INVESTIGATION ON PHYTOCHEMICAL CONSTITUENTS AND BIOLOGICAL POTENTIAL OF SOME TRADITIONAL MEDICINAL PLANTS

Thesis submitted to

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

IN CHEMISTRY UNDER THE

FACULTY OF SCIENCE

By

PRIYA RANI M.
(Reg. No. 4144)

Under the supervision of

Dr. K. P. Padmakumari Amma
&
Dr. K. G. Raghu

AGROPROCESSING AND NATURAL PRODUCTS DIVISION
CSIR - NATIONAL INSTITUTE FOR INTERDISCIPLINARY SCIENCE AND TECHNOLOGY (CSIR - NIIST)
THIRUVANANTHAPURAM – 695 019, KERALA, INDIA

JUNE 2014

DECLARATION

I hereby declare that the thesis entitled "**INVESTIGATION ON PHYTOCHEMICAL CONSTITUENTS AND BIOLOGICAL POTENTIAL OF SOME TRADITIONAL MEDICINAL PLANTS**" embodies the results of investigations carried out by me at Agroprocessing and Natural Products Division of CSIR-National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Thiruvananthapuram as a full time research scholar under the supervision of Dr. K. P. Padmakumari Amma and co-supervision of Dr. K. G. Raghu and the same has not been submitted elsewhere for any other 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.

Thiruvananthapuram
June, 2014

Priya Rani M.

June, 2014

CERTIFICATE

This is to certify that the work embodied in the thesis entitled '**INVESTIGATION ON PHYTOCHEMICAL CONSTITUENTS AND BIOLOGICAL POTENTIAL OF SOME TRADITIONAL MEDICINAL PLANTS'** has been carried out by **Ms. Priya Rani M.**, under my supervision and the co-supervision of Dr. K. G. Raghu at Agroprocessing and Natural Products Division, CSIR - National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Thiruvananthapuram in partial fulfillment of the requirements for the award of degree of Doctor of Philosophy in Chemistry under the Faculty of Science, Cochin University of Science and Technology, Cochin and the same has not been submitted elsewhere for any other degree. All the relevant corrections, modifications and recommendations suggested by the audience and the doctoral committee members during the pre-synopsis seminar of **Ms. Priya Rani M.** have been incorporated in the thesis.

Dr. K. G. Raghu
(Co-Thesis Supervisor)

Dr. K. P. Padmakumari Amma
(Thesis Supervisor)

ACKNOWLEDGEMENTS

It is with immense pleasure that I express my deep sense of gratitude to my research supervisors Dr. K. P. Padmakumari Amma and Dr. K. G. Raghu for suggesting the research topic and for their guidance, encouragement and constant support throughout my research.

I wish to thank Dr. Suresh Das, Dr. B. C. Pai and Professor T. K. Chandrashekar, present and former Directors, NIIST, Trivandrum, for providing necessary facilities for carrying out the work.

I would like to express my gratitude to Dr. A Sundaresan and Mr. M. M. Sreekumar, Chief Scientists, Agroprocessing and Natural Products Division, CSIR-NIIST for their support and encouragement extended to my work.

I wish to extend my sincere thanks to Smt. M. V. Reshma, Dr. P. Nisha, Dr. Jayamurthy, Mrs. Prasannakumari, Dr. B. S. Dileep Kumar, Dr. Priya S. and Mr. D. R. Soban Kumar for their help in my work. All the former and present members of Agroprocessing and Natural Products Division have been extremely helpful and co-operative and I am thankful to one and all for their kind gesture.

I am most grateful to Dr. Mangalam S. Nair, Dr. R. Luxmi Varma and Dr. K. V. Radhakrishnan, Scientists of Organic Chemistry Section and Dr. T. P. D. Rajan, Senior Scientist, Material Science and Technology Division (Convener, NIIST-CUSAT Research Council) for all their help and support.

I express my sincere thanks to Dr. P. A. Unnikrishnan, Asst. Professor, Dept. of Applied Chemistry, Cochin University of Science and Technology for his care and support as an external expert in Doctoral Committee.

I am also indebted to Dr A. Helen, Asst. Professor, Department of Biochemistry, University of Kerala for collaborative research work.

I would like to express my gratitude to Mrs. Soumini Mathew, Mrs. S. Viji, Mr. Saran P, Mr. Adarsh B., Ms. Athira, Mr. Praveen, Mr. Adarsh N. and Mr. George T. M. for recording all the spectra.

I wish to extent my sincere thanks to Ms. Chithra (University of Kerala) for collaborative research work and Ms. Fazira for her help, support and affection

I gratefully acknowledge the help and support received from Dr. Mahesh S. Krishna, Mr. Prathapan A., Dr. Nishanth Kumar, Mr. Lijo Cherian, Mr. Salin Raj P., Mr. Pratheesh S. Nair, Ms. Soumya R.S., Ms. Nisha V. M., Ms. Anusree S. S., Ms. Janu Chandran, Ms. Priyanka A., Ms. Vineetha V. P., Dr. Vandana Sankar, Ms. Riya Mariam Philip, Ms. Antu K. Antony, Ms. Reshma P.L., Ms. Namitha L. K., Ms. Indu Sasidharan, Ms. Shamla L.,

Mr. Arun K. B., Ms. Saranya, Ms. Dhanya R., Ms. Shyama H. P., Mr. Ajish K. R. etc. and all other friends of Agroprocessing and Natural products division.

I would like to express my gratitude to all the members of library, administrative, academic programme committee and technical staff of NIIST for their help and support

I extent my sincere thanks to my seniors and all friends in other divisions of NIIST for their help and support.

I express my gratitude to Dr. H. Biju (Taxonomist, JNTBGRI, Thiruvananthapuram, Kerala) for identifying the plants.

I express my sincere gratitude to all my teachers for their care and support.

I am also indebted to Council of Scientific and Industrial Research for financial assistance in the form of research fellowship.

Words are less though I am deeply indebted to my achan, amma, jyothy chettan, pradeep, vaishnu and all other family members for their immense and invaluable love and care.

Above all I praise and thank 'God Almighty' for everything.

PRIYA RANI M.

..... TO MY FAMILY

CONTENTS

ABBREVIATIONS

ROS	:	Reactive Oxygen Species
RNS	:	Reactive Nitrogen Species
DNA	:	Deoxy ribo nucleic acid
ATP	:	Adenosine triphosphate
O_2	:	Dioxygen
O_2^-	:	Superoxide anion
H_2O_2	:	Hydrogen peroxide
SOD	:	Superoxide dismutase
GPx	:	Glutathione peroxidase
CAT	:	Catalase
β-carotene	:	beta-carotene
Se	:	Selenium
Mn	:	Manganese
Zn	:	Zinc
PUFA	:	Polyunsaturated fatty acid
$OH^\bullet$	:	Hydroxyl radical
$^1O_2$	:	Singlet oxygen
$O_2^{\bullet-}$	:	Superoxide anion radical
Fe^{2+}	:	Ferrous ion
Fe^{3+}	:	Ferric ion
$OONO^-$	:	Peroxy nitrite anion
NO	:	Nitric oxide
$NO^\bullet$	:	Nitric oxide radical
NO^+	:	Nitrosonium ion
$ROO^\bullet$	:	Peroxy radical
ONOOH	:	Peroxy nitrous acid
$ONOO^\bullet$	:	Peroxy nitrite
H^+	:	Hydrogen ion

ADP	:	Adenosine diphosphate
MDA	:	Malondialdehyde
pH	:	Power of hydrogen
AIP	:	1-amino-3-iminopropene
α	:	Alpha
β	:	beta
γ	:	gamma
δ	:	delta
$^3O_2$	:	Triplet oxygen
TrxR	:	Thioredoxin reductase
IDD	:	Iodothyronine deiodinases
ArOH	:	Phenol
$ArO^{\bullet}$	:	Phenoxyl radical
IPP	:	Isopentenyl diphosphate
DMAPP	:	Dimethyl allyl diphosphate
GPP	:	Geranyl diphosphate
FPP	:	Farnesyl diphopshate
GGPP	:	Geranyl geranyl diphosphate
ED_{50}	:	Effective dose for 50% activity
KB cell	:	Nasopharyngeal cancer cells
p-coumaryl CoA	:	*para*-coumaryl Co-enzyme A
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
F-OH	:	Flavonoid molecule
BHA	:	Butylated hydroxy anisole
BHT	:	Butylated hydroxy toluene
WHO	:	World Health Organization
NSAIDS	:	Non-steroidal antiinflammatory drugs
COX	:	Cyclooxygenase
COX - 1	:	Cyclooxygenase -1
COX -2	:	Cyclooxygenase - 2
LTC4	:	Leukotriene C4
PGE2	:	Prostaglandin E2
PGF2	:	Prostaglandin F2
PGI2	:	Prostacyclin
TXA2	:	Thromboxane
NF-kB	:	Nuclear factor kappa B
IL-1	:	Interleukin-1
TNF-α	:	Tumor necrosis factor – alpha
HPLC	:	High performance liquid chromatography
HPTLC	:	High performance thin layer chromatography
C. rotundus	:	*Cyperus rotundus*
NMDA	:	N-methyl-D-aspartate receptor
GC	:	Gas chromatography
GC-MS	:	Gas chromatography – Mass spectrometry
LPS	:	Lipopolysaccharide
RAW 264.7	:	Mouse leukaemic monocyte macrophage cell line
mRNA	:	Messenger Ribonucleic acid
NMR	:	Nuclear magnetic resonance
TLC	:	Thin layer chromatography
DPPH	:	2, 2′-diphenyl-1-picrylhydrazyl

$DPPH^{\bullet}$	:	2, 2′-diphenyl-1-picrylhydrazyl radical
$CDCl_3$	:	Deuterated chloroform
CD_3COCD_3	:	Deuterated acetone
CD_3OD	:	Deuterated methanol
HRMS-FAB	:	High resolution mass spectroscopy- Fast atom bombardment
HRMS-ESI	:	High resolution mass spectroscopy-Electrospray ionization
SPSS	:	Statistical programme for social sciences for personal computer
UV-VIS	:	Ultraviolet-visible
FT-IR	:	Fourier Transform infrared
μ	:	Mu
g	:	gram
°C	:	Degree celcius
cm^{-1}	:	Centimeter inverse
MeOH	:	Methanol
MHz	:	Mega hertz
^{1}H	:	Proton
^{13}C	:	Carbon-13
d	:	doublet
dd	:	doublet of doublet
t	:	triplet
m	:	multiplet
J	:	Coupling constant
br	:	broad
m/z	:	Mass to charge ratio
$[\alpha]_D^{26}$	:	Specific rotation
λmax	:	Maximum wavelength
νmax	:	Maximum frequency
%	:	Percentage

$CHCl_3$	:	Chloroform
mg	:	Milligram
ml	:	Millilitre
µg	:	Microgram
µl	:	Microlitre
PTFE	:	Poly tetra fluoroethylene
R_T	:	Retention time
LOD	:	Limit of detection
LOQ	:	Limit of quantification
σ	:	Sigma
S	:	Slope
RSD	:	Relative standard deviation
Mo	:	Molybdenum
M	:	Molar
mM	:	Millimolar
min	:	Minute
nm	:	Nanometre
CC	:	Column chromatography
AAE	:	Ascorbic acid equivalent
L	:	Litre
λ	:	Lambda
MMP-1	:	Metalloproteinase-1
NHF	:	Normal human fibroblasts
R_f	:	Retention factor
r	:	Correlation coefficient
IC_{50}	:	Inhibitory concentration for 50% activity
PNPG	:	*p*-nitrophenyl-α-D-glucopyranoside
NBT	:	Nitroblue tetrazolium
PMS	:	Phenazine methosulphate
NADH	:	Nicotinamide adenine dinucleotide
MTT	:	3-(4, 5-dimethylthiazol-2-yl)-2, 5-

		diphenyltetrazoliumbromide
TCA	:	Trichloro acetic acid
SNP	:	Sodium nitroprusside
BHT	:	Butylated hydroxy toluene
eV	:	Electron volt
mm	:	millimeter
i.d.	:	inner diameter
NIST	:	National Institute for Standards and Technology
N	:	Normality
S. colais	:	*Stereospermum colais*
ASC	:	Acetone extract of *Stereospermum colais*
MSC	:	Methanol extract of *Stereospermum colais*
XO	:	Xanthine oxidase
XOI	:	Xanthine oxidase inhibition
$L^{\bullet}$	:	Lipid radical
$LOO^{\bullet}$	:	Lipid peroxy radical
LOOH	:	Lipid hydroperoxide
v/v	:	Volume by volume
w/v	:	Weight by volume
h	:	Hour
ppm	:	Parts per million
Tris-HCl	:	Tris-hydrochloride
$K_3(Fe(CN)_6)$	:	Potassium ferricyanide
$FeCl_3$	:	Ferric chloride
m.p.	:	Melting point
rel. int.	:	Relative intensity
g	:	Gyration
BSA	:	Bovine serum albumin
PBS	:	Phosphate buffered saline
nM	:	Nanomolar
Vc	:	Paw volume of control rats

Vt	:	Paw volume of test rats
MTCC	:	Microbial type culture collection
CFU	:	Colony forming unit
MIC	:	Minimum inhibitory concentration
SFO	:	Sun flower oil
SFO-O	:	Sun flower oil without extracts
NED	:	N-(1-naphthyl) ethylenediamine dihydrochloride
D_2O	:	Deuterated water
RB	:	Round bottom flask
DEPT	:	Distortionless enhancement by polarization transfer
mg/kg	:	Milligram per kilogram
LDL	:	Low density lipoprotein
VLDL	:	Very low density lipoprotein
STZ	:	Streptozotocin
TMPD	:	N, N, N′, N′-tetramethyl-*p*-phenylenediamine
DCFH-DA	:	2′, 7′-dichlorodihydrofluoresceindiacetate
FITC	:	Fluorescein isothiocyanate
DMEM	:	Dulbecco's modified eagle's medium
C2C12	:	Mouse myoblast cells
L6	:	Rat skeletal muscle cells
3T3L1	:	Mouse pre-adipocyte
FACS	:	Fluorescence activated cell sorting
ACE	:	Angiotensin converting enzyme
HHL	:	Hippuryl–L-histidyl–L-leucine
TBA	:	Thiobarbituric acid
FBS	:	Fetal bovine serum
2- NBDG	:	2-(7-Nitrobenz-2-oxa-1, 3-diazol-4-yl) amino-2-deoxy-D-glucose
CPCSEA	:	Committee for the Purpose of Control and Supervision of Experiments on Animals
$NaHCO_3$	:	Sodium bicarbonate

EDTA	:	Ethylene diamine tetra acetic acid
SeO_2	:	Selenium dioxide
K_2SO_4	:	Potassium sulphate
$CuSO_4$	:	Copper sulphate
H_2SO_4	:	Sulphuric acid
NaOH	:	Sodium hydroxide
TPC	:	Total phenolic content
EAG	:	Ethyl acetate extract of ginger
HA	:	Hippuric acid
DMSO	:	Dimethyl sulphoxide
DCF	:	2, 7-dichlorofluorescein
KRB	:	Kreb's ringer buffer
PPAR	:	Peroxisome proliferator activated receptors
HS	:	Horse serum
IBMX	:	Isobutyl methylxanthine
Fe	:	Iron
GLUTs	:	Glucose transporters
IDE	:	Image data explorer

Chapter 1

INTRODUCTION

1.1. NATURAL PRODUCTS

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. Natural products are attractive source of diverse functionalities, which exhibit potent biological activities. Hence many new drugs have been innovated based on natural products. Research on natural products got momentum with remarkable developments in the areas of separation sciences, spectroscopic techniques, *in vitro* assays etc. This introductory chapter imparts an overview of the various processes involved in natural products research, starting from extraction and isolation to elucidation of the structures of purified natural products and screening of bioactive potentials. Plant extracts have been used for centuries as a popular method for treating several health disorders. Over the last ten years, the study of those extracts has attracted attention in different fields of the biological sciences (Pezzoto, 1997). Investigation of traditionally used medicinal plants is valuable on two levels, first as a source of potential chemotherapeutic drugs and secondly, as a measure of safety for the continued use of medicinal plants. Plants and microorganisms provide the pharmaceutical industry with some of the most important sources of components for the research of new medications. In the last few decades much study has been directed at popular medicine, with the aim of identifying natural products with therapeutic properties (Hamburger and Hostetmann, 1991). According to World Health Organization

(Definition, diagnosis and classification; WHO 1999), 80% of the people in developing countries of the world rely on traditional medicine for their primary health care, and about 85% of traditional medicine involves the use of plant extracts.

1.2. IMPORTANCE OF AYURVEDA

Most natural products play a pivotal role in human disease therapy and prevention. Recently, Ayurvedic science is gaining more importance and popularity because of its amazing therapeutic potentials. Even from Vedic period, natural products remain a fertile source for the discovery of new drugs and recent reports implicates that around 80% drug molecules are of natural origin. Natural products from Indian traditional medicinal plants reported in Charaka Samhita and Ashtangahridayam contributed towards the upsurge in drug discovery. Ayurvedic system of medicine has an important role in bioprospecting new medicines from medicinal plants, which are considered as rich source of biological potentials (Anand and Neetu, 2011).

1.3. IMPORTANCE OF NATURAL PRODUCTS IN DRUG DISCOVERY

Throughout human history, importance of natural products in maintaining health has been tremendous (Kintzios and Barberaki, 2004). Natural products offer a virtually unlimited source of unique molecules and also serve as a reservoir for new potential drugs and drug prototypes. The search for new plant derived chemicals should thus be a priority in current and future efforts towards sustainable conservation and rational utilization of biodiversity. Secondary metabolites from natural sources have been considered as the most successful candidates of potential drug leads (Mishra and Tiwari, 2011). According to Hu *et al.,* (1995), only 5-15% of higher plants have been systematically investigated for the presence of bioactive compounds. Recognition of

natural products, uniquely well known for its chemical activity and biodiversity has fueled the current focus of the field of search for new drugs.

1.4. PHYTOCHEMICALS AS ANTIOXIDANTS

1.4.1. Introduction

Free radicals are generally originate from a large variety of normal and pathological metabolic transformations from host response to a disturbance of tissue integrity which include cellular damage, trauma etc. The term 'free radical' designates a group of compounds with greater activity and having impaired valence shell electronic configuration (Gupta *et al.,* 2006). Free radical chain reactions in the body are initiated mostly by reactive species possessing oxygen, reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical, hydrogen peroxide etc. (Halliwell, 1994) as well as nitrogen, reactive nitrogen species (RNS) such as nitric oxide, peroxy nitrite etc. Recently, there is a boom in the areas related to newer developments in the prevention of diseases especially the role of antioxidants and free radicals. Nature has bestowed each cell with effective protective mechanism against the harmful action of free radicals and antioxidants, which are substances that neutralize free radical reaction mechanisms (Devasagayam *et al.,* 2004). Antioxidants neutralize free radicals by eliminating the unpaired condition by accepting or donating an electron. They are used by the food industry to delay the oxidation process (Tsuda *et al.,* 1994). Free radical reaction mechanisms have been implicated in the pathology of several diseases such as atherosclerosis, neurodegenerative diseases, malaria, cancer, ageing etc. by altering lipids, DNA, proteins etc. (Halliwell and Gutteridge, 1997). Proteins are highly prone to free radical attack resulting in loss of enzyme activity. Free radicals inturn damage lipids

resulting in lipid peroxidation which leads to adverse alterations (Devasagayam *et al.,* 2004). DNA damage results in mutagenesis and carcinogenesis. Most of the organisms develop many defenses to protect themselves from free radical processes. The abnormalities in the antioxidant defense system and oxidative stress may lead to higher susceptibility to lipid peroxidation of low density lipoprotein (Morena *et al.,* 2000).

Oxygen is vital for all living cells and radicals derived from oxygen represent the most important class of radical species generated in living systems (Miller *et al.,* 1990). When cells use oxygen to generate energy, free radicals are created as a consequence of ATP (adenosine triphosphate) production by the mitochondria. ROS and RNS are produced as products of normal cellular metabolism and both the terms are collectively used for describing free radicals and other non-radical reactive species which are also called oxidants (Halliwell and Gutteridge, 2007).

Molecular oxygen (dioxygen, O_2) has unique electronic configuration and is itself a radical with unpaired electrons (Figure 1.1). Biological free radicals are highly unstable molecules that are available to react with various organic substrates such as proteins, DNA, lipids etc. Potential biological damages caused by ROS and RNS are termed oxidative and nitrosative stress respectively. Oxidative stress results from the metabolic reactions that use oxygen and represents a disturbance in the equilibrium status in living organisms (Kovacic & Jacintho, 2001).

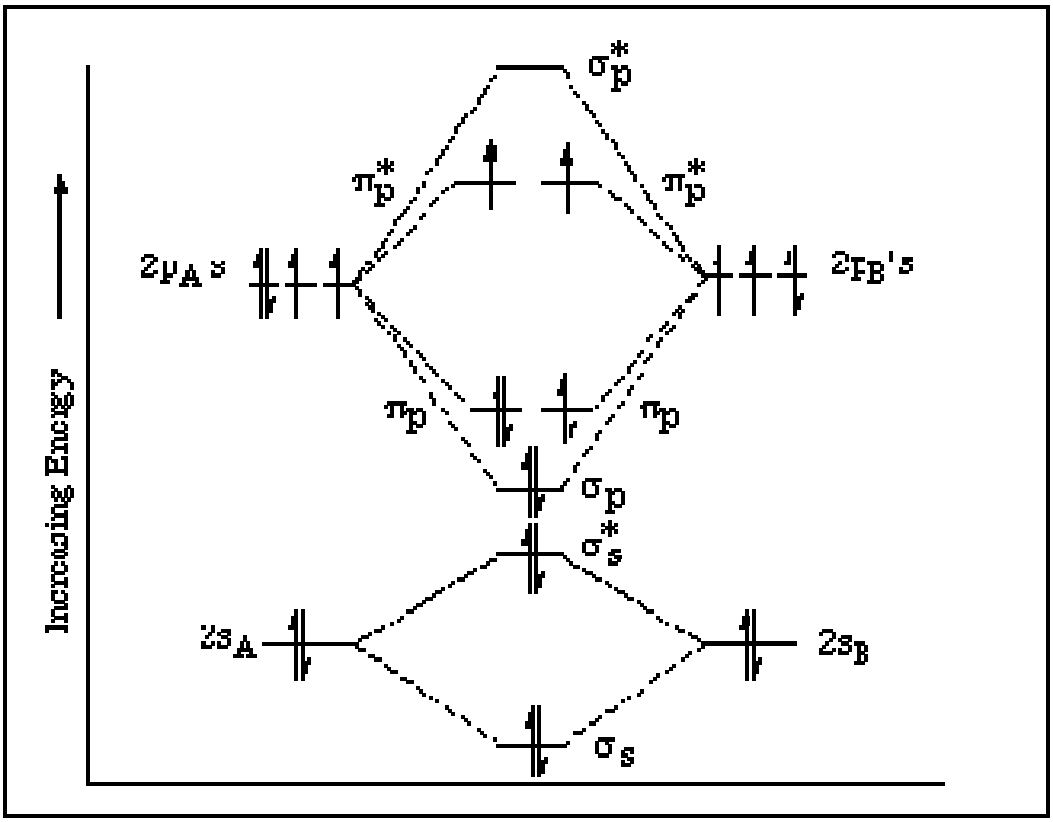


Figure 1.1: Molecular orbital diagram of oxygen molecule

1.4.2. Antioxidants

Antioxidants comprise a broad and heterogeneous family of compounds that share the common task of interfering with (stopping, retarding or preventing) the oxidation of an oxidizable substrate. Literally antioxidant is defined as "a substance that opposes oxidation or inhibits reactions promoted by oxygen or peroxides" (Webster, 2004). The most widely accepted explanation of an antioxidant is defined by Halliwell and Gutteridge (2007), as "*any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate*". To protect the body from harmful effects of free radicals and other oxidants, all aerobic organisms are endowed with powerful antioxidant systems. These include physical defenses, preventative and repair mechanisms and antioxidant defenses.

Antioxidants fight against the free radicals generated *in vivo*, thus preventing the organism against oxidative damage (Halliwell, 2001)

1.4.2.1. Endogenous antioxidants

There is a vast network of intracellular and extracellular antioxidants with diverse roles within each area of defense. Antioxidants that are produced within the body for defense as a result of normal metabolic processes are called endogenous antioxidants. Enzymes such as superoxide dismutase accelerates the conversion of superoxide (O_2^-) to H_2O_2 as shown in equation; $2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$ (Halliwell, 2001). Catalase converts H_2O_2 into water and oxygen which helps to dispose H_2O_2 generated by the action of the oxidase enzymes and glutathione peroxidase also remove H_2O_2 (Sies, 1996). The antioxidant enzymes, superoxide dismutase (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. The levels and locations of these antioxidant enzymes must be tightly regulated for cell survival (Mates *et al.,* 1999).

1.4.2.2. Exogenous antioxidants

Antioxidant compounds supplied through diet is termed as exogenous antioxidants. Dietary natural antioxidants have physiological significance to manipulate disease states and plays a vital role in the production of the antioxidant defense system by providing essential nutrient antioxidants such as vitamin E, C, β-carotene, flavonoids, trace metals (Se, Mn, Zn) etc. (Bouayed, 2010). Antioxidant plant phenols including flavonoids and essential minerals form important antioxidant enzymes. The interest in the role of dietary antioxidants in preventing many human diseases including cancer,

atherosclerosis, stroke, rheumatoid arthritis, neurodegeneration and diabetes is of great importance (Valko *et al.,* 2007). Polyunsaturated fatty acids (PUFA) having two or more double bonds are increasingly susceptible to free radical attack as the number of double bonds increases (Midori and Toshikazu, 1997). 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.

1.4.3. Oxidative stress

Free radicals and other reactive species are constantly generated in the human body. Oxidative stress is a harmful condition that occurs when there is an excess of ROS and decrease in antioxidant levels. This may cause tissue damage by physical, chemical that lead to tissue injury in human and causes different diseases viz. aging, cardiovascular diseases, cataract, diabetes, gastroduodenal pathogenesis, genetic disorders, inflammatory diseases etc. (Tian *et al.,* 2007). Oxidative stress causes oxidation of vital molecules and excess of ROS in body can damage cellular targets like DNA, protein, lipids etc. Dietary antioxidant and other essential dietary constituent depletion can also lead to oxidative stress (Halliwell, 2001).

1.4.4. Reactive oxygen species (ROS)

Molecular oxygen is being utilized by aerobic organisms as a terminal electron acceptor to enable metabolism of organic carbon for providing energy. ROS are generated in our body from oxygen by various endogenous systems and exposure to different pathophysiological states. They include superoxide anion (O_2^-), hydroxyl radicals (OH•), singlet oxygen ($^1O_2$) and non–free radical species such as hydrogen peroxide (H_2O_2) (Table 1.1) generally called oxidants are not free radicals, but can easily

lead to free radical reactions in living organisms. There are various forms of activated oxygen resulted from oxidative biological reactions or exogenous factors (Cerutti, 1991). Oxidative stress, a result of an imbalance between the antioxidant defense systems and the formation of ROS, may damage essential biomolecules such as proteins, DNA, and lipids. This damage may cause cellular injuries, death and exacerbates the development of several degenerative diseases associated with aging, cancer, cardiovascular diseases, diabetes and neurodegeneration (Ames *et al.,* 1993; Moskovitz *et al.,* 2002). The addition of one electron to dioxygen forms the superoxide anion radical ($O_2\bullet^-$) and occurs mostly within the mitochondiria of a cell (Cadenes and Sies, 1998). The mitochondrial electron transport chain is the main source of ATP in the mammalian cell and thus is essential for life. Another ROS, the hydroxyl radical, OH•, is the neutral form of the hydroxide ion that has high reactivity, making it a very dangerous radical. Under stress conditions, an excess of superoxide releases "free iron" from iron-containing molecules and the released Fe^{2+} can participate in the Fenton reaction, generating highly reactive hydroxyl radical ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^-$). Here $O_2\bullet^-$ acts as an oxidant and facilitates OH• production from H_2O_2 by making Fe^{2+} available for the Fenton reaction. The superoxide radical participates in the Haber-Weiss reaction ($O_2\bullet^- + H_2O_2 \rightarrow O_2 + OH\bullet + OH-$) which combines a Fenton reaction and the reduction of Fe^{2+} by superoxide, yielding Fe^{2+} and oxygen ($Fe^{3+} + O_2\bullet^- \rightarrow Fe^{2+} + O_2$). Peroxynitrite ($OONO^-$) formed in a rapid reaction between O_2^- and NO^- is also considered as active oxygen species (Liochev and Fridovich, 2002).

Biological antioxidants are natural molecules, which can prevent the uncontrolled formation of free radicals and activated oxygen species or inhibit their

reaction with biological structures (Chaudiere and Ferrari-Illiou, 1999). Biological antioxidants include antioxidative enzymes, such as superoxide dismutase, catalase, glutathione peroxidase and small nonenzymatic antioxidant molecules, such as glutathione, vitamins C and E etc. (Fridovich, 1999). The efficiency of the antioxidative defense system is altered under some pathological conditions and therefore ineffective scavenging and/or overproduction of free radicals may play a crucial role in determining tissue damage. Mechanisms responsible for the ROS mediated injuries to cells and tissues mainly include lipid peroxidation, oxidative DNA damage and protein oxidation. As a result, lipid and DNA oxidation have been the focus of investigations for many years. However due to their relatively high abundance, it is now recognized that proteins and lipids are the main targets for oxidants (Kayali *et al.,* 2007). The role of oxidative protein damage in the pathophysiology of human diseases is currently a topic of considerable interest, as oxidised proteins has been implicated in a wide spectrum of clinical conditions such as, diabetes, atherosclerosis, Alzheimer's disease, chronic lung disease, chronic renal failure, acute pancreatitis and sepsis (Dalle Donne *et al.,* 2003; Telci *et al.,* 2000). In order to cope up with the excess of free radicals produced upon oxidative stress, humans have developed sophisticated mechanisms in order to maintain redox homeostasis. These mechanisms include enzymatic and non-enzymatic antioxidant systems to scavenge or detoxify ROS, block their production or sequester transition metals which are the source of free radicals (Chaudiere & Ferrari-Iliou, 1999). This background has stimulated interest in the possibility of antioxidant supplements as a tool to prevent or slow down the progression of such diseases and indeed several investigations have confirmed the potential benefit of this strategy (Silva *et al.,* 2005).

Synthetic antioxidants have been in use as food additives for a long time, but safety concerns and reports on their involvement in chronic diseases have restricted their usage in food. Therefore, international attention has been directed toward natural antioxidants mainly from plant sources (Claudio and Hector, 2000). The depletion of endogenous scavenging compounds is occurred from the continuous increased production of reactive oxygen species during injury.

1.4.4.1. Beneficial effects of ROS

Free radicals and other reactive oxygen species are generated in the body as a result of stress, exercise, food habits, radiation etc. and serve important biological functions such as phagocytosis, cell signaling, apoptosis etc. 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 and involve physiological roles in cellular responses to noxia. At low/moderate concentrations, ROS invokes induction of a mitogenic response (Pacher *et al.,* 2007; Halliwell *et al.,* 2007). Free radicals are also involved in the generation of ATP from ADP and the discovery of redox sensitive transcription factors and that of NO•, a free radical produced enzymatically, plays a physiological role in vasodialation and neurotransmission (Yoshikawa *et al.,* 2000) and for killing intracellular pathogens and tumors. So free radicals and ROS having low or moderate concentration are significant to human health.

1.4.4.2. Deleterious effects of ROS

Free radicals and oxidants produced during aerobic metabolism can seriously alter the cell membranes and other structures such as lipids, DNA, proteins etc. Oxidative stress is a deleterious effect that arises when cells cannot adequately destroy the excess of

free radicals formed (Wells *et al.,* 2010). In other words, oxidative stress results from an imbalance between formation and neutralization of ROS/RNS. During lipid peroxidation, excess hydroxyl radicals and peroxynitrite can damage cell membranes and lipoproteins and leads to the formation of malondialdehyde (MDA) and conjugated diene compounds etc., which are cytotoxic and mutagenic. The MDA thus formed, reacts with the free amino group of proteins, nucleic acids and phospholipids to produce inter and intra molecular 1-amino-3-iminopropene (AIP) bridges and also produce a structural modification of these biomolecules (Halliwell and Gutteridge, 1989). Various oxidative stress induced diseases in humans is illustrated in figure 1.2.

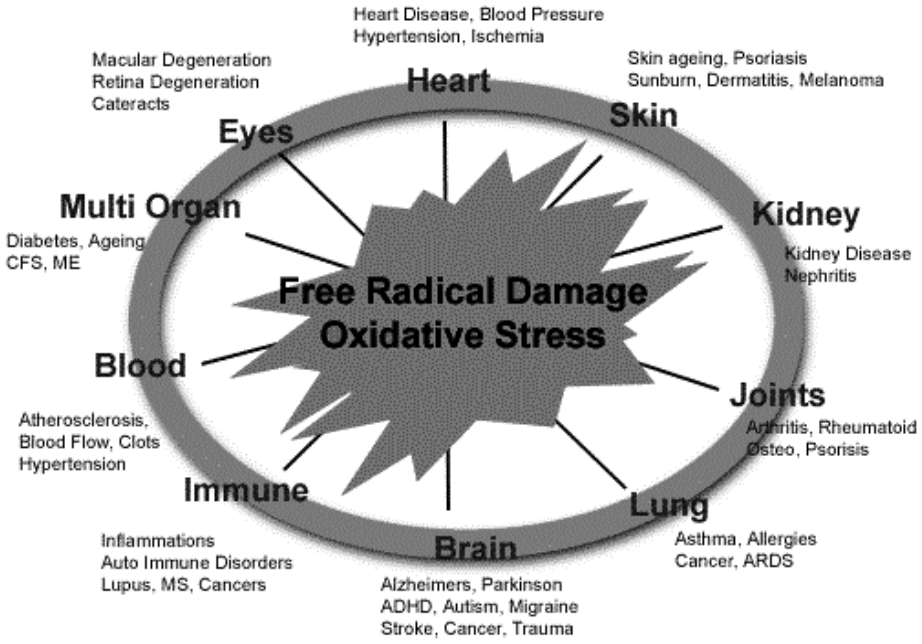


Figure 1.2: Oxidative stress induced diseases in humans

1.4.5. Reactive nitrogen species

Reactive nitrogen species are also formed as a byproduct of cellular metabolism and are known to act as secondary messengers controlling various physiological functions normally. Nitric oxide (NO) is a small molecule that contains one unpaired

electron on the $2\pi_y^*$ antibonding orbital and is therefore considered as a radical, NO•. RNS include various nitric oxide derived compounds such as NO^-, NO^+, higher oxides of nitrogen etc. (Table 1.1). RNS play crucial roles in living cells such as smooth muscle cells, nervous, cardiomyocytes, platelets etc. (Martinenz and Andriantsitohaina, 2009). It acts as a signaling molecule in various physiological processes including neurotransmission, defense mechanisms etc. Due to extraordinary properties, NO• was acclaimed as the "molecule of the year" in *Science Magazine* in 1992 (Koshland, 1992). Now these are considered as major components of oxidative burst and redox state regulation. The role of reactive nitrogen species and its effect on aging can attain considerable attention today. Some of the conditions associated with aging are cardiovascular disease, stroke, Alzheimer's disease etc. (Beck *et al.,* 1999; Lake-Bakaar *et al.,* 2001). The harmful effects caused due to overproduction of reactive nitrogen species is called nitrosative stress, which lead to nitrosylation reaction and alter the structure of proteins and inhibit their normal functions (Ridnour *et al.,* 2005; Valko *et al.,* 2001).

Table 1.1: Reactive oxygen and nitrogen species of biological interest (Devasagayam *et a*l., 2004)

Reactive species	Symbol	Half life (in sec)	Reactivity
Reactive oxygen species			
Superoxide	O_2^-•	10^{-6} s	Generated in mitochondria, in cardiovasculasr system and others
Hydroxyl	OH•	10^{-9} s	Very highly reactive, generated during iron overload and such
Hydrogen peroxide	H_2O_2	Stable	Formed in our body by large peroxide number of reactions and yields potent species like OH•
Peroxyl radical	ROO•	s	Reactive and formed from lipids, proteins, DNA, sugars etc. during oxidative damage
Singlet oxygen	$^1O_2$	10^{-6} s	Highly reactive, formed during photosensitization and chemical reactions
Reactive nitrogen species			
Nitric oxide	NO•	s	Neurotransmitter and blood pressure regulator, can yield potent oxidants during pathological states
Peroxynitrite	ONOO•	10^{-3} s	Formed from NO and superoxide, highly reactive
Nitrogen dioxide	NO_2	s	Formed during atmospheric pollution
Peroxynitrous acid	ONOOH	fairly stable	Protonated form of ONOO•

Both ROS and RNS species play well recognized dual role as toxic and beneficial compounds.

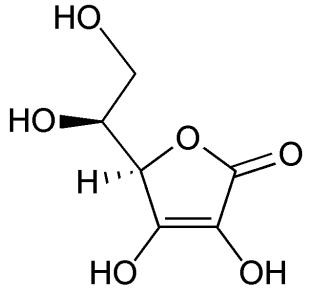
1.5. ROLE OF NATURALLY OCCURRING ANTIOXIDANTS IN HUMAN HEALTH SYSTEMS

Natural antioxidants that are present in plants are responsible for inhibiting or preventing the deleterious consequences 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 (Donaldson, 2004). Secondary metabolites are biologically active small molecules that are not required for viability but which provide a competitive advantage to the producing organism (Arryn *et al.,* 2013) and the process is called secondary metabolism. They are generally produced in response to climatic stress, infection, defense against predators, for propagation etc. Some of the pharmacologically active secondary metabolites are discussed below.

1.5.1. Vitamin C

Vitamin C, also known as ascorbic acid is a water soluble vitamin and is considered as an essential cofactor for α-ketoglutarate dependent dioxygenases (Traber and Stevans, 2011). Vitamin C provides protection against oxidative stress induced cellular damages by scavenging reactive oxygen species. Its reducing power being used in radical and non-radical redox reactions. Adjacent hydroxyl groups present in vitamin C can donate hydrogen atom to an oxidizing system and can scavenge radicals and act as a reducing agent. 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 is essential for neurotransmitter

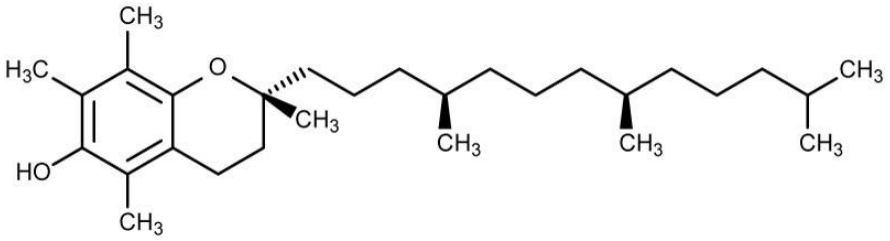
biosynthesis (Li and Schelhorn, 2007). Vitamin C is commonly found in citrus fruits, green vegetables, tomatoes etc. (Naidu, 2003).



Vitamin C

1.5.2. Tocopherols

Tocopherol is a naturally occurring chemical element and is commonly called as vitamin E, which is a fat soluble vitamin. The most common form of tocopherol is α-tocopherol, which is included in the diet to prevent the problems of central nervous system. Tocopherols are commonly found in spinach, nuts, vegetable oils etc. It is considered as a chiral compound with 3 chiral centres in its phytyl chain making a total of 8 stereocentres: α, β, γ, δ - tocopherol and α, β, γ, δ - tocotrienol (with double bonds in side chain) together known as chromanols. Both tocopherols and tocotrienols are effective in the inhibition of lipid oxidation in food and biological systems (Ronald and Junsoo, 2006). Owing to their ability to donate their phenolic hydrogen to lipid free radicals, tocopherols and tocotrienols are found to be potent antioxidants and the relative antioxidant efficacy is in the order α>β>γ>δ (Nguyen *et al.,* 2006). The activity may depend on the position of hydroxyl group in the sixth position of the chromane ring (Helmut and Wilhelm, 1995).

