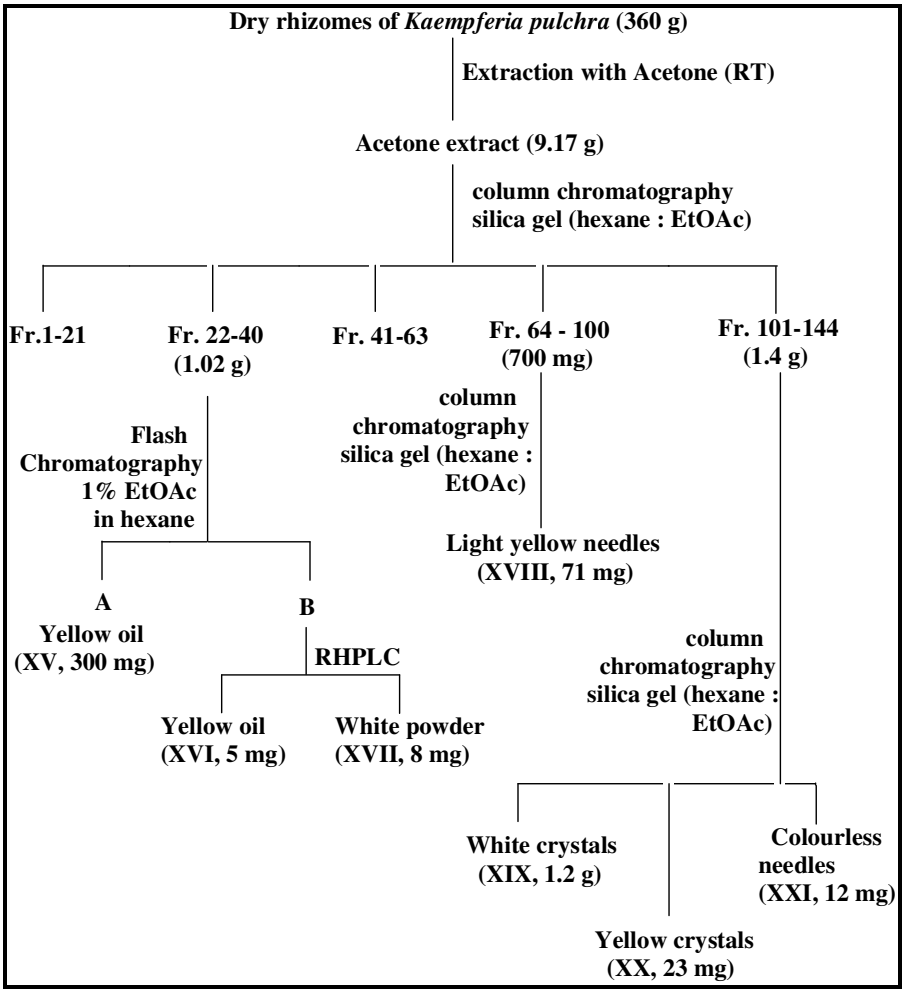
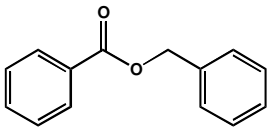


Figure 4.22: Pictorial representation of the isolation of compounds **XV – XXI**

4.4.2.1 Isolation of compound XV

Figure 4.22 depicts the isolation pathway for compound **XV**. It was obtained through flash chromatography of the second fraction pool with 1 % Ethyl acetate-hexane mixture. It was obtained as light yellow oil (300 mg). From various spectroscopic data and by comparison with the authentic

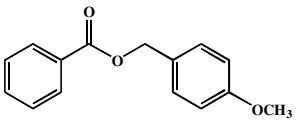
sample, compound **XV** was confirmed as benzyl benzoate whose structure is depicted below with complete spectral data.



FT-IR (neat, ν_{max}/cm^{-1})	:	1726, 1602, 1455, 1379, 1274, 1102, 712.
^1H NMR (300 MHz, $CDCl_3$)	:	δ 8.06 (2H, *d*, *J* = 7.4 Hz), 7.50-7.28 (8H, *m*), 5.32 (2H, *s*)
^{13}C NMR (75 MHz, $CDCl_3$)	:	δ 166.1, 135.9, 132.8, 130.0, 129.5 (2C), 128.4 (2C), 128.2 (2C), 128.0 (2C), 127.9, 66.4

4.4.2.2 Isolation of compound XVI

The remaining fractions after obtaining benzyl benzoate was subjected to recycling HPLC using chloroform as the solvent to yield compound **XVI** which was confirmed as *para* methoxy benzylbenzoate (yellow oil, 5 mg) from the various spectroscopic data. The structure and spectral details are given below.



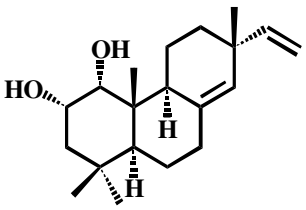
FT-IR (neat, ν_{max}/cm^{-1})	:	3749, 2928, 1720, 1651, 1559, 1541, 1508, 1457, 1269, 1105, 1027, 705
^1H NMR (300 MHz, $CDCl_3$)	:	δ 8.09 (2H, *d*, *J* = 8.0 Hz), 7.58-7.30 (5H, *m*), 7.00 - 6.91 (2H, *m*), 5.42 (2H, *s*), 3.86 (3H, *s*)
^{13}C NMR (75	:	δ 166.6, 157.6, 132.9, 130.5,

MHz, $CDCl_3$)		129.7 (2C), 129.5 (2C), 128.3 (2C), 124.5, 120.5, 110.5, 62.2, 55.5

4.4.2.3 Isolation of compound XVII

The isolation procedure is summarized in figure 4.22. Compound **XVII** was obtained as white powder (8 mg) by recycling HPLC using chloroform as the solvent. The compound was identified as the diterpene, sandaracopimaradien-1α,2 α-diol whose structural details are shown below.

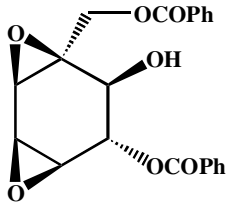
FT-IR (KBr, ν_{max}/cm^{-1})	:	3375, 1635, 1458, 1385, 1365, 1289, 1245, 1168, 1116, 1035, 1019, 995, 911, 906, 863
^1H NMR (300 MHz, $CDCl_3$)	:	δ 5.79 (1H, *dd*, *J* = 11.7, 10.8 Hz), 5.53 (1H, *s*), 4.99 - 4.92 (2H, *dd,dd*, *J* = 17.5 Hz), 4.22 (1H, *d*, *J* = 2.7 Hz), 3.85 (1H, *br s*), 2.12 (1H, *br t*, $J_1 = J_2 = 7$ Hz), 1.82 - 1.12 (11H, *m*), 1.06 (3H, *s*), 0.92 (3H, *s*), 0.87 (3H, *s*), 0.79 (3H, *s*)
^{13}C NMR (75 MHz, $CDCl_3$)	:	δ 148.3, 139.6, 133.9, 110.6, 73.4, 65.3, 46.1, 42.1, 39.1, 38.5, 37.4, 34.3, 33.4, 32.9, 29.3, 25.6, 21.9, 19.0, 18.3, 14.2
EI-MS m/z (rel.	:	304.2380 $[M]^+$ (100),



int.)		$C_{20}H_{32}O_2$, requires 304.2402
m.p. (°C)	:	88-90 °C, lit[37].90-92 °C

4.4.2.4 Isolation of compound XVIII

Compound **XVIII** was isolated from the fourth fraction pool (fractions 64-100) as summarized in figure 4.22. It was recrystallized from dichloromethane-hexane mixture and obtained as light yellow needles (71 mg). The compound was identified as 4-Benzoyloxymethyl-3,8-dioxatricyclo[5.1.0.$0^{2,4}$]-octane-5,6-diol-6-benzoate based on the spectral data shown below.



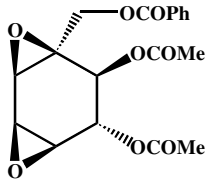
FT-IR (KBr, ν_{max}/cm^{-1})	:	3461, 1712, 1314, 1292, 1270, 1179, 1114, 1023, 922, 851, 709.
^{1}H NMR (500 MHz, $CDCl_3$)	:	δ 8.01-7.97 (4H, *m*), 7.56-7.53 (2H, *m*), 7.41-7.37 (4H, *m*), 5.42 (1H, *dd*, *J* = 4.3, 2.5 Hz), 4.62 (1H, *d*, *J* = 12.3 Hz), 4.48 (1H, *d*, *J* = 12.3 Hz), 4.30 (1H, *dd*, *J* = 10.3, 5.0 Hz), 3.74 (1H, *d*, *J* = 2.7 Hz), 3.60 (1H, *dd*, *J* = 3.4, 2.8 Hz), 3.46 (1H, *ddd*, *J* = 3.3, 2.5, 0.5 Hz), 2.82 (1H, OH),
^{13}C NMR (75 MHz, $CDCl_3$)	:	δ 166.2, 165.6, 133.8, 133.7, 130.0 (4C), 129.3, 129.1, 128.8 (2C), 128.7 (2C), 70.6, 67.1, 64.8, 56.8, 53.5, 51.4, 48.3.

FAB-MS m/z (rel. int.)	:	383.05 [M+1] (23), $C_{21}H_{18}O_7$, requires 382.11
m.p. (°C)	:	178-179°C, lit[30b]. 177-178 °C
$[\alpha]_D^{26}$ (ca, 0.659, $CHCl_3$)	:	-76.3 °, lit[30b]. -76 °

4.4.2.5 Isolation of compound XIX

Compound **XIX** was obtained as white crystals (1.2 g) after recrystallization from hexane-dichloromethane mixture. The isolation procedure is depicted in figure 4.22. The compound was identified as Crotepoxide (4-Benzoyloxymethyl-3,8-dioxatricyclooctane-5,6-diol-5,6-diac-etate) from the various spectral data as shown below.