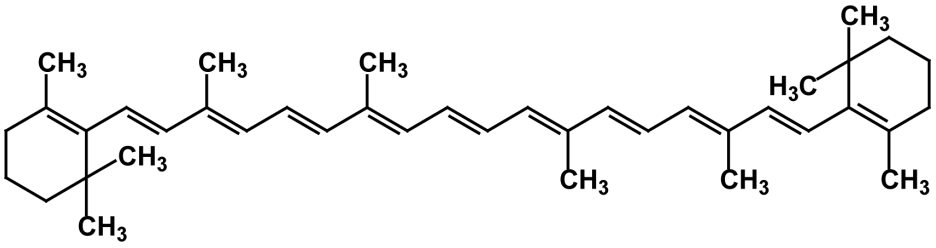


α-tocopherol

1.5.3. β-carotenes

β-carotenes are yellow coloured fat soluble member of carotenoids which are considered as pro-vitamins because they can be converted to active vitamin A. β-carotene is converted to retinol, which is essential for vision. It exerts antioxidant functions in lipid phase by quenching $^1O_2$ or free radicals (Helmut, 1995). The antioxidant property of carotenoids may be due to the presence of extended system of conjugated double bonds and depends on the formation of a carbon centered radical which protects the membranes from lipid peroxidation (Foote *et al.,* 1970). Foote *et al.,* (1971) found that the quenching of $^1O_2$ by *β*–carotene was due to an energy transfer from $^1O_2$ to *β*–carotene (Equation 1.1). This mechanism relies on the delocalization of the unpaired electrons of the peroxy and free radical species over the carotenoid conjugated polyene system (Burton and Ingold, 1984).

$$^1O_2 + \beta - \text{carotene} \rightarrow {}^3O_2 + 3\,\beta - \text{carotene}^* \text{ (excited state)} \quad \ldots\ldots\ldots \text{ Eqn 1.1}$$



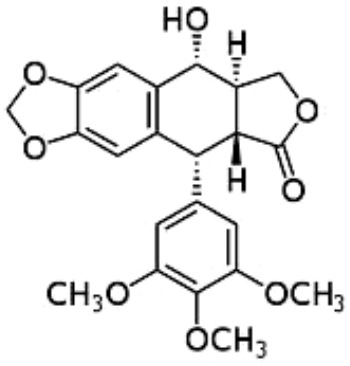
***β*–carotene**

1.5.4. Selenium (Se)

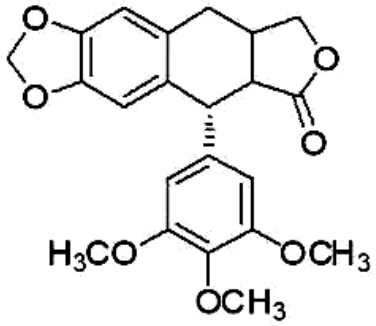
Selenium is an essential trace element found in sea food, cereals, milk, vegetables, fruits etc (Tinggi *et al.,* 1999). Selenium in biological materials and food can exist in both organic and inorganic form (Lobinski *et al.,* 2000; Dumont *et al.,* 2006). The increased interest in the study of Se is due to its presence in antioxidant enzymes such as glutathione peroxidase (GPx), thioredoxin reductase (TrxR) and iodothyronine deiodinases (IDD) (Tapiero and Townsend, 2003). Selenium has an important role in antioxidant selenoproteins for protection against oxidative stress induced by reactive oxygen and nitrogen species. Its importance in human health is well established and its deficiency can cause serious side effects such as Keshan disease (Beck, 2001). At low dose, health benefits of Se are antioxidant, anticarcinogenic, immunomodulator etc. and are considered as an essential factor in thyroid metabolism.

1.5.5. Lignans

Lignans are bioactive non-nutrient plant compounds widely distributed in plants (Julia *et al.,* 2010). They are formed as a result of coupling of monolignols *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol in which coniferyl alcohol being the most common monolignol used in lignin biosynthesis. Lignans by definition are dimers of phenylpropanoid (C_6-C_3) units linked by the central carbons of their side chains. Lignans are present in nuts, legumes, grains, fruits, vegetables etc. (Julia *et al.,* 2010). Podophyllotoxin and deoxypodophyllotoxin are two well known naturally occurring lignans.



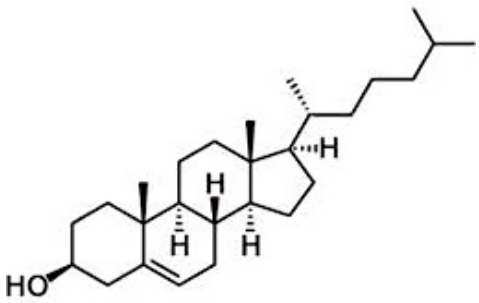
Podophyllotoxin



Deoxypodophyllotoxin

1.5.6. Steroids

Steroids are modified triterpenoids containing the tetracyclic ring system of lanosterol. Cholesterol exemplifies the basic structure of steroids. Steroids include a wide range of pharmacologically active compounds with slight changes in the side chain. Cholesterol typifies the fundamental structure. Many natural steroids together with a considerable number of synthetic and semisynthetic steroidal compounds are routinely employed (Dewick, 2009).



Cholesterol

1.5.7. Alkaloids

Alkaloids are secondary compounds containing one or more nitrogen atom derived from aminoacids or from the transamination process. Alkaloids impart a wide spectrum of physiological effects in plants and animals (Tadeusz, 2007). They are

derived from L-lysine, L-ornithine, L-tryptophan, L-histidine, L-phenyl alanine etc. Alkaloids in plants serve as chemo protective, antiherbivory agents or as growth regulators such as indole-3-acetic acid. First isolated alkaloid morphine, from opium poppy (*Papaver somniferum*) is considered as the most important applied one and is generally used as a narcotic analgesic. Reserpine (from *Rauwolfia serpentina*) is an antihypertensive alkaloid. Cocaine from coca plant (*Erythroxylum coca*) is a local anesthetic and a potent central nervous system stimulant. Colchinine from *Colchium autmuale* has been used to treat gout for 2000 years. Camptothecine, a quinoline alkaloid from Chinese tree (*Camptotheca accuminata*) is well known for its antiapoptic activity. Papaverine is used as a vasodialator. Atropine as a smooth muscle relaxant is used to dilate the pupil before eye examination and is also used for the treatment of ambylopia (lazy eye). Strychnine from *Strychnos nux-vomica* is a strong poison (Cordell, 1981).

1.5.8. Phenolic compounds

Phenolic compounds are a group of secondary plant metabolites having multifunctional roles in rhizospheric plant-microbe interactions and are produced in plants via shikimic acid through phenyl propanoid pathway (Figure1.3) (Santi *et al.,* 2010). The phenolic compounds found in plant cell walls and lignin have a unique chemical structure of C_6-C_3 (phenylpropanoid type) (Sarakanen and Ludwig, 1971). Naturally occurring phenolic acids contain two distinctive carbon frameworks: the hydroxycinnamic and hydroxybenzoic acid structures. Plant phenolic compounds have been studied extensively for their properties against oxidative damage leading to various degenerative diseases such as cardiovascular diseases, inflammation, cancer etc. Phenolic acids like ferulic, caffeic, protocatechuic, *p*-hydroxybenzoic, vanillic, syringic and *p*-

coumaric acid are the most common phenolic acids found in cereals and legumes. According to Harborne and Simmonds (1964), phenolics can be classified into groups based on the number of carbons in the molecule (Table 1.2). Phenolic antioxidants are considered as primary antioxidants which act as free radical scavengers. The free radical scavenging activity of phenolic compounds are mainly attributed to their ability to donate a hydrogen atom to reduce ROS radicals. Thus phenolic compounds (ArOH) get converted to oxidized phenoxyl radicals (ArO•) and are stabilized by resonance delocalization over the aromatic ring. For example, the reduction of peroxyl and hydroxyl radicals by phenolic compounds can be represented as follows: (Aruoma, 2003; Hensley *et al.,* 2004).

$$ROO\bullet + ArOH \rightarrow ROOH + ArO\bullet$$

$$HO\bullet \quad + ArOH \rightarrow HOH + ArO\bullet$$

Non-radical products may also be formed by the coupling of ROS radicals with phenoxy radicals (Pietta, 2000).

$$ROO\bullet + ArO\bullet \rightarrow ROOArO$$

The phenoxy radicals thus formed are relatively stable and further oxidation reactions are not easily initiated.

Table 1.2: Classification of phenolic compounds

Structure	Class
C_6	Simple phenolics
C_6-C_1	Phenolic acids and aldehydes
C_6-C_2	Acetophenones and phenyl acetic acids
C_6-C_3	Cinnamic acids, cinnamyl alcohols and cinnamyl aldehydes
C_6-C_1-C_6	Benzophenones
C_6-C_2-C_6	Stilbenes
C_6-C_3-C_6	Flavonoids
C_{18}	Betacyanins
C_{30}	Biflavonoids
C_6, C_{10} & C_{14}	Quinines
Dimers or oligomers	Lignans and neolignans
Oligomers or polymers	Tannins
Polymers	Phlobaphenes

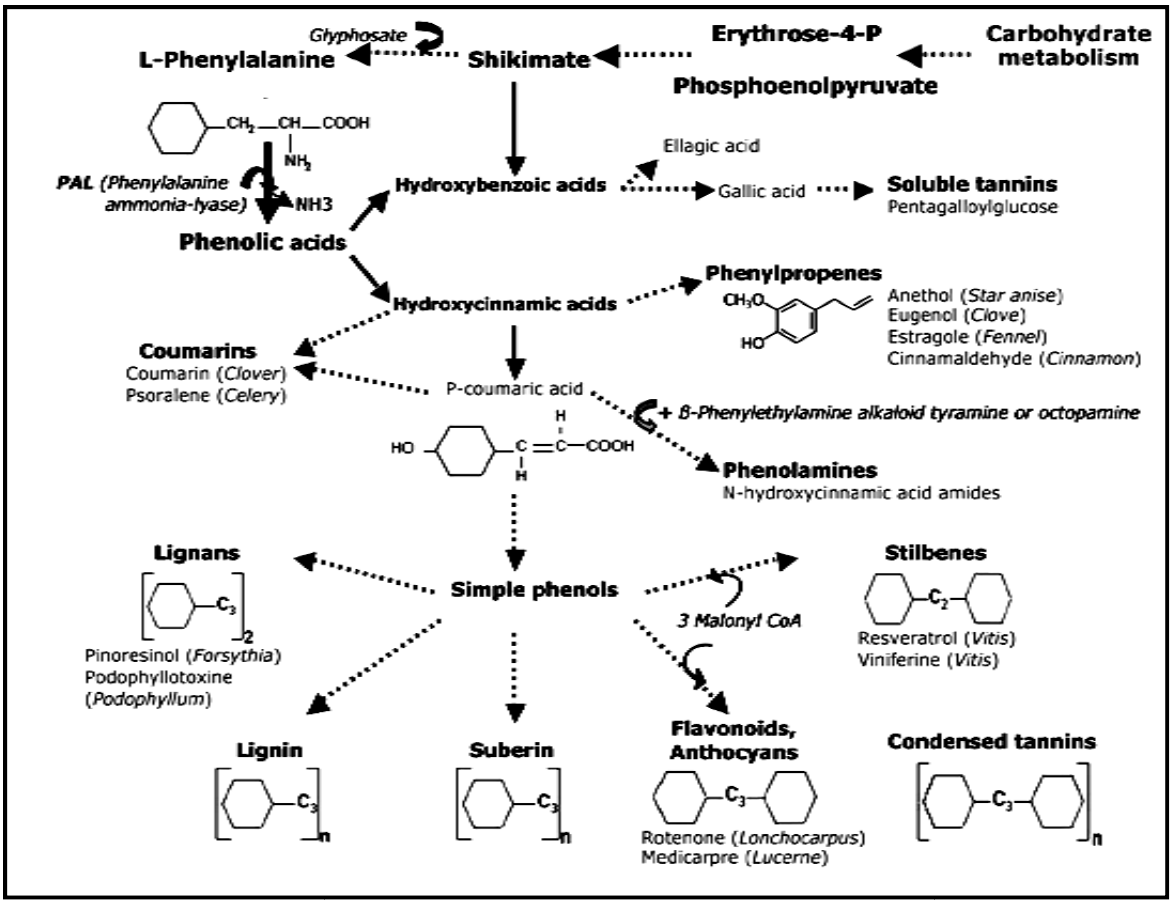


Figure 1.3: Simplified pathway of phenolic compound synthesis (Cecile *et al.,* 2012)

1.5.9. Terpenoids – An Introduction

Terpenoids are a diverse class of organic chemicals produced by a wide variety of plants, microorganisms and animals. They play a vital role in the chemical ecology of plants derived from C_5 isoprene units (McGarvey and Croteau, 1995). However terpenoids are often used as defense compounds that insects will use plant terpenes to ferret out specific host plants for their larvas. Terpenoids were originally known as terpenes and the suffix 'ene' in terpenes indicates the presence of olefinic double bonds. Based on the roles which terpenoids play in living organisms, it can be divided into three types: functional, defence and communication terpenoids.

1.5.9.1. Biosynthesis of isoprenoids or terpenoids

The glut of terpenoid compounds is biosynthetically formed by the head to tail addition of two simple precursor units, isopentenyl diphosphate (IPP, C_5 isoprene unit) and its allylic isomer dimethyl allyl diphosphate (DMAPP) (Figure 1.4). They condensed together to form geranyl diphosphate (GPP, C_{10}) by geranyl diphosphate synthase enzyme. The formed geranyl diphosphate further condensed with additional IPP units catalyzed by farnesyl diphopshate (FPP) synathase enzyme form successively larger prenyl diphosphates. FPP synthase sequentially adds two molecules of IPP to GPP to form the C_{15} diphosphate precursor of sesquiterpenes and triterpenes. Geranylgeranyl diphoshpate (GGPP) synthase adds three molecules of IPP to FPP to form the C_{20} diphosphate precursor of diterpenes and tetraterpenes (Koyama and Ogura, 1999). Monoterpenes (C_{10}) and sesquiterpenes (C_{15}), the constituents of essential oils, important flavouring agents in food and beverages exhibited a lot of pharmaceutical and therapeutic potentials (Mahmoud and Croteau, 2002).

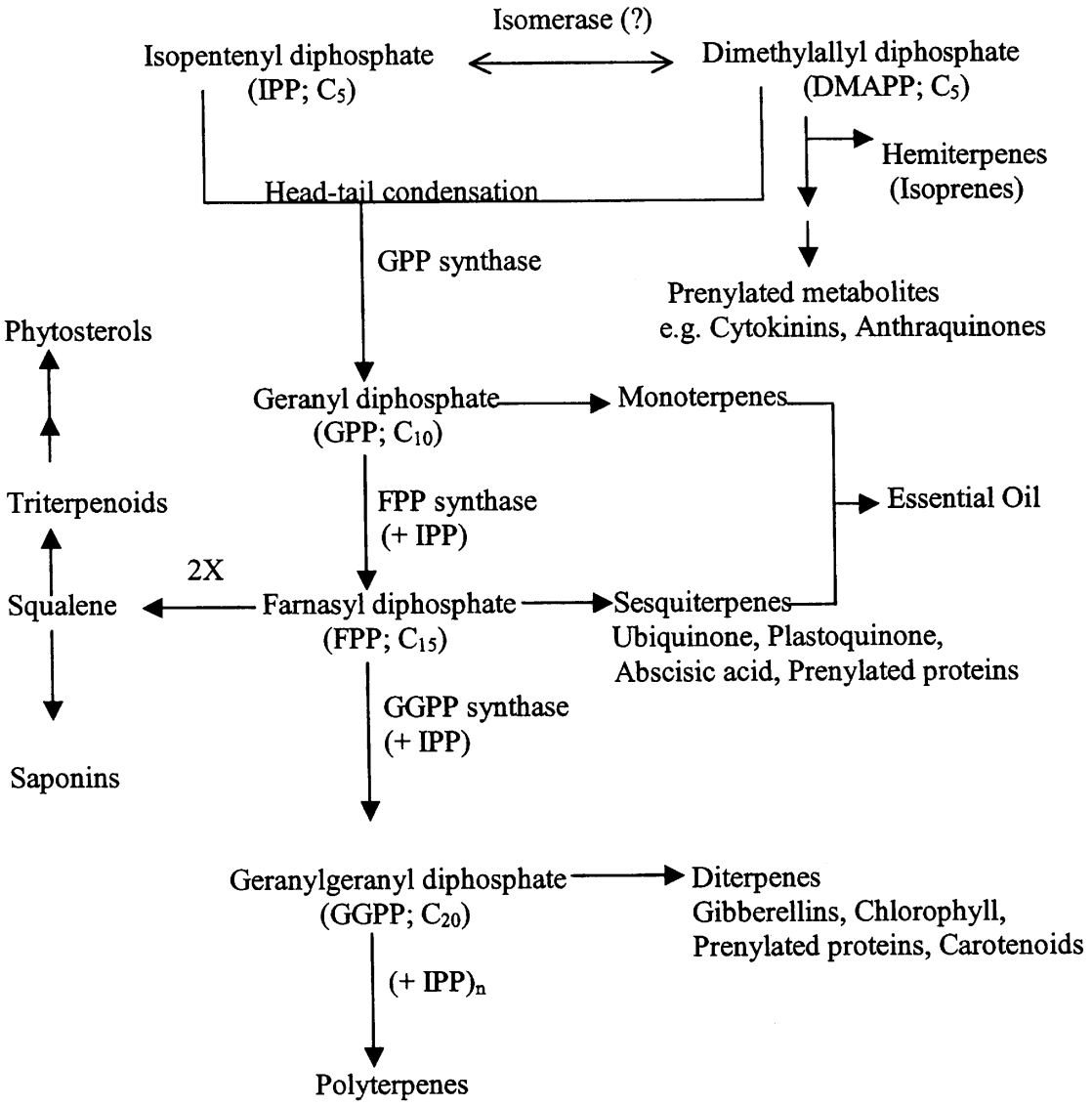


Figure 1.4: Biosynthetic pathway of terpenoids

The natural terpenoids mostly have the general formula $(C_5H_8)_n$ containing isoprene units joined in a head to tail fashion. Therefore these are also referred to as 'isoprenoids' (Table 1.3).

Table 1.3: Classification of terpenoids

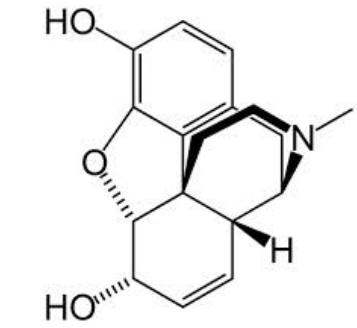
n	No. of carbon atoms	Molecular formula	Class of compound
2	10	$C_{10}H_{16}$	Monoterpenoid
3	15	$C_{15}H_{24}$	Sesquiterpenoid
4	20	$C_{20}H_{32}$	Diterpenoid
5	25	$C_{25}H_{40}$	Sesteterpenoid
6	30	$C_{30}H_{48}$	Triterpenoid
8	40	$C_{40}H_{64}$	Tetraterpenoid (Carotenoid)
> 8	> 40	$(C_5H_8)_n$	Polyterpenoid

1.5.9.2. Biological activities of terpenoids

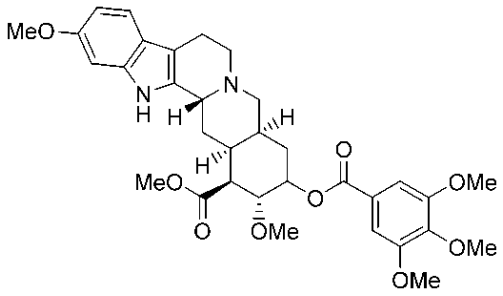
Extensive biological innovations have been carried out within the broad group of terpenoid family and the results showed the presence of a wide class of pharmacologically active constituents. Marine terpenoids with promising biological potentials were also observed. The sesquiterpenoids are a widespread group of substances occurring in many different organisms and form the largest class of terpenoids (Atta–ur-Rahman, 2005). Over 200 skeletal types are known and several thousand compounds of the class have been isolated and identified. Some of the first sesquiterpenoids with potent antitumor activity were vernolepin and vernomenin, from *Vernonia hymenolepis* reported by Kupchan and colleagues in 1968. They showed cytotoxicity (ED_{50}) against KB cell at 2 and 20 µg/ml respectively, and vernolepin also showed significant inhibitory activity against the Walker intramuscular carcinosarcoma 256 in the rat at 12 mg/kg (Hill, 1993). Besides cytotoxic and antitumor activity, sesquiterpenoids exhibit a rich variety of other

biological properties. The endoperoxide artemisinin (qinghaosu) (Klayman, 1985), isolated from the Chinese herb *Artemisia annua*, has been employed for the treatment of malaria. The main sesquiterpenoid mycotoxins are trichothecenes (Jarvis *et al.,* 1985), which are associated with a wide variety of human and animal toxic effects. They are phytotoxic compounds and potent anticancer agents, and some have antibacterial activity, such as verrucarin. The drimane sesquiterpenoids, warburganal and polygodial isolated from *Warburgia stuhlmannii*, are examples of insect antifeedant substances (Fraga *et al.,* 1991). Some of the structures of phytochemically important antioxidants are given in chart 1.1.

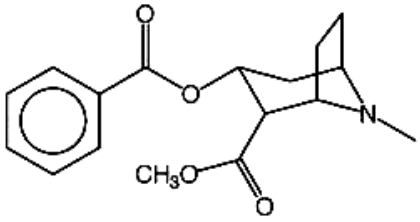
Chart 1.1: Phytochemically important antioxidants



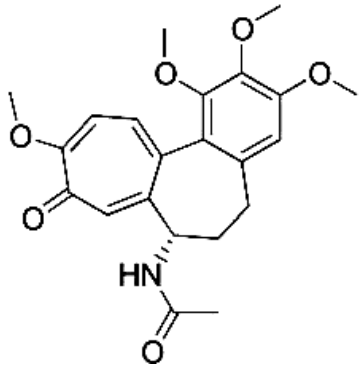
Morphine



Reserpine

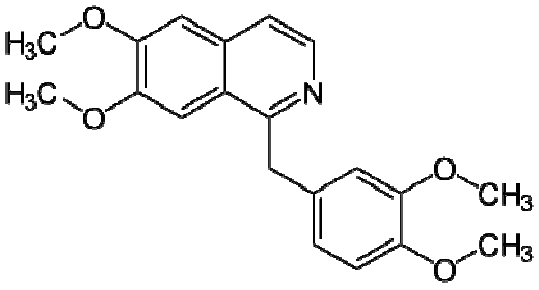


Cocaine

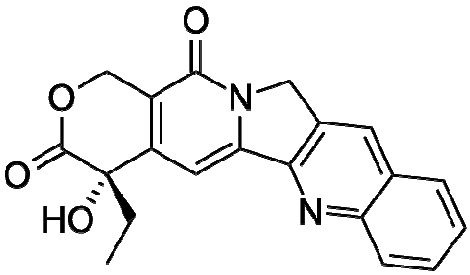


Colchinine

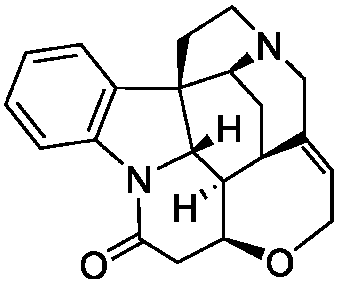
Chart 1.1. continued……



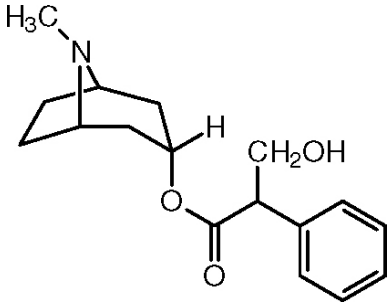
Camptothecin



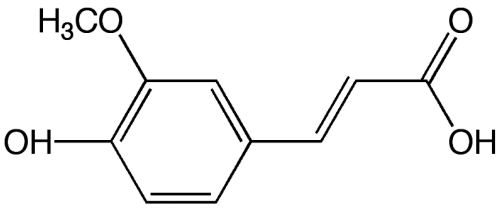
Papaverine



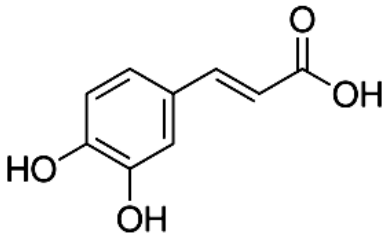
Atropine



Strychnine

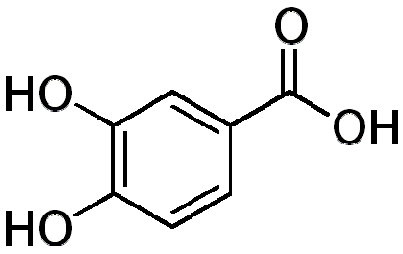


Ferulic acid

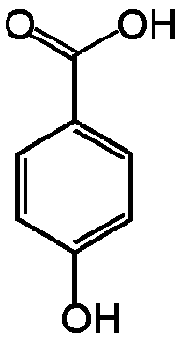


Caffeic acid

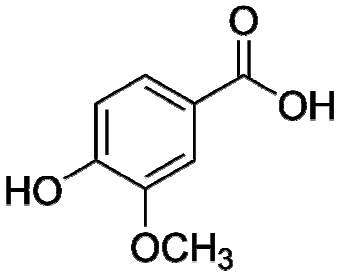
Chart 1.1. continued…….



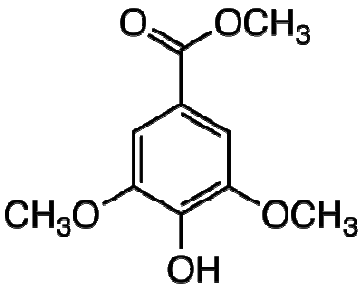
Protocatechuic acid



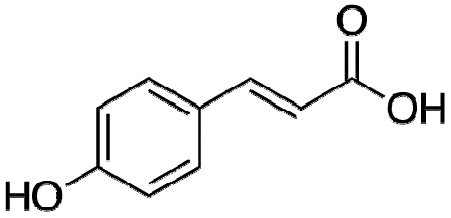
***p*-hydroxy benzoic acid**



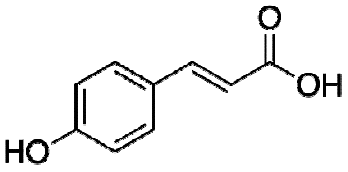
Vanillic acid



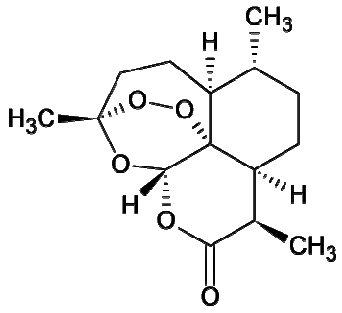
Syringic acid



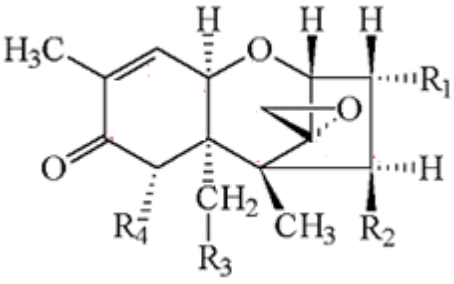
***p*-coumaric acid**



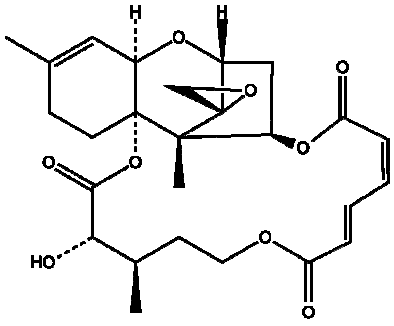
Vernolepin



Artemisinin

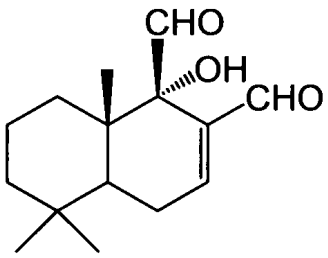


Trichothecenes

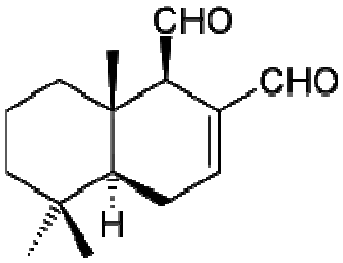


Verrucarin

Chart 1.1. continued…….



Warburganal

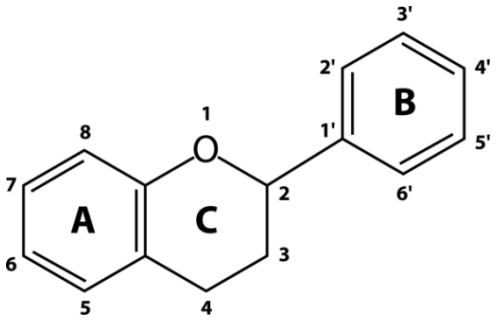


Polygodial

1.5.10. Flavonoids – An Introduction

Flavonoids are a group of secondary plant phenolic compounds with potent antioxidant and chelating properties. They are distributed throughout the plant kingdom and are responsible for the colour of flower and fruit. Flavonoids are mainly concentrated in fruits, vegetables, wines, cocoa, tea etc. Many of them posses low toxicity and can be widely used as antiinflammatory, antihepatotoxic, anticancer and antiulcer agents (Bors *et al.,* 1990; Colerige *et al.,* 1980). They can also inhibit enzymes such as α-glucosidase, α-amylase, aldose reductase, cycloxygenase, lipoxygenase, calcium ATPase, phosphodiasterase etc. Flavonoids are involved in floral pigmentation, symbiotic nitrogen fixation etc. in higher plants.

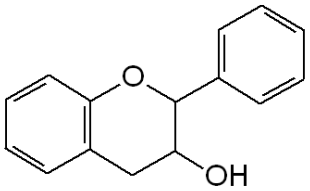
The basic structure of flavonoids includes two aromatic rings linked by a three-carbon aliphatic chain which normally has been condensed to form a pyran or less commonly, a furan ring. The heterocycle in the flavonoid backbone are generally called ring A, B and C.

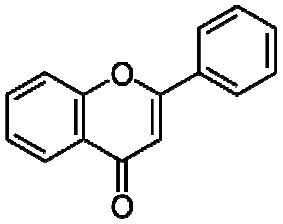
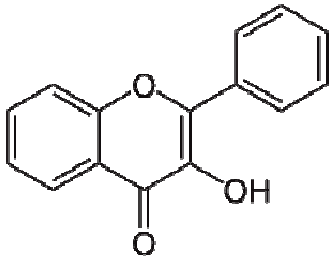
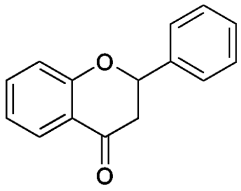
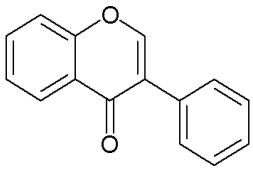
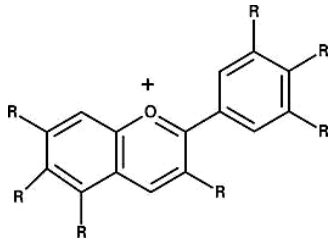


Basic structure of a flavonoid ring

Based on the molecular structure, flavonoids can be divided mainly into flavones, flavonols, flavonones, flavones, isoflavonoids, anthocyanins, flavan-3-ol etc. depicted in table 1.4. The classification is mainly based on the connection position of B and C rings, as well as the degree of saturation, oxidation and hydroxylation of the C ring etc. (Graf *et al.,* 2005). The individual differences within the group result from the variation in number and arrangement of hydroxyl groups as well as the nature and extent of glycoxylation. The presence of a planar structure characterizes a flavone moiety with double bond in the central aromatic ring and dihydroxylation in the 3′ and 4′ position of the B ring. Apigenin, a flavone is abundantly present in apple peels and quercetin, one of the best studied flavonoids is present in apples, broccoli, onions and berries. Anthocyanins are present mostly in tea, berries etc. whereas catechins are present in green tea.

Table 1.4: Classification of flavonoids (Rice evans *et al.,* 1996)

Class	General structure	Flavonoid	Source
Flavan-3-ol		(+)-catechin (-)-epicatechin Epigallocatechingallate	Tea

Flavone		Chrysin Apigenin Rutin Luteolin Luteolin glucoside	Fruits Parsley, celery Red wine, citrus, tomato skin Red pepper
Flavonol		Kaempferol Quercetin Myricetin Tamarixetin	Leek, broccoli, black tea Onion,broccoli, tomato, tea Cranberry grapes, red wine
Flavonone		Naringin Naringenin Taxifolin	Citrus, grapefruit Citrus fruits
Isoflavone		Genistin Genistein Daidzin	Soybean
Anthocyanidin		Apigenidin Cyanidin	Cherry, raspberry, strawberry

1.5.10.1. Biosynthesis of flavonoids

Basically, flavonoids derived from 1, 3-diphenylpropan-1-one (C_6-C_3-C_6) and biosynthesis involves the condensation of three molecules of malonyl-CoA with one molecule of *p*-coumaryl CoA ester catalyzed by chalcone synthase to form chalcone

intermediate, having a linear C_3 chain connecting the two rings (Martens and Mithofer, 2005). Chalcones and dihydrochalcones are classes of flavonoids that consist of two phenolic groups which are connected by an open three carbon bridge. Flavonones, derived from chalcone structure posses a three carbon bridge, which is part of an additional heterocycle six membered ring that involves one of the phenolic group on the adjacent ring. Based on these flavonones, all other flavonoid classes including flavonols, flavones, flavononols, anthocyanins etc. are generated (Figure 1.5) (Martens and Mithofer, 2005).

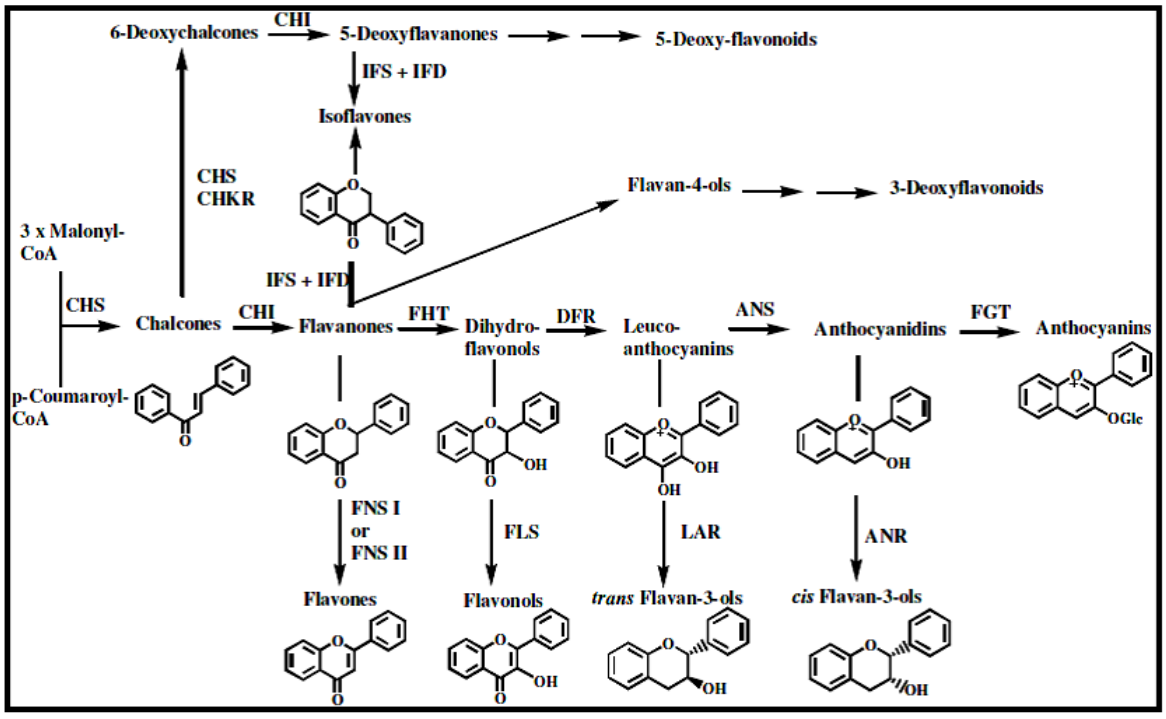


Figure 1.5. Scheme of general biosynthetic pathway of flavonoids

The enzymes involved are abbreviated as 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

1.5.10.2. Antioxidant potential of flavonoids

The most important effect of flavonoids is the scavenging of oxygen derived free radicals. The catechins and flavones seem to be most potent flavonoids for protecting the body against reactive oxygen species. Free radicals and reactive oxygen species are produced during normal oxygen metabolism and cause significant damages in the body cells and tissues (de Groot, 1994). Free radicals get oxidized by flavonoids forming a more stable less reactive radical and deactivate the radicals due to the high activity of the hydroxyl group (Pietta, 2000).

According to Hudson and Lewis (1983), a flavonoid moiety with a 3′, 4′-dihydroxy configuration gave strong antioxidant potential in addition to the presence of a 4-carbonyl group in the molecule. The free radical scavenging capacity of flavonoids is attributed to the presence of hydroxyl substituents.

F-OH+ R•→ F-O• + RH

where F-OH refers to a flavonoid, R• is a free radical and FO• is a less reactive free radical

Presence of hydroxyl group in the B ring attenuates the scavenging of ROS (Sekher Pannala *et al.,* 2001; Burda and Oleszek *et al.,* 2001) and RNS (Haenan *et al.,* 1997; Kerry and Rice Evans, 1999) by donating hydrogen and electron and giving rise to a stable flavonoid radical. A 3′, 4′ - catechol moiety in the B-ring strongly enhances lipid peroxidation potential, but A-ring correlates little with antioxidant activity. Another hydroxyl group in position 5 also contributes to antioxidant effects (Cholbi *et al.,* 1990).

The presence of heterocycle in the flavonoid moiety contribute to antioxidant

action by i) the presence of a free 3 –OH group ii) conjuation between the aromatic rings. The closed C ring may not be critical to the activity of flavonoids (Figure 1.6).

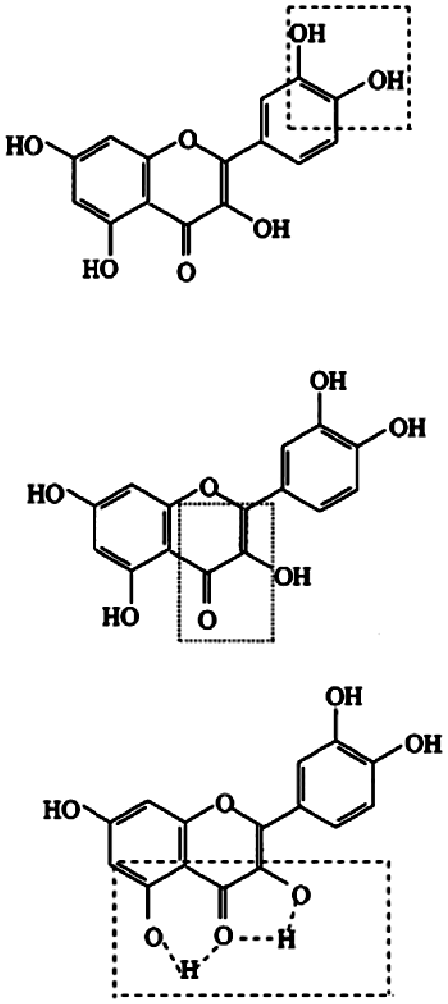


Figure 1.6: Position of hydroxyl groups on flavonoid ring structure

The proposed binding sites for trace metals to flavonoids are the catechol moiety in ring B, 3-hydroxyl, 4-oxo groups in the heterocyclic ring and the 4-oxo, 5-hydroxyl groups between the heterocyclic and the A rings (Cheng and Breen, 2000) (Figure 1.7).

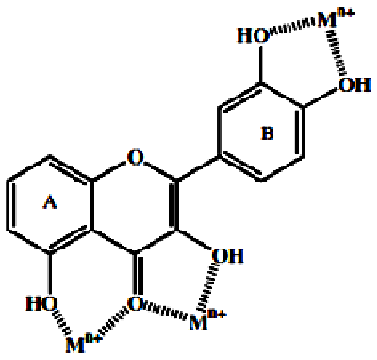


Figure 1.7: Binding sites for trace metals

1.5.11. Spices as antioxidants

Spices and herbs being used in food and medicinal mixtures act as antioxidants due to strong hydrogen donating activity (Lugasi *et al.,* 1995). Oxidation processes caused by ROS are a major cause for the deterioration of various food products like rapid development of stale flavors and rancidity which is considered as a primary mechanism of quality deterioration in lipid foods and oils (Guntensperger *et al.,* 1998) and have injurious physiological effects on human system. Usually synthetic antioxidants like butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT) etc. are used to decelerate the processes. Due to the uncertainties about their safety (Verhagen *et al.,* 1991; IARC 1987), demand for the discovery of safe antioxidants especially from natural origin is of great importance.

Since ancient times, spices have been added to food as whole spices or as ground spices to impart aromatic flavor. The advantageous antioxidant properties are due to many substances including vitamins, flavonoids, terpenoids, carotenoids, phytoestrogens, minerals etc. (Calucci *et al.,* 2003). 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 camphene, piperine, carvacrol, eugenol, lauric acid etc. in pepper; alanine, caffeic acid, campesterol, capsaicin, chlorogenic acid etc. in chilli; carnosol, carnosic acid, rosmanol, rosmaridiphenol, rosmadial and rosmariquinone and various methyl and ethyl esters of these substances in rosemary and sage; diarylheptanoid, gingerol, myrcene, myricetin, tryptophan and zingerone in ginger; curcumin, tetrahydrocurcumin, *p*-coumaric acid, protocatechuic acid, syringic acid etc. in turmeric (Chart 1.2) (Milan, 2006). On account of these, the application of natural products will gain more interest even in the future and it will be necessary to study their effects and interactions in more detail.