FT-IR (KBr, ν_{max}/cm^{-1})	:	1766, 1726, 1452, 1373, 1283, 1236, 1121, 1068, 1042, 1014, 921, 904, 863, 720
^1H NMR (300 MHz, $CDCl_3$)	:	δ 8.03 (2H, *d*, *J* = 7.4 Hz), 7.62-7.60 (1H, *m*), 7.49-7.44 (2H, *m*), 5.71 (1H, *d*, *J* = 9.0 Hz), 4.99 (1H, *d*, *J* = 9.0 Hz), 4.57 (1H, *d*, *J* = 12.1 Hz), 4.24 (1H, *d*, *J* = 12.1 Hz), 3.67 (1H, *d*, *J* = 2.6 Hz), 3.46 (1H, *dd*, *J* = 3.8, 2.7 Hz), 3.11 (1H, *dd*, *J* = 3.8, 1.4 Hz), 2.12 (3H, *s*), 2.03 (3H, *s*)

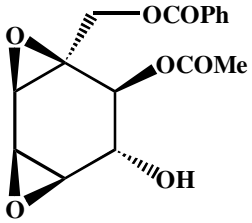


^{13}C NMR (75 MHz, $CDCl_3$)	:	δ 170.1, 169.8, 165.5, 133.6, 129.8 (2C), 129.2, 128.6 (2C), 70.4, 69.5, 62.5 59.4, 53.8, 52.6, 48.1, 20.7, 20.6.
FAB-MS m/z (rel. int.)	:	362.94 $[M]^+$ (23), $C_{18}H_{18}O_8$, requires 362.10
m.p. (°C)	:	152-153 °C, lit[30b]. 152-154°C
$[\alpha]_D^{26}$ (ca, 1.000, $CHCl_3$)	:	+64°, lit.[30b] +64°

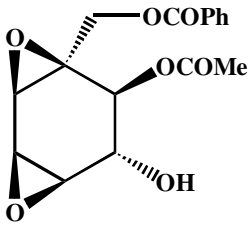
4.4.2.6 Isolation of compound XX

Compound **XX** was obtained as yellow crystals (23 mg) after crystallization from dichloromethane-hexane mixture. The isolation procedure is depicted in figure 4.22. The compound was identified as 4-Benzoyloxymethyl-3,8-dioxatricyclooctane-5,6-diol-5-acetate whose structure and spectral data are given below.

FT-IR (KBr, ν_{max}/cm^{-1})	:	3294, 1748, 1722, 1452, 1373, 1285, 1225, 1129, 1197, 1045, 928, 908, 852, 710
^1H NMR (300 MHz, $CDCl_3$)	:	δ 8.02 (2H, *d*, *J* = 7.4 Hz), 7.62-7.57 (1H, *m*), 7.49-7.44 (2H, *m*), 5.46 (1H, *d*, *J* = 9.0 Hz), 4.60 (1H, *d*, *J* = 12.1 Hz), 4.24 (1H, *d*, *J* = 12.1 Hz), 3.97 (1H, *dd*, *J* = 8.3,



5.6 Hz), 3.63 (1H, *d*, *J* = 2.5 Hz), 3.43 (1H, *dd*, *J* = 3.5, 3.0 Hz), 3.18 (1H, *dd*, *J* = 3.5, 1.0 Hz), 2.70 (1H, *d*, *J* = 5.5 Hz), 2.19 (3H, *s*).

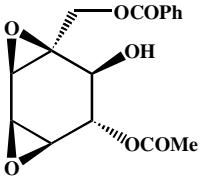


^{13}C NMR (75 MHz, $CDCl_3$)	:	δ 171.5, 165.9, 133.5, 129.7 (2C), 129.1, 128.5(2C), 73.3, 69.2, 62.6, 59.8, 54.7, 53.7, 48.1, 20.8
FAB-MS m/z (rel. int.)	:	321.11 [M+1] (20), $C_{16}H_{16}O_7$, requires 320.09
m.p. (°C)	:	136-137 °C, lit[30b]. 135-136°C
$[\alpha]_D^{26}$ (ca, 0.987, $CHCl_3$)	:	+35.8°, lit.[30b] +36°

4.4.2.7 Isolation of compound XXI

As depicted in figure 4.22, the compound **XXI** was obtained on repeated chromatographic separation of the fifth fraction pool (fractions 101-144). It was obtained as colourless needles upon recrystallization from dichloromethane-hexane mixture. Analysis of the spectral data led to the identification of the compound as 4-Benzoyloxymethyl-3,8-dioxatricyclooctane-5,6-diol-6-acetate shown below.

FT-IR (KBr, ν_{max}/cm^{-1}	:	3495, 1748, 1715, 1452, 1374, 1284, 1224, 1126, 1063, 913, 863, 716.



^{1}H NMR (300 MHz, $CDCl_3$)	:	δ 8.06 (2H, *d*, *J* = 7.4 Hz), 7.62-7.57 (1H, *m*), 7.49-7.44 (2H, *m*), 5.17 (1H, *dd*, *J* = 4.8, 2.5 Hz), 4.54 (1H, *d*, *J* = 12.2 Hz), 4.41 (1H, *d*, *J* = 12.2 Hz), 4.13 (1H, *dd*, *J* = 10.4, 4.8 Hz), 3.70 (1H, *d*, *J* = 2.7 Hz), 3.54 (1H, *dd*, *J* = 3.1, 3.0 Hz), 3.31 (1H, *dd*, *J* = 3.0, 2.7 Hz), 2.72 (1H, *d*, *J* = 10.4 Hz), 1.89 (3H, *s*)
^{13}C NMR (75 MHz, $CDCl_3$)	:	δ 169.9, 165.6, 133.6, 129.8 (2C), 129.3, 128.6 (2C), 69.7, 66.5, 64.7, 55.9, 53.7, 50.9, 48.0, 20.5
FAB-MS m/z (rel. int.)	:	321.11 [M+1] (30), $C_{16}H_{16}O_7$, requires 320.09
m.p. (°C)	:	142-143 °C, lit[30b]. 141-142°C
$[\alpha]_D^{26}$ (ca, 0.658, $CHCl_3$)	:	-58.2°, lit.[30b] -58°

4.5 References

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Chapter 5

Isolation of the biologically active compound nimbolide from *Azadirachta indica* (Neem) leaves and bioevaluation for anticancer and antioxidant capacity

5.1 Introduction

Figure 5.1: Photograph of *A. indica* leaves with seeds

The promising properties of *Azadirachta indica* (commonly known in India as Neem; Family: Meliaceae) have been realized worldwide.[1a] Almost all parts of the tree offer tremendous potential for medicinal, agricultural and industrial exploitation. Various parts of the *A. indica* tree are used in traditional *Ayurvedic* medicines in India. Some of the traditional uses[1b] of different parts of the tree is given in Table 5.1. There are a number of formulations in *Ayurvedic* system of medicine which contain *A. indica*. In addition to its therapeutic efficacies, *A. indica* has already established its potential as a source of naturally occurring insecticides, pesticides and agrochemicals. The environmental compatibility of the *A. indica* products, the lack of resistance developed to them, their harmless nature against non target

organisms and lack of toxicity, all have significantly enhanced the use of *A. indica* in integrated pest control. For thousands of years the beneficial properties of the *A. indica* tree have been recognized in India and it is perhaps the country's most utilized useful traditional medicinal plant.[2]

Table 5.1: Medicinal properties of different parts of neem tree

Part	Medicinal uses
Leaf	Treatment of leprosy, eye problems, intestinal worms, anorexia, skin ulcers.
Flowers	Bile suppression, elimination of intestinal worms
Twig	Relieves cough, asthma, piles, spermatorrhoea, diabetes
Bark	Analgesic, antipyretic.
Fruit	Relieves piles, intestinal worms, urinary disorder, eye problem, diabetes etc
Gum	Effective against ring worms, scabies, wounds etc.
Seed pulp	Leprosy and intestinal worms.
Oil	Leprosy and intestinal worms.
Root, bark, leaf, flower and fruit together	Biliary afflictions, itching, skin ulcer, burning sensation and leprosy.

A. indica tree is native to the Indian subcontinent and South East Asia. There are two closely related species, *A. indica* and *A. azedarch*. The former is popularly known as Indian neem or Indian lilac and the later as the Persian lilac.[3] *A. indica* is a large, hardy, fast growing tree, 10-15m in height with spreading branches. Leaves are 20-40 cm long, are pinnately branched and have ovate-lanceolate, bright green leaflets, measuring up to 5cm in length and 1.5cm in width. Leaves have disagreeable odour and very bitter taste.[4] Almost all parts of the *A. indica* tree (stem, bark, roots, leaves, gum, seeds, fruits, flowers etc) have been in use as traditional medicine for house hold remedies against various ailments.[5] These spectacular properties of *A. indica* have attracted organic chemists, biologists, clinicians and agriculturalists

around the world to undertake systematic research on this unique plant in various directions.[3]

It is reported that the aqueous extract of *A. indica* leaves significantly reduces blood sugar levels and prevent adrenaline and glucose induced hyperglycemia[6] as well as decreases hyperglycemia in streptozotocin induced diabetes.[7] *A. indica* leaf extract has shown significant antiulcer and antisecretory effects in rats.[8] It also possess antimalarial activity. Extracts of the leaves have been studied for their effect on *in vitro* and *in vivo* growth and development of the human malarial parasite *Plasmodium falciparium* and found to have highly appreciable results on parasites resistant to antimalarial drugs such as chloroquine and pyrimethamine.[9] The aqueous extract of the leaf showed antioxidant activities.[10] The chemoprotective property of the leaf extract was also confirmed.[11] The antioxidant activity of *A. indica* seed extract has also been demonstrated.[12] *A. indica* is very effective against common skin diseases like acute and chronic eczema, ring worm and scabies.[13] The acetone extract of the leaf showed significant anxiolytic activity in rats.[14] The ethanol extract of the stem bark and root bark showed hypotensive, spasmolytic and diuretic activities. The chemical constituent sodium nimbidinate, was found to be a potent diuretic agent in dogs.[15] Oral administration of an aqueous extract of *A. indica* leaf also showed antifertility effects in mice.[16] The anti-inflammatory activity and antihypertensive effect of *A. indica* has also been reported.[17]

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

(i) Isoprenoids like diterpenoids and triterpenoids including protomeliacin, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin type compounds and nimbin, salanin and azadirachtin.