Chart 1.2: Important spices as antioxidants

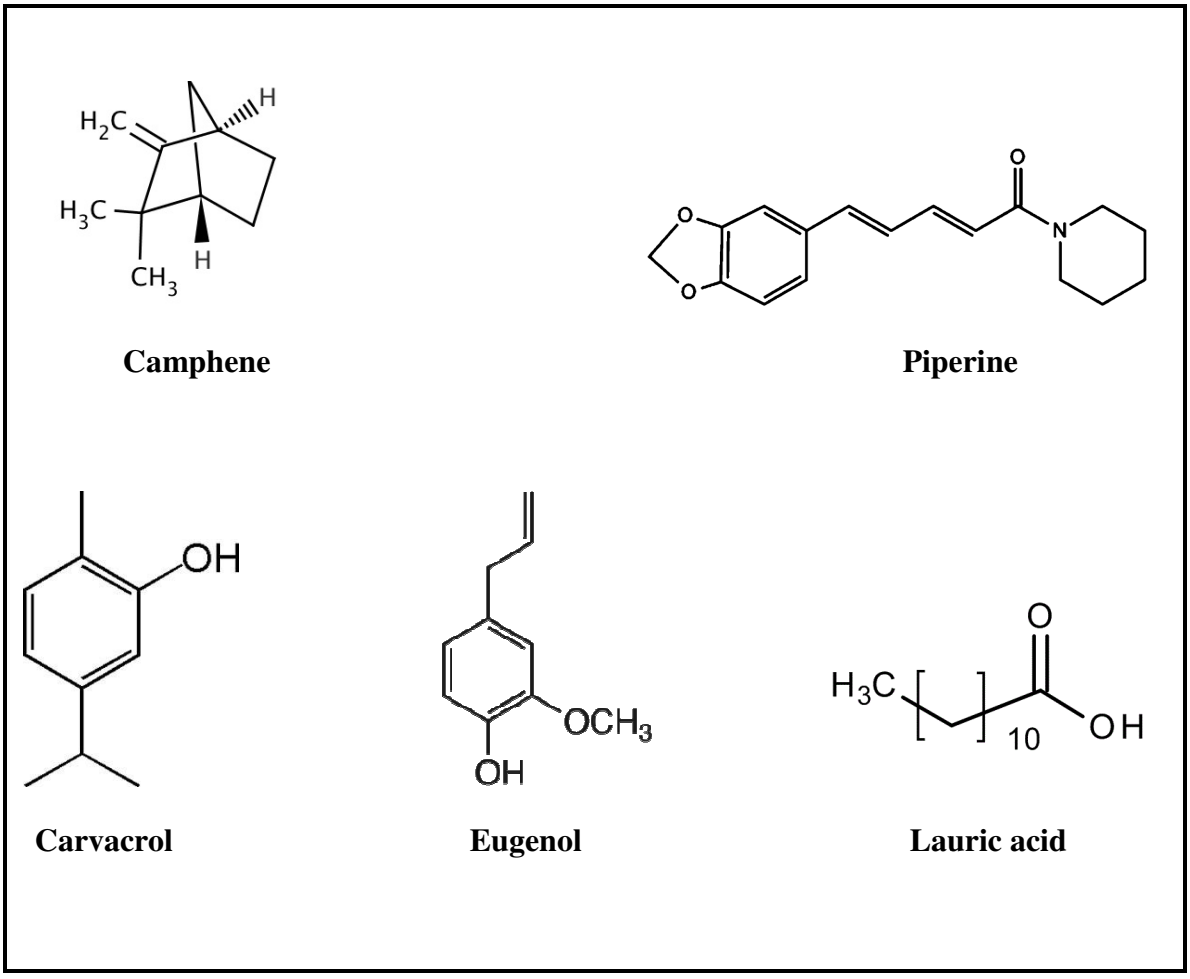
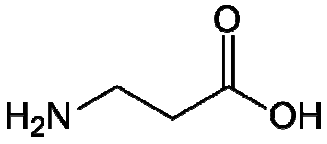
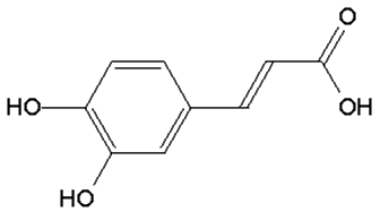


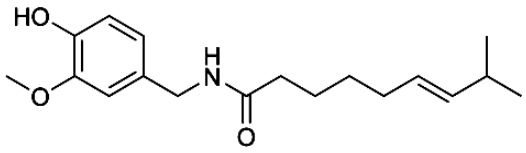
Chart 1.2 continued……



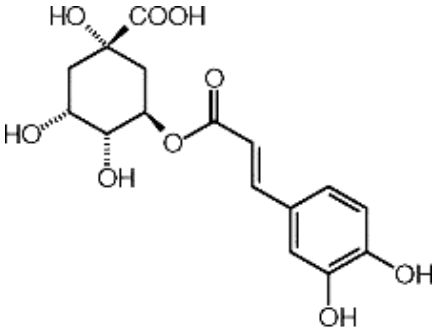
Alanine



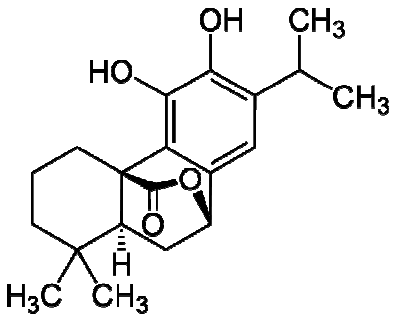
Campesterol



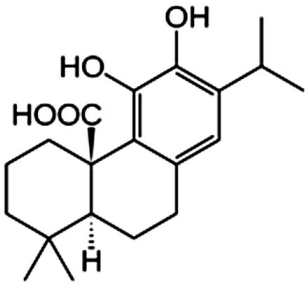
Capsaicin



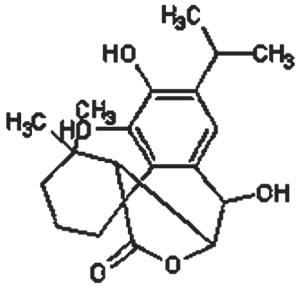
Chlorogenic acid



Carnosol

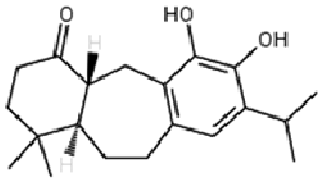


Carnosoic acid

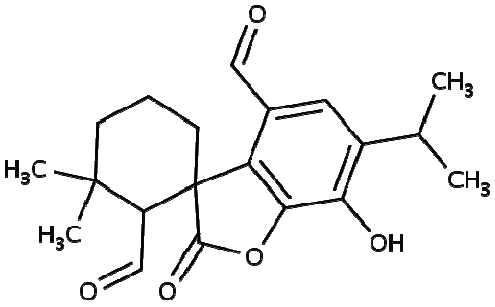


Rosmanol

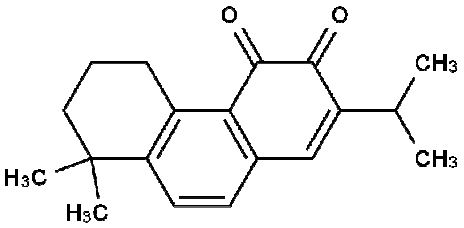
Chart 1.2 continued……



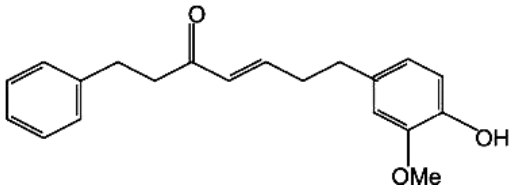
Rosmaridiphenol



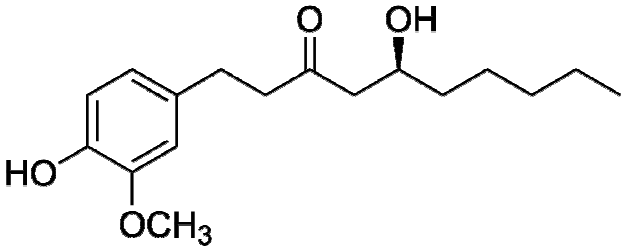
Rosmadial



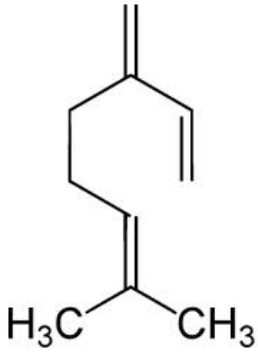
Rosmariquinone



Diaryl heptanoid

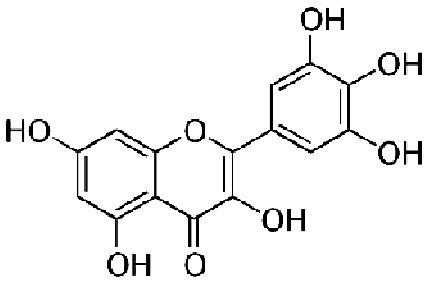


Gingerol

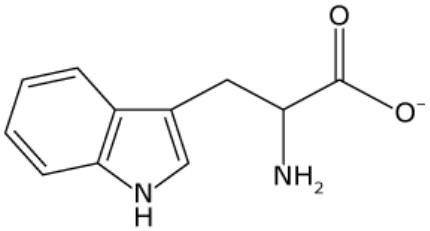


Myrcene

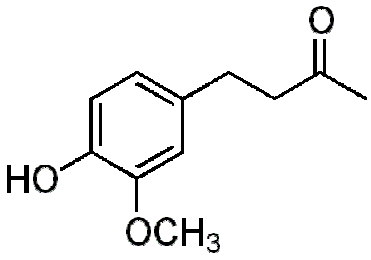
Chart 1.2 continued……



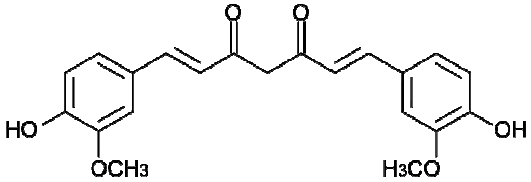
Myricetin



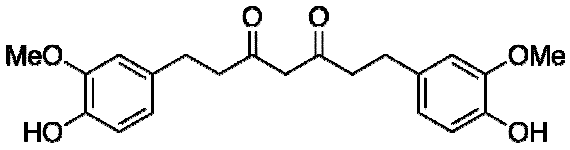
Tryptophan



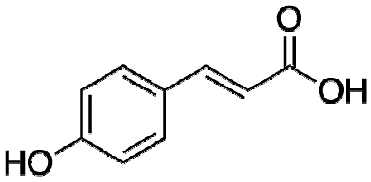
Zingerone



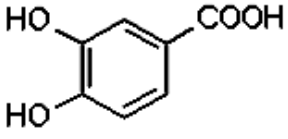
Curcumin



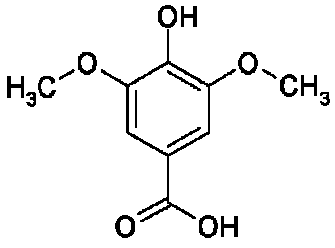
Tetrahydro curcumin



***p*-Coumaric acid**



Protocatechuic acid



Syringic acid

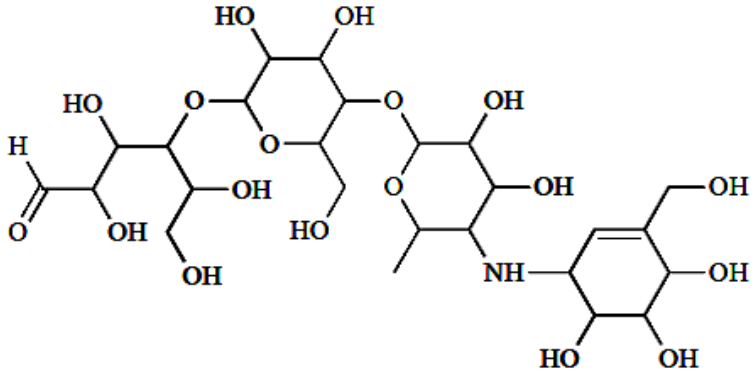
1.6. DIABETES

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycaemia resulting from defects in insulin secretion or insulin action and affects various organs (Definition, diagnosis and classification; WHO 1999). The long term effects of diabetes mellitus include progressive development of the specific complications of retinopathy with potential blindness, renal failure, food ulcers, nephrophathy, embryopathy etc. (Standards of medical care in diabetes 2013). Various pathogenic process in diabetes include the destructiom of β-cells of pancreas with insulin deficiency or resistant to insulin action. Clinically, diabetes is divided into four types; type 1 diabetes resulting from β-cell destruction, type 2 diabetes resulting from a progressive insulin secretory defect on the background of insulin resistance, gestation diabetes mellitus diagnosed during pregnancy and diabetes due to genetic defects in β-cell function (Verspohl, 2012).

Type 2 diabetes is the common form of diabetes and is characterized by disorders of insulin action. Inhibition of α-glucosidase and α-amylase enzymes involved in the digestion of carbohydrates can significantly reduce the post prandial hyperglycaemia and can be considered as an important strategy in the management of blood glucose level in type 2 diabetes (Tundis *et al.,* 2010). Natural α-glucosidase and α-amylase inhibitors from plant sources offer an attractive strategy for the control of hyperglycaemia by delaying the digestion of starch and sucrose. Diverse biological constituents offer a unique renewable source for the discovery of potent diabetic drugs. More than 800 plants are used for the treatment of diabetes throughout the world (Pushparaj *et al.,* 2000). Therapeutic approach involves the use of natural products which helps to retard the

absorption of glucose through the inhibition of carbohydrate hydrolyzing enzymes, α-amylase and α-glucosidase in the digestive tract. Consequently, enzyme inhibitors reduce the rate of glucose absorption and blunt post-prandial plasma glucose rise (Chiasson 2006; Chen 2006).

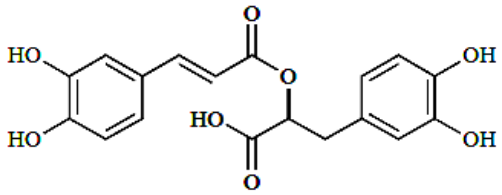
Acarbose, a natural product obtained by the fermentation process of *Actinoplanes* (Truscheit *et al.,* 1981) is a pseudotetrasaccharide with unsaturated cyclitol [2,3,4-trihydroxy-5-(hydroxymethyl)-5, 6-cyclohexene in a D-*gluco* configuration] attached to the nitrogen of 4-amino-4, 6-dideoxy-D-glucopyranose, which is linked α-(1→4) to maltose showcased promising activities in the inhibition of α-glucosidase and α-amylase enzymes (Muller *et al.,* 1980).



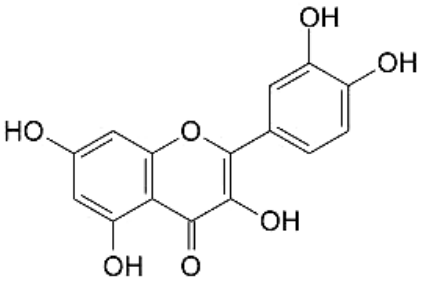
Acarbose

Polyphenol compound like rosmarinic acid showed more potent hypoglycaemic effect and have been used in traditional medicine to treat diabetes (Eddouks *et al.,* 2002). Flavonoids such as quercetin, myricetin and kaempferol (Tadera *et al.,* 2006); triterpenes such as oleanolic acid, arjunolic acid, asiatic acid, corosolic acid and 23-hydroxyursolic acid (Hou *et al.,* 2009); tannins such as pedunculagin, strictinin, sanguiin H-5, lambertianin and sanguiin H-6 (Li *et al.,* 2007) showed more potent inhibition patterns (Chart 1.3).

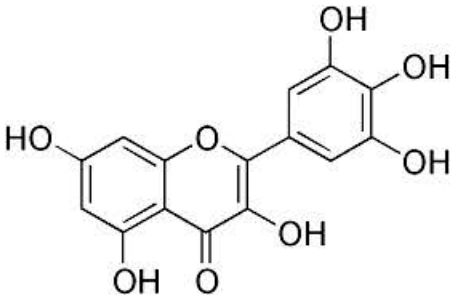
Chart 1.3: Important antidiabetic phytochemicals



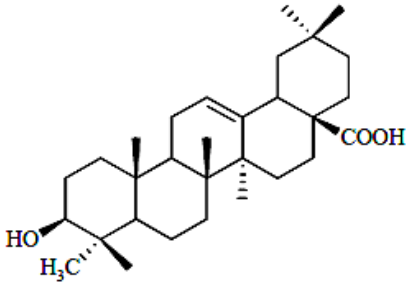
Rosmarinic acid



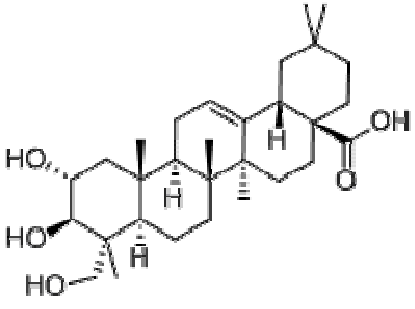
Quercetin



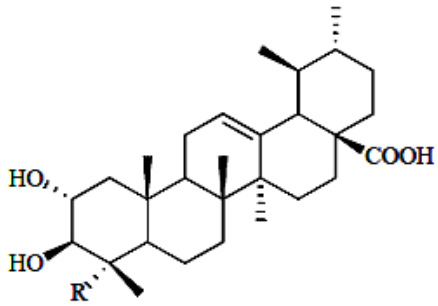
Myricetin



Oleanolic acid

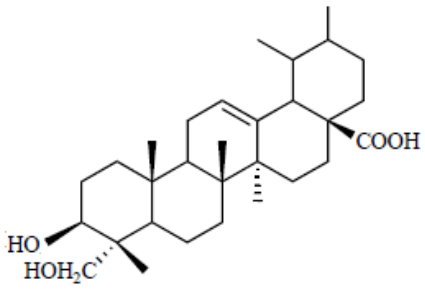


Arjunolic acid

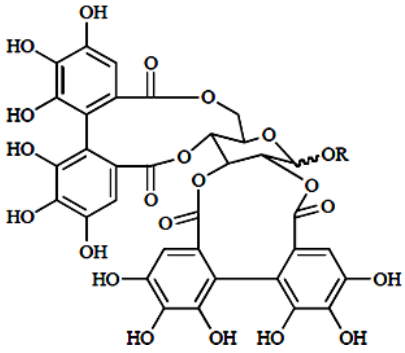


Asiatic acid, R = CH_2OH
Corosolic acid, R = CH_3

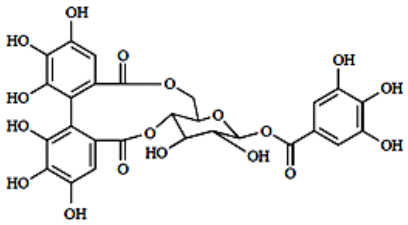
Chart 1.3 continued……



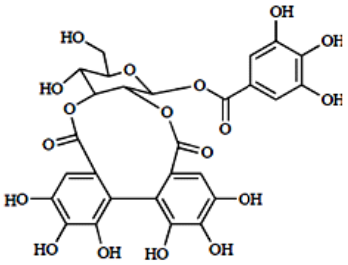
23-hydroxy ursolic acid



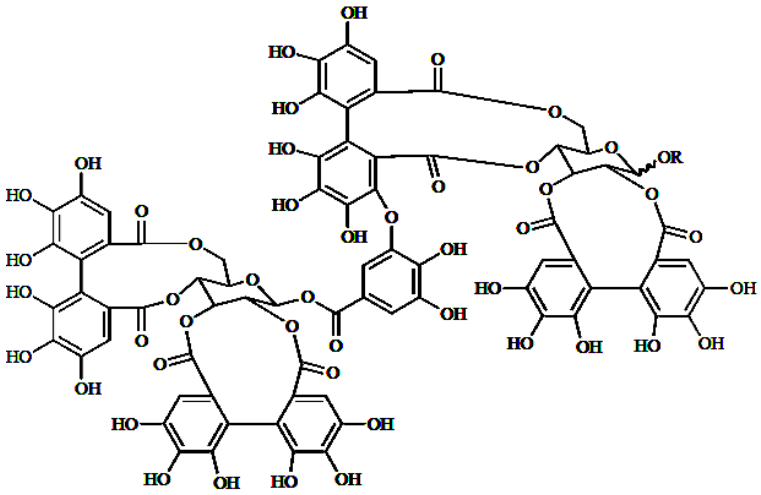
Pedunculagin



Strictinin



Sanguin H-5



Lambertianin R = β-galloyl
Sanguin H-6 R = α-galloyl

1.7. INFLAMMATION

Inflammation, a pattern representing response to injury involves the accumulation of cells and exudates in irritated tissues which allows protection from further damage (Gaofeng *et al.,* 2006). It involves a complex series of intra and extra cellular biological responses and the critical cell-cell interaction is accomplished by numerous cell communicator substances (cytokines) from injured tissue and subsequent responser cells. However, when uncontrolled, initiated by an abnormal stimulus or occurring for prolonged duration of time, inflammation may become a disease process. This appears to be the underlying basis of inflammation mediated diseases (Hurst and Barrette, 1989). The use of non-steroidal antiinflammatory drugs (NSAIDs) medication is still the mainstay of many clinicians for joint and spine related inflammation.

The inflammatory pathway (Figure 1.8) is a complex biochemical pathway which produces inflammatory mediators during injury. A major component involved in this pathway is arachidonic acid pathway because arachidonic acid is immediately released from traumatized cellular membranes and transformed into prostaglandins and thromboxanes partly through the enzymatic action of cyclooxygenase, COX. There are two types of COX enzymes COX-1 and COX-2 (Fitzgerald, 2004; Hostanska *et al.,* 2002). The NSAIDs can block the action of COX enzymes and thereby prevent the production of inflammatory prostaglandins and thromboxanes. But the non selective use of NSAIDs cause adverse side effects including gastrointestinal upset, gastritis, ulceration, haemorrhage etc. (Harris and Von Schacky, 2004). Because of the significant adverse effects shown by steroidal and NSAIDs medications, plant derived antiinflammatory agents are more popular with their relatively fewer side effects.

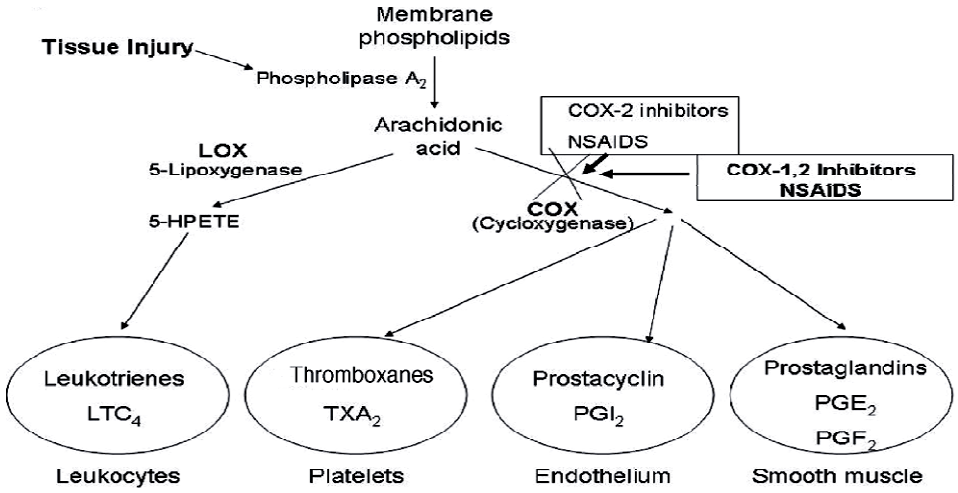


Figure 1.8: The arachidonic acid pathway activated to initiate the local inflammatory response

The enzymes involved are abbreviated as 5-HPETE - 5-hydroperoxyeicosatetraenoic acid; LTC4 - leukotriene C4; PGE2 = prostaglandin E2; PGF2 - prostaglandin F2; PGI2 - prostacyclin; TXA2 = thromboxane (Maroon *et al.,* 2010)

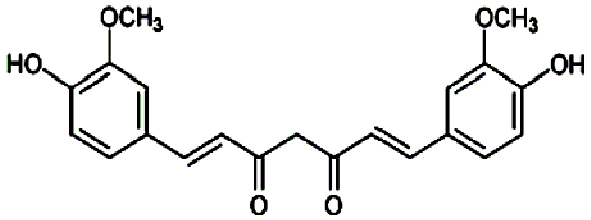
1.7.1. Plant derived antiinflammatory agents

Nutraceutical preparation from plants has been used for ages to obtain effective pain relief and herbal medications are of utmost importance now a days. Since ancient times, man used phytochemicals to curtail the inflammatory process. For example, ancient Egyptian, Greek and Indian civilizations use bark of willow tree as an analgesic, antipyretic and antiinflammatory agent (Setty and Sigal, 2005). Some of the commonly used antiinflammatory phytochemicals are,

1.7.1.1. Curcumin

Curcumin, a polyphenol derived from the rhizomes of *Curcuma longa* (turmeric) has been traditionally used as a coloring and flavoring agent. In Ayruvedic system of medicine, curcumin is used as an antiinflammatory agent, for the treatment of digestive disorders and to enhance wound healing (Srivastava and Srimal, 1985). Study by Zandi and Karin (1999) suggested the efficiency of curcumin in the treatment of cystic fibrosis

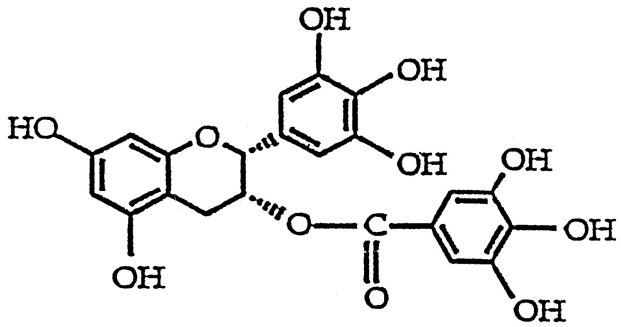
because of its antiinflammatory activity. Curcumin inhibit inflammation by suppressing nuclear factor kappa B (NF-kB) or by restricting various activators of NF-kB (Bengamark, 2006; Plummer *et al.,* 1999), arachidonic acid metabolism and by regulating the activity of several enzymes by inhibiting COX-1 and COX-2 (Bengamark, 2006). Antiinflammatory activity of curcumin in comparison with non steroidal medications such as phenyl butazone showed better activity with no side effects (Badria *et al.,* 2002).



Curcumin

1.7.1.2. Green tea

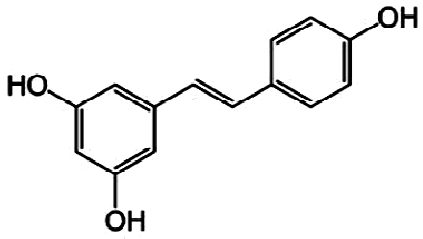
The main constituents in green tea include catechin, epigallocatechin-3-gallate etc. and more recently it is used in the treatment of arthritis as an antiinflammatory agent. Epigallocatechin-3-gallate suppresses IL-1 induced proteoglycan release and attenuates activation of NF-kB (Ghosh *et al.,* 1998). Increased consumption of green tea in Asia led to cardiovascular, neuroprotective and cancer prevention properties (Tijburg *et al.,* 1997).



Epigallocatechin-3-gallate

1.7.1.3. Resveratrol

Resveratrol is a plant based polyphenol found in the skins of red wine grapes and found to have significant antiinflammatory, antioxidant and DNA protective actions (Maroon, 2010). According to Elmali *et al.,* (2007), animals with intra-articular injection of resveratrol protect cartilage and reduces the inflammatory reaction in simulated knee osteoarthritis. Resveratrol is found to have specific inhibition on TNF-α and IL-1b–induced NF-kB activation and shows antiinflammatory activity by suppressing COX-2 enyzmes by blocking NF-kB activation.



Resveratrol

1.8. ANTIMICROBIAL ACTIVITY

Infectious diseases are considered as the second leading cause of death in developing countries and third in developed countries (Nathan, 2004). Recent years, the use of traditional medicinal plants for primary health has increased worldwide. Plants are very rich in secondary metabolites that have found antimicrobial properties and these compounds play a great role in controlling infectious diseases. Scientists are in search of new phytochemicals that could be developed as useful antimicrobial drugs for the treatment of infectious diseases. Plant derived antimicrobial molecules posses a relatively broad spectrum of activity and are used for various diseases caused by microorganisms (Craig, 1998).

1.8.1. Antimicrobial agents from plants

Currently, most of the antimicrobial agents are natural products or potent semi synthetic variations thereof (Chopra *et al.,* 2002). Based on various reports, there are around 90 antibacterial drugs that became commercially available worldwide. Out of these, 79% are natural product origin (Baker *et al*., 2007). The useful major groups of antimicrobial phytochemicals can be divided into several categories that include polyphenols, terpenes, alkaloids, tannins, flavonoids etc. Some of the phytochemicals with antibacterial activity are described below and the structures are given in chart 1.4.

1.8.1.1. Alkaloids

Heterocyclic compounds containing nitrogen are called alkaloids and its antimicrobial activity has been reported from years back. Alkaloid extract from *Sida acuta* showed antibacterial activity with cryptolepine as major component (Karou *et al.*, 2006). Berberine, a planar quarternary alkaloid is potentially effective against trypanosoma and plasmodia. The mechanism of action is attributed to their ability to intercalate with DNA (Philippson and O'Neill, 1987).

1.8.1.2. Phenolic compounds

Phenolic compounds are widely distributed in the plant kingdom and many of them possess antimicrobial activity. The presence of hydroxyl groups related to the relative toxicity to microorganism showed that increased hydroxylation results in increased toxicity. For example, catechol has two -OH groups and pyrogallol has three -OH groups, shown to be toxic to microorganism, with evidence that increased hydroxylation results in increased toxicity (Geissman, 1963). Essential oil, possessing a C_3 side chain at a lower level of oxidation is often cited as antimicrobial as well. For

example, eugenol is considered bacteriostatic against both fungi (Duke, 1985) and bacteria (Thomson, 1978).

1.8.1.3. Flavonoids

Flavonoids and hydroxylated phenolic compounds are synthesized by plants in response to microbial infections and are considered as effective antimicrobial substances against a wide variety of micro organisms. Isobonducellin from *Caesalpinia pulcherrima* containing a cis (Z)-double bond possessing antimicrobial activity and quercetin derivatives showed antiviral properties. A large class of flavonoids, sesquiterpenoid alcohols, triterpenoids and quinic acid caffeates product from plants may also be useful as antimicrobials (Hu and Chen, 1997) and the activity may be attributed to the formation of a complex with extra cellular and soluble proteins, which then binds to bacterial cell wall (Tsuchiya *et al.,* 1996). Flavonoids lacking hydroxyl groups on their β-rings are more active against microorganisms and the microbial target is the membrane with –OH groups. The major flavonoids showing antimicrobial activity include alpinumisoflavone, galangin (Cowan, 1999), licochalcone A, apigenin, luteolin etc.

1.8.1.4. Terpenes

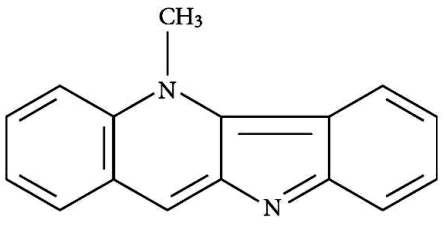
Most of the terpenes are able to alter membrane fluidity and efflux of ions. The antimicrobial properties of aromatic volatile oils from medicinal plants have been recognized since antiquity and the presence of phenolic components like thymol and carvacrol are responsible for the broad spectrum of antimicrobial activities (Katerere *et al.,* 2003). Terpenes are active against bacteria. Imberbic acid, a pentacyclic triterpenoid acid showed potent activity against *Mycobacterium fortuitum* and *Staphylococcus aureus*. Cycloartane type triterpenes showed antimicrobial activity against vancomycin-resistant

enterococci (Gutierrez-Lugo *et al.,* 2002). The mechanism of action of terpenes is speculated to involve membrane disruption by the lipophilic compounds.

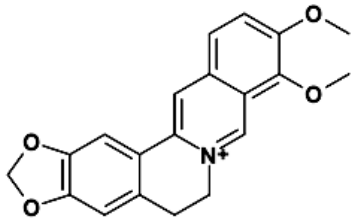
1.8.1.5. Coumarins

Coumarins are well known for its antimicrobial activity from very long back. It has to be noted that coumarins are the only class of antibacterial compounds for which a specific target (DNA gyrase) can be suggested and is based on the studies on action of novobiocin (Lewis and Ausubel, 2006). Novobiocin, a clinically using antibacterial drug, is a microbial derived coumarin. Warfarin, a well known coumarin have antiviral effect (Berkada, 1978). Coumarin was found to inhibit *Candida albicans in vitro* and has been found to stimulate macrophages (Casley Smith *et al.,* 1997), showed indirect negative effect on infections. Phytoalexins produced in carrots show response to fungal infection and can be presumed to have antifungal activity (Hoult and Paya, 1996).

Chart 1.4: Antimicrobial agents from plants

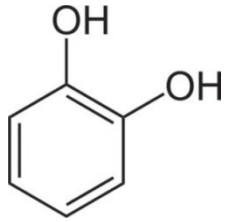


Cryptolepine

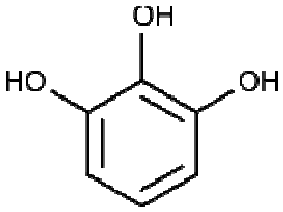


Berberine

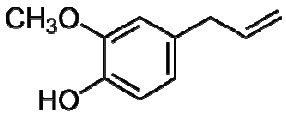
Chart 1.4 continued…….



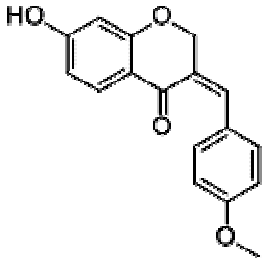
Catechol



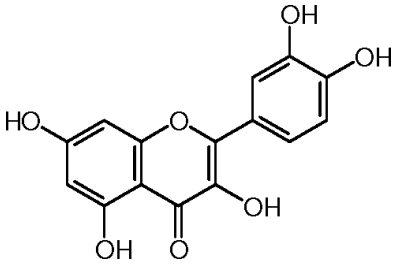
Pyrogallol



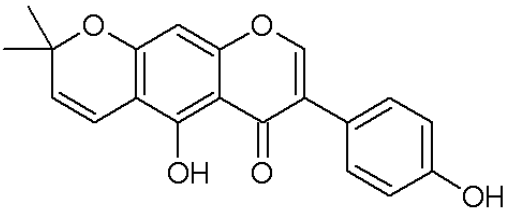
Eugenol



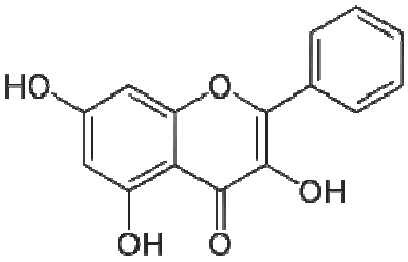
Isobonducellin



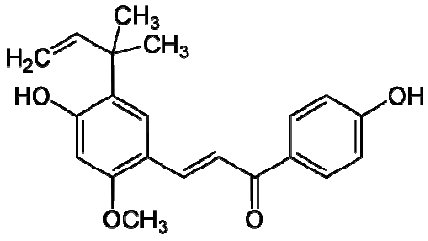
Quercetin



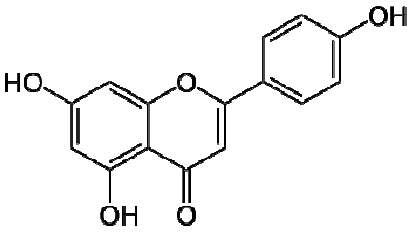
Alpinumisoflavone



Galangin

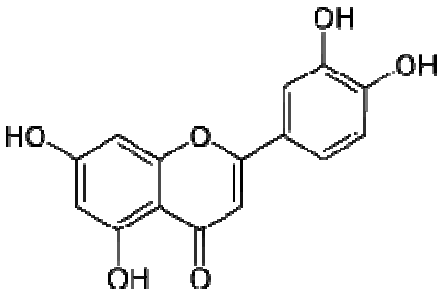


Licochalcone A

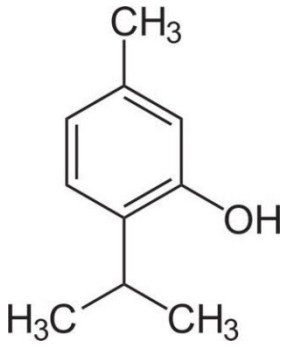


Apigenin

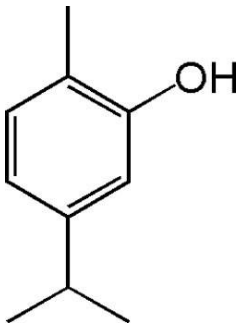
Chart 1.4 continued…….



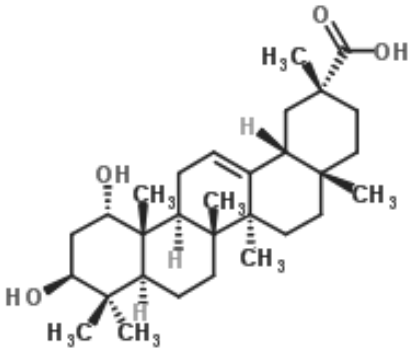
Luteolin



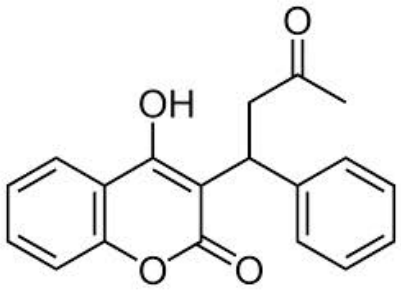
Thymol



Carvacrol



Imberbic acid



Warfarin

1.9. RELEVANCE AND OBJECTIVES OF THE PRESENT STUDY

Medicinal plants constitute a highly endowed and least explored treasure of bioactive compounds. The role of phytochemicals in present day life is getting more significant and is of vital importance in accordance with changing lifestyles. This demands detailed phytochemical and pharmacological investigations on plants along with standardization and evidence based validation of herbal products. In India, Ayurvedic therapeutic formulations are extensively used for the treatment of chronic diseases such as rheumatism, atherosclerosis etc. The present study is meant to screen three pharmacologically important indigenous medicinal plants such as *Cyperus rotundus* (Family: Cyperaceae), *Stereospermum colais* (Family: Bignoniaceae) and *Zingiber officinale* (Family: Zingiberaceae) for bioactive molecules and for antioxidant, antidiabetic, antiinflammatory, antimicrobial potential etc. The work also deals with the chemical profiling and structural characterization of active compounds using chromatographic and spectroscopic methods. Chemical and cell culture based assays of isolated compounds is supposed to accelerate the chance of developing a lead molecule for further trials. It is expected that the result of present work will increase the relevance of above mentioned medicinal plants in a pharmacological perspective.

There are difficulties to give a detailed picture of natural products in a very concise manner. But an attempt has been made to bring out the potentialities of biologically active natural products with special reference to antioxidant, antimicrobial, antidiabetic and antiinflammatory compounds from terrestrial plants in Chapter 1. A detailed discussion on *Cyperus rotundus* of Cyperaceae family with special emphasis on the phytochemicals present in them covers Chapter 2. The sesquiterpenoids isolated from

the rhizomes of *Cyperus rotundus* were identified using spectral methods and quantified using chromatographic methods such as HPLC, HPTLC etc. Comparative antioxidant potential of these sesquiterpenoids was also measured. Chapter 3 deals with the phytochemical investigation and biological activity studies on the roots of *Stereospermum colais*. In chapter 4, biological potential of the rhizomes of *Zingiber officinale* of Zingiberaceae family is discussed. Finally, summary and conclusion are also enclosed.

REFERENCES

1. Ames, B. N.; Shigrenaga, M. K.; Hagen, T. M. *Proc. Natl. Acad. Sci. U.S.A* **1993**, *90,* 7915-7922.

2. Anand, C.; Neetu, S. J. *Ayur. Int. Med*. **2011**, *2*, 179-186.

3. Arryn, C.; Salman, A.; Justin, N. *J. Antibiot.* **2013**, *66*, 387-400.

4. Aruoma, O. I. *Mut. Res*. **2003**, *523*, 9-20.

5. Atta-ur-Rahman (Ed.) Studies in Natural Product Chemistry, **2005**, *30*, 714.

6. Badria, F. A.; El-Farahaty, T.; Shabana, A. A.; Hawas, S. A.; El-Batoty, M. F. *Alt. Complement. Ther.* **2002,** *8,* 341-348.

7. Baker, D. D.; Chu, M.; Oza, U.; Rajgarhia, V. *Nat. Prod. Report* **2007**, *24*, 1225-1244.

8. Beck, K. F.; Eberhardt, W.; Frank, S.; Huwiler, A.; Mebmer, U. K.; Muhl, H.; Pfeilshifter, J. *J. Exp. Biol*. **1999**, *202,* 645-653.

9. Beck, M. A. *J. Am. Coll. Nutr***. 2001***,* *20*, 384S-388S.

10. Bengmark, S. J. *Parenter Enteral Nutr.* **2006,** *30,* 45-51.

11. Berkada, B. *J. Irish Coll. Phys. Surg*. **1978**, *22*(Suppl.), 56.

12. Bors, W.; Heller, W.; Michael, C.; Saran, M. *Methods Enzymo*. **1990**, *186*, 343-355.

13. Bouayed, J. *Curr. Nutr. Food Sci*. **2010,** *6*, 13-18.

14. Burda, S.; Oleszek, W. *J. Agric. Food Chem.* **2001**, *49*, 2274-2779.

15. Burton, G. W.; Ingold, K. U. *Science* **1984,** *224*, 569-573.

16. Calucci, L.; Pinzono, C.; Zandomeneghi, M.; Capocchi, A. *J. Agric. Food Chem.* **2003**, *51*, 927-934.

17. Casley-Smith, J. R.; Casley-Smith, J. R. O'Kennedy R., Thornes, R. D. (ed.), John Wiley & Sons, Inc., New York, N.Y. **1997**, *9*, 348.

18. Cecile, A. E.; Philippe, L.; Luc de, L. D.; Ludivine, L. *Biotechnol. Agron. Soc. Environ*. **2012**, *16*, 393-404.

19. Cerutti, P. A. *Eur. J. Clin. Invest.* **1991**, *21,* 1-5.

20. Chaudiere, J.; Ferrari-Iliou, R. *Food Chem. Toxicol.* **1999**, *37*, 949-962.

21. Chen, X.; Zheng, Y.; Shen, Y. *Curr. Med. Chem.*, **2006**, *13*, 109-116.

22. Cheng, I. F.; Breen. K. *Biometals*, **2000**,*13*, 77-83.

23. Chiasson, J. L. *Endocr. Pract.* **2006**, *1*, 25-30.

24. Cholbi M. R.; Paya, M.; Alcaraz, M. J. *Experientia* **1990**, *47*, 195-199.

25. Chopra, I.; Hesse, L.; O Neil, A. J. *J. Appl. Microbiol*. **2002**, *92*, 4S-15S.

26. Claudio, C.; Hector, F. *Food Res. Int*. **2000**, *33*, 357-365.

27. Colerige Smith, P, O.; Thomas, P.; Scurr, J. H.; Dormandy, J. A. *Br. Med. J*. **1980**, *296*, 1726-1727.

28. Cordell, G. A. Introduction to alkaloids: A Biogenetic Approach. Wiley & Sons, New York, **1981**, 1055.

29. Cowan, M. M. *Clin. Microbiol. Rev.* **1999**, *12*, 564-582.

30. Craig, W. A. *Clin. Infect. Dis.* **1998***, 26*, 1-10.

31. Dalle-Donne, I.; Giustarini, D.; Colombo, R.; Rossi, R.; Milzani, A. *Trends Mol. Med.* **2003**, *9*, 169–176.

32. Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications: Report of a WHO Consultation Part 1: Diagnosis and Classification of Diabetes Mellitus, World Health Organization, Department of Noncommunicable Disease Surveillance, Geneva. **1999**, 1-49.

33. de Groot, H. *Hepatogastroenterology*, **1994**, 41, 328-332.

34. Devasagayam, T. P. A.; Tilak, J. C.; Boloor, K. K.; Sane, K. S.; Saroj, S. G.; Lele, R. D. *J. Assoc. Phys. India* **2004**, *52*, 794-804.

35. Dewick, P. M. Medicinal Natural Products: A Biosynthetic Approach, 3rd Edition. UK: John Wiley & Sons, Ltd. **2009**, 236-237.

36. Donaldson, M. S. *Nutr. J.* **2004**, *3*, 19-25.

37. Duke, J. A. Handbook of medicinal herbs. CRC Press, Inc., BocaRaton, Fl., **1985**, *677*.

38. Dumont, E.; Vanhaecke, F.; Cornels, R. *Anal. Bioanal. Chem.* **2006**, *385*, 1304-1323.

39. Eddouks, M.; Maghrani, M.; Lemhadri, A.; Ouahidi, M.L.; Jouad, H. *J. Ethnopharmacol*., **2002**, *82*, 97-103.

40. Elmali, N.; Baysal, O.; Harma, A.; Esenkaya, I.; Mizrak, B. *Inflammation* **2007**, *30*, 1-6.

41. Fitzgerald, G. A. *N. Engl. J. Med*. **2004**, *351*, 1709-1711.

42. Foote, C. S.; Chang, Y. C.; Denny, R. W. *J. Am. Chem. Soc*. **1970,** *92*, 5216-5218.

43. Foote, C. S.; Denny, R. W.; Weaver, L.; Chang, Y.; Peters, *Ann. N. Y. Acad. Sci*. **1971,** *171*, 139-145.

44. Fraga, B. M. in Terpenoids; Charlwood, B. V., Banthorpe, D. V., Eds., Methods in Plant Biochemistry, Academic Press, **1991**, *7*, 145-185.

45. Fridovich, I. *Ann N. Y. Acad. Sci.* **1999,** *893*, 13-18.

46. Gaofeng, Y.; Mark, L.; Wahlqvist; Guoqing, H.; Min, Y.; Duo, L. *Asia Pac. J. Clin. Nutr*. **2006**, *15*, 143-152.

47. Geissman, T. A. in M. Florkin and E. H. Stotz (ed.), Elsevier, New York, N.Y. **1963**, *9*, 265.

48. Ghosh, S.; May, M. J.; Kopp, E. B. *Annu. Rev. Immunol*. **1998**, *16*, 225-260.

49. Graf, B. A.; Milbury, P. E.; Blumberg, J. B. *J. Med. Food* **2005**, *8*, 281-290.

50. Guntensperger, B.; Hammerli-Meier, D. E.; Escher, F. E. *J. Food Sci*. **1998**, *63*, 955-957.

51. Gupta, S.; Agarwal, A.; Krajcir, N.; Alvarez, J. G. *Reprod. Biomed. Online* **2006**, *13*, 126-134.

52. Gutierrez-Lugo, M. T.; Singh, M. P.; Maiese, W. M.; Timmermann, B. N. *J. Nat. Prod.* **2002**, *65*, 872-875.

53. Haenen, G. R.; Paquay, J. B.; Korthouwer, R. E.; Bast, A. Biochem. *Biophys. Res. Commun.* **1997**, *236*, 591-593.

54. Halliwell, B. *The Lancet*, **1994**, *344*, 721-724.

55. Halliwell, B. *Biochem. Soc. Trans*. **2007**, *35*, 1147-1150.

56. Halliwell, B. Encyclopedia of Life Sciences Nature Publishing Group, **2001**, 1-7.

57. Halliwell, B.; Gutteridge, J. M. C. Free Radicals in Biology and Medicine, Clarendon Press, Oxford, Ed.2, **1989**, 96-98.

58. Halliwell, B.; Gutteridge, J. M. C. (eds), Free Radicals in Biology and Medicine, Oxford University Press, Oxford, **1997**.

59. Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*. 4th edition, Clarendron Press, Oxford, UK, **2007.**

60. Hamburger, M.; Hostetmann, K. *Phytochemistry* **1991**, *30*, 3864-3884.

61. Harborne, J. B.; Simmonds, N. W. Biochemistry of Phenolic Compounds, Academic Press, London, **1964**, *101*.

62. Harris, W. S.; Von Schacky, C. *Prev. Med*. **2004**, *39*, 212-220.

63. Helmut, S.; Wilhelm, S. *Am. J. Clin. Nutr.* **l995**, *62* (suppl), l3l5S-132lS.

64. Hensley, K.; Mou, S.; Pye, Q. N.; Dixon, R. A.; Summner, L. W.; Floyd, R. A. *Curr. Topics in Nutraceutical Res*. **2004**, *2,* 13-26.

65. Hill, R. A. in The Chemistry of Natural Products; Thomson, R. H., Ed. Blackie Academic & Professional, **1993**, 106-139.

66. Hostanska, K.; Daum, G.; Saller, R. *Anticancer Res*. **2002**, *22*, 2853-2862.

67. Hou, W.; Li, Y.; Zhang, Q.; Wei, X.; Peng, A.; Chen, L.; Wei, Y. *Phytother. Res.*, **2009**, *23*, 614-618.

68. Hoult, J. R. S.; Paya. M. *Gen. Pharmacol*. **1996**, *27*, 713-722.

69. Hu, J.P.; Calomme, M.; Lasure, A.; DeBruyne, T.; Pieters, A.; Vlietinick, A.; Vanden Berghe, D.A. *Biol. Trace Elem. Res*. **1995**, *47*, 327-331.

70. Hu, L.; Chen, Z. *Phytochemistry*, **1997**, *44*, 1287-1290.

71. Hudson, B. J. F.; Lewis, J. I. *Food Chem.* **1983,** *10*, 47-55.

72. Hurst, J. K.; Barrette, W. C. J. *Crit. Rev. Biochem. Mol. Biol.* **1989**, *24*, 271-328.

73. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Overall Evaluation of Carcinogenicity: An Updating of IARC Monographs. International Agency for Research on Cancer Lyon, WHO, Lyon, **1987**, *59*.

74. Jarvis, B. B.; Pavanasasivam, G.; Bean, G. A. in Trichothecenes and other mycotoxins Lacey, J., Ed., John Wiley & Sons Ltd., **1985**, 221-231.

75. Julia, P.; Johanna, D.; Herman, A.; Augustin, S.; Paul, J.; Marjorie, L.; McCullough. *Nutr. Rev*. **2010**, *68*, 571-603.

76. Karou, D.; Savadogo, A.; Canini, A.; Yameogo, S.; Montesano, C.; Simpore, J.; Colizzi, V.; Traore, A. S. *Afri. J. Biotech.* **2006**, *5*, 195-200.

77. Kayali, R.; Cakatay, U.; Tekeli, F. *Mech. Ageing Dev*. **2007**, *128*, 365-369.

78. Katerere, D. R.; Gray, A. I.; Nash, R. J.; Waigh, R. D. *Phytochemistry* **2003**, *63*, 81-88.

79. Kerry, N.; Rice-Evans, C. *J. Neurochem.* **1999**, *73*, 247-253.

80. Kintzios, S.; Barberaki, M. CRC Press, Boca Raton, FL. **2004**.

81. Klayman, D. L. *Science* **1985**, *228*, 1049-1055.

82. Koshland, D. E. *Science* **1992**, *258*, 1861-1861.

83. Kovacic, P.; Jacintho, J. D. *Curr. Med. Chem.*, **2001**, *8*, 773-796.

84. Koyama, T.; Ogura, K. In: Cane, D. E. (ed.) Pergamon Press, Oxford. **1999**, *2*, 69-96.

85. Kupchan, S. M.; Hemingway, R. J.; Werner, D.; Karim, A.; Mc Phail A. T.; Sim, G. A. *J. Am. Chem. Soc*. **1968**, *90*, 3596-3597.

86. Lake-Bakaar, G.; Sorbi, D.; Mazzoccoli, V. *Dig. Dis. Sci*. **2001**, *46,* 1072-1076.

87. Lewis, K.; Ausubel, F. M. *Nature Biotech.* **2006**, *24*, 1504-1507.

88. Li, Y.; Schellhorn, H. E. *J. Nutr.* **2007**, *137*, 2171-2184.

89. Li, H.; Tanaka, T.; Zhang, Y. J.; Yang, C. R.; Kouno, I. *Chem. Pharm. Bull*., **2007**, *55*, 1325-1331.

90. Liochev, S. I.; Fridovich, I. *Redox Rep.* **2002**, *7*, 55-57.

91. Lobinski, R.; Edmonds, J.; Suzuki, K.; Uden, P. *Pure Appl. Chem.* **2000**, *72*, 447-461.

92. Lugasi, A.; Dworschak, E.; Hovari, J. Federation of the European Chemists' Society. Proceedings of the European Food Chemists. VIII, Vienna, Austria, September. **1995**, *3,* 639–643.

93. Mahmoud, S. S.; Croteau, R. B. *Trends Plant Sci*. **2002**, ***7,*** 366-373.

94. Maroon, J. C.; Bost, J. W.; Maroon, A. *Surg. Neurol. Int*. **2010**, *1*, 80.

95. Martens, S.; Mithofer, A. *Phytochemistry* **2005**, *66*, 2399-2407.

96. Martínez, M. C.; Andriantsitohaina, R. *Antioxid Redox Signal.* **2009**, *11,* 669-702.

97. Mates, J. M.; Perez G. C.; de Castro, N. *Clin. Biochem.* **1999**, *32*, 595-603.

98. McGarvey, D.J.; Croteau, R. Terpenoid metabolism; *Plant Cell* **1995**, *7*, 1015-1026.

99. Midori, H.; Toshikazu, Y.; Masayasu, I. Food and free radicals Proceedings of the first symposium on food and free radicals, Springer, **1997**.

100. Milan, S. J. *Food Comp. Anal*. **2006**, *19*, 531-537.

101. Miller, D. M.; Buettner, G. R.; Aust, S. D. *Free Rad. Biol. Med.* **1990**, *8*, 95-108.

102. Mishra, B. B.; Tiwari, V. K. *Eur. J. Med. Chem.* **2011**, *46*, 4769-4807.

103. Morena, M.; Cristol, J. P.; Canaud, B. *Blood Purif*. **2000**, *18*, 191-199.

104. Moskovitz, J.; Yim, K. A.; Choke, P. B. *Arch. Biochem. Biophys.* **2002**, *397*, 354-359.

105. Muller, L.; Junge, B.; Frommer, W.; Schmidt, D.; Truscheit, E. in *Enzyme Inhibitors*; Brodbeck, U., Ed.; Verlag Chemie: Weinheim, BRD **1980**, 109-122.

106. Naidu, A. K. *Nutr. J.* **2003**, *2*, 1-10.

107. Nathan, C. *Nature **2004**, 431*, 899-902.

108. Nguyen, L. A.; He, H.; Pham-Huy, C. *Int. J. Biomed. Sci.* **2006**, *2*, 85-100.

109. Pacher, P.; Beckman, J. S.; Liaudet, L. *Physiol. Rev*. **2007**, *87*, 315- 424

110. Pezzoto, J. M. *Biochem. Pharmacol*. **1997**, *2*, 121-133.

111. Phillipson, J. D.; O'Neill, M. *J. Acta Pharm. Nord*. **1987**, *1*, 131-144.

112. Pietta, P. G. *J. Nat. Prod*. **2000**, *63*, 1035-1042.

113. Plummer, S. M.; Holloway, K. A.; Manson, M. M.; Monks, R. J. L.; Kaptein, A.; Farrow, S.; Howells, L. *Oncogene* **1999,** *18,* 6013-6020.

114. Pushparaj, P.; Tan, C. H.; Tan, B. K. H. *J. Ethnopharmaco*l. **2000**, *72*, 69-76.

115. Rice evans, C. A.; Miller, N. J.; Paganga, G. *Free Radic. Biol. Med*. **1996**, *20*, 933- 956.

116. Ridnour, L. A.; Isenberg, J. S.; Espey, M. G.; Thomas, D. D.; Roberts, D. D.; Wink, D. A. *Proc. Natl. Acad. Sci. U.S.A.*, **2005**, *102*, 13147-13152.

117. Ronald R. E.; Junsoo L.; Vitamin E, food chemistry, composition and analysis, Taylor and Francis Publication, **2004**.

118. Santi, M. M.; Dipjyoti, C.; Satyahari, D. *Plant Signal Behav.* **2010**, *5*, 359-368.

119. Sarkanen, K.V.; Ludwig, C.H. Lignins: occurance, formation, structure and reactions. New York: Wiley-Interscience. **1971**.

120. Sekher Pannala, T. S.; Chan , P. J.; Brien, O.; Rice-Evans, C. A. *Biochem. Biophys. Res. Commun*. **2001**, *282*, 1161-1168.

121. Setty, A. R.; Sigal, L. H. *Semin. Arthritis Rheum.* **2005**, *34,*773-784.

122. Sies, H. (ed.) Antioxidants in disease mechanisms and therapy, Academic Press, New York, **1996**.

123. Silva, B. A.; Ferreres, F.; Malva, J. O.; Dias, A. C. P. *Food Chem*. **2005**, *90*, 157-167.

124. Srivastava, R.; Srimal, R. C. *Ind. J. Med. Res.* **1985**, *81*, 215-223.

125. Standards of medical care in diabetes, American Diabetes Association, **2013**, 1.

126. Tadera, K.; Minami, Y.; Takamatsu, K.; Matsuoka, T. *J. Nutr. Sci. Vitaminol*. **2006**, *52*, 149-153.

127. Tadeusz, A. Alkaloids-Secrets of Life: Alkaloid Chemistry, Biological Significance, Applications and Ecological role. Elsevier, **2007**, 1 ed, 1-315.

128. Tapiero, H.; Townsend, D. M. *Biomed. Pharmacother*. **2003**, *57*, 134-144.

129. Telci, A.; Cakatay, U.; Salman, S.; Satman, I.; Sivas, A. *Diabetes Res. Clin. Pract*. **2000**, *50*, 213-223.

130. Thomson, W. A. R. (ed.). McGraw-Hill Book Co., Maidenhead, United Kingdom, **1978**, *49*.

131. Tian, Y. Y.; Jiang, B.; An, L. J.; Bao, Y. M. *Eur. J. Pharmacol*. **2007**, *568*, 142-148.

132. Tijburg, L. B.; Mattern, T.; Foltz, J. D.; Weisgerber, U. M.; Katan, M. B. *Crit. Rev. Food Sci. Nutr*. **1997**, *37*, 771-785.

133. Tinggi, U. *J. AOAC Int*. **1999**, *82*, 364-367.

134. Traber, M. G.; Stevens, J. F. *Free Radic. Biol. Med*. **2011**, *51,* 1000-1013.

135. Truscheit, E.; Frommer, W.; Junge, B.; Muller, L.; Schmidt, D.; Wingender, W. *Angew. Chem. Int. Ed. Engl.*, **1981**, *20*, 744-761.

136. Tsuchiya, H.; Sato, M.; Miyazaki, T.; Fujiwara, S.; Tanigaki, S.; Ohyama, M.; Tanaka, T.; Iinuma, M. 1996. *J. Ethnopharmacol*., **1996**, *50*, 27–34.

137. Tsuda, T.; Ohshima, K.; Kawakishi, S.; Osawa, T. *J. Agric. Food Chem*. **1994**, *42*, 248-251.

138. Tundis, R.; Loizzo, M. R.; Menichini, F. *Mini Rev. Med. Chem.* **2010**, *10*, 315-331.

139. Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T.; Mazur, M.; Telser, J. *Int. J. Biochem. Cell Biol.* **2007,** *39,* 44-84.

140. Valko, M.; Morris, H.; Rapta, P.; Mazur, M.; Bilton, R. F. *Biochim. Biophys. Acta*, **2001**, *1527*, 161-166.

141. Verhagen, H.; Schilderman, P. A. E. L.; Kleinjans, J.C.S. *Chem. Biol. Interact.* **1991**, *80*, 109-134.

142. Verspohl, E. J. *Pharmacol. Rev*. **2012**, *64*, 188-237.

143. Webster's Third New International Dictionary, Antioxidant. Unabridged; Merraiam-Webster: 2002, http://unabridged.merriamwebster.com, **2004**, 9.

144. Wells, P. G.; McCallum, G. P.; Lam, K. C.; Henderson, J. T.; Ondovcik, S. L. *Birth Defects Res. C Embryo Today* **2010**, 90, 103-109.

145. Yoshikawa, T.; Toyokuni, S.; Yamamoto, Y.; Naito, Y. (eds) Free Radicals in Chemistry Biology and Medicine, OICA International, London, **2000**, 580.

146. Zandi, E.; Karin, M. *Mol. Cell. Biol*. **1999**, *19*, 4547-4551.

Chapter 2

PHYTOCHEMICAL INVESTIGATION, HPTLC, RP-HPLC ANALYSIS AND ANTIOXIDANT SCREENING OF RHIZOMES OF *Cyperus rotundus*

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 Cyperaceae family occupy an important place in Ayurvedic system of medicine. Many Cyperaceae plants have been investigated for phytochemical constituents and evaluated for their biological potentials. *Cyperus* is an important genus of Cyperaceae family and many plants under this genus are used in the traditional system of medicine. Our findings of the phytochemical constituents of *Cyperus rotundus*, its HPLC and HPTLC analysis, screening of antioxidant potential etc. constitute the subject matter of Chapter 2. Literature reviews on Cyperaceae plants in general and *Cyperus rotundus* specifically was carried out to understand the current status and various species in it which is summarized in the following introduction.

2.1. INTRODUCTION

The family Cyperaceae is the third largest family of monocotyledonous graminoid flowering plants commonly known as sedges and comprising about 90 genera and 5,500 species. The species are widely distributed in tropical Asia and South America. The plant stems are usually 3-angled and solid; the leaves are alternate commonly in 3 ranks usually with a closed sheating base and a parallel-veined, strap-shaped blade (Taylor,

1983). The flowers are very minute and are bisexual or unisexual and the species vary greatly in size. Literature survey on the chemical composition and biological activities of the various *Cyperus* species reported so far is discussed here briefly and the structures of different isolated phytochemical constituents are given in chart 2.1.

Cyperaceae plants were investigated for flavonoids and most of the plants contain glycoflavones, tricin, luteolin, quercetin etc. as constituents. Previous reports indicates the presence of apigenin, luteolin, tricin etc. and aurone type compounds such as aureusidin (4, 6, 3′, 4′-tetrahydroxyaurone), sulphuretin in Egyptain *Cyperus* species (El Habashy *et al*., 1989). *Cyperus longus* is an evergreen perennial plant growing to 1.2 m and having irregular shaped root. Essential oil composition was studied (Abdenour *et al*., 2012) and led to the identification of β-himachalene, α-humulene and γ-himachalene as major components. The oil showed quantitative antibacterial activity against gram (+) ve and gram (-) ve bacteria. Leaves contain tricin and luteolin-7-arabinosyl glucoside as major constituents (Harnborne, 1971).

Cyperus alopecuroides is a perennial stout leafy herb, found to be native to the ancient Egyptian swamps and widely distributed in tropical areas. Chromatographic separation of methanolic extract of *C. alopecuroides* inflorescence led to the identification of 12 phenolic components such as scopoletin, isoliquiritigenin-4′-methyl ether, luteolin-5, 3′-dimethyl ether, luteolin-7, 3′-dimethyl ether, aureusidin-4-methyl ether, apigenin, luteolin, transferulic acid, luteolin-4′-O-β-D-glucopyranoside, luteolin-7-O-β-D-glucopyranoside, quercetin-3-O-β-D-glucopyranoside, apigenin-7-O-neohesperidoside, kaempferol-3-O-rutinoside, quercetin-3-O-rutinoside etc. (Sayed *et al*., 2006).

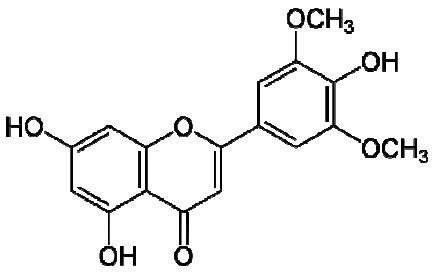
Cyperus articulates is a traditional marshland plant grown in Africa and Latin America used in the treatment of wide variety of human diseases such as headache, epilepsy, migraine etc. (Schultes and Raffauf, 1990). Water extract of *C. articulatus* concentration dependently reduced epileptiform discharges and N-methyl-D-aspartate receptor (NMDA) induced depolarisations in the rat cortical wedge preparation and further studies supports the anticonvulsant properties of methanol extract *in vivo* (Ngo *et al*., 2001). GC-MS analysis of the essential oil of *C. articulatus* showed the presence of mustakone and caryophyllene oxide as major components (Zoghbi *et al*., 2008).

Cyperus glomeratus is an annual rarely perennial plant native to Europe and Asia (Webb *et al*., 1980). Hydrodistillation and GC-MS analysis of fresh underground parts yielded around 107 constituents such as caryophyllene oxide, humulene epoxide, β-caryophyllene, α-humulene etc. as major components. Chemotaxonomically important compound cyperene was also identified from the oil in minor amounts (Lazarevic *et al*., 2010).

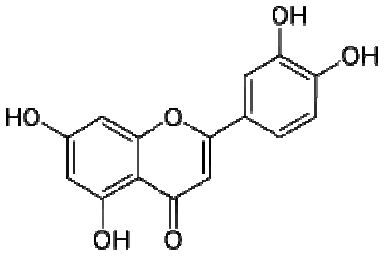
Cyperus scariosus is popularly known as nagarmotha in unani medicine and widely distributed in India. Being used in aromatherapy and in perfume industry, the roots are highly ranked in India. Traditionally, the plant rhizomes are used to cure diarrhoea, epilepsy, gonorrhea, syphilis and liver damage. In unani system of medicine, it is used as diuretic and emmenagogue (Kabiruddin and Makhzan, 2010). Hydrodistilled isolation of essential oil yielded cyperenol, cyperenone, cyperotondone, isopatchoul-4(5)-en-3-one, isopatchoulenone, isopatchoula-3,5-diene, patchoulenol, rotundene, isopatchoul-3-ene, rotundenol, scariodone, β-selinene, cyperine, isopatchoulene as constituents. Leptosidin-6-0-β-L-rhamnopyranoside, stigmasta-5, 24(28)-diene-3-β-O-α-

L–rhamnopyranosyl–O-β-D-arabinopyranoside etc. were isolated from leaves (Chatterjee and Pakrashi, 2009; Anonymous, Wealth of India, 2001). A number of compounds including sesquiterpenes (Fengming *et al*., 2004), quinones (Abdel-Mogib *et al*., 2000; Abdel-Razik *et al*., 2005), stilbenoids (Morikawa *et al*., 2002), coumarins (Antonio *et al*., 1993), phenyl propanoids (Abdel-Mogib *et al*., 2000) etc. have been isolated from different *Cyperus* species.

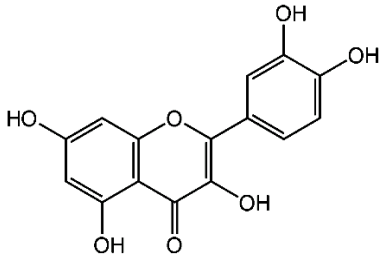
Chart 2.1: Phytochemical constituents isolated from genus *Cyperus*



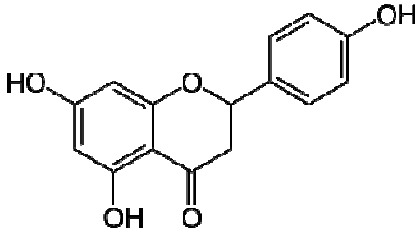
Tricin



Luteolin

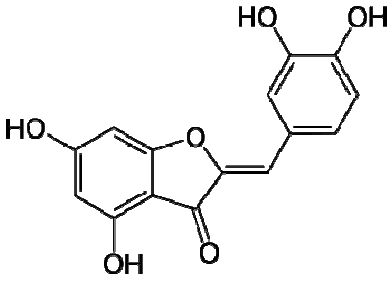


Quercetin

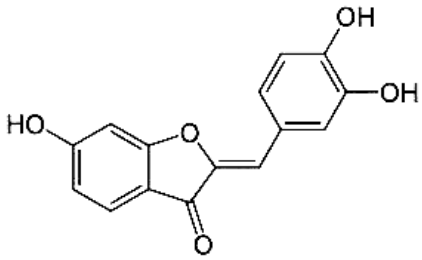


Apigenin

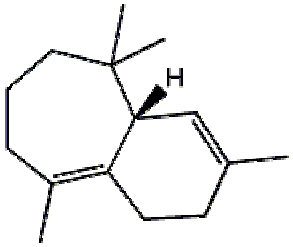
Chart 2.1 continued……..



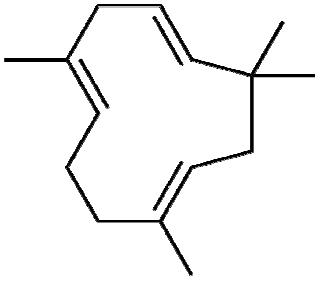
Aureusidin



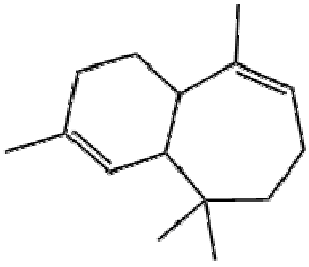
Sulphuretin



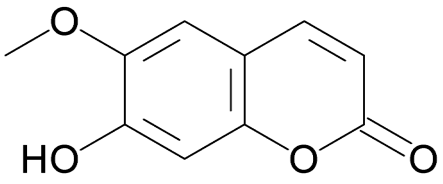
β-himachalene



α-humulene

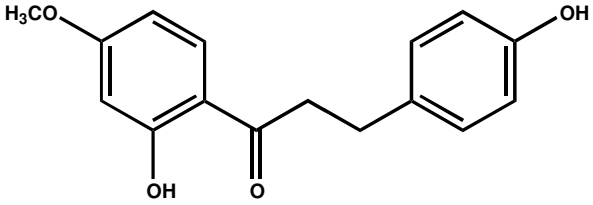


ν-himachalene

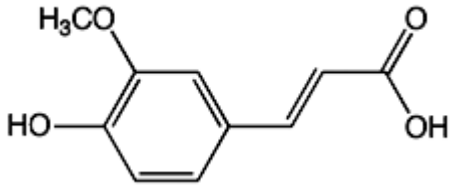


Scopoletin

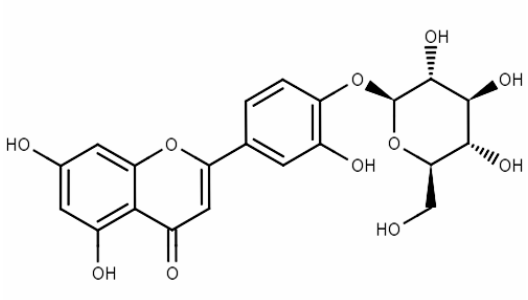
Chart 2.1 continued……..



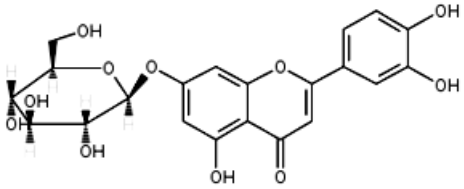
Isoliquiritegenin-4′-methyl ether



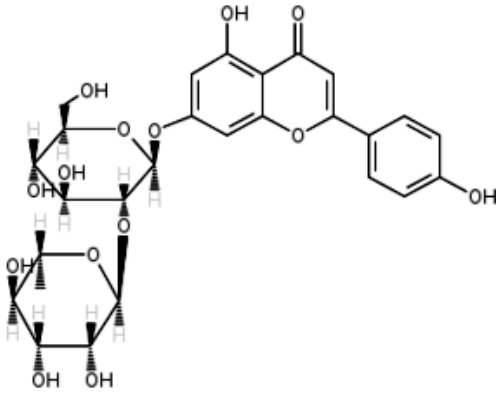
Ferulic acid



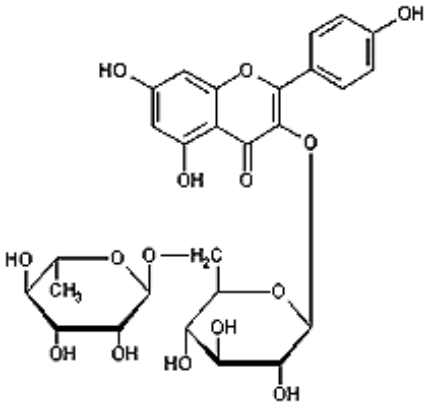
Luteolin-4'-O-β-D-glucopyranoside



Luteolin-7-O-β-D-glucopyranoside

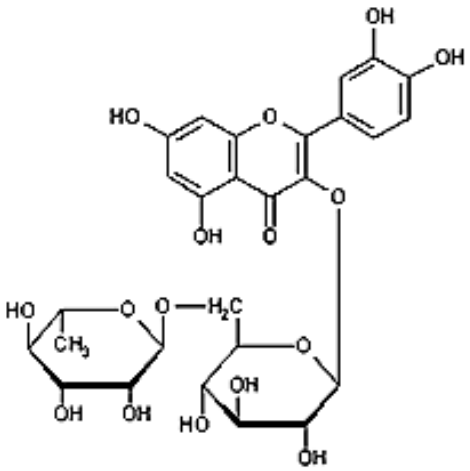


Apigenin-7-O-neohesperidoside

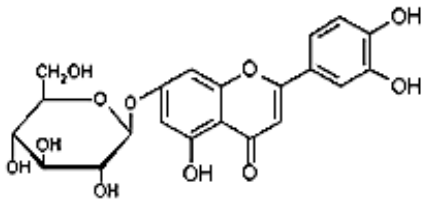


Kaempferol-3-O-rutinoside

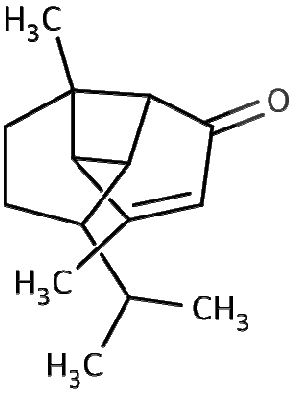
Chart 2.1 continued……



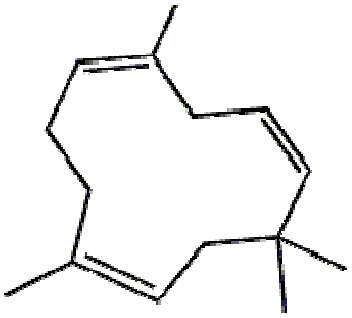
Quercetin-3-O-rutinoside



Luteolin-7-O-glucoside

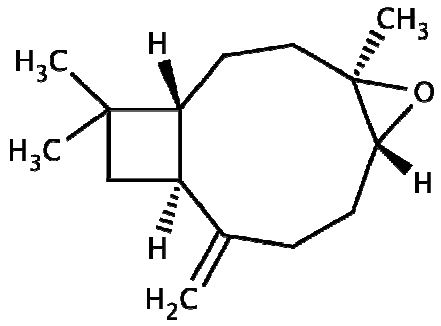


Mustakone

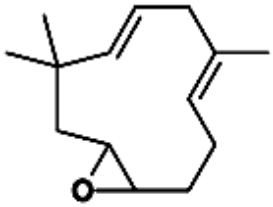


Caryophyllene

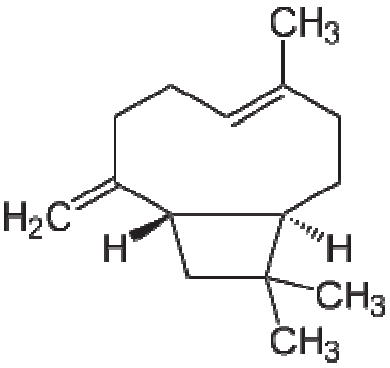
Chart 2.1 continued……



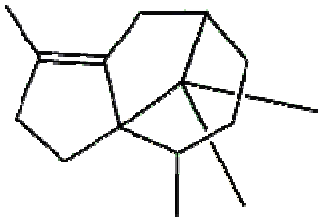
Caryophyllene oxide



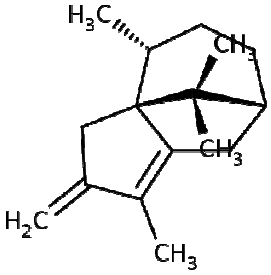
Humulene epoxide



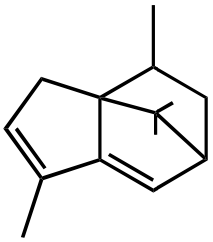
β-caryophyllene



Cyperene

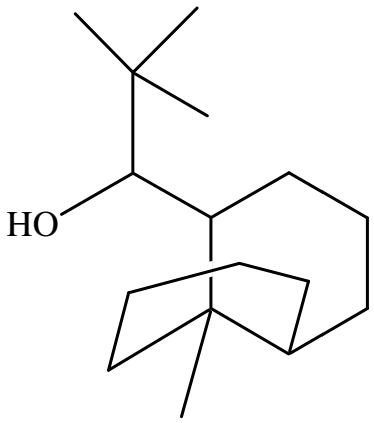


Cyperotundone

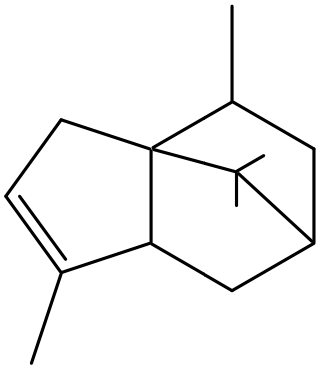


Isopatchoula-3,5-diene

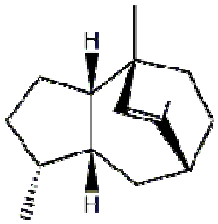
Chart 2.1 continued……



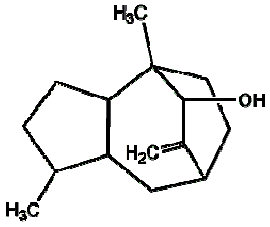
Patchoulenol



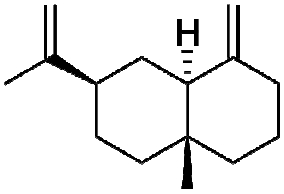
Isopatchoul-3-ene



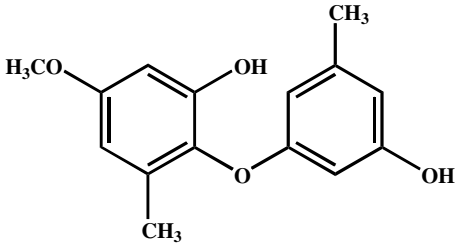
Rotundene



Rotundenol



β-Selinene



Cyperine

2.2. AIM AND SCOPE OF THE PRESENT INVESTIGATION

Cyperus rotundus L. (Family: Cyperaceae; *C. rotundus)* is a weed widespread in temperate, tropical and subtropical regions of the world (Figure 2.1). It is an erect

perennial glabrous grass like sedge (known as *musta* in Sanskrit and *muthanga* in Malayalam) with slender subterranean stolons ending in ovoid or cylindrical brown edible tubers. It is well-known for its traditional medicinal uses described for exerting antiinflammatory (Heo and Lee, 2005), antipyretic, analgesic (Seo *et al*., 2001), antidiarrhoeal (Gupta *et al*., 1971) and antimalarial effects (Uddin *et al*., 2006; Weenen *et al*., 1990). Although, the rhizomes were also used as hypotensive, estrogenic, antiemetic (Heo and Lee, 2005), remedy for dysentery and women's diseases (Boulos, 1983). In ancient ayurvedic literature (Charaka Samhita – 3000 B.C.), *C. rotundus* tubers were listed together with nine other plant species as lekhania drugs, which were capable of de-fatting adipose or muscular tissues (Thebtaronth *et al*., 1995). In Kerala, the rhizomes of *Cyperus rotundus* rhizomes were used in ayurvedic systems of medicine. 'Lagugandhar Churna', a common compound preparation used for the treatment of diarrhoea and dysentery contains rhizomes of *C. rotundus* (Anshu *et al*., 2010). The plant has long been used as a folk medicine and as a food. Despite the bitter taste of the tubers, they are edible and have nutritional value. Extracts of *C. rotundus* were reported to lower glycemia in a rodent model of diabetes (Trivedi and Mann, 1972). Rhizomes also contain proteins and traces of Mg, V, Cr, Mn and Co (Duan *et al*., 1993).

Figure 2.1: *Cyperus rotundus* plant and rhizomes

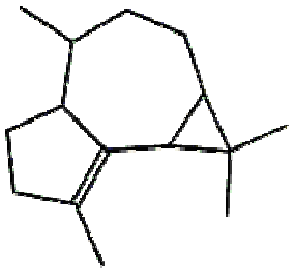
2.2.1. Literature review - *Cyperus rotundus*

Rhizomes of *C. rotundus* on steam distillation is reported to yield essential oil of yellow colour with a characteristic odour and the major components identified were 5-oxo isolongifolene, α-gurjunene, (z)-valerenyl acetate, α-selinene etc. At high concentrations, the oil showed good activity against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosai* (Anupam *et al*., 2011). Olaiduppo *et al*. (2009) has determined the essential oil composition of *C. rotundus* from South Africa and identified around 43 components with *β*-pinene as the major component**.** The essential oil composition of rhizomes of *C. rotundus* from Tunisia was evaluated by Soumaya *et al*., (2008) and the oil contained cyperene, α-cyperone, isolongifolen-5-one, rotundene and cyperorotundene as principal components. Essential oil showed antifungal properties also.

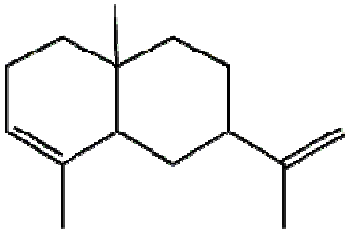
Many pharmacologically active constituents were identified from *C. rotundus* rhizomes. In an earlier report, a new cerebroside 1-O-(β-D-glucopyranosyloxy)-(2S, 3R,

4E, 8Z)-2-[(20R)-20-hydroxylignoceranoylamino]-4,8-tetradecene-3-diol with potent antiproliferative effect on vascular smooth muscle cell was isolated (Pei *et al*., 2010). Phytochemical investigation on the aerial parts of *C. rotundus* afforded a steroid glycoside sitosteryl (6-0-hentriacontanoyl)-β-D-galactopyranoside and three furochromones, khellin, visnagin and ammiol. These compounds showed cytotoxicity against L5178y mouse lymphoma cells and were active in the brine shrimp lethality test (Hanaa *et al*., 2007). A study by Jun *et al*., (2012) reported the presence of two sesquiterpenoids rotundusolide A & B, one triterpenoid rotundusolide C etc. in the ethanol extract of *C. rotundus* rhizomes. Ethyl acetate extract showed antioxidant, antibacterial, cytotoxic and apoptotic activities. Furthermore, ethyl acetate extract suppressed growth and proliferation of L1210 cells derived from murine lymphoblastic leukaemia (Soumaya *et al*., 2008). α-cyperone isolated from the tubers of *C. rotundus* showed antiinflammatory activity by inhibiting LPS induced COX-2 expression and PGE_2 production through the negative regulation of NFκB signalling in RAW 264.7 cells. Additionally, it also down regulated the production and mRNA expression of the inflammatory cytokine IL-6 (Seung *et al*., 2013). Another report demonstrates the antiplatelet effect of nootkatone, isolated from the rhizomes which can be used for the therapeutic benefit for the prevention of platelet associated cardiovascular diseases (Eun *et al*., 2011). Zhu *et al*. (1997) described ulcer inhibitory effect of decoction from the rizhomes of *C*. *rotundus*, related to inhibition of gastric motility. Column chromatographic separation of rhizomes of *C. rotundus* yield flavonoids such as kaempferol, luteolin, pinoquercetin etc. (Xu *et al*., 2010). Some of the structures of compounds isolated from *C. rotundus* are given in chart 2.2.