(ii) Non isoprenoids which include proteins, aminoacids, carbohydrates, sulfur compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcones, coumarins, tannins, aliphatic compounds etc.

Although a large number of compounds have been isolated from various parts of neem tree, especially from the seeds, only a few of them have been investigated so far for biological activity.[18,19] Structures of some of the important compounds isolated from *A. indica* is given in chart 5.1 and some important compounds isolated from the leaves are given in chart 5.2.

Chart 5.1: Structures of important compounds (known) isolated from *A. indica*

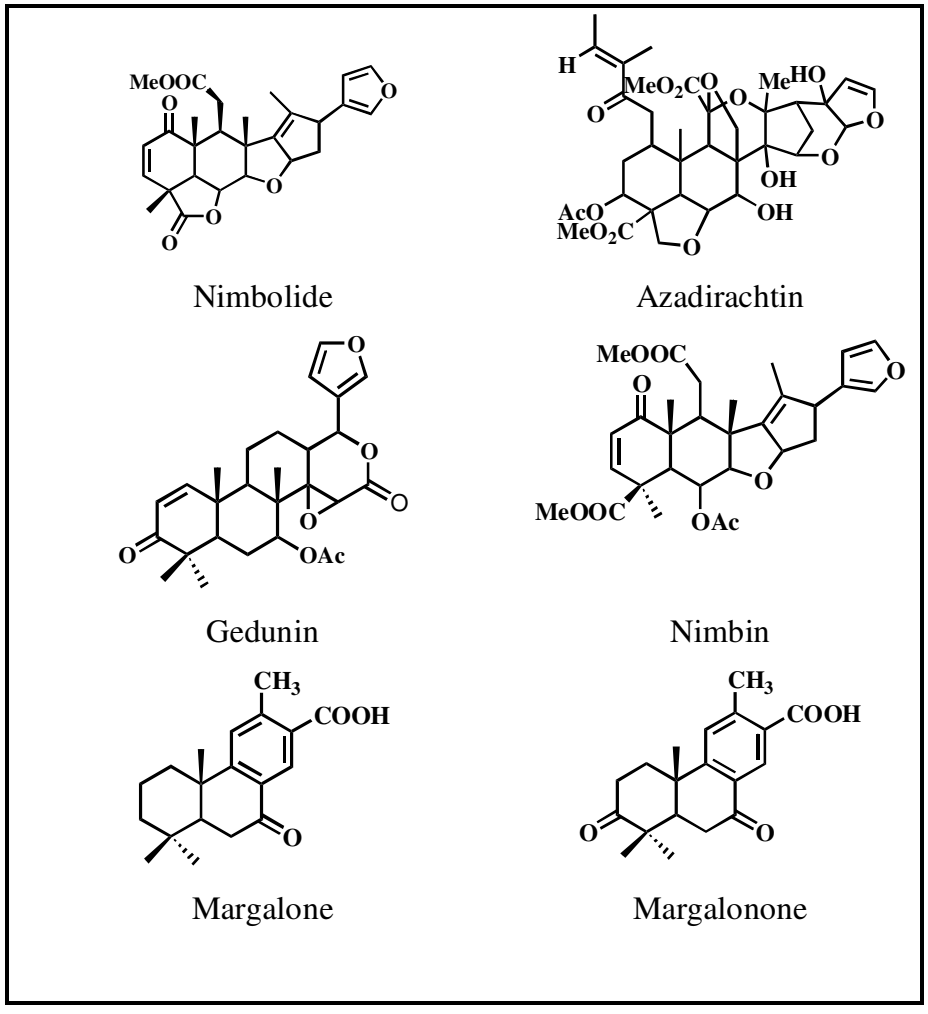
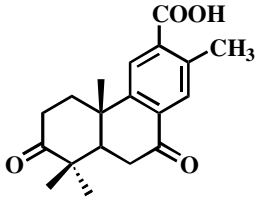
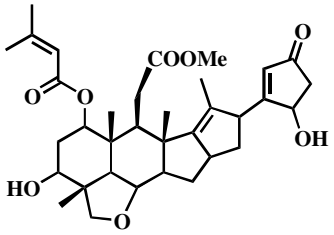


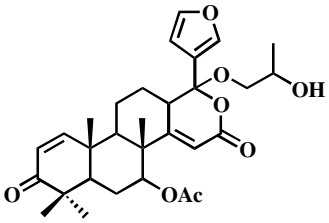
Chart 5.1 continued…



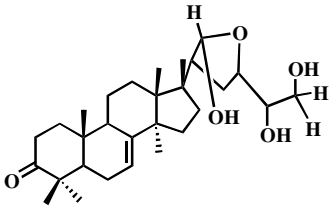
Isomargolonone



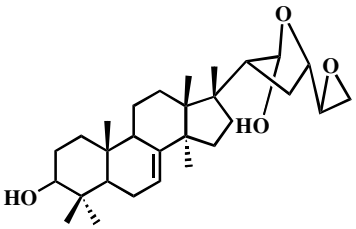
Isoazadirolide



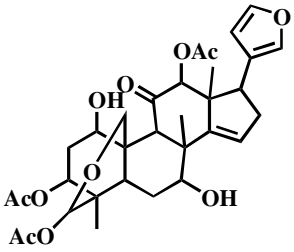
Mahmoodin



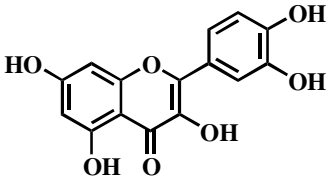
Melianodiol



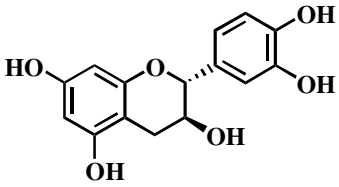
Melianol



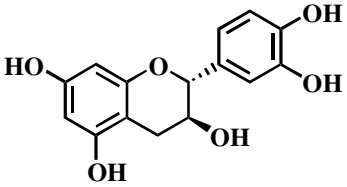
Azadirachtanin



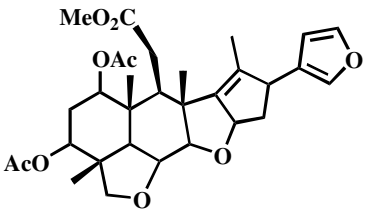
Quercetin



Catechin

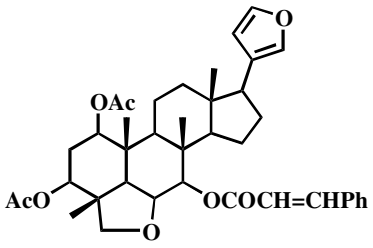


(-)-Epicatechin

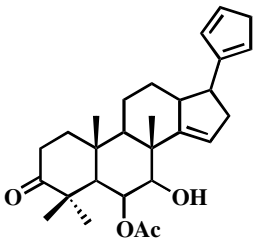


Salanin

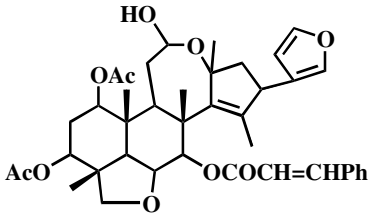
Chart 5.1 continued…



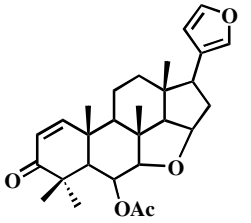
Nimbolin A



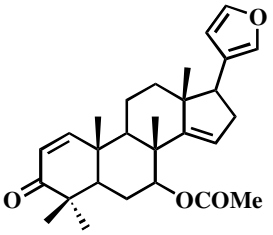
Meldenin



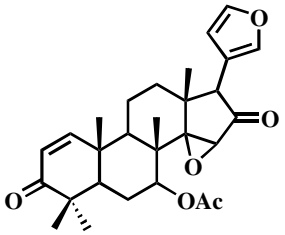
Nimbolin B



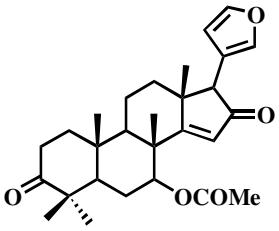
Vepinin



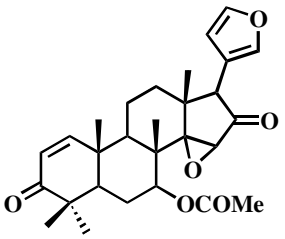
Azadirone



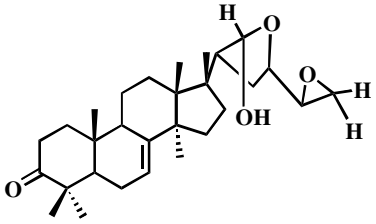
Nimbinin



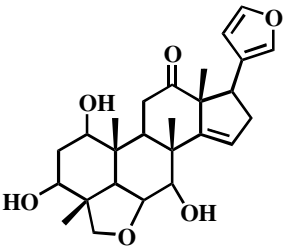
Azadiradione



Epoxyazadirone

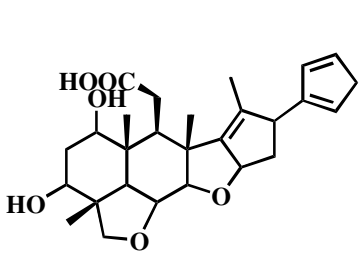


Melianone

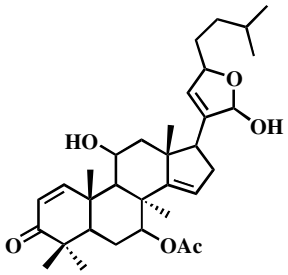


Nimbidin

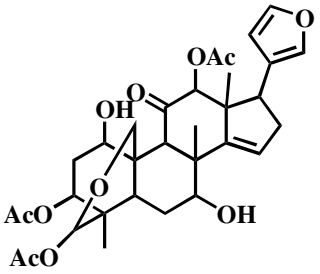
Chart 5.1 continued…



Salannic acid

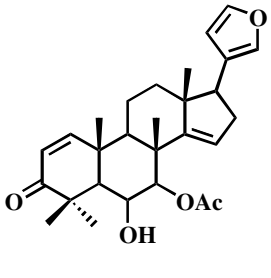


Azadirachtol

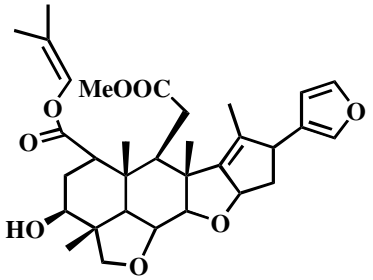


Azadirachtanin

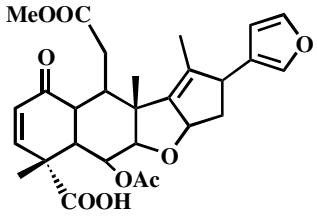
Chart 5.2: Structures of important compounds (known) isolated from the leaves of *A. indica*



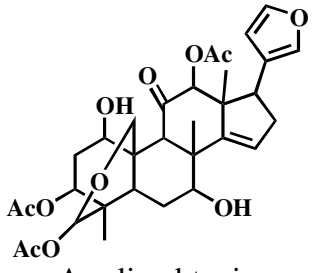
Nimocinol



2',3'-Dehydosalannol

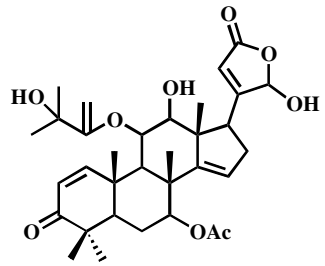


4-Epinimbin

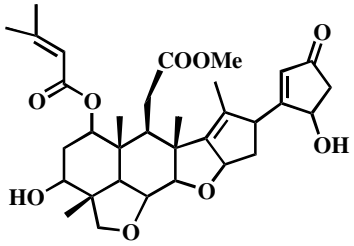


Azadirachtanin

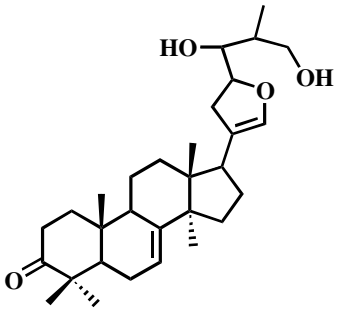
Chart 5.2 continued…



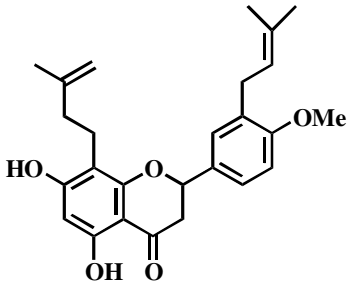
Isonimbocinolide



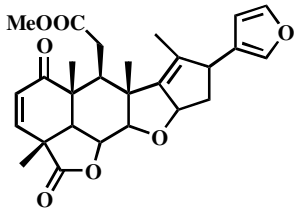
Isoazadirolide



Nimbocinone



Nimbaflavone



Nimbolide

5.2 Aim and scope of the present investigation

From the review of literature, it was clear that there are detailed reports on the biological activity of the compounds isolated from the seeds. But less known is the activity of the components isolated from the leaves. Only few reports are available on the anti-cancer studies of nimbolide, which is one of the major constituent of *A. indica* leaf.[20] Therefore it was of interest to study the anti cancer activity of nimbolide and antioxidant activity of different leaf extracts. During an earlier study conducted in our group on the leaves of *A. indica*[21] a methodology for the rapid isolation of nimbolide was found. From this study, pure sample of nimbolide was also available that could be used for TLC comparison purposes.

5.3 Extraction of *A. indica* leaves and isolation and characterization of nimbolide

5.3.1 Plant material and extraction

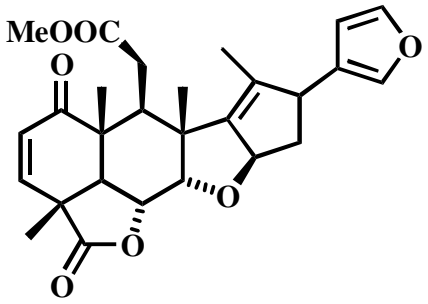
A. indica leaves were collected from NIIST campus, dried and powdered. 450 g of the powdered leaves was extracted successively with hexane, acetone and methanol at room temperature. Solvents were removed under reduced pressure using a rotary evaporator to get 12 g, 21 g and 32 g of the hexane, acetone and methanol extracts respectively. TLC was performed on the extracts along with a standard sample of nimbolide which indicated that acetone extract contained maximum amount of nimbolide. Thus the acetone extract was subjected to column chromatographic separation for obtaining nimbolide.

5.3.2 Isolation of nimbolide from the acetone extract

The acetone extract (21 g) was then subjected to careful column chromatography using silica gel (400 g, 100-200 mesh) starting with hexane as eluant and thereafter gradually raising the polarities with ethyl acetate depending on the separation obtained after examining the fractions by TLC. 360 fractions of 80-90 ml were collected which were finally pooled to ten fraction pools according to the similarities in TLC's. The first fractions contained β-carotene as a major constituent upon examination of the TLC with a standard sample of β-carotene.

The seventh fraction pool (150-189; 1 g) obtained on elution with 25% ethyl acetate in hexane contained a major compound upon examination by TLC. On crystallization in dichloromethane-ethyl acetate mixture to get white crystals were obtained (116 mg, m.p: 204-205 °C). The compound was analyzed using various spectroscopic techniques. ^{1}H NMR spectrum (Figure 5.2) 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 a sharp singlet

integrating for three protons at δ 3.54. Multiplet between δ 7.32–7.22 confirmed the presence of three protons in the furan ring. The α protons of the α, β-unsaturated ketone was observed as the doublet integrating for one proton at δ 5.92 (J = 11.8 Hz). Further by comparison of the Mass, ^{1}H and ^{13}C NMR data with the values reported earlier[22] it was confirmed that the compound was **nimbolide** whose structure is shown below.