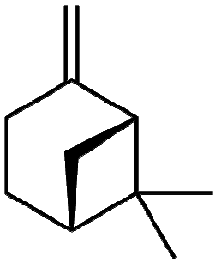
Chart 2.2: Compounds isolated from *Cyperus rotundus*



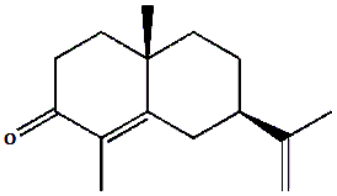
α-gurjunene



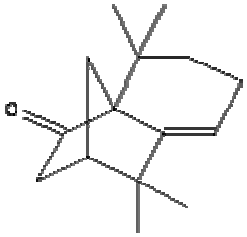
α-selinene



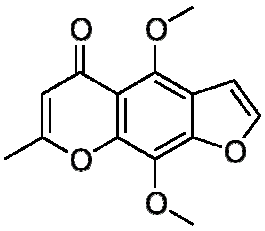
β-pinene



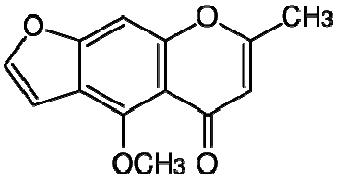
α-cyperone



Isolongifolen-5-one

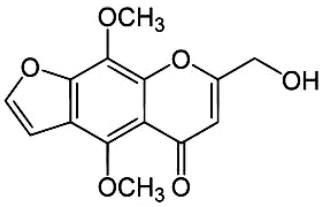


Khellin

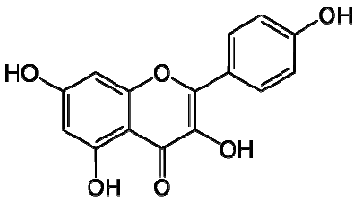


Visnagin

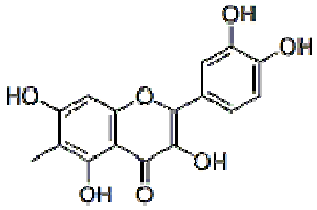
Chart 2.2 continued……



Ammiol



Kaempferol



Pinoquercetin

2.3. EXPERIMENTAL

2.3.1. Collection of plant material

The rhizomes of *Cyperus rotundus* (1.25 kg) were obtained from CSIR-NIIST campus, Thiruvananthapuram, Kerala (8°28′59″ N and 76°55′00″ E) and were identified and authenticated by Dr. H. Biju (Taxonomist, JNTBGRI, Palode, Thiruvananthapuram, Kerala). A voucher specimen (No. 034/2011) has been deposited in the herbarium for further use. The rhizomes collected were cleaned, dried at 50°C and powdered.

2.3.2. Chemicals and reagents used

Ammonium molybdate, deuterated solvents for NMR, silica gel 60 F_{254} TLC aluminium sheets, silica gel HPTLC plates (Kieselgel 60 F_{254}, 20 cm × 20 cm, 0.2 mm thickness), HPLC grade methanol etc. were purchased from E. Merck, Mumbai, India and sodium phosphate from Alfa Aesar, India was purchased. HPLC grade acetonitrile was obtained from Rankem India Ltd and HPLC water was purified on a Milli-Q system (Millipore India Pvt Ltd, Bangalore, India). 2,2′-diphenyl-1-picrylhydrazyl radical

(DPPH•), gallic acid etc. were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA). All other reagents used were of standard analytical grade.

2.3.3. General experimental details

Nuclear magnetic resonance spectroscopy was performed on a 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 (Merck, Mumbai, India). 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. Mass spectra were recorded by mass spectral analysis in HRMS-FAB at 5000 resolution using JMS 600H (JEOL) mass spectrometer and HRMS-ESI spectrum was performed at a resolution of 61800 using a Thermo Scientific Exactive mass spectrometer. UV-VIS spectrum and absorbance were measured using a Shimadzu 1601 UV-VIS spectrophotometer (UV 1601, Kyoto, Japan) and Synergy 4 Biotek multiplate reader (Biotek Instruments Inc., Highland Park, P.O. Box 998, Winooski, Vermont-0504-0998, USA) using spectroscopic grade methanol as solvent. IR spectra were taken with Alpha FT-IR, Bruker Optics. Melting points were determined with a Büchi melting point apparatus. Analytical thin layer chromatography was performed on silica gel 60 F_{254} TLC aluminium sheets. The TLC plates were kept in an air oven for 15 min at 80-100°C prior to use and the spots were visualized under UV lamp or in an iodine chamber. The plant materials collected were dried in a hot air oven (Model No, BC/PC/48, Bhuvaneswari Corporation, Chennai, India) at 50°C. Column chromatography was carried out using 100-200 mesh silica gel (Merck) and eluted using varying polarities of hexane-ethyl acetate mixtures. The solvents were evaporated using a

Büchi rotary evaporator. All the solvents used were distilled prior to use. Salkowski and Shinoda test were done using standard procedures. Specific rotations were recorded on Rudolph Research Analytical Autopol I automatic polarimeter (Hackettstown, NJ 07840, USA).

High performance liquid chromatography (HPLC) was performed on a Waters liquid chromatography equipped with a Rheodyne injector (Cotati, California, USA) fitted with a 20 µl sample loop and a Waters 2487 (M/s Waters GESMBH, Hietzinger Hauptstrasse 145, A 1130, Vienna, Austria, Europe) UV detector. The system is interfaced with a computer for data acquisition and control (Millennium software). The separation of compounds was made on a µ-Bondapak C18 column (150 mm × 4.6 mm, 5.0 µm) at room temperature using HPLC grade acetonitrile (Rankem) and water was purified using a Milli-Q system (Millipore India Pvt Ltd, Bangalore, India). High performance thin layer chromatography was performed on silica gel HPTLC plates (Kieselgel 60 F_{254}, 20 cm × 20 cm, 0.2 mm thickness, Merck, Darmstadt, Germany) washed with methanol before use and kept at 60°C for 30 min for the analysis. The samples were spotted in the form of bands of width 6 mm by means of a Camag Linomat V (Switzerland) fitted with a Hamilton microliter syringe. A constant application rate of 0.1 µl/s was employed and the space between the two bands was maintained as 5 mm. After spotting, the plates were developed in an ascending manner with hexane-ethyl acetate (SD Fine chemicals, Mumbai, India) solvent system of suitable polarity in a pre-saturated development chamber. The plates were dried in an air oven after development at a temperature of 50°C and scanned using a TLC Scanner 3 (Camag) in absorbance-reflectance mode. The radiation source used for analysis was a deuterium lamp emitting a

continuous UV spectrum between 200 and 300 nm. The slit dimension was kept at 5 mm × 0.45 mm and 10 mm/s scanning speed was employed. Analysis was performed in an airconditioned room maintained at 22°C and 65% relative humidity. Various concentrations of isolated compounds and extract were analyzed a minimum of three times. Data processing was performed with 'win CATS' planar chromatography manager software (version 1.4.3) and UV spectra of the isolated compounds were obtained using Camag TLC Scanner 3.

2.3.4. Extraction

The fresh rhizomes of *Cyperus rotundus* (1.25 kg) collected were cleaned and dried in an air oven. The dried rhizomes were coarsely powdered using a blender and the powdered rhizomes (500 g) of *C. rotundus* were extracted with acetone (2 L) at room temperature. The extraction was carried out by immersing the powdered material in acetone for overnight and decanted the solvent. The extraction procedure continued for 4 days and the acetone extracts obtained were combined. The removal of solvent at 40°C under reduced pressure in a rotary evaporator yielded 30 g of crude acetone extract. Pure compounds were obtained from the crude extract using column chromatographic method.

2.3.5. Column chromatographic separation of acetone extract

Column chromatography was done using 100-200 mesh silica gel and acetone extract dissolved in minimum amount of hexane was loaded on the top of the column. The column was eluted successively with varying polarities of hexane-ethyl acetate mixtures (staring from 100% hexane and ending with 100% ethyl acetate). The pictorial representation of the isolation procedure of compounds **I – V** is given in figure 2.2.

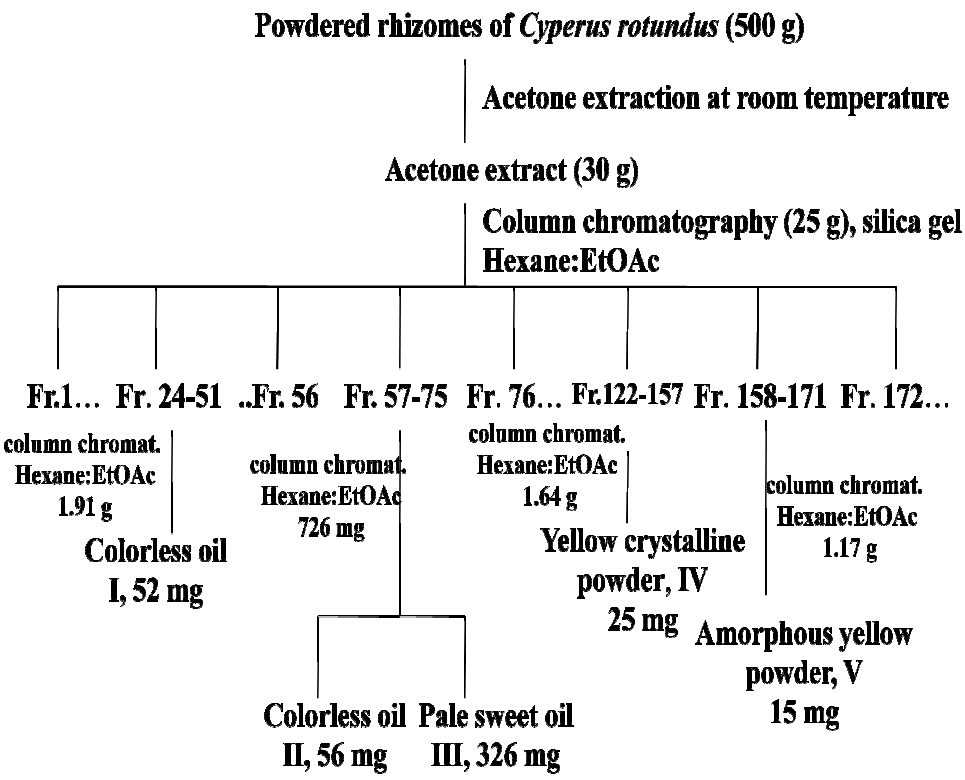


Figure 2.2: Pictorial representation of the isolation of compounds **I-V**

2.3.5.1. Isolation of compound I

Compound **I** was isolated on elution with 5% ethyl acetate in hexane (5:95) as colourless oil. Various spectroscopic techniques such as UV, IR, ^{1}H NMR, ^{13}C NMR, HRMS etc. were used for structural elucidation and compared with literature values confirmed the structure as **solavetivone**, a sesquiterpenoid. The structure of compound **I** is given below:

UV (MeOH, nm) λ_{max} : 246

FT-IR (ν_{max}/cm^{-1}) : 2919, 2853, 2385, 2351, 1711, 1600, 900

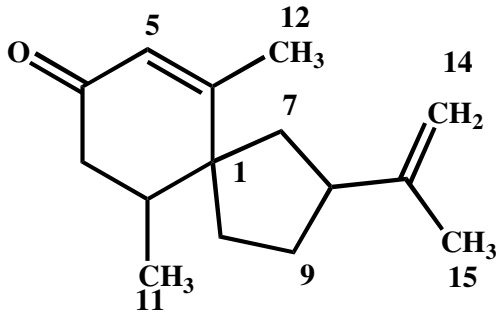
^{1}HNMR($CDCl_3$ 300 MHz) : δ 5.59 (1H, s, C5-H), 4.77 (2H, s, =CH_2), 2.48-2.41 (1H, m C8-H), 2.42-2.29 (2H, m, C3-H), 2.12- 2.02 (1H, m, C2-H), 1.89- 1.43 (2H, m, C10-H), 1.77 (3H, s, C-15 Me), 1.68 (3H, s,

C12-Me), 1.29-1.25 (2H, m, C7-H), 1.29-1.18 (2H, m, C9-H), 1.02 (3H, d, *J*=6.0 Hz, C11-Me)

^{13}C NMR ($CDCl_3$, 75 MHz) : δ 198.26 (C-4), 161.31 (C-6), 148.15 (C-13), 127.81 (C-5), 108.20 (C-14), 54.68 (C- 1), 47.77 (C-8), 44.91 (C-3), 36.42 (C-2), 34.83 (C-10), 32.78 (C-7), 31.92 (C-9), 21.49 (C-15), 19.64 (C-11), 15.81 (C-12)

HRMS-FAB m/z (rel.int.) : 219.65 $[M+H]^+$ $C_{15}H_{22}O$ requires 218.34

$[\alpha]_D^{25}$ (c, 1.000, MeOH) : -123°, lit. $[\alpha]_D^{25}$ -119 ° (Coxon *et al*., 1974)



I

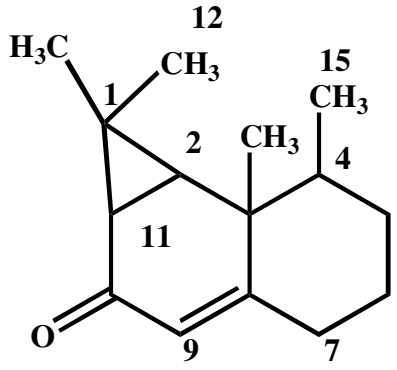
2.3.5.2. Isolation of compound II

Compound **II** was isolated on elution with 5% ethyl acetate in hexane (5:95) as colourless oil which on crystallization with hexane yielded pure crystals. Various spectroscopic techniques such as IR, ^{1}H NMR, ^{13}C NMR, HRMS etc. were used for structural elucidation and compared with literature values confirmed the structure as **aristolone**, a sesquiterpenoid.

UV (MeOH, nm) λ_{max} : 242

FT-IR (ν_{max}/cm^{-1}) : 2920, 2853, 2385, 2351, 1711

^{1}H NMR ($CDCl_3$, (500 MHz)	:	δ 5.67 (1H, s, C9-H), 2.12-2.10 (2H, m, C7-H), 1.96-1.91 (2H, m, C6-H), 1.88 (1H, d, *J= 2.5 Hz*, C11-H), 1.68-1.63 (1H, m, C4-H), 1.23-1.21 (2H, m, C5-H), 1.19 (3H, s, C12-H), 1.04 (1H, d, *J*=7.5 *Hz*, C2-H), 0.96 (3H, s, C13-H), 0.95 (3H, s, C14-H), 0.82 (3H, d, *J*= 5.5 *Hz*, C15-H)
^{13}C NMR ($CDCl_3$, 125 MHz)	:	δ 211.08 (C-10), 138.84 (C-8), 121.20 (C-9), 45.38 (C-3), 34.86 (C-4), 33.73 (C-2), 33.67 (C-7), 30.49 (C-11), 28.37 (C-5), 27.01 (C-2, 6), 24.81 (C-1), 19.55 (C-15), 18.06 (C-14), 16.82 (C-13), 15.53 (C-12)
HRMS-FAB m/z (rel.int.)	:	218.56 $[M]^+$ $C_{15}H_{22}O$ requires 218.34
m.p. (°C)	:	61-62°C, lit. 62-63°C (Edward *et al*., 1969)



II

2.3.5.3. Isolation of compound III

Compound **III** was isolated on elution with 10% ethyl acetate in hexane (10:90) as pale oil with sweet smell. Analysis of UV, IR, ^{1}H NMR, ^{13}C NMR and mass spectral

studies of this compound and comparison to the literature values, confirmed it to be **nootkatone**, a sesquiterpenoid.

UV(MeOH, nm) λ_{max} : 245

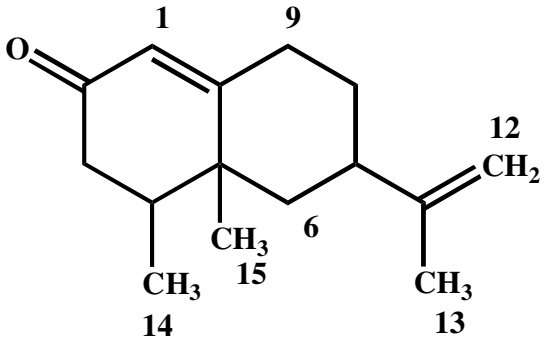
FT-IR (ν_{max}/cm^{-1}) : 2921, 2852, 1711, 1556

^{1}H NMR ($CDCl_3$, 500 MHz) : δ 5.69 (1H, s, C1-H), 4.66 (2H, d, *J*= 10 Hz, C12-H), 2.73-2.48 (2H, m, C9-H), 2.47-2.44 (1H, m, C7-H), 2.42-2.17 (2H, m, C3-H), 1.97-1.89 (1H, m, C4-H), 1.86-1.83 (2H, m, C8-H), 1.67 (3H, s, C13-H), 1.18 (3H, s, C15-H), 1.06-0.94 (3H, m, C14-H), 0.92-0.90 (2H, m, C6-H)

^{13}C NMR ($CDCl_3$, (125 MHz) : δ 199.66 (C-2), 170.60 (C-10), 148.98 (C-11), 124.62 (C-1), 109.23 (C- 12), 43.90 (C-3), 42.01 (C-7), 40.42 (C-5), 40.28 (C-6), 39.30 (C-8), 33.00 (C- 9), 31.59 (C-4), 20.78 (C-13), 16.81 (C-15), 14.86 (C-14)

HRMS-FAB m/z (rel.int.) : 218.35 $[M]^+$ $C_{15}H_{22}O$ requires 218.34

$[\alpha]_D^{25}$ (c, 0.981, $CHCl_3$) : +194° lit. $[\alpha]_D^{25}$ + 193.5° (Krepinsky *et al*., 1968)



III

2.3.5.4. Isolation of compound IV

Compound I**V** was isolated on elution with ethyl acetate in hexane (20:80) as yellow crystalline powder. Various spectroscopic techniques such as UV, IR, ^{1}H NMR, ^{13}C NMR, HRMS etc. were used for structural elucidation and compared with literature values confirmed the structure as **quercetin**, a flavonoid. The structure of the compound is given below:

UV(MeOH, nm) λ_{max} : 256, 372

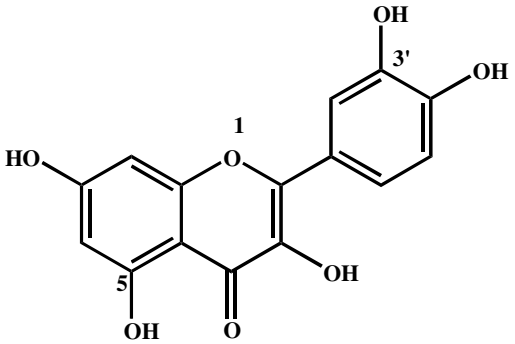
FT-IR (ν_{max}/cm^{-1}) : 3397, 2919, 1621, 1462, 1378

^{1}H NMR (CD_3OD, 500 MHz) : δ 12.24 (5H, s, OH), 7.69 (1H, d, *J*= 1.5 Hz, C2′-H), 7.59 (1H, dd, J_1= 1.5 Hz, J_2= 5 Hz, C6′-H), 6.88 (1H, d, *J*= 8.5 Hz, C5′-H), 6.39 (1H, d, *J*=1 Hz, C8-H), 6.19 (1H, d, *J*= 1.5 Hz, C6-H)

^{13}C NMR (CD_3OD & $CDCl_3$, 125 MHz) : δ 179.90 (C-4), 167.82 (C-7), 160.75 (C-5), 157.06 (C-9), 151.19 (C-2), 148.58 (C-4′), 146.22 (C-3′), 139.71 (C-3), 126.75 (C-1′), 124.64 (C-6′), 119.15 (C-5′), 118.89 (C-2′), 102.20 (C-10), 97.57 (C-6), 95.42 (C-8)

HRMS-FAB m/z (rel.int.) : 302.48 $[M]^+$ $C_{15}H_{10}O_7$ requires 302.24

m.p. (°C) : 316-317°C, lit. 317-318°C (Aarti *et al*., 2012)



IV

2.3.5.5. Isolation of compound V

Compound **V** was isolated on elution with 50% ethyl acetate in hexane (50:50) as yellow amorphous powder. Various spectroscopic techniques such as UV, IR, ^{1}H NMR, ^{13}C NMR, HRMS etc. were used for structural elucidation and compared with literature values confirmed the structure as **amentoflavone**, a biflavonoid. The structure of the compound is given below:

UV(MeOH, nm) λ_{max} : 269, 335

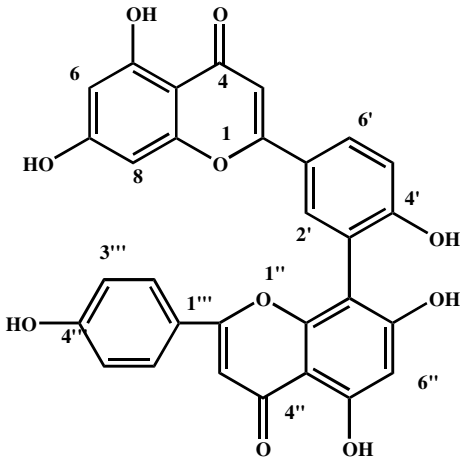
FT-IR (ν_{max}/cm^{-1}) : 3380, 3172, 2923, 1657, 1611, 1570, 1428, 1360, 1287, 1167, 1030, 947

^{1}H NMR ((CD$_3$)$_2$CO 500 MHz) : δ 13.03 and 12.86 (1H each, s, 2 x OH), 10.18-9.62 (4H, s, 4 x OH), 7.96 (1H, d, *J*=2.0 Hz, C2′-H), 7.90 (1H, dd, J_1= 2, J_2=8.5 Hz, C6′-H), 7.52 (2H, d, *J*= 8.5 Hz, C2″′, 6″′-H), 7.13 (1H, d, *J*=8.5 Hz, C5′-H), 6.71 (2H, d, *J*=8.5 Hz, C3″′, 5″′-H), 6.61 (1H, s, C3-H), 6.55 (1H, s, C3″-H), 6.39 (1H, d, *J*= 1.5 Hz, C8-H), 6.35 (1H, s, C6″-H), 6.12 (1H, d, *J*= 2 Hz, C6-H)

^{13}C NMR ((CD$_3$)$_2$CO 125 MHz) : δ 182.13 (C-4″), 181.73 (C-4), 164.13 (C-7), 163.86 (C-2″), 163.81 (C-2), 161.83 (C-7″), 161.51 (C-5″), 161.19 (C-5), 160.69 (C-4″′), 159.08 (C-4′), 157.44 (C-9), 154.69 (C-9″), 131.12 (C-6′), 127.72 (C-2″′, 6″′), 127.31 (C-2′), 121.68 (C-1″′), 121.65 (C-1′), 119.74 (C-3′), 115.95 (C-5′), 115.36 (C-3″′,5″′), 103.89 (C-8″), 103.77 (C-10″), 103.29 (C-10), 102.74 (C-3″), 102.20 (C-3), 98.41 (C-6″), 98.33 (C-6), 93.43 (C-8)

HRMS-FAB m/z (rel.int.) : 539.47 $[M+H]^+$ $C_{30}H_{18}O_{10}$ requires 538.45

m.p. (°C) : 255-256°C lit. 254-255°C (Lobstein *et al*., 1988)



V

2.3.6. Quantification of isolated sesquiterpenoids using HPTLC and reverse phase HPLC methods

Column chromatographic method led to the isolation of three sesquiterpenoids having same molecular formula and there are previous reports that most of the properties of *Cyperus rotundus* were attributed to the presence of sesquiterpenoids present in the rhizomes (Thebtaronth *et al*., 1995; Jeong *et al*., 2000). Hence, HPTLC and reverse phase HPLC methods were done for the quantification of sesquiterpenoids present in the plant material.

2.3.6.1. Optimization of chromatographic conditions for the analysis of sesquiterpenoids

2.3.6.1.1. Preparation of sample solutions

A reference stock solution with a concentration of 1 mg/ml of isolated compounds and acetone extract (5 mg/ml) was prepared in methanol. Stock solutions were diluted in

such a way to obtain various concentrations of solavetivone, aristolone and nootkatone in methanol (0.1-1 µg/ml) as working solutions for HPTLC and HPLC analysis. The solutions were filtered through 0.45 µm PTFE filter prior to analysis.

2.3.6.1.2. Standardisation of HPTLC analytical method

Silica gel HPTLC plates were washed with methanol before use and kept at 60°C for 30 min for the analysis. 5 µl of various concentrations of the samples (0.1, 0.5 and 1 µg/ml) were spotted as bands of width 6 mm by means of a Camag Linomat V (Switzerland) fitted with a Hamilton microliter syringe. A constant application rate of 0.1 µl/s was employed and the space between the two bands was maintained as 5 mm. The spots were developed using hexane-ethyl acetate solvent mixture of varying polarity and 30% ethyl acetate-hexane solvent system gave better separation and was finally fixed. A presaturated Camag glass twin trough development chamber using 30% ethyl acetate-hexane solvent was used to develop the spots. After spotting the plates were dried in an air oven at a temperature of 50°C and scanned using a TLC Scanner 3 (Camag) in absorbance–reflectance mode. The radiation source used for the analysis was a deuterium lamp emitting a continuous UV spectrum between 200 and 300 nm. The slit dimension was kept at 5 mm × 0.45 mm and 10 mm/s scanning speed was employed. Analysis was performed in an air-conditioned room maintained at 22°C and 65% relative humidity. Chromatographic conditions were optimized to obtain maximum resolution of compounds. Various concentrations of isolated compounds (0.1-1 µg/ml) and extract were analyzed a minimum of three times. Data processing was performed with win CATS planar chromatography manager software (version 1.4.3). The mean ± standard deviation of three replications was calculated.

2.3.6.1.3. Standardisation of HPLC-UV analytical method for sesquiterpenoids

The integrated high performance liquid chromatography was performed on a Waters liquid chromatography equipped with a Rheodyne injector and a Waters 2487 UV detector. The system is interfaced with a computer for data acquisition and control (Millennium). The separation of compounds was made on a µ Bondapak C-18 column (150 mm × 4.6 mm, 5.0 µm) at room temperature. The mobile phase used was a mixture of acetonitrile–water (60:40) pumped at a flow rate of 1 ml/min and the sample was chromatographed at a series of wavelength from 200 to 300 nm. Retention time of the three sesquiterpenoids obtained was so close and the compounds showed better resolution at 245 nm. Peak identification of extracts was based on comparing the retention time (R_T) values of individual components. The different peaks of sesquiterpenoids in extract were quantified by comparing the peak area with that of isolated one. The mean ± standard deviation of three replications was calculated.

2.3.6.2. Method validation

The chromatographic methods validated were linearity, detection limit, quantification limit, precision and accuracy. Validation was performed using adequate statistics estimated.

Comparison between retention time and spectra of the peaks ascertained the specificity of the method. By comparing the spectral levels at three different levels, i.e., start, apex and end positions assessed the peak purity of the chromatogram. Linearity of HPLC and HPTLC methods were obtained by determining the detector responses against a series of varying concentrations of isolated compounds. Five analyses per concentration were conducted and plotted calibration diagrams. All the components showed good

linearity in a relatively wide concentration range. The linear range, regression equation and correlation coefficient of each analytes were summarized.

Limits of detection (LOD) and quantification (LOQ) of the methods were calculated using the equations LOD = 3.3 σ/S and LOQ = 10 σ/S where σ is standard deviation of response and S is the slope of calibration curve. The limit of detection is the lowest concentration of an analyte in a sample that can be detected but cannot be used for the quantification as it falls below the linear range and limit of quantification is the lowest concentration of the analyte in a sample that can be quantified with acceptable precision and accuracy under the conditions of operation.

Repeatability was determined by the measurement of instrumental, inter and intra-assay precision. Instrumental precision was measured by scanning the same spot of a single concentration seven times. The repeatability or intra assay precision was studied by analyzing repeatedly, in the same laboratory and on the same day, at three concentrations (0.1, 0.5 and 1 µg/ml). Intermediate precision included the analysis of the same samples and each of them analyzed three times a day over three days in three same concentrations. The relative standard deviation (RSD) was taken as a measure of precision and the intraday and interday precisions by the method are summarized.

Accuracy of the methods was determined by standard addition techniques. Known amounts of isolated sesquiterpenoids in a range of low, medium and high concentrations were added to preanalyzed extract samples and were analyzed under the optimized conditions. Addition experiments for each concentration were performed in triplicate and the accuracy was calculated as the % of analyte recovered. Three analyses per concentration were performed and mean ± SD was determined.

2.3.6.3. HPTLC versus HPLC

The reliability of HPTLC densitometric analysis was verified by analyzing five independently prepared acetone extracts of *C. rotundus* by HPTLC and HPLC methods. Each sample was analyzed in triplicate and mean values were compared by matched pair Student's T-test. The observed T value (T_{obs}) was calculated by,

$$T_{(obs)} = \frac{|\bar{d}|}{\sqrt{\sum d_i^2 - 1/n(\sum d_i)^2/n(n-1)}} \quad \text{……. Eqn 2.1}$$

where d_i is the difference between two pairs of measurements for the same observation.

2.3.7. *In vitro* radical scavenging and antioxidant capacity evaluation of sesquiterpenoids

2.3.7.1. Radical scavenging assay using DPPH radical

The radical scavenging efficacy of isolated sesquiterpenoids and extract was evaluated in terms of purple colored DPPH radical spectrophotometrically (Brand Williams *et al*., 1995). Different installments of the sample solution (2-10 µg/ml) were mixed with 1.5 ml of methanolic solution of DPPH (25 mg/L) radical and the reaction mixture was incubated in dark for 30 minutes. The degree of decrease of purple color indicates the radical scavenging potential of the added substance. After 30 min incubation, absorbance was measured at 517 nm against control. Gallic acid served as the standard. The antioxidant capacity of samples to scavenge DPPH radical was calculated by the equation,

$$\text{Scavenging effect (\%)} = \frac{A_0 - A_1}{A_0} \times 100 \quad \text{…… Eqn 2.2}$$

where A_0 is the absorbance of blank sample (containing each reagent sample solution) and A_1 is the absorbance of samples. The percentage radical scavenging activity was plotted against concentration to obtain 50% inhibition of the drug scavenging DPPH radical (IC_{50}).

2.3.7.2. Total antioxidant activity

Antioxidant capacity by phosphomolybdenum reagent was evaluated by the method of Prieto *et al*. (1999) and is based on the reduction of Mo (VI) – Mo (V) by sesquiterpenoids resulting in the formation of a green phosphate/Mo (V) complex at acidic pH. An aliquot of 0.3 ml of sample solution (from 1 mg/ml) was combined with 3 ml of reagent solution (0.6 M conc. sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate in 100 ml distilled water) in test tubes. The tubes were capped and incubated at 95°C for 90 min. After cooling to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank. Methanol (0.3 ml) in place of samples served as the blank. Ascorbic acid was used as the standard and the total antioxidant capacity is expressed as ascorbic acid equivalents (g AAE/100 g of dry rhizomes).

2.4. STATISTICAL ANALYSIS

The experimental results are expressed as the mean ± standard deviation (SD) of three parallel measurements. The results were subjected to one way analysis of variance performed by using the statistical program SPSS, version 11.5 (SPSS, Chicago, IL, USA). Duncan's multiple range test was conducted for comparison of means at $P \leq 0.05$.

2.5. RESULTS AND DISCUSSION

2.5.1. Plant material and extraction

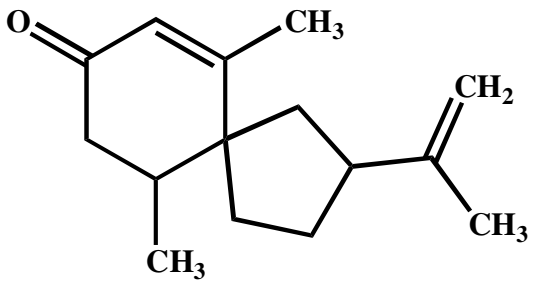
Cyperus rotundus, species of Cyperaceae family are widely grown in Kerala, India. The rhizomes collected were dried and powdered. Powdered rhizomes (500 g) were extracted with acetone (2 L) at room temperature and the extraction procedure continued for 4 days and the acetone extracts obtained were combined. The removal of solvent at 40°C, under reduced pressure in a rotary evaporator yielded 30 g of crude acetone extract. Thin layer chromatography of extracts was performed using varying polarities of solvents and column chromatography was used for the isolation of pure compounds.

2.5.2. Isolation and characterization of compounds from acetone extract

Acetone extract (25 g) was subjected to careful column chromatography with 100-200 mesh silica gel (500 g) started 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 195 fractions of 70-80 ml were collected and were further pooled according to their similarities in TLC into 8 major fraction pools. The initial fraction pools were found to have less polar components (most probably mixtures of essential oil components). Second fraction pool (24-51; 1.91 g) with a UV active spot as major component was purified further by column chromatography using hexane-ethyl acetate mixture (95:5) yielded compound **I** (52 mg) as colorless mobile oil. It was analyzed using various spectroscopic techniques.

Chloroform solution of compound **I** responded positively with Salkowski reaction which forms a reddish brown coloration at the interface showed the presence of

terpenoids and the structure was supported by spectral data. The UV spectrum (Figure 2.3) had λ_{max} (MeOH) at 246 nm which was consistent with the enone system. The IR spectrum revealed an α, β-unsaturated carbonyl group at 1711 cm^{-1} and terminal methylene group at 900 cm^{-1}. No hydroxyl group was observed. The ^{1}H NMR spectrum (Figure 2.4) showed a secondary methyl group at δ 1.02 as a doublet (J = 6.0 Hz) (Structure **I**), a methyl group attached to β-position of an α, β-unsaturated carbonyl group as a singlet (δ 1.68), an isopropenyl group was obtained at δ 1.77 and olefinic protons were obtained δ 4.77 and 5.59 as singlets. Further, presence of fifteen carbon atoms can be confirmed from the fifteen signals in the ^{13}C NMR spectrum (Figure 2.5) at δ 198.26, 161.31, 148.15, 127.81, 108.20, 54.68, 47.77, 44.91, 36.42, 34.83, 32.78, 31.92, 21.49, 19.64 and 15.81. The molecular formula of the compound was deducted as $C_{15}H_{22}O$ on the basis of HRMS-FAB at m/z 219.65 $[M+H]^{+}$ (cal. 218.34) (Figure 2.6) classified this compound as a sesquiterpene. From all the above data as well as comparison with the literature reports (Akiyoshi *et al*., 1982), the structure of the compound **I** was assigned as **solavetivone** as shown here. This is the first report of a vetispirane derivative, solavetivone (**I**) in *C. rotundus*. The compound showed a specific rotation of -123° similar to the literature reports (Coxon *et al*., 1974). Previously, solavetivone was reported as a minor constituent of essential oil of *Cyperus rotundus* by GC-MS method (Oladipupo *et al*., 2009). It has been isolated for the first time as a phytoalexin and a major stress metabolite from infected potato tubers (Coxon *et al*., 1974). It has been also identified as a minor volatile component of tobacco leaves (Fujimori *et al*., 1977).