NIMBOLIDE

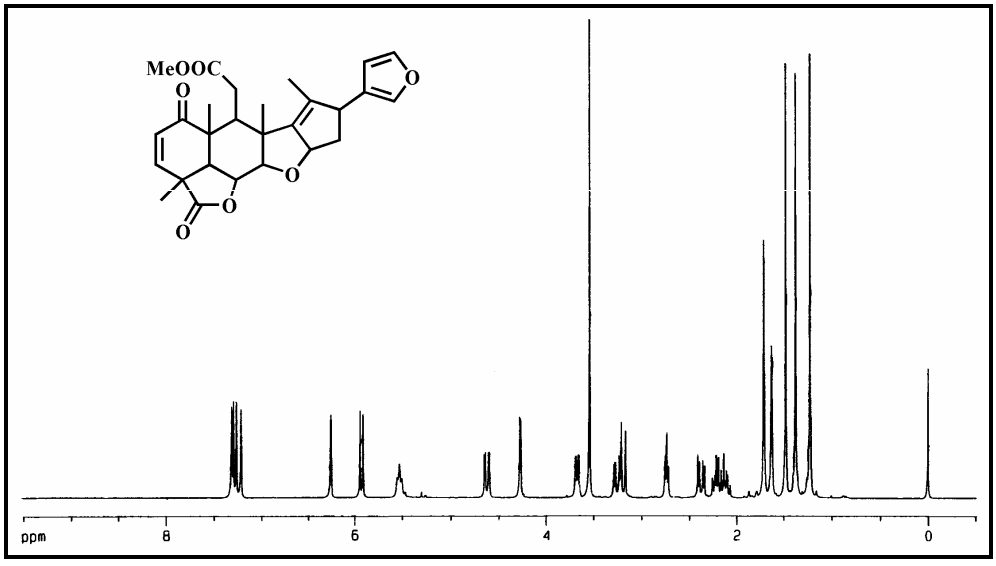


Figure 5.2: ^{1}H NMR spectrum of **nimbolide**

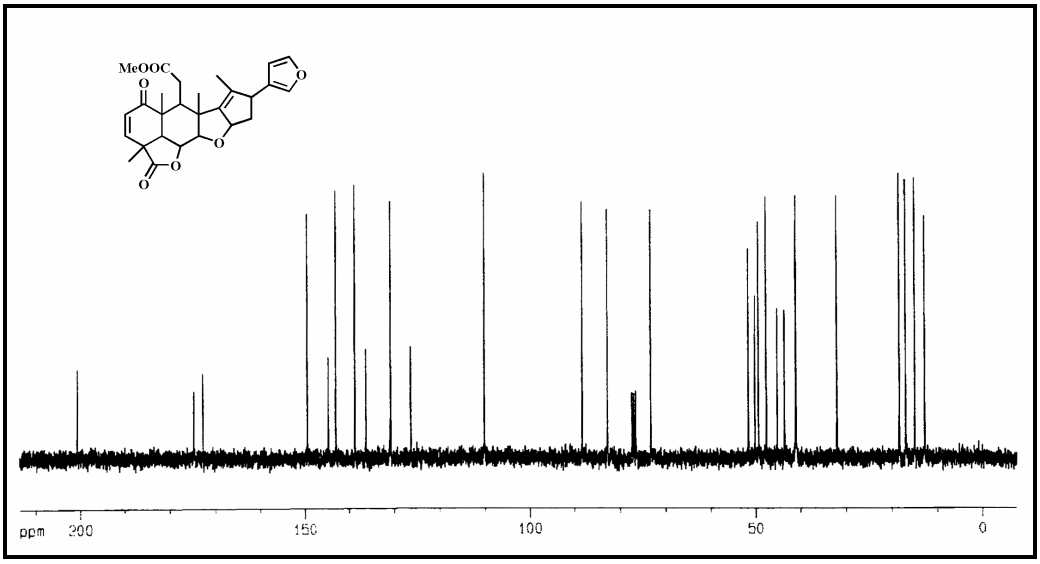


Figure 5.3: ^{13}C NMR spectrum of **nimbolide**

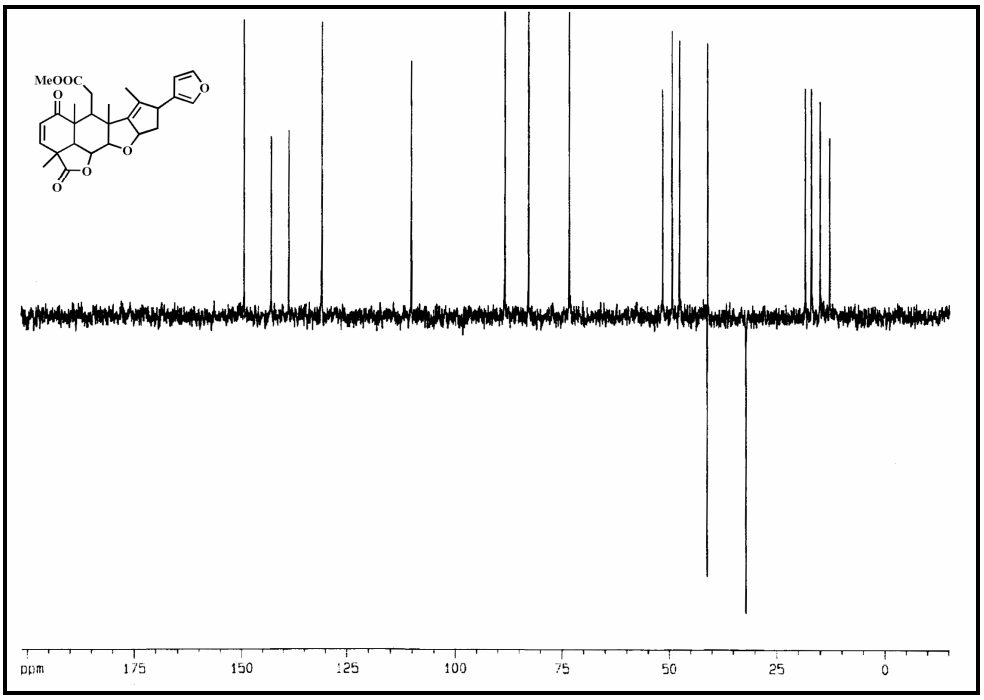


Figure 5.4: DEPT – 135 NMR spectrum of **nimbolide**

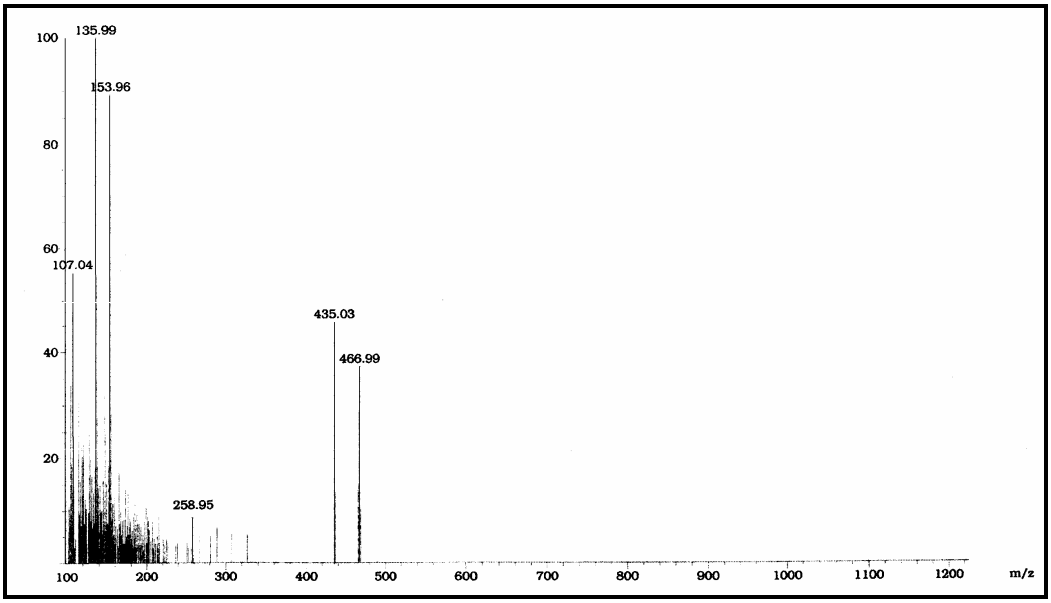


Figure 5.5: Mass spectrum of **nimbolide**

5.4 Biological activity studies of *Azadirachta indica*

5.4.1 *In-vitro* cytotoxicity of nimbolide

The *in vitro* cytotoxicity of nimbolide against human cancer cell line was determined in collaboration with RRL, Jammu. The cell lines studied include colon (colo-205, HCT-15, HT-29, SW-620); liver (Hep-2); lung (A-549) and prostate (Du-145). It was observed that nimbolide showed more than 50% growth inhibition at 1 X 10^{-6} M against four cell lines (HCT-15, HT-29, Hep-2 and Du-145) and 37% growth inhibition for the cell line colo 205. Its effect was evaluated at higher concentrations in case of seven cell lines and the growth inhibition was found to be greater than 50%. The growth inhibition was also studied at 2.5 x 10^{-7} M, 5 x 10^{-7} M and at concentrations lower than 1 x 10^{-6} M and in all cases it was less than 50 %. The results of the colon cell lines were compared with 5-fluorouracil and in all cases nimbolide was found to be better than the reference drug. In case of liver cell line (Hep-2), nimbolide showed better result than 5-fluorouracil but it was less active than Mitomycin C. The lung (A-549) cell line showed better effect with the

compound as compared to Paclitaxel at 1 x 10^{-5} M. The results of colon cell lines indicate that in all these cases, the compound was better than the reference drug. The details are shown in Table 5.2. A graphical representation of the cytotoxicity is depicted in figure 5.6.

Table 5.2: % Growth inhibition of cancer cells by nimbolide

Sample	Concn. (M)	Cell line / tissue						
		Colo-205	HCT-15	HT-29	SW-620	Hep-2	A-549	Du-145
		Colon	Colon	Colon	Colon	Liver	Lung	Prostate
		Growth inhibition (%)						
Nimbolide	2.5 X 10^{-7}	14	16	3	0	14	12	0
	5 X 10^{-7}	22	33	19	20	52	15	12
	1 X 10^{-6}	37	62	59	49	62	34	55
	2 X 10^{-6}	**85**	**85**	**58**	**81**	**70**	**54**	**70**
	3 X 10^{-6}	84	87	66	81	65	63	71
	4 X 10^{-6}	83	88	77	82	67	49	64
	1 X 10^{-5}	87	89	89	84	63	78	89
5-Flurourasil	1 X 10^{-5}	26	64	34	40	26		
Mitomycin C	1 X 10^{-6}					87		67
Paclitaxel	1 X 10^{-6}						59	

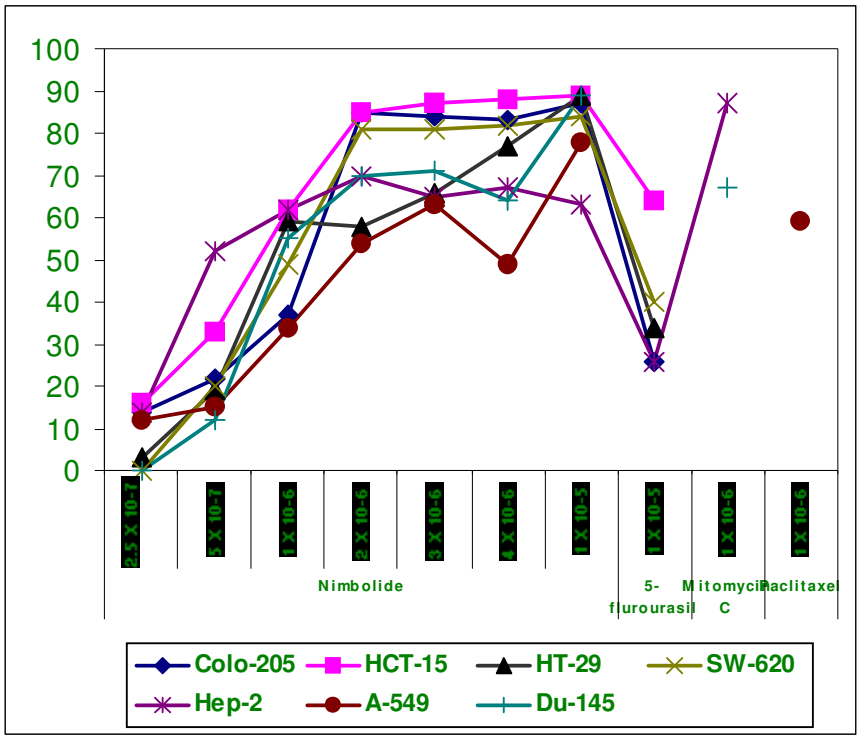


Figure 5.6: Cytotoxicity by nimbolide

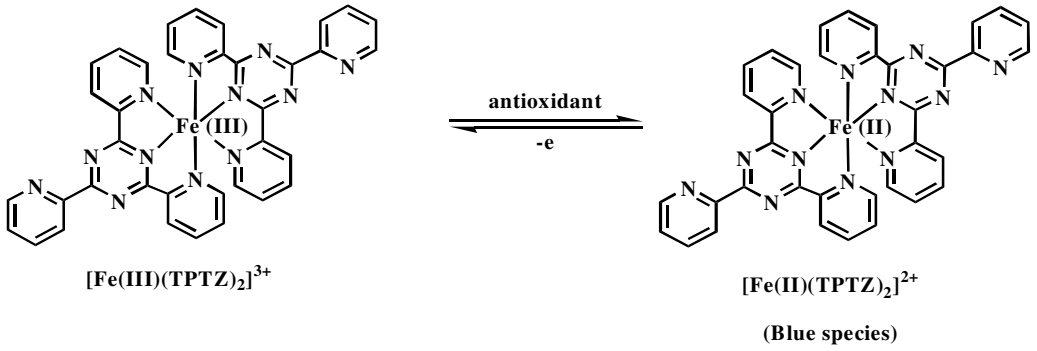
The results indicate that the compound nimbolide obtained from the medicinal plant *Azadirachta indica* has significant cytotoxicity against human colon cancer cell lines derived from different tissues. The cytotoxic effect is dose dependent and degree of growth inhibition was cell line specific. Therefore, nimbolide has the potential for further development as an anticancer drug.

5.4.2 Antioxidant activity of *Azadirachta indica* using the ferric reducing antioxidant power (FRAP) assay

FRAP assay[23] provides information on the reducing ability of polyphenols which seems to be an important factor for dietary antioxidant activity and free radical scavenging ability of these compounds. In this method, carotenoids which are known antioxidants do not participate and

therefore do not contribute to the ferric reducing ability. As a result, the method is commonly used for the routine analysis of single antioxidants and total antioxidant activity of plant extracts rich in carotenoids.[24]

FRAP assay is based on electron transfer reaction. The FRAP assay is a simple and reproducible method which can be applied to study the antioxidant activity of plasma or antioxidants. Here a ferric salt (Fe [III] $[TPTZ]_2$ Cl_3) is used as an oxidant (TPTZ is 2,4,6 tripyridyl s-triazine). This assay depends on the reduction of ferric tripyridyl triazine (Fe (III)-TPTZ) complex to ferrous tripyridyl triazine (Fe (II) TPTZ) by a reductant at low pH which has intense blue color which is monitored at 593 nm.[23] Results are expressed in trolox equivalents. Trolox, a powerful antioxidant is 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). It is a water soluble derivative of Vitamin E



Hexane, acetone and methanolic extracts were analyzed for their antioxidant activities by FRAP assay. The ferric reducing ability is expressed in gram equivalents of Trolox/100g of extracts. As per this assay, the methanol extract contained 17.6 ± 2.36 mg Trolox equivalent/100 g of dry leaves, whereas the hexane and the acetone extracts respectively contained 8.96 ± 1.14 mg and 5.36 ± 1.06 mg Trolox equivalent/100 g dry leaves of *A. indica*. Thus the methanol extract has the highest percentage of reductones

that could contribute largely to the antioxidant activity of the extracts. Nimbolide did not show any antioxidant activity by the FRAP assay.

The extracts as well as nimbolide were also tested for the free radical scavenging activity by DPPH$^{\bullet}$.

5.4.3 DPPH$^{\bullet}$ radical scavenging capacity

The hexane, acetone and methanol extracts of the leaves of *A. indica* as well as nimbolide were evaluated for free radical scavenging capacity. The samples were tested at a concentration range of 2500-4000 ppm. Among the extracts, the methanol extract exhibited the highest free radical scavenging capacity (92 % at 4000 ppm) with an EC_{50} value of 1830 ppm whereas the hexane and acetone extracts showed free radical scavenging capacity of 64 % and 39 % at 4000 ppm respectively (their respective EC_{50} values are 3100 and 3480 ppm). Thus the free radical scavenging capacity decreases in the order methanol extract > hexane extract > acetone extract. Nimbolide, isolated from the acetone extract of the leaves of *A. indica* did not show any DPPH free radical scavenging capacity even at higher concentrations. The plot of the DPPH$^{\bullet}$ radical scavenging capacity of the extracts at different concentration is presented in figure 5.6.

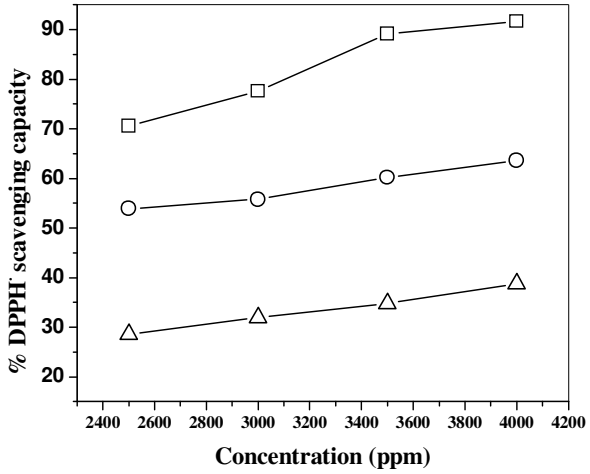


Figure 5.6: DPPH radical scavenging capacities at different concentrations of (□) methanol extract; (○) hexane extract and (△) acetone extract of *A. indica* leaves.

5.4 Experimental

General experimental procedures and chemicals used are as reported in chapter 2 of this thesis. RPMI-1640 medium, glutamine, streptomycin, fetal bovine serum, penicillin, gentamicin, TPTZ and Trolox were purchased from Sigma-Aldrich. All solvents and reagents used were of spectroscopic grade.

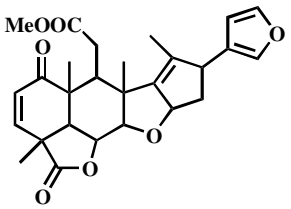
5.4.1 Extraction

Fresh *A. indica* leaves were collected from NIIST campus. It was then thoroughly cleaned, chopped into small pieces and dried for 48 hours at 50 ºC in a convection air drier. This was coarsely powdered and 450 g of this material was subjected to extraction at room temperature (27 °C) using hexane, followed by acetone and methanol. Each extract was then concentrated under reduced pressure in a rotary evaporator.

5.4.2 Isolation of nimbolide from the acetone extract

The acetone extract (21 g) was dissolved in 1:1 mixture of hexane:ethylacetate and then loaded on to a column packed with silica gel (400 g, 100-200 mesh). The column was eluted starting with 5 % ethyl acetate in hexane and then gradually increasing the polarity. A total of 162 fractions of 40-50 ml each were collected and pooled together in 22 fraction pools according to the similarities in TLC. The seventh fraction pool contained a major compound on examination by TLC. The fraction was kept for crystallization in dichloromethane-ethyl acetate mixture. 116 mg of pure white crystals of nimbolide was obtained upon recrystallisation. The structure was assigned from the spectral data as shown below.

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



^{1}H NMR (300 MHz, $CDCl_3$)	:	δ 7.32 – 7.22 (3H, *m*), 6.26 (1H, *s*), 5.92 (1H, *d*, *J* = 11.8 Hz), 4.66 (1H, *dd*, *J* = 3.67, 12.5 Hz), 4.27 (1H, *d*, *J* = 3.6 Hz), 3.67 (1H, *d*, *J* = 8.6 Hz), 3.54 (3H, *s*), 3.23-3.16 (2H, *m*), 2.74 (1H, *t*, $J_1 = J_2$ = 5.6 Hz), 2.37 (1H, *dd*, *J* = 5.8, 16.2 Hz), 2.21-2.13 (2H, *m*), 1.70 (3H, *s*), 1.47 (3H, *s*), 1.37 (3H, *s*), 1.22 (3H, *s*)
^{13}C NMR (75 MHz, $CDCl_3$)	:	δ 200.7, 174.9, 172.8, 149.4, 144.7, 143.0, 138.7, 136.3, 130.8, 126.4, 110.2, 88.2, 82.8, 73.3, 51.6, 50.2, 49.3, 47.6, 45.1, 43.5, 41.1, 41.0, 32.0, 18.4, 17.0, 15.0, 12.7
DEPT-135 (75 MHz, $CDCl_3$)		δ (i) 149.5, 143.1, 138.8, 130.9, 110.3, 88.3, 82.8,
(i) CH, CH_3	:	73.4, 51.7, 49.4, 47.7, 41.1,
(ii) CH_2	:	18.5, 17.1, 15.1, 12.8.
		(ii) 41.2, 32.1
FAB-MS m/z (rel. int.)	:	257.12 [M+1] (100), $C_{15}H_{12}O_4$, requires 256.07
m.p. (°C)	:	204-205 °C, lit[21]. 204-205 °C

5.4.3 *In-vitro* cytotoxicity assessment of nimbolide

A stock solution of $2X10^{-2}$ M of nimbolide was prepared in DMSO. The stock solutions were serially diluted to obtain working test solutions with

complete growth medium (RPMI-1640 medium with 2mM glutamine, 100µg/ml streptomycin, pH 7.4, sterilized by filtration and supplemented with 10% fetal bovine serum and 100 units/ml penicillin before use) containing 50 µg/ml of gentamicin to obtain working test solutions. The working test solutions were not filtered /sterilized but microbial contaminations was controlled by addition of gentamicin in complete growth medium used for dilution of stock solutions to prepare working test solutions.