I

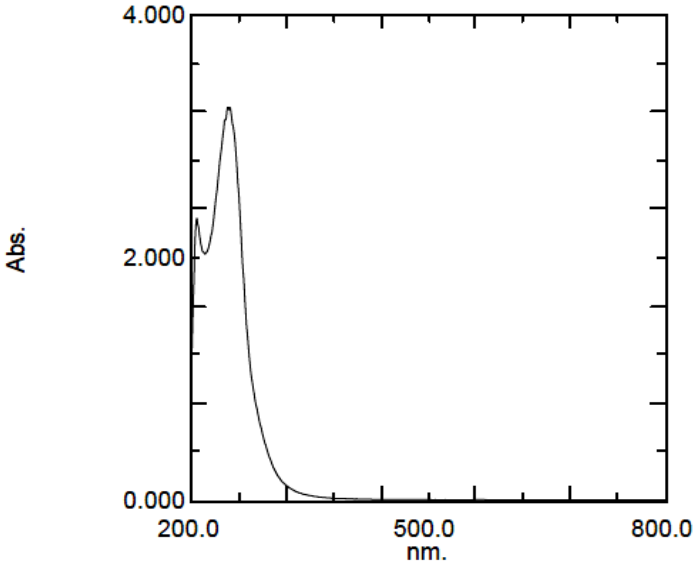


Figure 2.3: UV spectrum of compound **I**

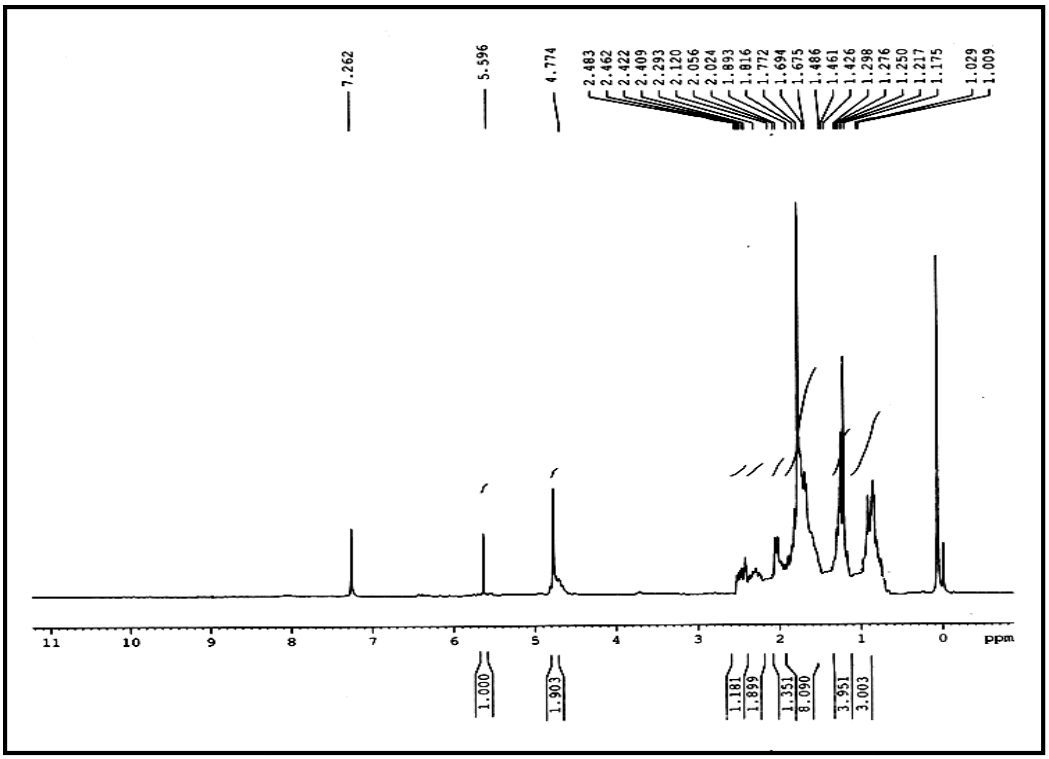


Figure 2.4: ^{1}H NMR spectrum of compound **I**

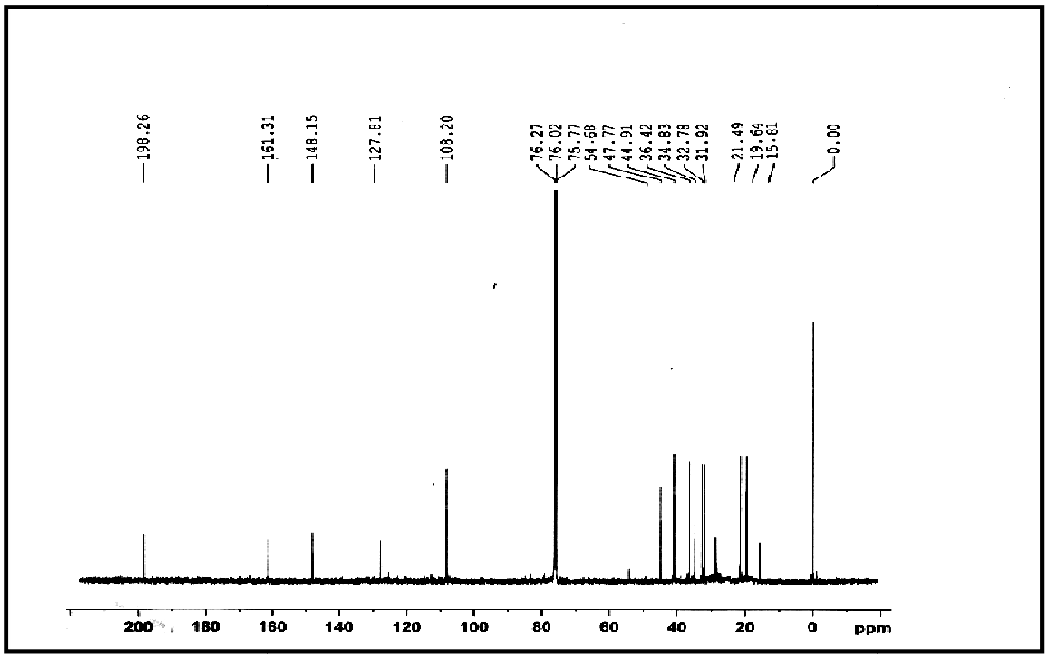


Figure 2.5: ^{13}C NMR spectrum of compound **I**

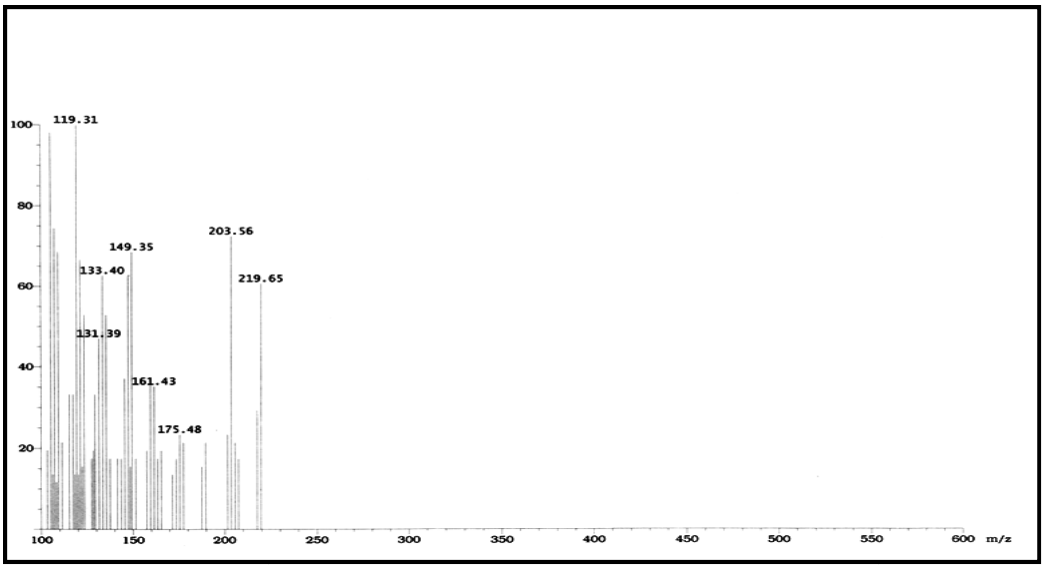
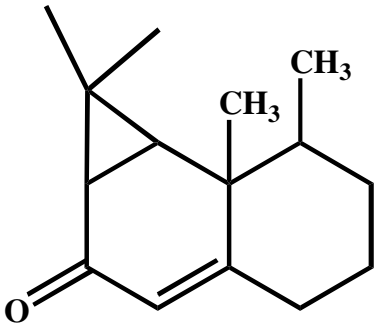


Figure 2.6: Mass spectrum of compound **I**

The fourth fraction pool (57-75; 726 mg) with two inseparable uv spot on further purification by column chromatography using 5% ethyl acetate-hexane (5:95) yielded compound **II** (56 mg) which on crystallization with hexane yielded pure crystals of compound **II** (40 mg, m.p. 61-62°C) and with 10% ethyl acetate-hexane mixture (10:90) yielded compound **III** as pale oil with sweet smell (326 mg) in pure forms. Both the compounds answer positively to Salkowski reaction which forms a reddish brown coloration at the interface showed the presence of terpenoids. The compounds obtained were analyzed by various spectroscopic techniques. Ultraviolet spectrum shows an absorption at 242 nm (Figure 2.7) and the IR spectrum showed an α, β-unsaturated carbonyl group at 1711 cm^{-1}. Terminal methyl groups were observed at 2920, 2853, 2385 and 2351 cm^{-1}. ^{1}H NMR spectrum (Figure 2.8) showed an olefinic proton at δ 5.67 as a singlet. Furthermore, at δ 1.04 a pair of doublets could be attributed to the cyclopropyl hydrogen at C-2 and a further doublet at δ 1.88 was assigned to the cyclopropyl proton at C-11. The three tertiary methyl groups were clearly evident at δ

0.96, 0.95 and 1.19 as singlets while the secondary methyl group was appeared at δ 0.82 as a doublet (*J*= 5.5 Hz). Further, presence of fifteen carbon atoms can be confirmed from the fifteen signals in the ^{13}C NMR spectrum (Figure 2.9) at δ 211.08, 138.84, 121.20, 45.38, 34.86, 33.73, 33.67, 30.49, 28.37, 27.01, 24.81, 19.55, 18.06, 16.82 and 15.53, The molecular formula of compound **II** was deducted as $C_{15}H_{22}O$ on the basis of HRMS-FAB (Figure 2.10). Molecular weight calculated was 218.34 and the obtained was 218.56 $[M]^+$. From all the above data as well as comparison with the literature reports (Carboni *et al*., 1965), the structure of the compound **II** was assigned as **aristolone** as shown here.



II

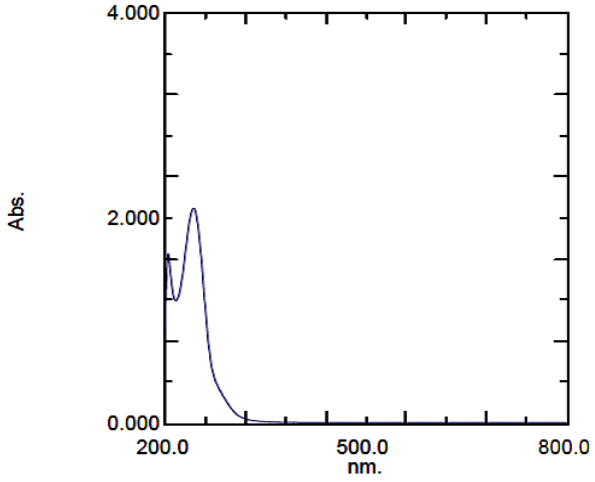


Figure 2.7: UV spectrum of compound **II**

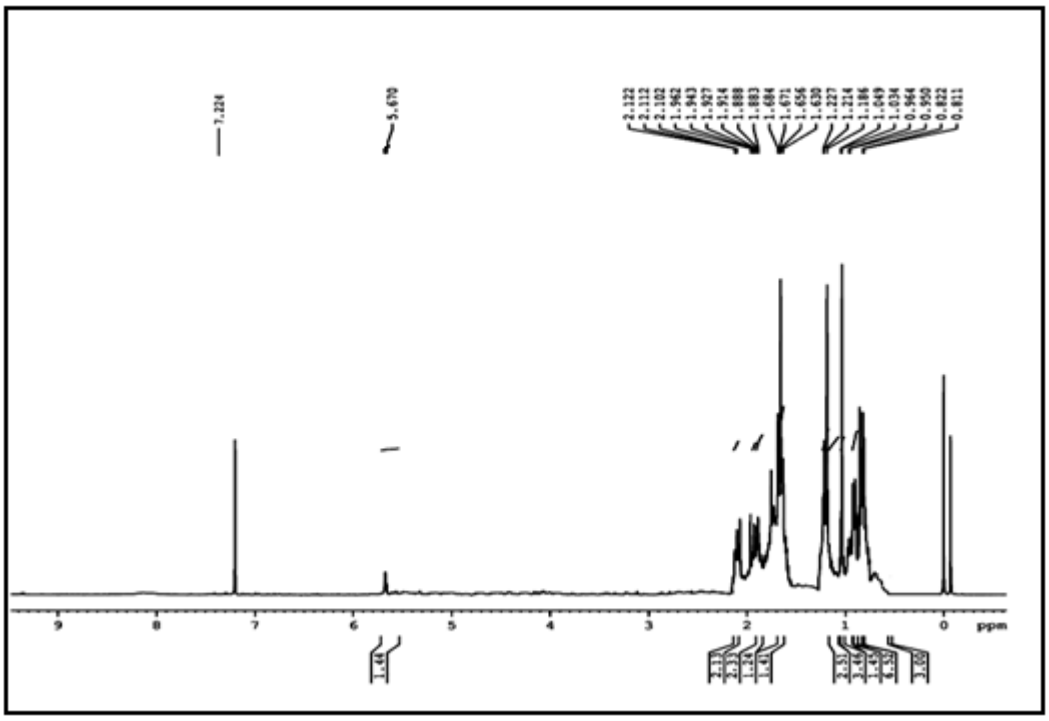


Figure 2.8: ^{1}H NMR spectrum of compound **II**

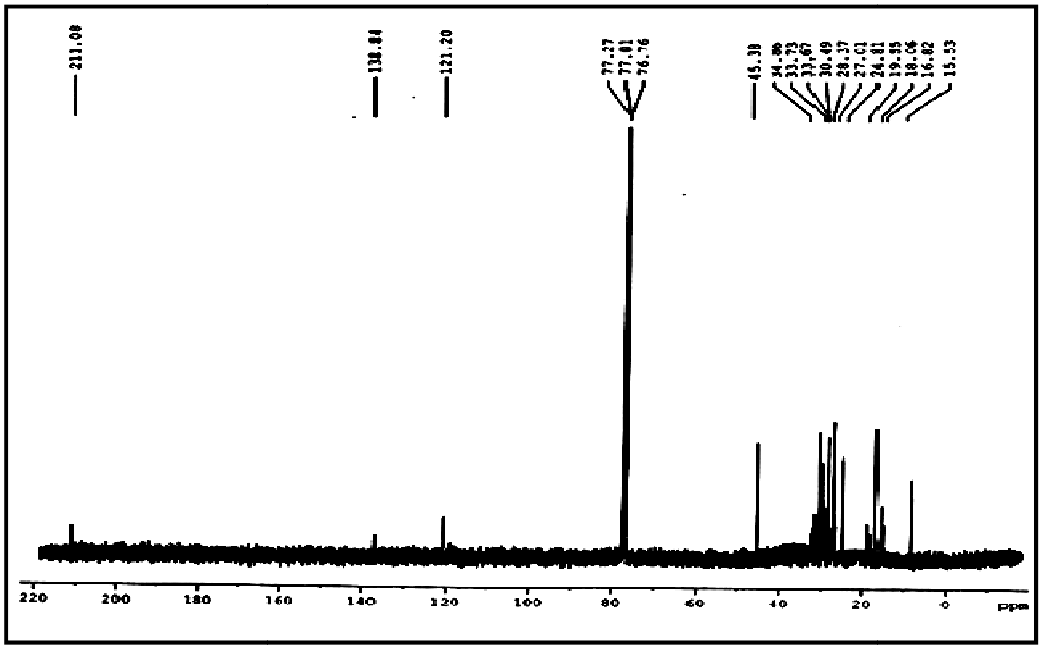


Figure 2.9: ^{13}C NMR spectrum of compound **II**

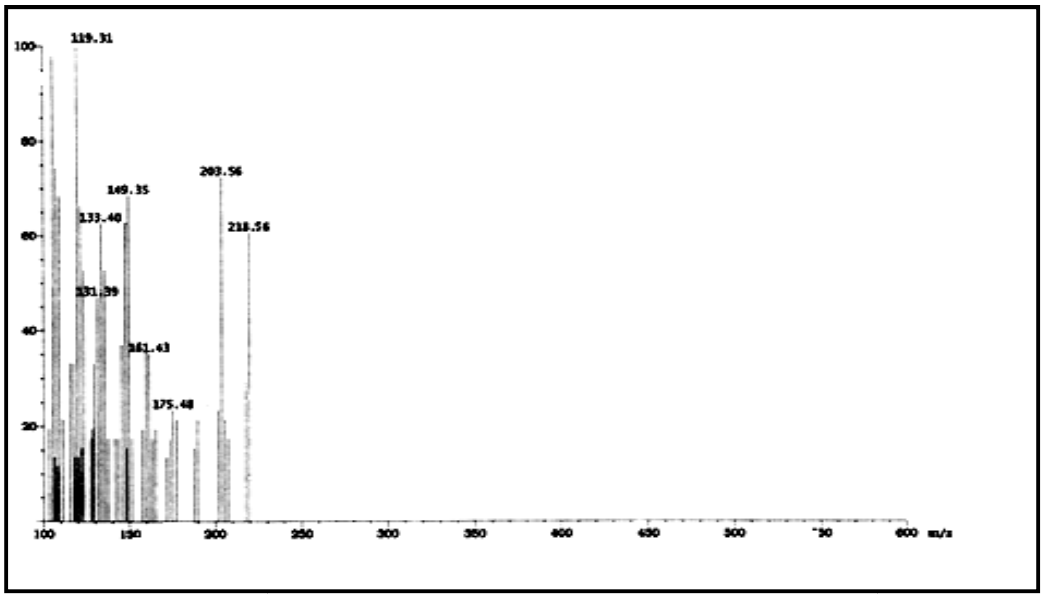
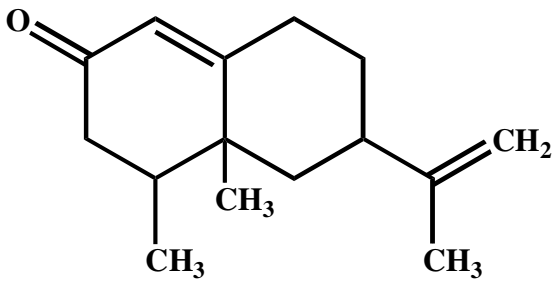


Figure 2.10: Mass spectrum of compound **II**

Compound **III** was obtained as a pale oil with sweet smell. Its UV spectrum shows an absorption peak at 245 nm (Figure 2.11). IR spectrum deduced characteristic peaks at 1711 cm^{-1} indicating an α, β-unsaturated carbonyl group. Terminal methyl groups were appeared at 2921, 2852 and 1556 cm^{-1}. ^{1}H NMR spectrum (Figure 2.12) showed an olefinic proton at δ 5.69 as a sharp singlet. A secondary methyl group was observed at δ 1.06-0.94 as a multiplet and a tertiary methyl group at δ 1.18 as a singlet. An isopropenyl group was observed at δ 1.67 as a singlet. Presence of fifteen carbon atoms can be confirmed from the fifteen signals in the ^{13}C NMR spectrum (Figure 2.13) at δ 199.66, 170.60, 148.98, 124.62, 109.23, 43.90, 42.01, 40.42, 40.28, 39.30, 33.00, 31.59, 20.78, 16.81 and 14.86. Based on HRMS-FAB (Figure 2.14) obtained at m/z 218.35 $[M]^{+}$ (cal. 218.34), the molecular formula of compound **III** was deducted as $C_{15}H_{22}O$. The compound showed a specific rotation of +194° similar to the literature reports (Krepinsky *et al*., 1968). From all the above data as well as comparison with the

literature reports (Miyazawa *et al*., 2000), the structure of compound **III** was assigned as **nootkatone** as shown here.



III

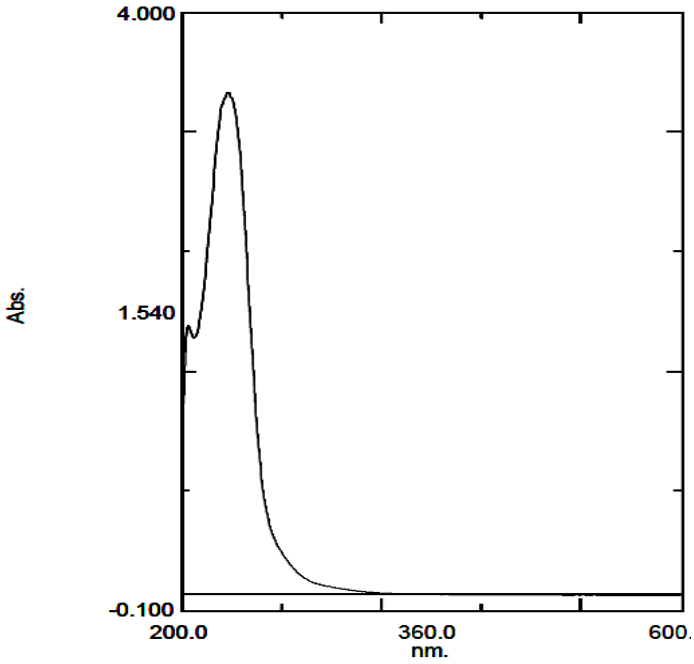


Figure 2.11: UV spectrum of compound **III**

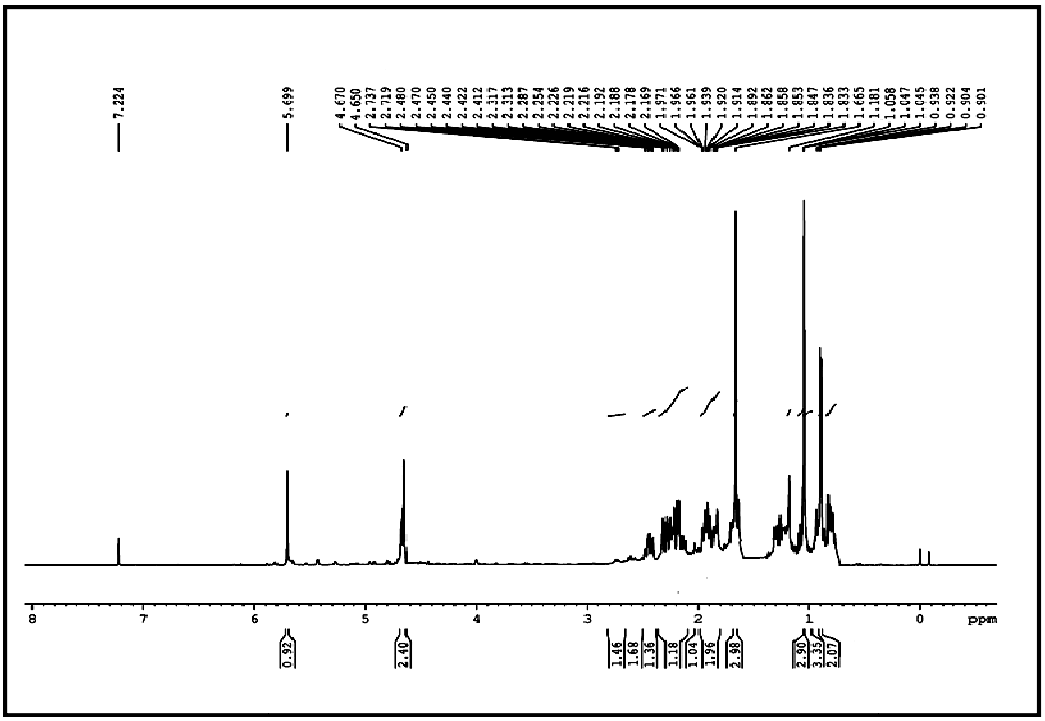


Figure 2.12: ^{1}H NMR spectrum of compound **III**

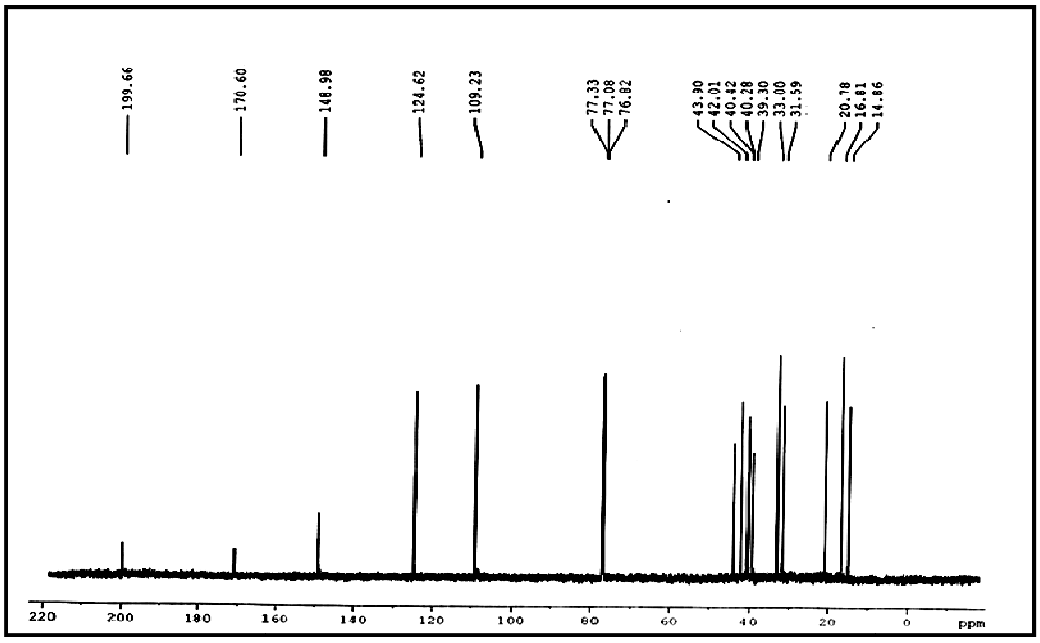


Figure 2.13: ^{13}C NMR spectrum of compound **III**

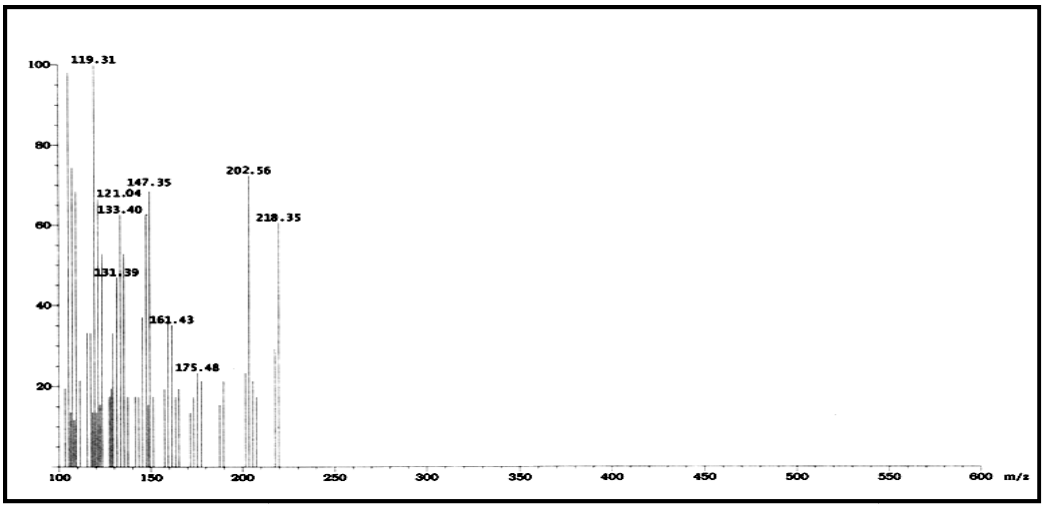
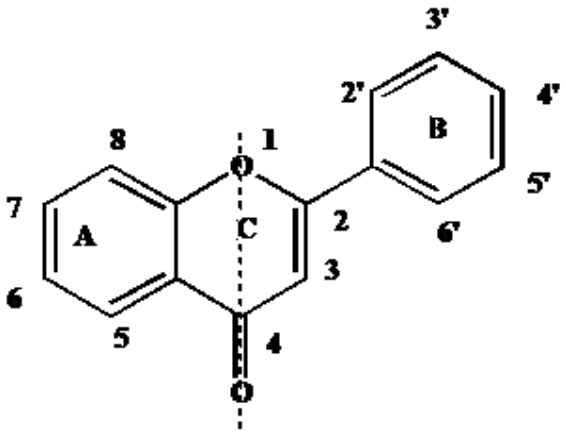


Figure 2.14: Mass spectrum of compound **III**

The sixth (122-157; 1.64 g) fraction pool was purified by column chromatography using hexane-ethyl acetate mixture (80:20-60:40) to yield compound **IV** (25 mg; m.p: 316-317°C) as yellow crystalline powder. It was analysed by various spectroscopic methods.

An alcoholic solution of compound **IV** imparted positively to Shinoda test which forms an orange colour indicates that **IV** is a flavonoid derivative. The UV-visible spectrum of the compound showed strong absorptions below 400 nm. 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 2.15).



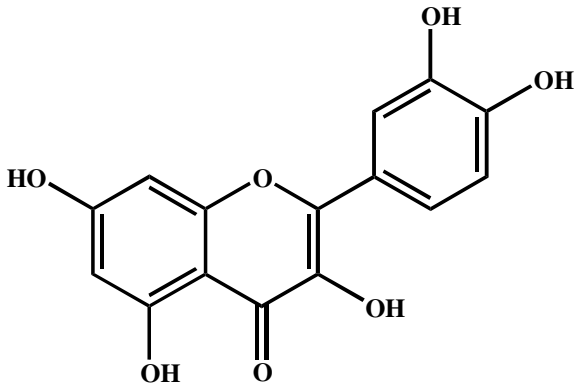
Benzoyl **Cinnamoyl**

Figure 2.15

The UV spectrum of flavonoids is readily distinguished by the presence of a low intensity band I absorption which often appears as a shoulder to band II peak (Markham and Mabry, 1975). The UV spectrum of compound **IV** was suggestive of a flavonoid with a flavonol ring system from the low intensity band I peak (372 nm) appearing as a shoulder to band II peak (256 nm). There are previous reports that the UV spectra of flavonoids are unaffected by changes in the oxygenation and substitution patterns in the B-ring. However, increased oxygenation and substitution in the A ring results in a bathochromic shift in the band II absorption. The band II absorption of compound **IV** was observed at 256 nm indicating oxygenation of the A ring (Figure 2.16). To confirm the structure of **IV**, other spectroscopic techniques were employed (Markham *et al*., 1975). The IR spectrum of compound **IV** showed absorption at 3397 cm^{-1} (hydroxyl) and 1621 cm^{-1} (carbonyl). The ^{1}H NMR spectrum (Figure 2.17) showed a sharp singlet at δ 12.24 indicates the presence of 5 phenolic –OH groups. This sharp peak is the result of the strong hydrogen bonding between the carbonyl group at the C-4 position and the 3 & 5 -OH groups. B ring aromatic protons were observed at δ 6.88 as a doublet (J = 8.5 Hz), δ

7.59 as a doublet of a doublet (J_1 = 1.5 Hz, J_2= 5 Hz) and δ 7.69 as a doublet (J = 1.5 Hz) integrating for three protons concluded that the B ring of compound **IV** is disubstituted. Two singlets obtained at δ 6.19 and 6.39 integrating for 1 proton each indicated that the protons were at position 6 & 8, with hydroxyl groups at positions 5 & 7 in the A ring. Band II bathochromic shift in the UV spectrum also supported this. In the ^{13}C NMR spectrum (Figure 2.18), the signal at δ 179.90 confirmed the presence of carbonyl carbon. The signal at δ 151.19 could be attributed to C-2 whereas the signal at δ 139.71 corresponds to C-3 carbon adjacent to the carbonyl group. Furthermore, the ^{13}C NMR spectrum clearly showed 15 carbon signals at δ 179.90, 167.82, 160.75, 157.06, 151.19, 148.58, 146.22, 139.71, 126.75, 124.64, 119.15, 118.89, 102.20, 97.57 and 95.42. The HRMS-FAB spectrum (Figure 2.19) of compound **IV** was more informative and gave the molecular ion peak at m/z 302.48 $[M]^+$. The melting point of the compound was found to be 316-317°C.

Based on all the spectral data and by comparing the values with those reported in the literature (Subramanyam Naidu *et al*., 2012), compound **IV** was identified as **quercetin** ($C_{15}H_{10}O_7$). Quercetin has been reported for treating allergies, asthma, bacterial infections, gout, eye disorders, hypertension etc. (Abigail *et al*., 2012).



IV

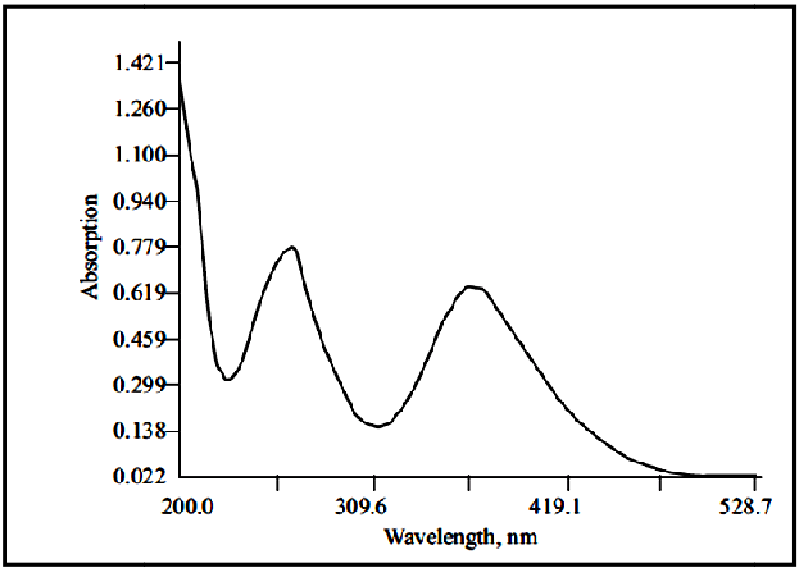


Figure 2.16: UV spectrum of compound **IV**

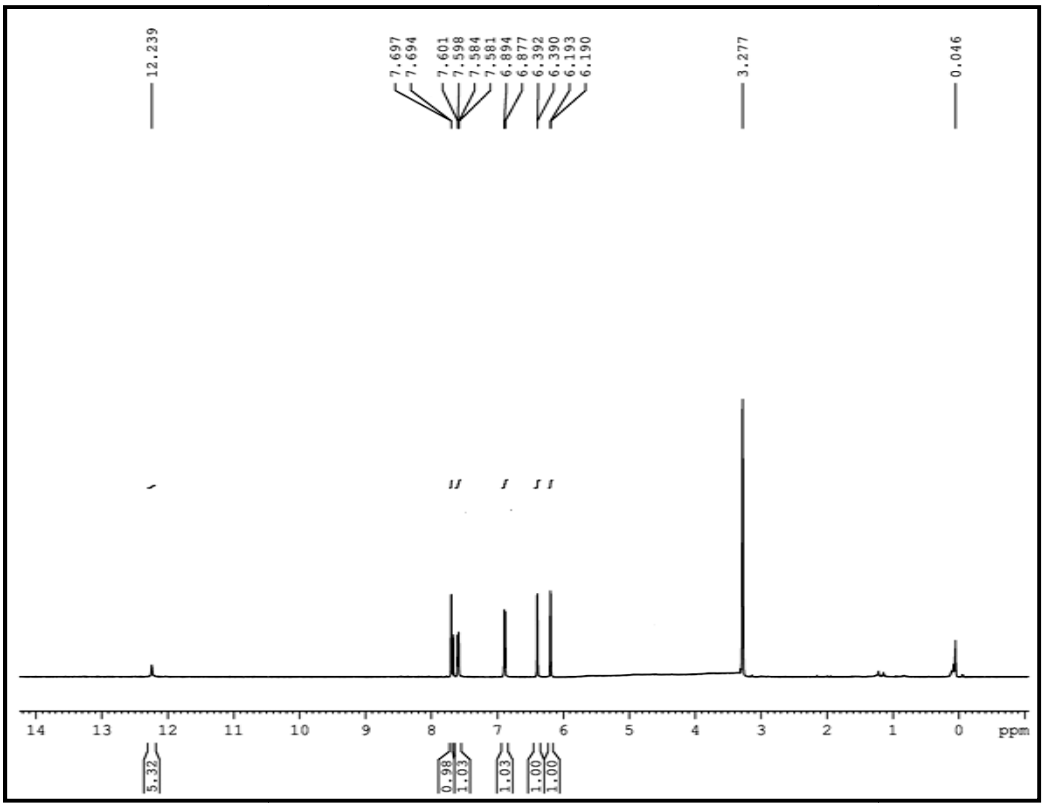


Figure 2.17: ^{1}H NMR spectrum of compound **IV**

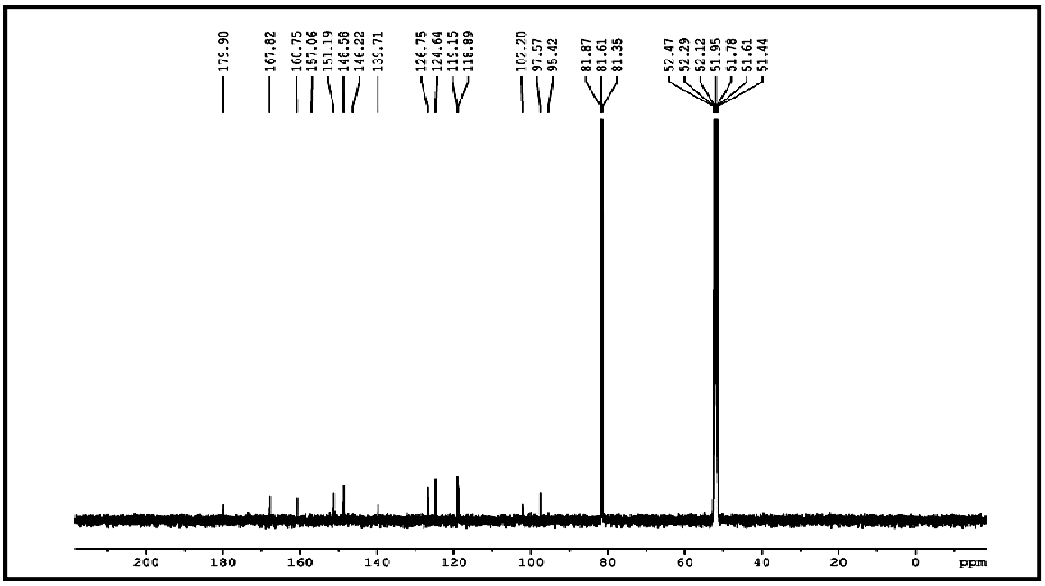


Figure 2.18: ^{13}C NMR spectrum of compound **IV**

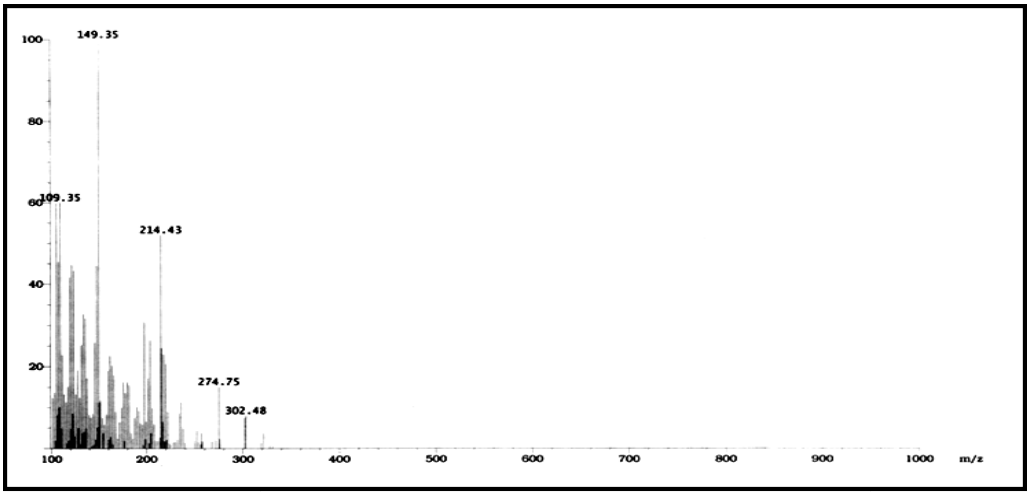
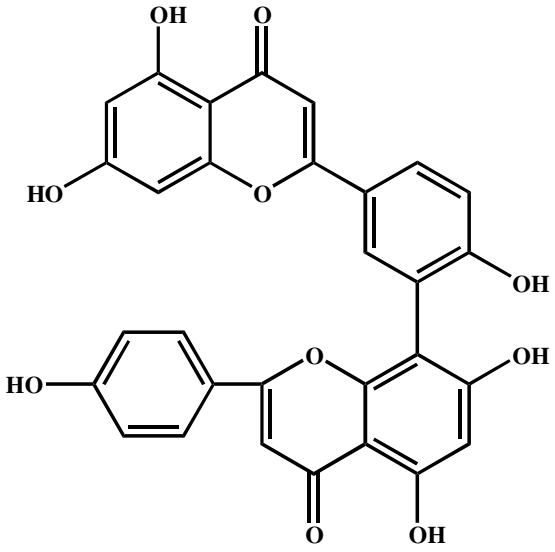


Figure 2.19: Mass spectrum of compound **IV**

The seventh fraction pool (158-171; 1.17 g) indicated another uv active spot in TLC which on column chromatography separation with hexane-ethyl acetate mixture (50:50-40:60) yielded amorphous yellow powder of compound **V** (15 mg, m.p: 255-256°C) which responded positively to Shinoda test imparted an orange colour indicated the presence of a flavonoid moiety. The uv spectrum (Figure 2.20) showed absorption

maxima at 335 and 269 nm in methanol. IR specrum showed characteristic peak at 3380 cm^{-1} indicates the presence of hydroxyl groups, carbonyl group at 1657 cm^{-1} and *p*-substituted phenyl ring at 947 cm^{-1}. The ^{1}H NMR spectrum (Figure 2.21) showed 6 phenolic hydroxyl groups at δ 10.18-9.62, 12.86 and 13.03 as singlets. A set of aromatic protons integrating for one proton were obtained as a singlet at δ 6.35, doublets at δ 6.12 (*J*= 2 *Hz*), 6.39 (*J*= 1.5 Hz), 7.13 (*J*= 8.5 Hz) and 7.96 (*J*= 2 Hz); doublet of a doublet at δ 7.90 (J_1= 2.0, J_2= 8.5 Hz). Another aromatic protons integrating for two protons were obtained at δ 6.71 (*J*= 8.5 Hz) and δ 7.52 (*J*= 8.5 Hz) as doublets. In the ^{13}C NMR spectrum (Figure 2.22), the signal at δ 182.13 and 181.73 confirmed the presence of two carbonyl carbons. The signal at δ 163.86 and 163.81 could be attributed to C2″ and C2 whereas the signal at δ 102.74 and 102.20 corresponds to C3″ and C3 carbon adjacent to the carbonyl group. Furthermore, the ^{13}C NMR spectrum clearly showed 30 carbon signals at δ 182.13, 181.73, 164.13, 163.86, 163.81, 161.83, 161.51, 161.19, 160.69, 159.08, 157.44, 154.69, 131.12, 127.72 (2C), 127.31, 121.68, 121.65, 119.74, 115.95, 115.36 (2C), 103.89, 103.77, 103.29, 102.74, 102.20, 98.41, 98.33 and 93.43. The mass spectrum of compound **V** gave the molecular ion peak at m/z 539.47 $[M+H]^+$ (Figure 2.23), from which the molecular formula was found to be $C_{30}H_{18}O_{10}$. From all the above data as well as comparison with the literature reports (Yun *et al*., 2003) compound **V** was found to be a biflavonoid, **amentoflavone**. It has been previously reported from *Cyperus roundus* (Xu, 2010), which posses antiinflammatory (Hee *et al*., 1998) property and can be able to suppress UVB-induced matrix metalloproteinase-1 (MMP-1) expression in normal human fibroblasts (NHF) (Lee *et al*., 2012).