In vitro cytotoxicity against human cancer cell lines was determined[23] using 96-well tissue culture plates. The cells were grown in tissue culture flasks in complete growth medium at 37ºC in an atmosphere of 5% CO_2 and 90% relative humidity in a carbon dioxide incubator. The cell suspension of required cell density (1-2 lakhs/ml) depending on the mass doubling time of cell lines was prepared in complete growth medium for determination of cytotoxicity. Aliquots of 100 µl of cell suspension were added to each well on a 96- well tissue culture plate. The cells were incubated for 24 hours. The blank wells contained complete medium in place of suspension. Simultaneously, control experiment with positive controls containing known anticancer agent 5-fluorouracil was also carried out.

The test materials (100µl in each well) were added after 24 hours to the wells containing cell suspension and blank wells. The cells were allowed to grow in presence of test material by further incubating the plates for 48 hours. At the end of incubation period the cell growth was stopped by gently layering trichloroacetic acid (50% TCA, 50µl /well) on top of the medium in all the wells. The plates were incubated at 4 °C for 1 hour. The plates were washed five times with distilled water to remove TCA, growth medium, low molecular weight metabolites, serum proteins etc and was air-dried.

The cell growth was measured by staining with Sulphorhodamine B. Sulphorhodamine B (SRB, 0.4% in 1% acetic acid, 100µl/ well) was added to each well and plates were allowed to stand at room temperature for 30

minutes. The plates were washed with 1% acetic acid four times and then dried. Tris-HCl buffer (0.01 M, pH 10.5, 100µl/well) was added to each well to solubilize the dye. The plates were shaken gently for 10mts on a shaker and the optical density was recorded on ELISA reader at 540 nm. The cell growth was determined by subtracting mean OD value of respective blank from the mean OD value of experimental set. Percent growth in absence of any test material as 100 % and in turn percent growth inhibition in presence of test material was calculated. All the experiments were carried out in quadruplicate.

5.4.4 Trolox equivalent antioxidant capacity by FRAP method

The automated method for measuring the Ferric Reducing Antioxidant power (FRAP)[19] was used for the present study. The reagents include (i) Acetate buffer, 300 mmol/l, pH 3.6, (ii) 10 mmol/l 2,4,6-Tripyridyl-s-triazine (TPTZ) in 40 mmol/l HCl and (iii) 20 mmol/l $FeCl_3$ x $6H_2O$ in distilled water. The FRAP working solution is the mixture of the above three solutions in the ratio 25:2.5:2.5. The working solution must be always freshly prepared. 1.5 ml of the working solution was mixed with 0.1 ml of the extract. It was incubated at 37 °C for 20 min. Trolox was the standard and a standard curve was drawn by reading the absorbances at 593 nm. The results are expressed in trolox equivalents.

5.5 References

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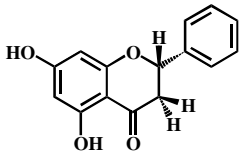
SUMMARY AND CONCLUSION

Knowledge of medicinal plants used in traditional systems of medicine is of great value as biologically active compounds isolated from them can serve as leads for the development of novel drugs used in the modern system of medicine. It has been estimated that more than 60% of world population still depend on crude plant drug preparations to tackle various health problems. Several medicinal plants belonging to the Zingiberaceae family are extensively used in '*Ayurveda*' and '*Sidha*' systems of medicine practiced in India to combat many diseases. This thesis involves a study of four medicinal plants; three belonging to the Zingiberaceae family, viz., *Alpinia galanga*, *Alpinia calcarata* and *Kaempferia pulchra* as well as the well known medicinal plant *Azadirachta indica* as the fourth.

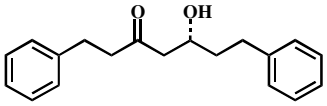
Chapter 1 gives an overview of biologically active natural products with special reference to anticancer and antioxidant compounds from terrestrial sources. A brief outline of carcinogenesis and the details of some naturally occurring anticancer compounds is portrayed here. The role played by free radicals/reactive oxygen species in the initiation of atherosclerosis, carcinogenesis, ageing, rheumatism, inflammation etc., and the importance of naturally occurring antioxidants for the control of such free radicals is also outlined in the first chapter. Since, the three main plants studied belong to Zingiberaceae family, a brief outline of the important compounds isolated from this family is also included in this chapter.

Chapter 2 deals with the phytochemical investigation and biological activity studies on the medicinal plant *A. galanga*. Here, a brief outline of the genus *Alpinia* is given along with the detailed survey of literature on the phytochemistry and biological activity of *A. galanga*. Isolation of pinocembrin (**I**), 1,7-Diphenyl-5-hydroxy-3-heptanone (**II**), galangin (**III**) and kaempferide (**IV**) from the rhizomes of *A. galanga* is being reported for the

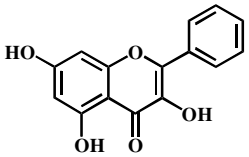
first time. The structures of the compounds isolated from *A. galanga* are given below.



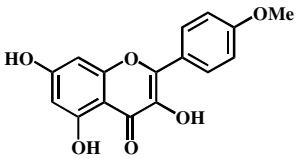
Pinocembrin (**I**)



1,7-Diphenyl-5-hydroxy-3-heptanone (**II**)



Galangin (**III**)



Kaempferide (**IV**)

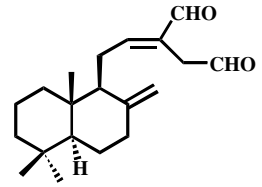
The flavonoid pinocembrin (**I**) was found to be an apoptotic inducer on colon cancer cell lines. We have shown that the medicinal plant *A. galanga* contain galangin (**III**) as the major compound in its rhizomes. The antioxidant capacity studies on the ethanol and acetone extracts of the rhizomes as well as on the pure flavonoids galangin (**III**) and kaempferide (**IV**) were estimated using various *in vitro* methods viz., total phenolic content, total flavonoid content, total antioxidant capacity, DPPH free radical scavenging capacity, superoxide radical scavenging capacity, hydroxyl radical scavenging capacity, scavenging of hydrogen peroxide, reducing power, metal chelating activity, β -carotene bleaching method, inhibition of lipid peroxidation and nitric oxide scavenging capacity is also discussed in detail in chapter 2. The kinetics of the reaction of the extracts and the pure flavonoids galangin and Kaempferide towards DPPH free radical has been explored in detail in order to understand the antioxidant behavior and is also discussed in this chapter.

Our studies showed that the rhizomes of *A.galanga* possessed antioxidant capacity as is evident from the results of various *in vitro* assays. In the free radical scavenging assay determined by DPPH˙ method, extracts as

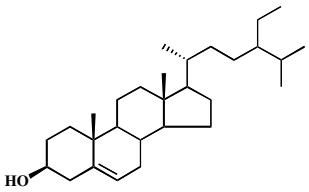
well as galangin and kaempferide showed only moderate scavenging ability and were lower than that of the standards quercetin and BHA. Eventhough galangin possessed better radical scavenging ability than kaempferide, the kinetic studies revealed that as concentration increases, the radical scavenging activity of galangin decreases, thereby giving an idea about the pro-oxidant nature of galangin. Both galangin and kaempferide were superoxide radical scavengers, even better than the standard compounds. The most powerful scavenging property by the extracts as well as the compounds was the hydroxyl radical scavenging property. Also, it is interesting to note that even though galangin and kaempferide were found to be powerful antioxidants than the crude extracts in most of the assays, in H_2O_2 scavenging and Fe^{2+} metal chelation, the extracts were far more active than the flavonoids themselves. The acetone extract (AGA) was highly efficient in H_2O_2 scavenging even better than the standard BHA and the isolated flavonoids. The antioxidant activity determined by the β -carotene bleaching method showed that kaempferide and acetone extract possessed greater ability to prevent β -carotene bleaching even better than BHA. The extracts as well as the flavonoids also possessed moderate nitric oxide scavenging capacity, inhibition of lipid peroxidation and reducing power. Thus, it could be surmised from the results of various assays discussed in chapter 2 that the rhizomes of *A. galanga* possessed very good antioxidant properties.

Chapter 3 is divided into two sections. The first section includes the phytochemical investigation and structural elucidation of various compounds isolated from *A. calcarata* as well as the antioxidant studies on the rhizomes. The compounds isolated include (*E*)-Labda-8(17),12-diene-15,16-dial (**V**); β-sitosterol (**VI**); (*trans*) 3-methoxy-5-hydroxy stilbene (**VII**); pinocembrin (**VIII**, same as compound **I**), 1,7-diphenyl-5-hydroxy-3-heptanone (**IX**, same as compound **II**), galangin-3-methyl ether (**X**), 1,7-diphenyl-5-hydroxy-6-heptene-3-one (**XI**); galangin (**XII**, same as compound **III**); pinobanksin

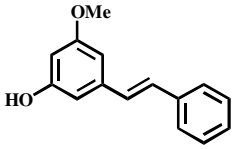
(**XIII**) and Zerumin A (**XIV**). Among the ten compounds isolated, all except Zerumin A are being reported for the first time from *A. calcarata*. The structures of the compounds isolated from *A. calcarata* are given below.