V

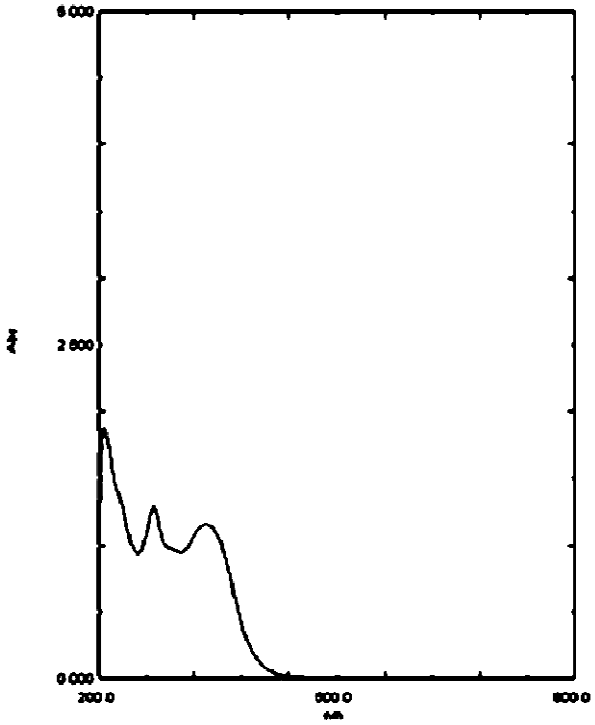


Figure 2.20: UV spectrum of compound **V**

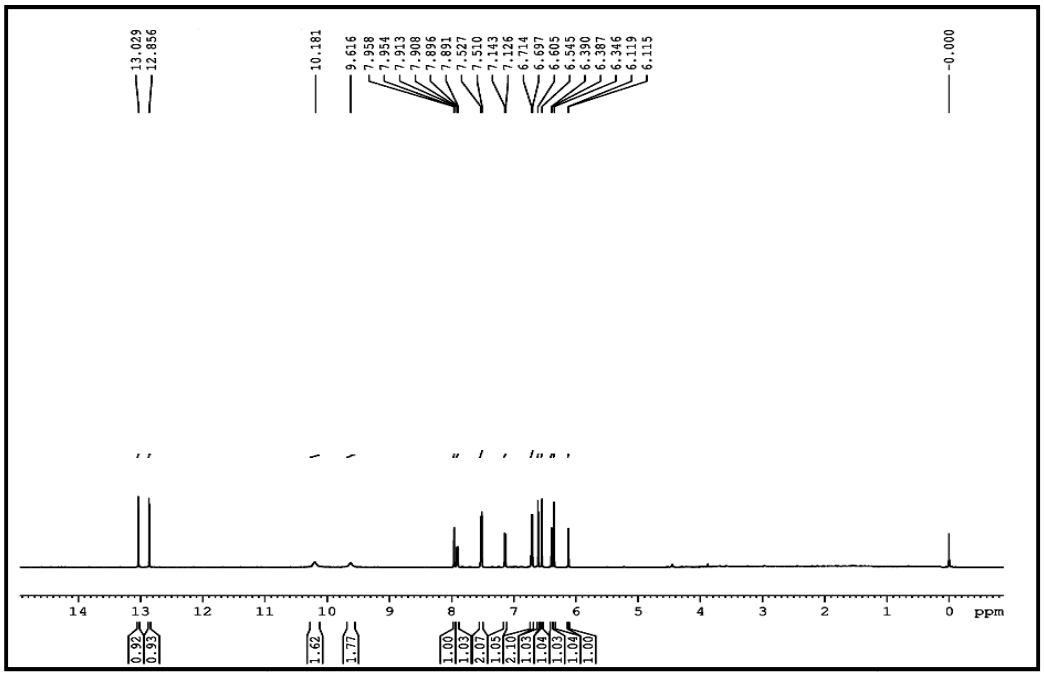


Figure 2.21: ^{1}H NMR spectrum of compound **V**

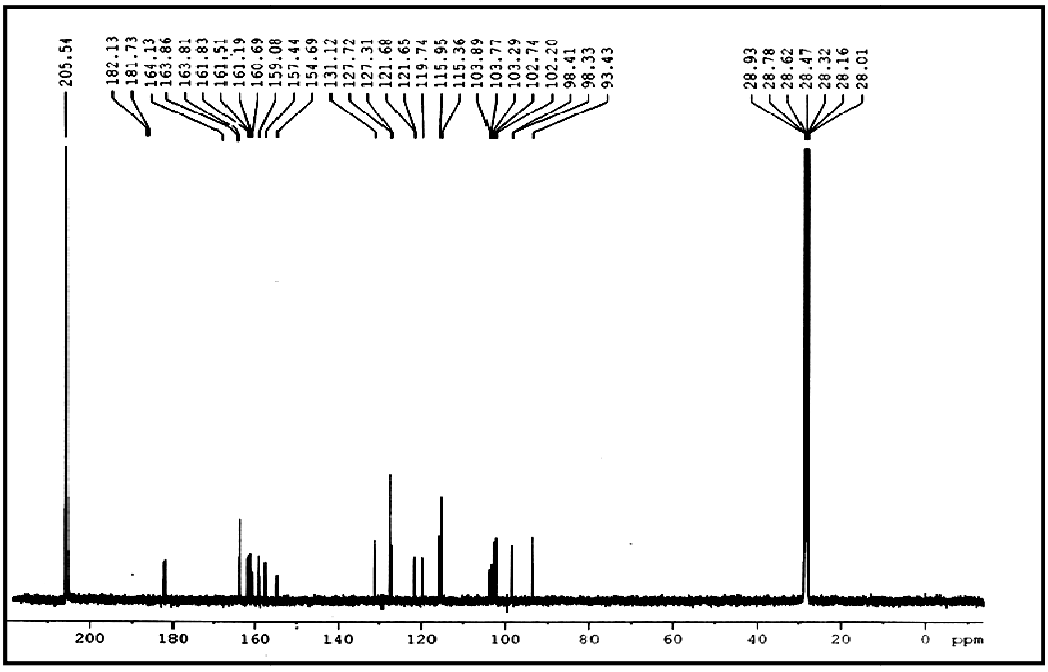


Figure 2.22: ^{13}C NMR spectrum of compound **V**

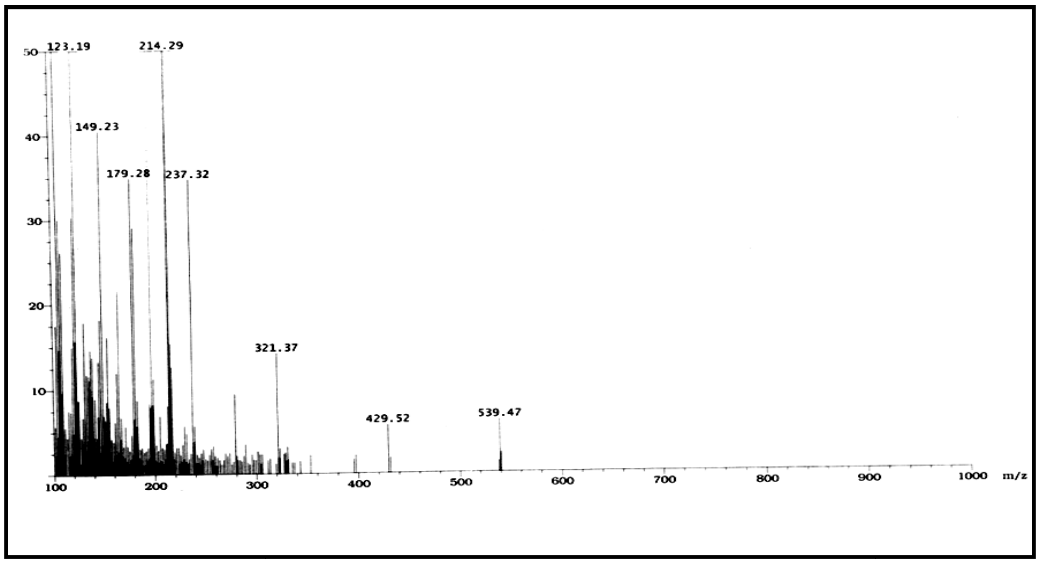


Figure 2.23: Mass spectrum of compound **V**

2.5.3. HPTLC and RP-HPLC analysis of sesquiterpenoids

2.5.3.1. Optimization of HPTLC chromatographic conditions

Different concentrations (0.1, 0.5 and 1 µg/ml) of solavetivone, aristolone and nootkatone along with acetone extract of *C. rotundus* were spotted in an HPTLC plate and developed with mobile phases of different polarities. The mobile system of 30% ethyl acetate-hexane resulted in sharp, symmetric and well resolved peaks at a wavelength of 245 nm and an R_f value of 0.77 for solavetivone, 0.70 for aristolone and 0.55 for nootkatone. Since the R_f values of solavetivone and aristolone are close, they seem to be merged in the chromatogram. The HPTLC chromatograms of the compounds recorded at 245 nm is shown in figure 2.24. The calibration curve was found to be linear. Peak area and concentration were subjected to linear regression analysis to calculate the calibration equation and correlation coefficients. The peaks corresponding to solavetivone, aristolone and nootkatone in the HPTLC profile of acetone extract was identified by comparing its R_f values and spectrum. The amounts of solavetivone,

aristolone and nootkatone present in the plant material are 0.2, 0.4 and 0.5% respectively (Table 2.1).

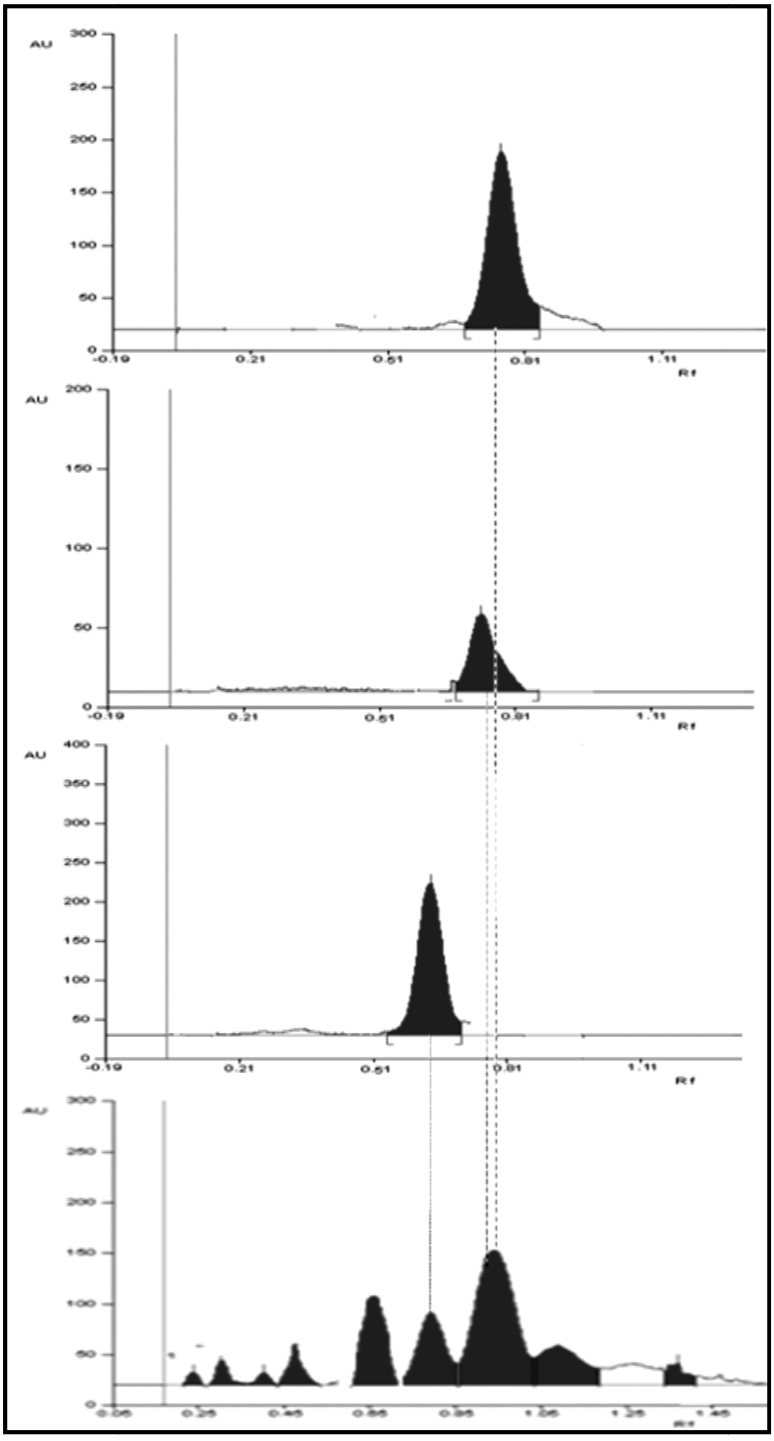


Figure 2.24: HPTLC chromatogram of solavetivone, artistolone, nootkatone and acetone extract

2.5.3.2. RP-HPLC-UV analysis of solavetivone, aristolone and nootkatone

A simple isocratic program was used to elute three sesquiterpenoids in a single run within a reasonable period of time. In order to get reproducible retention time, prior to next injection, the column was solvent conditioned by passing the initial solvent through the column until the baseline get stabilized. Acetonitrile and water mixtures with varying ratios were tried as mobile phases using reverse phase column for profiling the isolated compounds and acetone extract. An isocratic mobile phase of acetonitrile–water (60:40) was optimized so as to obtain a complete sesquiterpenoid profile. The spectra of sesquiterpenoids were obtained by using a UV detector. As maximally efficient detection can be obtained by selecting the wavelength where the compound has the maximum absorption and the compounds were detected at a wavelength of 245 nm. With this optimized conditions solavetivone was found to elute at retention time of 10.51 min, aristolone at 8.87 min and nootkatone at 8.78 min as a symmetric and well resolved peak (Figure 2.25). The amounts of solavetivone, aristolone and nootkatone were quantified as 0.2, 0.4 and 0.5% respectively in plant material (Table 2.1). The method selectivity was assessed by evaluating the similarity (≥ 95%) between UV spectra at start, middle and end of the compound peaks.

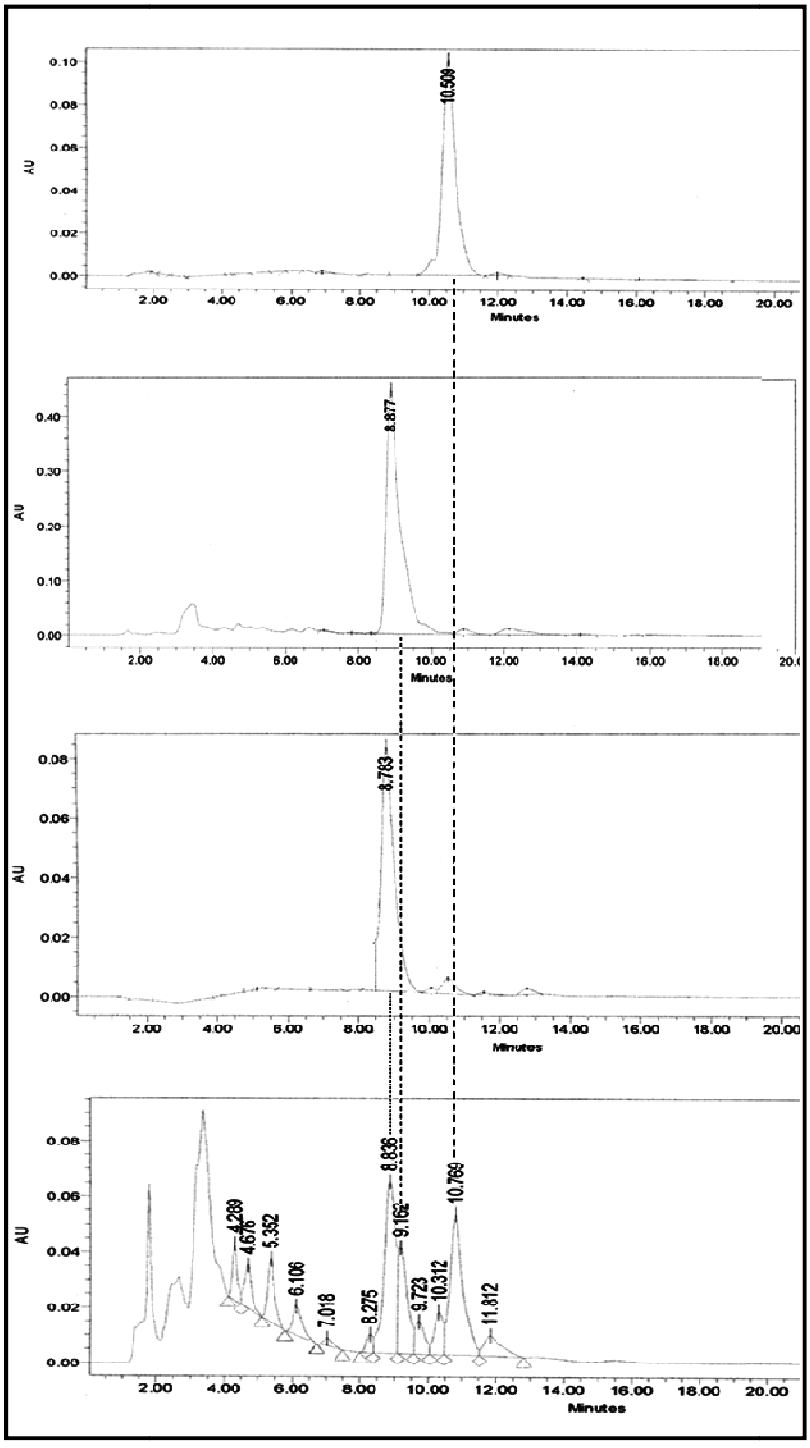


Figure 2.25: HPLC chromatogram of solavetivone, aristolone, nootkatone and acetone extract

2.5.4. Validation of chromatographic methods

The developed HPTLC and HPLC methods were validated in terms of their linearity, limit of detection and quantification, precision and accuracy.

2.5.4.1. Linearity

Linearity between the detector responses and concentration of solavetivone, aristolone and nootkatone in HPTLC and HPLC (0.1-1 µg/ml) were evaluated. All the isolated compounds show a correlation coefficient within the range of r = 0.98. The amount of each isolated sesquiterpenoid in plant material, correlation coefficient, LOD and LOQ of the methods etc are shown in Table 2.1.

Table 2.1: Amount in plant material, linearity, limits of detection and quantification of HPTLC and HPLC analysis of solavetivone, aristolone and nootkatone

Parameters	Solavetivone		Aristolone		Nootkatone	
	HPTLC	**HPLC**	**HPTLC**	**HPLC**	**HPTLC**	**HPLC**
Amount present in plant material	0.2%	0.2 %	0.4%	0.4%	0.5%	0.5%
Regression equation[a)]	y=1660 +2562x	y=55479.98 +1080000x	y=156.79 +2808.7x	y=12446400x - 2114330	y=292.27 +450.98x	y=20070800x - 2857550
Linear range ($\mu g ml^{-1}$)	0.5 - 1	0.1 - 1	0.5 - 1	0.5 - 1	0.1 - 0.5	0.1 - 0.5
Correlation coefficient (r)	0.9846	0.9889	0.9809	0.9910	0.9914	0.9984
LOD ($\mu g ml^{-1}$)	1.08	0.60	0.79	0.98	0.23	0.46
LOQ ($\mu g ml^{-1}$)	3.56	2.02	2.61	3.23	0.79	1.52

[a)]y=peak area, x= amount of substance added (µg)

2.5.4.2. Precision

Instrumental, intra and inter assay precision are presented in terms of % RSD. The relative standard deviation (RSD) was taken as a measure of precision. The intraday and inter day precisions for the R_T and the peak areas by the method are summarized in Table 2.2. The RSD was found to be low and inter and intraday precision were satisfactory for all analytes. The values were in acceptable range.

Table 2.2: Precision (RSD %) of sesquiterpenoids

Precision	**Solavetivone**		**Aristolone**		**Nootkatone**	
	HPTLC	**HPLC**	**HPTLC**	**HPLC**	**HPTLC**	**HPLC**
Instrumental	0.76	0.99	0.98	1.01	1.01	1.23
Repeatability	0.92	1.38	1.14	1.84	1.13	1.71
Intermediate precision	1.67	1.67	1.96	1.49	1.97	1.58

2.5.4.3. Recovery

To verify the accuracy of the method, recovery studies were performed by the method of addition of known amounts of isolated compounds to extract solution. Various concentrations (10-100%) of solavetivone, aristolone and nootkatone were added to previously analyzed extract solution (5 mg/ml) and the recovery was calculated and it was repeated three times. A reliable accuracy (99.2-105.6%) was shown by this method (Table 2.3). It can be seen that the proposed method has an adequate degree of accuracy for the determination of sesquiterpenoids.

Table 2.3: Recovery studies on solavetivone, aristolone and nootkatone

Parameter	Concentration (%)	% Recovery	
		HPTLC	HPLC
Excess of solavetivone added	10	99.8	104.6
	30	101.7	100.6
	100	103.6	101.8
Excess of aristolone added	10	99.2	101.2
	30	102.4	99.8
	100	104.8	102.8
Excess of nootkatone added	10	100.8	99.9
	30	102.7	103.4
	100	105.6	102.9

2.5.4.4. HPTLC vs HPLC

For five pairs of analysis the T_{obs} was 1.71 which was found to be lower than the value obtained from student's distribution table for a risk factor of 5%. The results showed that there is no statistically significant difference between HPTLC and HPLC analytical methods.

2.5.5. Radical scavenging and antioxidant potential of sesquiterpenoids

As discussed in the introductory chapter, antioxidants are a wide group of biologically active molecules. A number of screening tests including analytical methods are necessary for the evaluation of the effectiveness of antioxidants in a sample. During metabolic processes, a number of deleterious free radicals and reactive oxygen species

(ROS) are produced in the human body. A better antioxidant can scavenge all these free radicals and ROS. As a result, different methods are necessary to evaluate the antioxidant effects. Total antioxidant activity and DPPH radical scavenging potential were screened for comparing the activities of isolated sesquiterpenoids along with acetone extract and are discussed in the following sections.

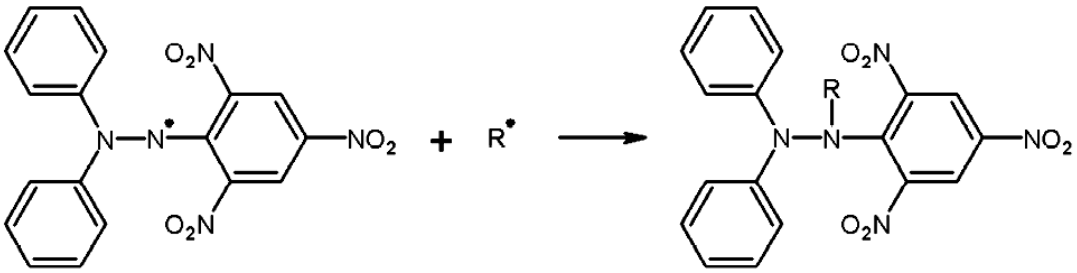
2.5.5.1. DPPH radical scavenging activity

DPPH• scavenging assay is routinely practiced for the assessment of free radical scavenging potential of an antioxidant molecule and considered as one of the standard and easy colorimetric methods for the evaluation of antioxidant properties of pure compounds. This method has been used extensively to predict the antioxidant activity because of the relatively short time required for analysis. DPPH is a stable free radical which accepts one electron or hydrogen radical to become a diamagnetic molecule. The reduction capacity of DPPH radicals was determined by the decrease in its absorbance at 517 nm (Sanchez, 2002). The radical scavenging capacity is expressed in terms of IC_{50} value which can be defined as the concentration of an antioxidant required to decrease the free radical concentration by 50% (IC_{50}) (Kanner *et al*., 1994). In DPPH• assay, the antioxidants reduce DPPH• to yellow coloured diphenyl picryl hydrazine and the extent of the reaction will depend on the antioxidant hydrogen donating ability (Scheme 2.1).

$$[DPPH]^{\cdot} + (AH) \longrightarrow DPPH\text{-}H + A^{\cdot}$$

$$[DPPH]^{\cdot} + A^{\cdot} \longrightarrow DPPH\text{-}A$$

$$A^{\cdot} + A^{\cdot} \longrightarrow A\text{-}A$$



DPPH• (Purple) **DPPH** (Yellow)

Scheme 2.1: Reaction of antioxidant with DPPH free radical

Radical scavenging potential of the isolated compounds and extract was compared with standard gallic acid, BHT and BHA. A low value of IC_{50} or a high percentage of radical scavenging activity indicates strong antioxidant activity. Among the three sesquiterpenoids isolated, the potent radical scavenger was nootkatone (IC_{50} 22.01 nM) preceeded by aristolone (IC_{50} 24.22 nM) and solavetivone (IC_{50} 31.28 nM) compared with standard gallic acid (IC_{50} 8.76 nM), BHT (16.96 nM) and BHA (15.58 nM). Acetone extract also showed good radical scavenging potential. Figure 2.26 shows the plot of concentration of each sample against the %DPPH radical scavenging capacity and it is clear that the isolated compounds showed a concentration dependent scavenging capacity by quenching DPPH free radicals.

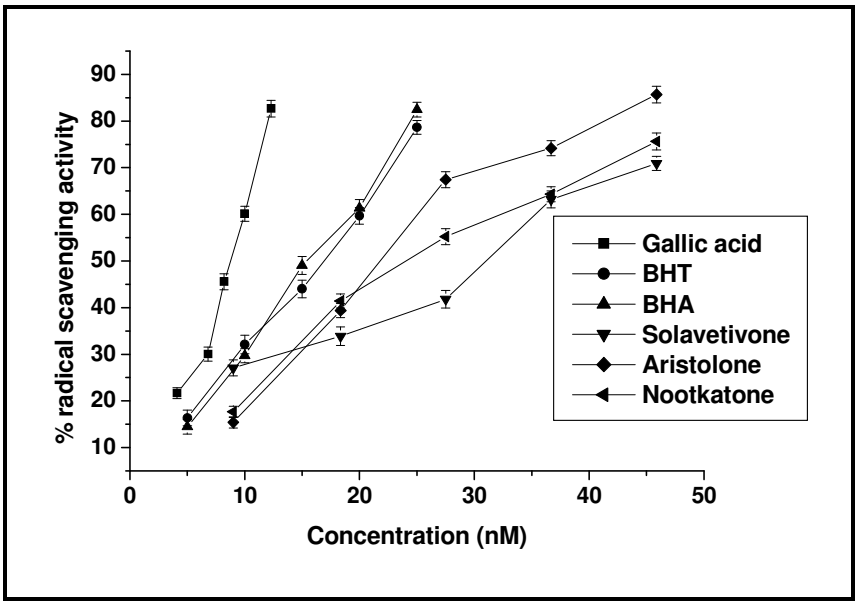


Figure 2.26: DPPH radical scavenging capacity of sesquiterpenoids at different concentrations

2.5.5.2. Total antioxidant activity by phosphomolybdenum method

The antioxidant capacity of the compounds was measured spectrophotometrically through phosphomolybdenum method (Prieto *et al*., 1999), which was based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate Mo (V) compounds with a maximum absorption at 695 nm. A higher absorbance indicates higher antioxidant activity. It is a quantitative method in which the total antioxidant activity is expressed as gram equivalents of ascorbic acid. The antioxidant activities of isolated compounds and extract ranked in the order nootkatone > acetone extract > aristolone > solavetivone on the basis of absorbance. The results also showed that nootkatone posses a total antioxidant capacity of 1.58 ± 0.2 g ascorbic acid equivalents/100 g dry weight of rhizomes, acetone extract showed 1.52 ± 0.09 g ascorbic acid equivalents/100 g dry weight of rhizomes, aristolone showed 0.62 ± 0.01 g ascorbic acid equivalents/100 g dry weight of rhizomes and solavetivone showed 0.28 ± 0.03 g

ascorbic acid equivalents/100 g dry weight of rhizomes. From the results obtained, nootkatone posses more antioxidant potential. In phosphomolybdenum assay hydrogen and electron transfer occurs from antioxidant to Mo (VI) complex. The data presented here indicated that the antioxidant activity of sesquiterpenoids may act in a similar fashion as reductones by donating the electrons and reacting with free radicals to convert them to more stable product and terminate free radical chain reaction (Gordon, 19901).

REFERENCES

1. Aarti, C.; Anil, U.; Shukla. R. N. *J. Pharm. Biomed. Sci*. **2012**, *25*, 179-181.
2. Abdel-Mogib, M.; Basaif, S. A.; Ezmirly, S.T. *Pharmazie*, **2000**, *55*, 693-695.
3. Abdel-Razik, A. F.; Nassar, M. I.; El-Khrisy, E.D.A.; Dawidar, A. A. M.; Mabry, T. J. *Fitoterapia*, **2005**, *76*, 762-764.
4. Abdenour, A. O.; Susana, L.; Abdelhay, A.; Amin, L.; Carmen, R.; Antonio, H.; Rafael, P.; Pilar, C. *Food Res. Int.* **2012**, *45*, 313–319.
5. Abigail, J. L.; David, S. J.; Thunder. *J. Am. Soc. Nutr. Adv. Nutr*. **2012**, *3*, 39–46.
6. Akiyoshi, S.; Takane, F.; Kunio, K. *Phytochemistry,* **1982**, *21*, 2986-2987.
7. Anonymous. The Wealth of India. New Delhi; Council of Scientific and Industrial Research: **2001**, *2*, 334.
8. Anshu, R.; Vartika, R.; Sayyada, K.; Subha, R.; Ajay Kumar Singh, R.; Shanta, M.; Pandey, M.M. *Indian J. Trad. Knowledge* **2010**, *9*, 576-580.
9. Antonio, D.; Eliseo, R.; Paola, S.; Antonia, S.; Ildefonsa. S. A. *Biochem. Syst. Ecol.* **1993**, *21*, 305.
10. Anupam, B.; Bisht, G. R. S.; Mamta, S.; Richa, G.; Vinod, S. *Int. J. Res. Pharm. Biomed. Sci*. **2011**, *2*, 661-665.

11. Boulos. Reference Publications, Algonac **1983**, *286*, 82.

12. Brand-Williams, W.; Cuvelier, M. E.; Berset, C. *Lebens. Wissen. Tech*. **1995**, *28*, 25-30.

13. Carboni, S.; Da Settimo, A.; Malaguzzi, V.; Marsili, A; Pacini, P. L. *Tetrahed. Lett*. **1965**, 3017-3021.

14. Chatterjee, A.; Pakrashi, S. C. The Treatise on Indian Medicinal Plants. New Delhi; National Institute of Science Communication and Information Resources, Council of Scientific and Industrial Research: **2009**, *6*, 40.

15. Coxon, D. T.; Price, K. R.; Howard, B.; Osman, S. F.; Kalan, E. B.; Zacharius, R. M. *Tetrahed. Lett*. **1974**, *34*, 2921-2924.

16. Duan. Y.; Liu. C.; Luo. Y.; Guo. B.; Cao. Z. *Zhangguo Yaoxne Zazhi* **1993**; *28*, 396.

17. Edward, P.; Ronald, W. B. William, D.W. *Canad. J. Chem*. **1969**, *47*, 831-840.

18. El-Habashy, I.; Mansour, R. M. A., Zahran M. A., El-Hadidi, M. N., Saleh N. A. M. *Biochem. Syst. Ecol*. **1989**, *17*, 191-195.

19. Eun, J. S.; Dong, U. L.; Jong, H. K. Sun, M. L.; Yeong, S. K.; Yi-Sook, J. *J. Ethnopharmacol*. **2011**, 135, 48–54.

20. Fengming, X.; Toshio, M.; Hisashi, M.; Kiyafumi, N.; Masayuki. Y. *J. Nat. Prod*. **2004**, *67*, 569.

21. Fujimori, T.; Kasuga, R.; Kaneko, H.; Noguchi, M. *Phytochemistry* **1977**, *16*, 392-394.

22. Gordon, M. F. In Food Antioxidants; Hudson, B. J. F., Ed.; Elsevier Applied Science: London, U.K., **1990l,** 1-18.

23. Gupta, M. B.; Palit, T. K.; Singh, N.; Bhargawa, K. P. *Indian J. Med. Res*. **1971**, *59*, 76-82.

24. Hanaa, M. S.; Mahmoud, H. M.; Salwa, F. F.; Gamal, A. M.; Peter, P. *Nat. Prod. Res*. **2007**, *21*, 343–350.

25. Harborne, J. B. *Phytochemistry* **1971**, *10*, 1569-1574.

26. Hee, K. K.; Kun, H. S.; Hyeun, W. C.; Sam, S. K.; Hyun, P. K. *Arch. Pharm. Res*. **1998**, *21*, 406-410.

27. Heo, H. J.; Lee, C.Y. *J. Agri. Food Chem*. **2005**, *53*, 1984-1989.

28. Jeong, J.; Miyamoto, T.; Inagaki, M.; Kim, Y.C.; Higuchi, R. *J. Nat. Prod*. **2000**, *63*, 673-675.

29. Jun, L. Y.; Yan, P. S. *Planta Med*. **2012**, *78*, 59–64.

30. Kabiruddin, H., Makhzan ul M. (Kitabul Advia). New Delhi; Idara Kitab us Shifa: **2010**, 583-584.

31. Kanner, J.; Frankel, E.; Granit, R.; German, B.; Kinsella, J. E. *J. Agri. Food Chem.* **1994**, *42*, 64-69.

32. Krepinsky, J.; Motl, O.; Dolejs, L.; Novotny, L.; Herout,V.; Bates, R. B. *Tetrahedron Lett.*, **1968**, *29*, 3315-3318.

33. Lazarevic, J.; Radulovic, N.; Palic, R.; Zlatkovic, B. *J. Ess. Oil Res*. **2010**, *22*, 578-581.

34. Lee, C. W.; Na, Y.; Park, N. H.; Kim, H. S.; Ahn, S. M.; Kim, J. W.; Kim, H. K.; Jang, Y. P. *Appl. Biochem. Biotechnol*. **2012**, *166*, 1137-47.

35. Lobstein-Guth, A.; Briacon-Schied, F.; Victoire, C.; Haag-Berrurier, M. *Planta Med*. **1988**, *55*, 555.

36. Markham, K. R.; Mabry, T. J. In *The Flavonoids*, Harborne, J. B.; Mabry, T. J.; Mabry, H. (Eds.), Chapman and Hall, London, **1975,** 45-77.

37. Miyazawa, M.; Nakamura, Y.; Ishikawa, Y. *J. Agric. Food Chem.* **2000**, *48*, 3639-3641.

38. Morikawa, T.; Xu, F.; Matsuda, H.; Yashikawa. M. *Heterocycles*, **2002**, *57*, 1983-1988.

39. Ngo B. E.; Schmutz, M.; Meyer, C.; Rakotonirina, A.; Bopelet, M.; Portet, C.; Jeker, A.; Rakotonirina, S.V.; Olpe, H. R.; Herrling, P. *J. Ethnopharmacol.* **2001**, *76*, 145–150.

40. Oladipupo, A.; Lawal; Adebola, Oyedeji O. *Molecules* **2009**, *14,* 2909-2917.

41. Pei, L.; Li, L.; Yu, P. T..; Jin, A. D.; Nian, Y.Y. *Chin. Chem. Lett*. **2010**, *21*, 606–609.

42. Prieto, P.; Pineda, M.; Aguilar, M. *Anal. Biochem*. **1999**, *269*, 337-341.

43. Sanchez, C.M. *Food Sci. Tech. Int.* **2002**, *8*, 121-137.

44. Sayed, H. M.; Mohamed, M. H.; Farag, S. F.; Mohamed, G. A.; Ebel, R.; Omobuwajo, O. R. M.; Proksch, P. *Bull. Pharm. Sci*. Assiut University, **2006**, *29*, 9-32.

45. Schultes, R. E.; Raffauf, R. F. The Healing Forest: Medicinal and toxic plants of the Northwest Amazonia. Dioscorides Press, Portland, **1990**, 156.

46. Seo, W. G.; Pae, H. O.; Oh, G.S.; Chai, K.Y.; Kwon, T.O.; Yun, Y.G.; Kim, N.Y.; Chung, H.T. *J. Ethnopharmacol*. **2001**, *76*, 59-64.

47. Seung H. J.; Su, J. K.; Bo-Gyu, J.; Kyung-Tae, L.; Seon-Pyo, H.; Myung Sook, O.; Dae Sik, J.; Jung-Hye, C. *J. Ethnopharmacol*. **2013**,*147*, 208–214.

48. Soumaya, K.; Jerome, L.; Ines, B.; Mohamed, B. S.; Jihed, B.; Ines, S.; Ridha, M.; Kamel, G.; Daniel, B.; Leila, C. G. *Chem. Biodiversity* **2008**, *5*, 729-742.

49. Soumaya, K.; Mohamed, B. S.; Ilef, L.; Ines, B.; Jihed, B.; Wissem, B.; Ines, S.; Aicha, N.; Ribai, B.; Marie, G. D. F.; Kamel, G.; Leila, C. G. *Bioresource Tech*. **2008**, *99*, 9004-9008.

50. Subrahmanyam Naidu, P.V.; Prakash. M. M. S. Kinthada; Kalyani P.; Muralidhar, P. *Int. J. Pharm. Biomed. Sci.* **2012**, *3*, 24-27.

51. Taylor, T. M. C. British Columbia Provincial Museum, The Sedge family (Cyperaceae) *Science*, **1983**, 375.

52. Thebtaranonth, C.; Thebtaranonth, Y.; Wanauppathamkul, S.; Yuthavong, Y. *Phytochemistry* **1995**, *40*, 125-128.

53. Trivedi, V. P.; Mann, A. S. Q. *J. Crude Drug Res*. **1972**, *12*, 1988-1999.

54. Uddin, S. J.; Mondal, K.; Shilpi, J. A.; Rahman, M.T.; *Fitoterapia* **2006**, *77*, 134-136.

55. Webb D. A, Tutin, T. G.; Heywood V. H., Burges N. A., Moore D. H., Valentine D. H.; Walters S. M., (Eds.) Flora Europae, University Press, Cambridge, UK **1980**, *5*, 284-288.

56. Weenen, H.; Nkunya, M. H. H.; Bray, D.H.; Mwasumbi, L. B.; Kinabo, L. S.; Kilimali, V. A. E. B.; Wijnberg, J. B. P. A. *Planta Med*. **1990**, *56*, 371-373.

57. Xu, Y.; Zhang, Z. Z.; Mou, Z. M. *Chin. Pharm. J*. **2010**, *45*, 818-821.

58. Yun, L. L.; Wan, Y.W. *Chin. Pharm. J*. **2003**, *55*, 71-75.

59. Zhu, M.; Luk, H. H.; Fung, H. S.; Luk, C. T. *Phytother. Res*. **1997**, *11*, 392-394.

60. Zoghbi, M.; Das G. B.; Andrade, E. H. A.; Carreira, L. M. M.; Rocha, E. A. S. *J. Ess. Oil Res*. **2008**, *20*, 42-46.

Chapter 3

PHYTOCHEMICAL INVESTIGATION AND SCREENING OF BIOLOGICAL POTENTIAL OF *Stereospermum colais*

This chapter deals with the studies on the roots of *Stereospermum colais*, a Bignoniaceae plant belonging to the genus *Stereospermum* which is used extensively in Ayurveda. The study of the chemical constituents of the roots, antioxidant and radical scavenging potential of acetone and methanol extracts, evaluation of the inhibition of enzymes by crude extracts as well as the individual compounds, screening of antiinflammatory and antimicrobial potential of isolated molecules etc. are discussed in the present chapter.

3.1. INTRODUCTION - Genus *Stereospermum*

Stereospermum belongs to the family Bignoniaceae is a genus of trees and many plants have been studied so far for various biological potentials. *Stereospermum* is one of the big genus in the family native to India and is used most commonly as an antiinflammatory and antimicrobial agent by traditional healers in Ayurveda and comprised of about 55 species worldwide (Chopra *et al*., 1956)

Steresopermum chelenoides is a large sized deciduous tree and distributed in the central parts of India. The decoction of the root is used as antipyretic and is useful in the treatment of asthma, cough and excessive thirst. Spectroscopic analysis led to the isolation of two quinones stereochenol A and stereochenol B, two naphthoquinones sterekunthal B and sterequinone C (Mohammed *et al*., 2006), *p*-hydroxy benzaldehyde

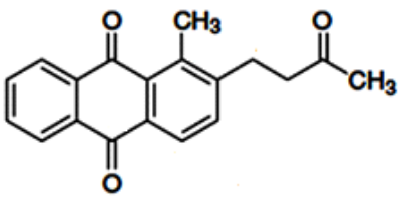
and stigmasterol from methanolic extract of stem bark of *S. chelenoides* (Mohammed *et al*., 2006).

Stereospermum personatum is used in the Ayurvedic Indian traditional system of medicine and widely advocated as diuretic, antiinflammatory, anticancer agents etc. The plant is also well known for its antimicrobial, antiprotozoal and antiinflammatory properties. Bioassay guided fractionation led to the isolation of anthraquinones sterequinones F-H (Sampath Kumar *et al*., 2003), a naphthoqinone sterequinone I, 2(4′-hydroxyphenyl) ethyl undecanoate, 2(4′-hydroxyphenyl) ethyl nonacosanoate, a new 3, 4, 5-trimethoxycinnamylether-2-methoxy-4-(3′-(3″,4″,5″-trimethoxyphenyl) allyloxymethyl) phenol, norvibutinal, lapachol, dehydro-R-lapachone, coniferaldehyde, sinapaldehyde, pinoresinol, 7′-hydroxydivanillyltetrahydrofuran, (-)-secoisolariciresinol, (-)-olivil and (+)-cycloolivil (Sampath Kumar *et al*., 2005). The components showed radical scavenging and xanthine oxidase inhibition potential.

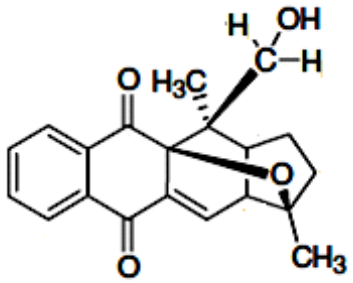
Stereospermum acuminatissimum is a tall tree found in tropical Africa and planted for ornamental purposes (Hutchinson, 1963). Phytochemical investigation on *Stereospermum acuminatissimum* stem bark resulted in the isolation of 2-(4′-hydroxyphenyl) ethyl dotriacontanoate, octacosan-1, 28-dioldiferulate and triacontan-1, 30-dioldiferulate, ursolic acid, pomolic acid, quinovic acid, oleanolic acid, (+)-cycloolivil, coniferaldehyde, 2-(4′-hydroxyphenyl) ethyl undecanoate, 2-(4′-hydroxyphenyl) ethylnonacosanoate, pinnatal, stereochenol B, sterekunthal B, sterequinone B, sterequinone F, sterequinone H, zenkequinone B, zenkequinone A, sterequinone C, norvibutinal, *p*-coumaric acid, methyl caffeate, caffeic acid, psilalic acid, syringaldehyde, specioside, verminoside, tyrosol, eutigoside, ellagic acid, atranorin etc.