(**V**)



(**VI**)



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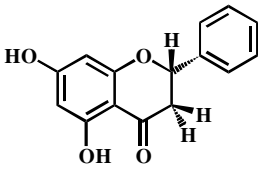

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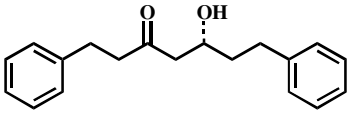

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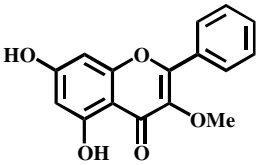
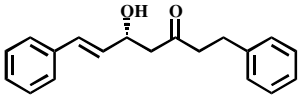
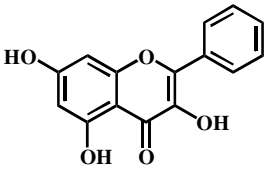


(**X**)



(**XI**)



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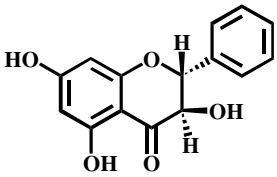

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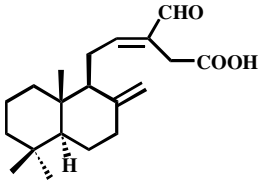


(**XIV**)

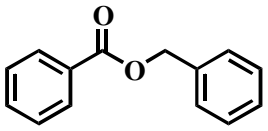
Pinocembrin was found to be the major constituent of the rhizomes of *A. calcarata*. The antioxidant activity of the ethanol and acetone extracts of *A. calcarata* and the compounds isolated form it was analyzed using various *in vitro* models. The extracts as well as the isolated flavonoids were efficient in scavenging certain free radicals and reactive oxygen species. The extracts showed very little DPPH˙ and superoxide radical scavenging properties and

the pure compounds showed no activity. However, the *trans* stilbene (compound **VII**) was very effective in scavenging H_2O_2. Pinocembrin, pinobanksin and galangin-3-methyl ether also showed good H_2O_2 scavenging property. The extracts as well as the isolated compounds showed moderate reducing power, but lower than that of the standard. Even though the crude extracts showed antioxidant capacity by DPPH$^{\bullet}$ free radical scavenging, hydroxyl radical scavenging and H_2O_2 scavenging, in other sets of tests measuring the chelating power, nitric oxide scavenging, prevention of β-carotene bleaching and the inhibition of lipid peroxidation, neither the extracts nor the compounds showed very positive results.

As in herbal formulations, both *A. galanga* and *A. calcarata* are used without distinction, a comparison between the chemical constituents and biological activity appeared significant. This has been carried out and therefore, Section B of Chapter 3 compares *A. galanga* and *A. calcarata* in terms of chemical constituents and antioxidant activity. From the comparison, it has become clear that *A. galanga* is more efficient as an antioxidant than *A. calcarata*. Also, the antioxidative flavonoid galangin was obtained in larger amounts from *A. galanga* whereas in *A. calcarata* it was obtained only in very smaller amounts. On the other hand, pinocembrin, an apoptosis inducer in human colon cancer cells, was obtained in larger amounts from the rhizomes of *A. calcarata* than from the rhizomes of *A. galanga*, thus indicating that the two species are quite different in terms of their chemistry.

Chapter 4 describes the phytochemical investigation of another Zingiberaceae plant viz., *Kaempferia pulchra* belonging to the genus *Kaempferia*. Here, a brief outline of the genus *Kaempferia* is portrayed aong with a detailed literature survey of *K. pulchra*. Phytochemical investigation of the rhizomes of *K. pulchra* led to the identification of seven compounds including four cyclohexane diepoxides. The compounds isolated are benzyl benzoate (**XV**), *para* methoxy benzyl benzoate (**XVI**), sandaracopimaradien-

1α,2α-diol (**XVII**), 4-Benzoyloxymethyl-3,8-dioxatricyclo[5.1.0.$0^{2,4}$]-octane-5,6-diol-6-benzoate (**XVIII**), Crotepoxide (**XIX**), 4-Benzoyloxymethyl-3,8-dioxatricyclo[5.1.0.$0^{2,4}$]-octane-5,6-diol-5-acetate (**XX**) and 4-Benzoyloxy methyl-3,8-dioxatricyclo[5.1.0.$0^{2,4}$]-octane-5,6-diol-6-acetate (**XXI**). Of these, all the compounds except (**XVII**) are being isolated for the first time from the rhizomes of *K. pulchra*. Even though the extracts as well as the pure compounds did not show any antioxidant capacity, preliminary experiments on crotepoxide (**XIX**), and compound **XX** has confirmed anticancer activity on breast cancer cell lines.



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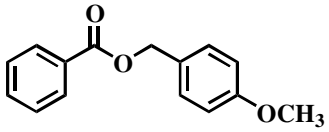
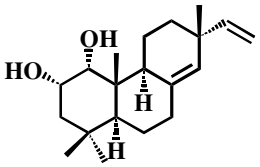
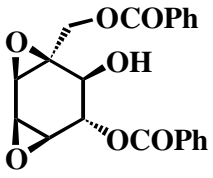


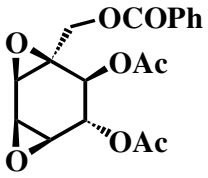
(**XVI**)



(**XVII**)



(**XVIII**)



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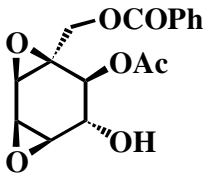
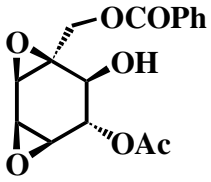


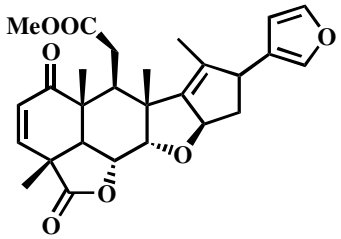
(**XX**)



(**XXI**)

Chapter 5 describes the isolation of the compound nimbolide from the leaves of *Azadriachta indica* (Neem) belonging to the family Meliaceae and the bioevaluation of nimbolide as a potential anticancer agent. The results

indicate that the nimbolide has significant cytotoxicity against human colon cancer cell lines derived from different tissues. The cytotoxic effect is dose dependent and degree of growth inhibition was cell line specific. Evaluation of the leaf extracts as well as pure nimbolide as antioxidants using ferric reducing antioxidant power and DPPH free radical scavenging assay was also carried out. Eventhough the extracts showed positive results, nimbolide did not show any antioxidant activity.



Nimbolide

In conclusion *Alpinia galanga*, *Alpinia calcarata* and *Kaempferia pulchra*, three medicinal plants belonging to the Zingiberaceae family have been analysed in detail for their phytochemical constituents. Also, the positive results obtained from biological activity studies such as anticancer activity and antioxidant activity on the isolated compounds/extracts add on to the medicinal properties of these Zingiberaceae plants. Apart from that, the bioactive compound nimbolide isolated from *A. indica* (neem) leaves has been shown to have very good anticancer activity on colon cancer cell lines.

List of publications

1. **P. S. Hema** and Mangalam S. Nair. Flavonoids and other constituents from the rhizomes of *Alpinia calcarata*. *Biochem. Syst. Ecol*. **2009**, *37*, 52-54.
2. Ajaikumar B. Kunnumakkara, Haruyo Ichikawa, Preetha Anand, Chiramel J. Mohankumar, **Padmanabhan S. Hema**, Mangalam S. Nair and Bharat B. Aggarwal. Coronarin D, a labdane diterpene, inhibits both constitutive and inducible nuclear factor-κB pathway activation, leading to potentiation of apoptosis, inhibition of invasion, and suppression of osteoclastogenesis. *Mol. Cancer Ther*. **2008**, *10*, 3306-3316.
3. M. A. Sureshkumar, Mangalam Nair, **P. S. Hema**, John Mohan and T. R. Santhoshkumar. Pinocembrin triggers bax-dependent mitochondrial apoptosis in colon cancer cells. *Mol. Carcinogen*. **2007**, *46*, 231-241.

Manuscripts submitted

1. **P. S. Hema** and Mangalam S. Nair. Investigation of antioxidant capacity of acetone and ethanol extracts of the rhizomes of *Alpinia galanga* L. (Zingiberaceae). (Submitted to *Bioresource Technology*).
2. **P. S. Hema**, M. Priya Rani and Mangalam S. Nair. Cyclohexane diepoxides and other constituents from the rhizomes of *Kaempferia pulchra*. (Submitted to *Biochemical Systematics and Ecology*).
3. **P. S. Hema** and Mangalam S. Nair. Flavonoid characterization and *in-vitro* antioxidant capacity of *Alpinia calcarata*. (Submitted to *Journal of the Science of Food and Agriculture*).

Posters presented

1. **P. S. Hema** and Mangalam S. Nair. Isolation of anticancer and antioxidant compounds from *A. galanga* (*Medicinal Chemistry Research* **2006**, *15(1-6)*, 150-151), Poster No. 29, 3rd International Symposium on Current Trends in Drug Discovery Research (CTDDR-2007) held at CDRI, Lucknow on February 17-21, 2007.
2. **Hema, P. S**, Mangalam S. Nair, Saxena, A. K, Madhulika Bhagat, Shanmugavel. M. *In vitro* cytotoxicity of the limonoid nimbolide from *Azadirachta indica* on various human cancer cell lines. Poster No. P051, Joint conference on Building bridge, Forging bonds for 21st century Organic Chemistry and Chemical Biology (OCCB-2006) held at NCL, Pune on January 7-9, 2006.
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