(Kamdem Soup *et al*., 2012). The structures of isolated constituents of various *Stereospermum* species are given in chart 3.1.

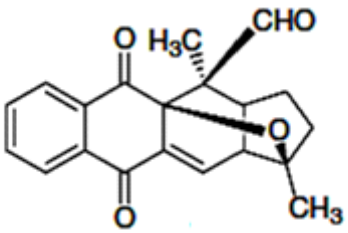
Chart 3.1: Phytochemical constituents of *Stereospermum* species



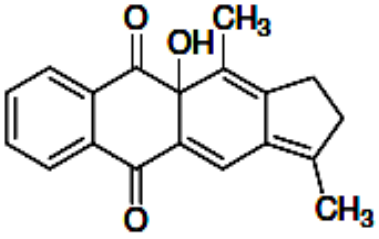
Stereochenol A



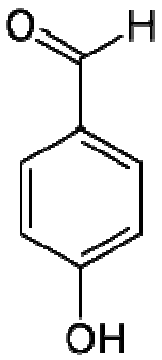
Stereochenol B



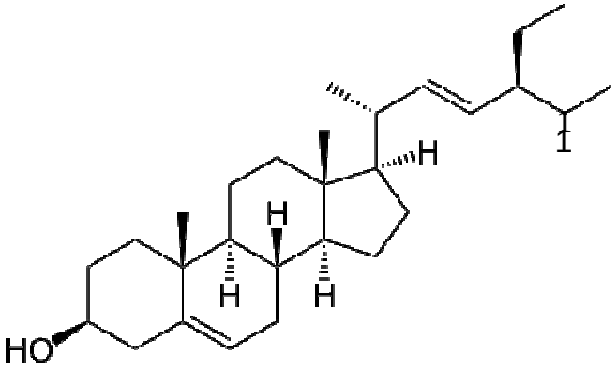
Sterekunthal B



Sterequinone C

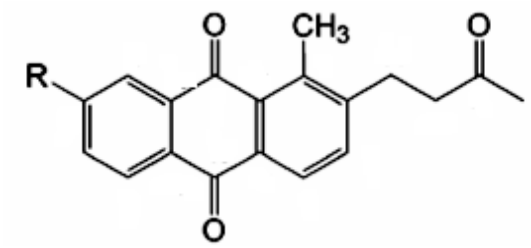


***p*-hydroxy benzaldehyde**

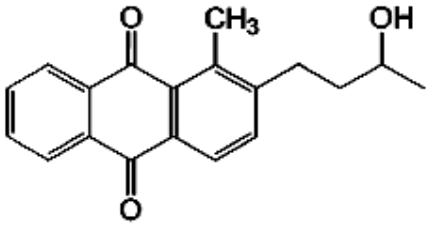


Stigmasterol

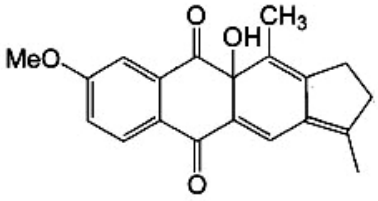
Chart 3.1. continued…. ..



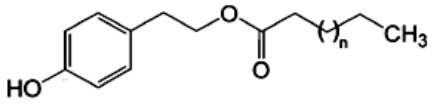
Sterequinone F, R= H
G, R= OCH_3



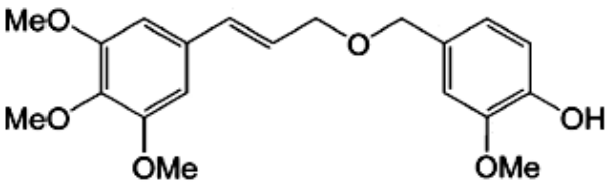
Sterequinone H



Sterequinone I

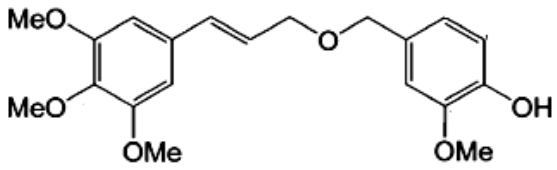


2(4′-hydroxyphenyl) ethyl undecanoate n= 7
2(4′-hydroxyphenyl) ethyl nonacosanoate n= 25

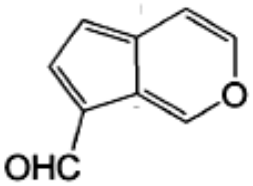


3, 4, 5-trimethoxycinnamyl ether

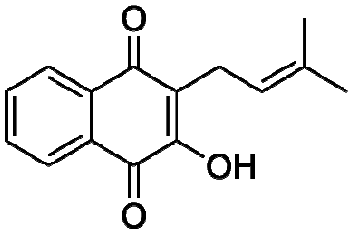
Chart 3.1.continued……



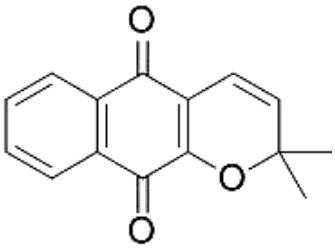
2-methoxy-4-(3′-(3″,4″,5″-trimethoxyphenyl) allyloxymethyl) phenol



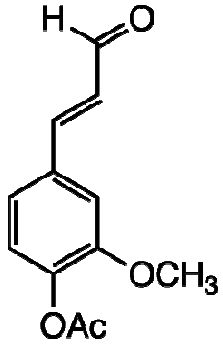
Norvibutinal



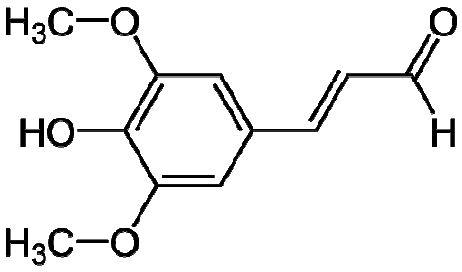
Lapachol



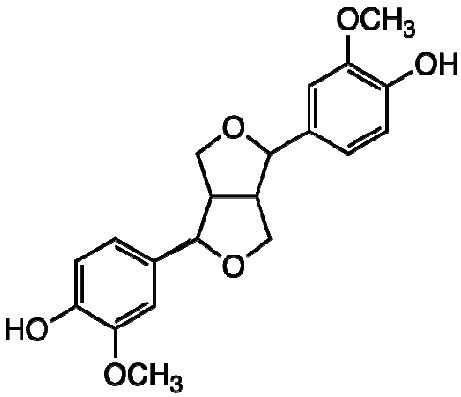
Dehydro-R-lapachone



Coniferaldehyde

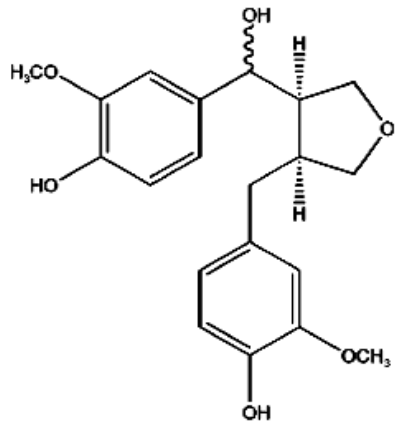


Sinapaldehyde

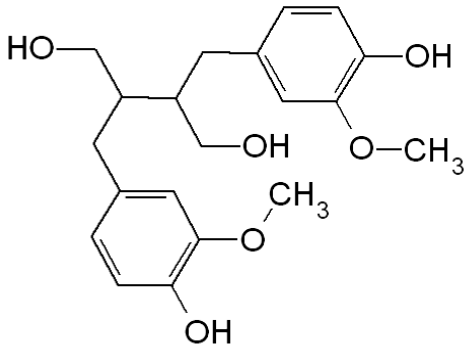


Pinoresinol

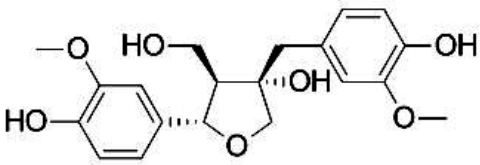
Chart 3.1.continued…. ..



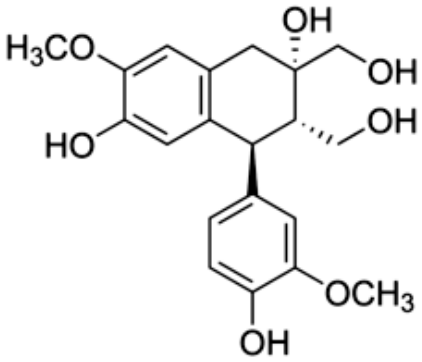
7′-hydroxydivanillyltetrahydro furan



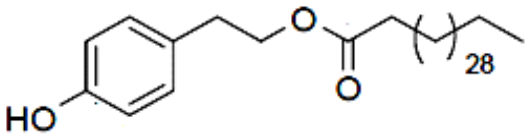
Secolarisciresinol



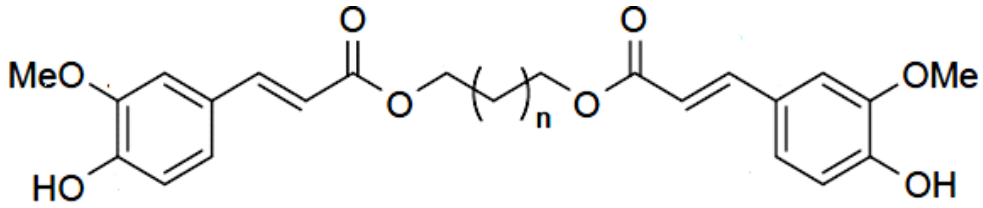
Olivil



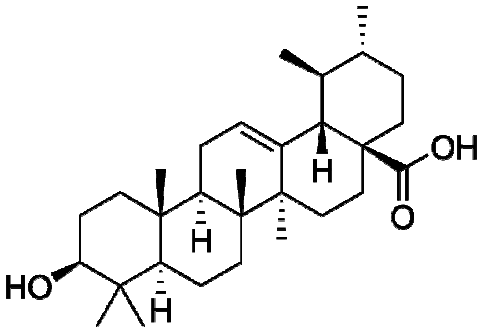
Cycloolivil



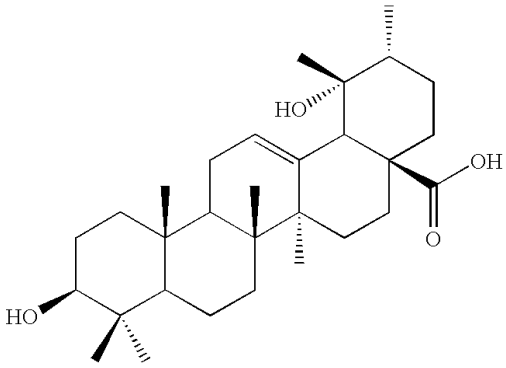
2-(4′-hydroxy phenyl) ethyl dotriacontanoate



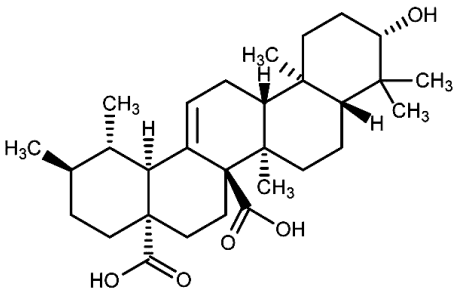
Octacosan- 1, 28-dioldiferulate n = 26
Triacontan- 1, 30-dioldiferulate n = 28



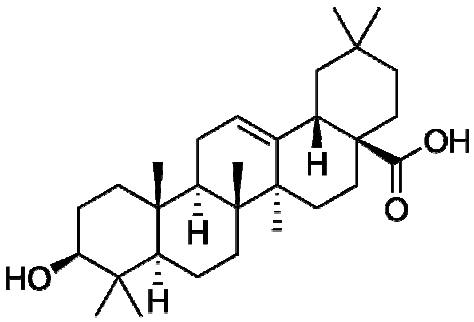
Ursolic acid



Pomolic acid

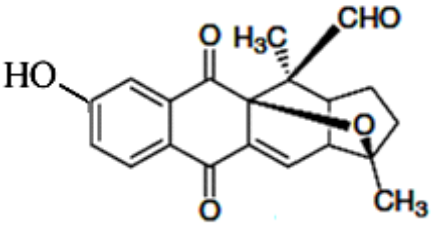


Quinovic acid

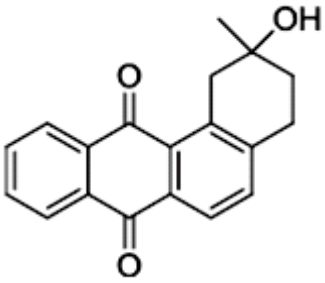


Oleanolic acid

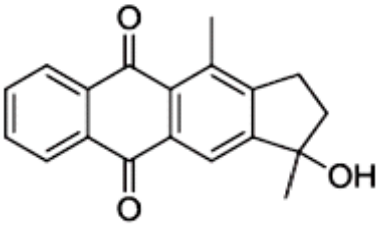
Chart 3.1. continued….. .



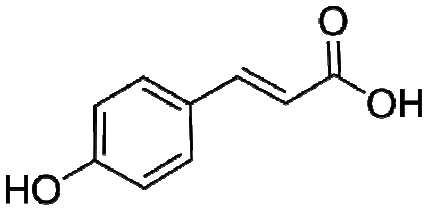
Pinnatal



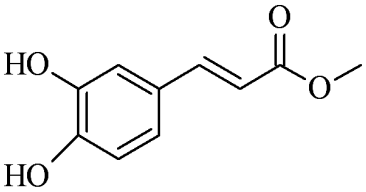
Zenkequinone B



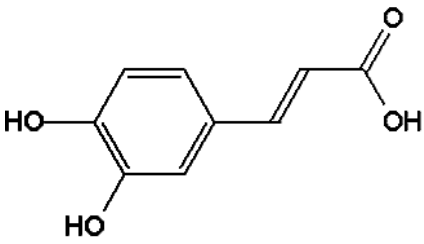
Zenkequinone A



***p*-Coumaric acid**

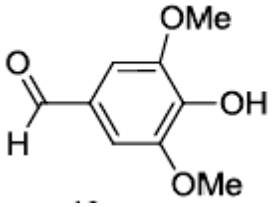


Methyl caffeate

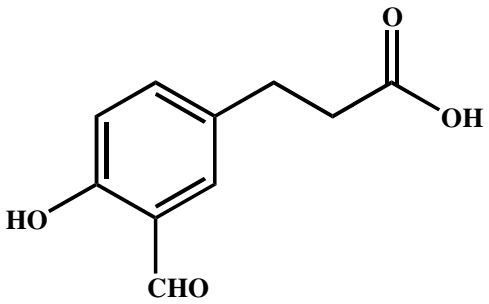


Caffeic acid

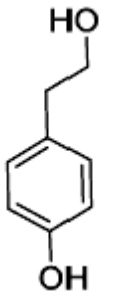
Chart 3.1. continued……



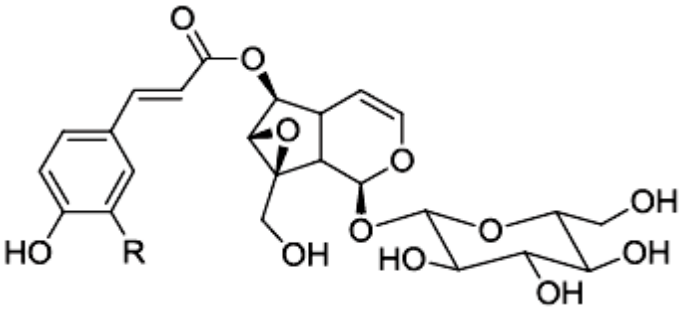
Syringaldehyde



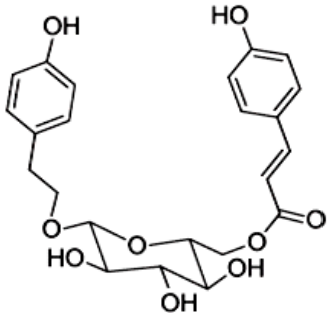
Psilalic acid



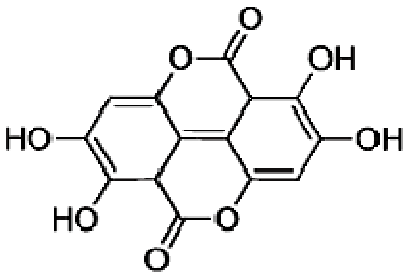
Tyrosol



Specioside R = H
Verminoside R = OH

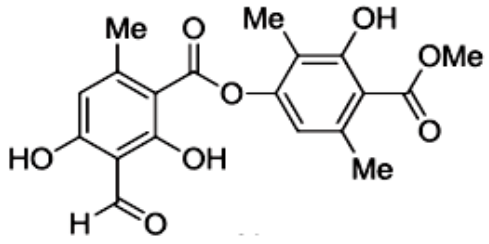


Eutigoside



Ellagic acid

Chart 3.1. continued……



Atranorin

3.2. AIM AND SCOPE OF THE PRESENT INVESTIGATION

Stereospermum colais (known as *pathiri* in Malayalam) is a large straight stemmed deciduous tree 18-30 m in height and 2.8 m in girth found throughout in moist regions of India up to an altitude of about 1200 m (Figure 3.1). The roots are brown, cylindrical, hard and heavy with transversely extended lenticels (Warrier, 1996). The root of this plant is used in reputed Dasamula; an ayurvedic formulation is a combination of ten medicinal plants of which mainly roots are employed. Each of the plants is endowed with incredible medicinal properties and they act synergistically in combination. The roots are bitter, diuretic, lithontriptic, expectorant, cardio tonic, aphrodisiac, antiinflammatory, antidiabetic, antibacterial, febrifuge and tonic. They are useful in vitiated conditions of vata, dyspepsia, haemorrhoids, hyper acidity etc. (Warrier, 1996).

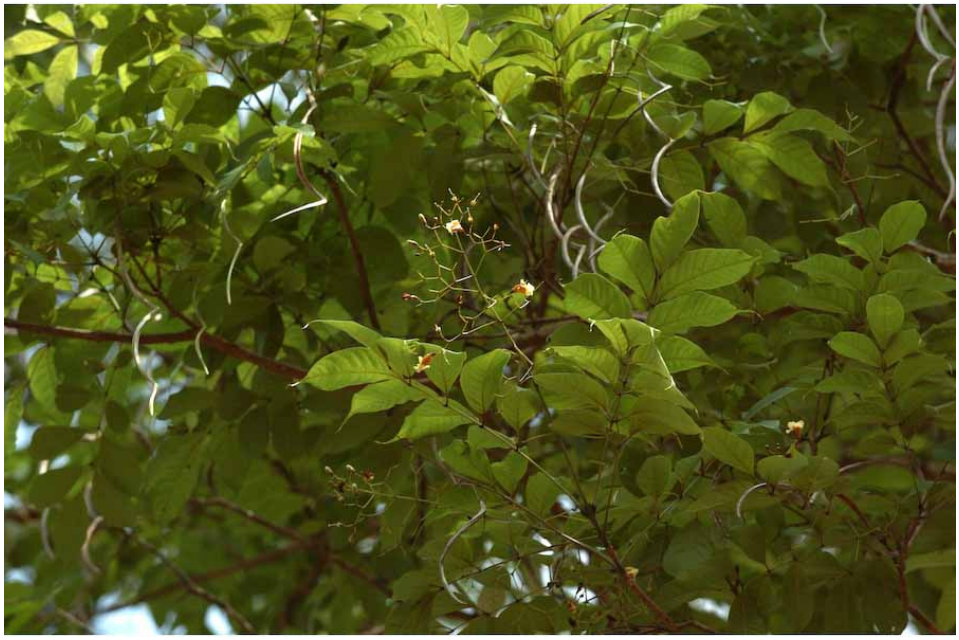


Figure 3.1: *Stereospermum colais* plant

3.2.1. *Stereospermum colais* – Literature survey

A few literature reports are available related to the scientific validation of the biological activities of *Stereospermum colais.* The plant extract reported to posses anticancer potential in Walker 256 tumor system (Ram & Mehrotra, 1993). Antioxidant and wound healing capacity studies on different extracts of *Stereospermum colais* were also reported (Vijaya Bharathi *et al*., 2010).

3.3. EXPERIMENTAL

3.3.1. Collection of plant material

Stereospermum colais roots (2.5 kg) were collected from local areas of Thiruvananthapuram, Kerala on April 2011 and were identified and authenticated by Dr. H. Biju (Taxonomist, JNTBGRI, Palode, Thiruvananthapuram, Kerala). A voucher specimen (042/2011) has been deposited in the herbarium for future reference. The roots

were cleaned and dried in an air oven at 50°C (moisture content 11%) and coarsely powdered.

3.3.2. Chemicals and reagents used

α-amylase from *Aspergillus oryzae*, α-glucosidase type 1 from Baker's yeast, dinitrosalicylic acid, 2, 2′-diphenyl-1-picryl hydrazyl (DPPH) radical, gallic acid, curcumin, xanthine oxidase, allopurinol, *p*-nitrophenyl-α-D-glucopyranoside (PNPG), albumin from bovine serum, ciprofloxacin, amphotericin B, nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), nicotinamide adenine dinucleotide (NADH), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide], xanthine, carrageenan, indomethacin etc. were purchased from Sigma Aldrich (St. Louis, MO, USA). Acarbose was obtained from Serva Electrophoresis GmbH (Mannheim, Germany). Tween 20 and ascorbic acid were purchased from Sarabhai Chemicals, Baroda. *p*-anisidine was purchased from SD Fine Chemical Pvt Ltd, Mumbai, India. Greiss reagent and ferrous chloride were bought from Fluka Chemical Pvt Ltd, Mumbai, India. Trichloro acetic acid (TCA), linoleic acid, sodium nitroprusside (SNP), sodium chloride, D-glucose, tris HCl, anhydrous sodium phosphate monobasic and anhydrous sodium phosphate dibasic etc. were purchased from Sisco Research Laboratories Pvt Ltd, Mumbai, India. Potassium ferricyanide was obtained from Central Drug House, New Delhi. All microbiological reagents were from Hi-Media Laboratories Limited, Mumbai, India. Butylated hydroxy toluene (BHT), ferric chloride and potassium phosphate buffer were purchased from Central Drug House, New Delhi, India. Refined sunflower oil was purchased locally. All other chemicals employed were of standard analytical grade.

3.3.3. General experimental details

General experimental procedures are as reported in chapter 2 of this thesis.

3.3.4. Essential oil composition of *Stereospermum colais* roots

Coarsely powdered roots of *S. colais* (100 g) were subjected to hydrodistillation using Clevenger type apparatus. The oil obtained was analysed on an analytical Shimadzu QP 5050A GC/MS and Hewlett Packard 5890 Gas chromatograph. Both GC and GC/MS were operated using the same conditions and the column used was fused silica capillary column (50 x 0.2 mm i.d., film thickness 0.25 μm) coated with cross linked methyl silicone. The oven temperature was kept at 80-200°C at the rate of 2°C/min. Flame Ionization Detector was used at a temperature of 300°C and injection temperature was 250°C. Helium was used as the carrier gas and ionization voltage used was 70 eV.

Components were identified by comparing the retention time of authentic samples, confirmed by comparing the retention indices and the mass spectra of the samples with standard library (NIST) and library generated in the laboratory (Davies, 1990; Jenning, 1980). The percentage composition of the identified constituents was computed from the GC peak area in methyl silicon column.

3.3.5. Extraction

Powdered roots (1.25 kg) were subjected to successive cold extraction using acetone and methanol as solvents at room temperature. Solvents were evaporated using rotary evaporator at 50°C under reduced pressure and yield of acetone (ASC) and methanol (MSC) extracts were calculated. Pure compounds were obtained from acetone extract using column chromatographic separation. Antioxidant studies were carried out using acetone and methanol extracts.

3.3.6. Antioxidant studies on acetone and methanol extracts of *S. colais*

Acetone (ASC) and methanol (MSC) extracts obtained from successive extraction were used for antioxidant evaluation using different assays.

3.3.6.1. Xanthine oxidase inhibition activity

Xanthine oxidase inhibitory activity was assayed spectrophotometrically under aerobic conditions (Owen & Johns, 1999). The assay mixture consisted of 1 ml of test solution containing varying concentration of standard allopurinol (4–15 μg/ml), ASC (0.6-4 μg/ml) and MSC (5-40 μg/ml), 2.9 ml of phosphate buffer (0.1 M, pH 7.5) and 0.1 ml of xanthine oxidase enzyme solution (0.01 units/ml in phosphate buffer, pH 7.5 which was prepared immediately before use). After pre-incubation at 25°C for 15 min, the reaction was initiated by the addition of 2 ml of substrate solution (150 mM xanthine in the same buffer). The assay mixture was incubated at 25°C for 30 min. The reaction was then stopped by the addition of 1 ml of 1 N hydrochloric acid and the absorbance was measured at 290 nm using a uv-visible (UV-VIS) spectrophotometer. A blank was also prepared in the same way, but the enzyme solution was added to the assay mixture after adding 1 N hydrochloric acid. The assay was done in triplicate. XOI activity was expressed as the percentage inhibition of XO in the above assay system and was calculated using equation 2.2. The % inhibition was plotted against the sample or standard concentration to obtain the amount of antioxidant necessary to decrease the initial concentration to 50% (IC_{50}). A lower IC_{50} value indicates greater antioxidant activity.

3.3.6.2. Determination of lipid peroxidation by linoleic acid emulsion system

The peroxidative effect of *Stereospermum colais* extracts in linoleic acid emulsion system was determined by the thiocyanate method (Duh *et al*., 1997). A concentration of 0.1 mg/ml of ASC and MSC in methanol was mixed with linoleic acid emulsion in potassium phosphate buffer (0.02 M, pH 7.0). Linoleic acid emulsion was prepared by mixing and homogenizing 155 µl linoleic acid, 175 µg Tween 20 as emulsifier and 50 ml 0.02 M phosphate buffer. The reaction mixture was incubated at 37°C. Aliquots of 0.1 ml were taken at various intervals during incubation from the mixture. The degree of oxidation was measured by sequentially adding ethanol (5 ml, 75% v/v), ammonium thiocyanate (0.01 ml, 30% w/v) and ferrous chloride (0.1 ml, 0.02 M in 3.5% HCl w/v) to sample solution (0.1 ml) and then noted the absorbance at 500 nm. Solutions without added extracts were used as blank samples. BHT was used as the standard. The degree of oxidation was measured every 24 h and the data were the average of triplicate analysis. The inhibition of lipid peroxidation in percent was calculated by equation 2.2.

3.3.6.3. Antioxidant activity of extracts of *Stereospermum colais* root under accelerated storage of sunflower oil

Acetone and methanol extracts (60 mg each) of *S. colais* roots were separately added into 100 g of refined sunflower oil at a concentration of 600 ppm and the oil samples were stirred for 30 min at 50°C for uniform dispersion. A control (without the addition of any antioxidant extract) was also prepared at same analytical conditions. The samples were stored at accelerated conditions (at 65°C for 15 days). The analysis was carried out periodically after every 3 days. The oxidative deterioration was followed by *p*-anisidine value. *p*-anisidine value was made by the reaction of oil sample in isooctane solution (100 ml) with *p*-anisidine reagent (0.25% in glacial acetic acid). The samples

were made to react with 1 ml of *p*-anisidine reagent to produce color compound and the absorbance was noted at 350 nm using a uv-vis spectrophotometer (Firestone,AOCS Cd18-90). *p*-anisidine value was calculated using the equation,

$$p\text{-anisidine value} = \frac{25\,[1.2\,(A_s) - (A_b)]}{M} \qquad \text{……. Eqn 3.1.}$$

where, A_s = absorption of fat solution after reaction with *p*-anisidine, A_b = absorption of solution of fat and M = weight of oil.

3.3.6.4. Determination of DPPH free radical scavenging activity

Varying concentrations of ASC and MSC were prepared and the assay has been done according to the procedure given in Chapter 2 section 2.3.7.1 of this thesis.

3.3.6.5. Nitric oxide radical scavenging activity

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. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. Sodium nitroprusside (1 ml, 10 mM) in PBS was mixed with different concentration of ASC (2-10 µg/ml) and MSC (10-50 µg/ml) prepared in methanol and incubated at 25°C for 180 min and the samples were allowed to react with 100 µl Greiss reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 3% phosphoric acid). The absorbance of chromophores formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine dichloride was read at 546 nm and referred to the absorbance of curcumin, used as a positive control treated in the same way with Griess reagent (Balakrishnan *et al*., 2009) using equation 2.2.

3.3.6.6. Superoxide radical scavenging activity

Measurement of superoxide radical scavenging capacity was done by using the method of Liu *et al*. (1997) with slight modifications. According to this, superoxide anions were generated in a non-enzymatic phenazine methosulphate-NADH (PMS-NADH) system through the reaction of PMS, NADH and oxygen and it was assayed by the reduction of NBT. In this assay, the superoxide anions were generated in tris-HCl buffer (100 µM, pH 7.4, 3 ml) containing 0.75 ml of NBT (300 µM) solution, 0.75 ml of NADH (936 mM) and 0.3 ml of different concentrations of extracts. The reaction was initiated by adding 0.75 ml of PMS (120 mM) solution to the mixture. After 5 minutes of incubation at room temperature, the absorbance at 560 nm was measured against blank in a uv-vis spectrophotometer. Ascorbic acid was used as the positive control. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging capacity and the percentage inhibition was calculated using equation 2.2 and expressed in terms of IC_{50} values.

3.3.6.7. Total reducing power

The reducing power of the extracts was determined by the method of Oyaizu (1986) with slight modifications. Extracts with different concentrations along with standard 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 minutes and add 2.5 ml of 10% TCA to the solution. The mixture was shaken well and 2.5 ml from this solution was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$. The absorbance was read at 700 nm. Gallic acid served as the standard. Increase in absorbance indicates increase in reducing power which in turn

shows the reducing property of the samples and hence an increase in the antioxidant activity. A graph was plotted with absorbance against concentration.

3.3.7. Column chromatographic separation of acetone extract

Acetone extract was subjected to column chromatography using 100-200 mesh silica gel for screening active components. The extract was adsorbed on 100-200 mesh silica gel using dichloromethane and loaded on to the column. The column was eluted successively with hexane and ethyl acetate mixtures of increasing polarities (starting from 100% hexane and ending with 100% ethyl acetate). The pictorial representation of the isolation procedure of compounds **VI-XII** is depicted in figure 3.2.

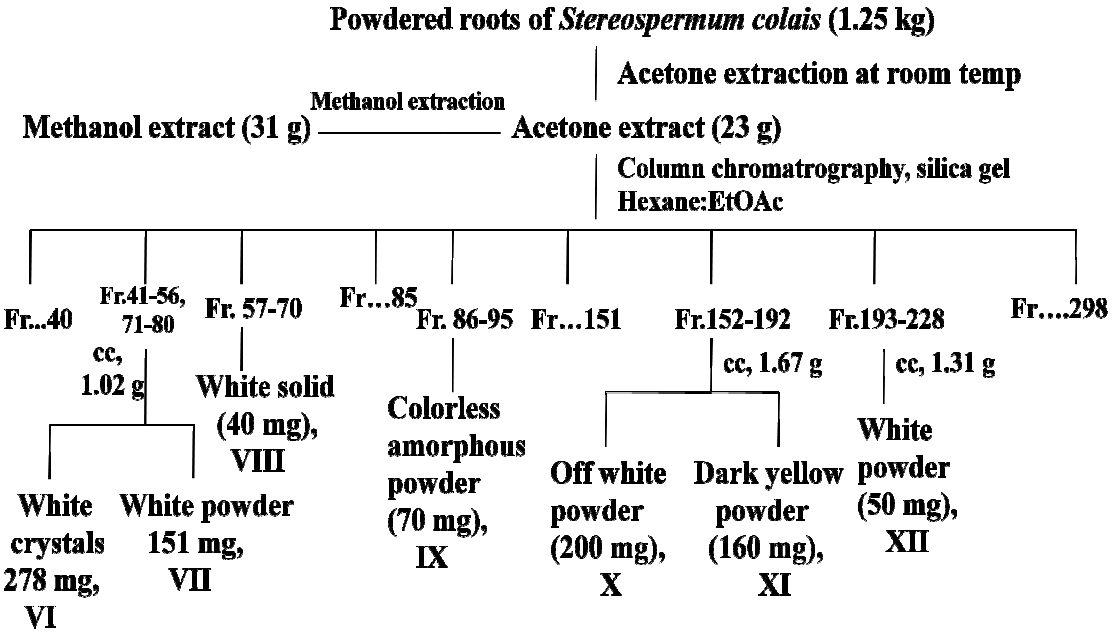
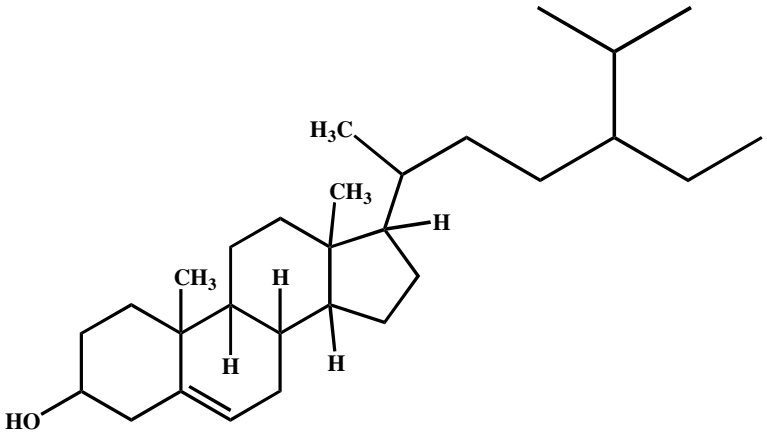


Figure 3.2: Pictorial representation of the isolation procedure for compounds **VI-XII**

3.3.7.1. Isolation of compound VI

The isolation procedure of compound **VI** is depicted in figure 3.2. It was obtained on column chromatographic separation of the fraction with 5% ethyl acetate-hexane (5:95) solvent system as white crystals (278 mg). The melting point of the compound

gave a value of 136-137°C. IR, ^{1}HNMR, ^{13}CNMR and mass spectral data confirmed the structure as **β-sitosterol**, whose structure is as shown.



VI

3.3.7.2. Isolation of compound VII

Figure 3.2 depicts the isolation procedure of compound **VII**. It was obtained on column chromatographic separation of the fraction with 5% ethyl acetate-hexane mixture (5:95) to give 151 mg of compound **VII** as white powder. From various spectroscopic data, on comparison to literature values confirmed it to be **2-(4′-hydroxyphenyl) ethyl undecanoate**.

FT-IR (ν_{max}/cm^{-1}) : 2916, 2848, 1730, 1520, 1462, 1178

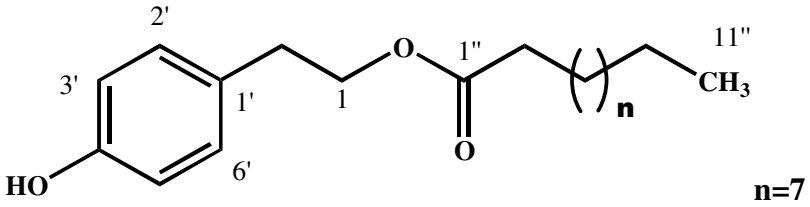
^{1}H NMR ($CDCl_3$, 500 MHz) : δ 7.08 (2H, d, *J*=8 Hz, H-2′ and 6′), 6.77 (2H, d, *J*=8 Hz, H-3′ and 5′), 5.35 (1H, s, -OH), 4.23 (2H, t, J_1=J_2= 7 Hz, H-1), 2.86 (2H, t, J_1=J_2= 7 Hz, H- 2), 2.35 (2H, t, J_1=J_2=7.5 Hz, H-2″), 1.65-1.57 (2H, m, H-3″), 1.25 (14H, s, H-4″ to 10″), 0.88 (3H, t, J_1=J_2=6.5 Hz, H-11″)

^{13}C NMR ($CDCl_3$, : δ 173.88 (C-1″), 154.20 (C-4′), 130.05 (C-2′ and 6′), 129.35 (C-

125 MHz 1′), 115.31 (C-3′ and 5′), 64.92 (C-1), 34.28 (C-2), 31.93 (C-2″), 29.67-22.70 (C-3″ to 10″), 14.12 (C-11″)

DEPT-135 ($CDCl_3$, 125 MHz) : (i) CH, CH_3 : δ 129.98, 129.73, 115.36 (2 CH), 14.13 (ii) CH_2 : δ 65.08, 34.38, 31.93, 29.48, 29.45, 29.38, 29.33, 29.28, 29.14, 29.08, 22.70

HRMS-FAB m/z (rel.int.) : 307.48 $[M+H]^+$ $C_{19}H_{30}O_3$ requires 306.46



VII

3.3.7.3. Isolation of compound VIII

Compound **VIII** was obtained also as a white solid (40 mg) and the isolation procedure is as shown in figure 3.2. The structure was assigned by various spectral studies and the structure of compound **VIII** was found to be **2-(4′-hydroxyphenyl) ethyl pentadecanoate** and the structure is as shown below.

FT-IR (ν_{max}/cm^{-1}) : 2916, 2848, 1737, 1462, 1178, 761

^1H NMR ($CDCl_3$, CD_3COCD_3, 500 MHz) : δ 7.08 (2H, d, *J*=8.5 Hz, H-2′ and 6′), 6.77 (2H, d, *J*=8 Hz, H-3′ and 5′), 4.81 (1H, s, -OH), 4.23 (2H, t, $J_1=J_2$= 7 Hz, H-1), 2.86 (2H, t, $J_1=J_2$= 7 Hz, H-2), 2.35 (2H, t, $J_1=J_2$= 7.5 Hz, H-2″), 1.59-1.54 (2H, m, H-3″), 1.26 (22H, brs, H-4″ to 14″), 0.88 (3H, t, $J_1=J_2$=6.75 Hz, H-15″)

^{13}C NMR ($CDCl_3$, : δ 172.48 (C-1″), 155.01 (C-4′), 128.96 (C-2′ and 6′), 127.82 (C-

CD_3COCD_3 1′), 114.48 (C- 3′ and 5′), 64.09 (C-1), 32.02 (C-2), 31.01 (C-2″),

125 MHz) 28.64-21.75 (C-3″ to 14″), 13.03 (C-15″)

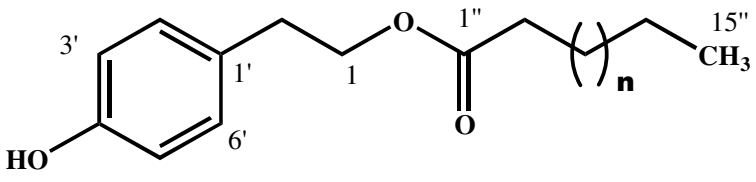
DEPT-135 : (i) CH, CH_3: δ 130.58 (2 CH), 116.28 (2 CH), 14.74

$CDCl_3$, CD_3COCD_3 (ii) CH_2 : δ 65.85, 32.70, 31.06, 30.90, 30.75, 30.68, 30.59,

125 MHz) 30.48, 30.32, 30.22, 30.14, 29.95, 26.70, 25.71, 23.47

HRMS-FAB m/z : 363.19 $[M+H]^+$ $C_{23}H_{38}O_3$ requires 362.58

(rel.int.)



n=11

VIII

3.3.7.4. Isolation of compound IX

The isolation of compound **IX** is summarized in figure 3.2. It was obtained as colourless amorphous powder (70 mg). IR, ^{1}HNMR, ^{13}CNMR and mass spectral data and on comparison with literature data, confirmed the structure as **5α-ergostan-7, 22-dien-3β-ol** whose structure is shown below.

FT-IR (ν_{max}/cm^{-1}) : 3282, 2933, 2865, 1658, 960

^{1}H NMR ($CDCl_3$, : δ 5.92 (1H, s, -OH), 5.35-5.36 (1H, m, H-7), 5.15 (1H, dd, J_1= 9

500 MHz) Hz, J_2= 5 Hz, H-23), 5.01 (1H, dd, J_1= 8.5 Hz, J_2= 5 Hz, H-22),

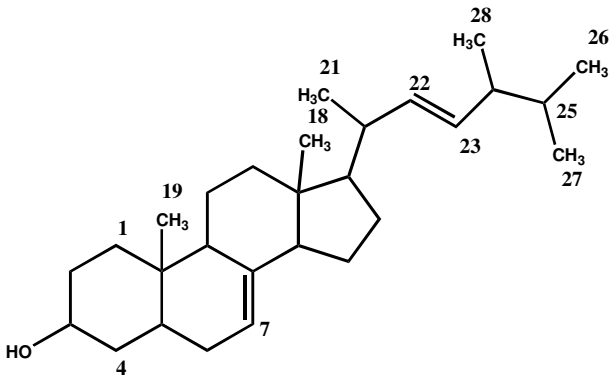
3.55-3.51 (1H, m, H-3), 2.32-2.24 (1H, m, H-9), 2.03-1.99 (1H,

m, H-14), 2.02-1.86 (2H, m, H-6), 1.86-1.84 (1H, m, H-20), 1.82-

1.67 (1H, m, H-24), 1.69-1.67 (3H, m, H- 4,5), 1.66-1.54 (4H, m, H-15,16), 1.51-1.49 (1H, m, H-25), 1.49-1.44 (2H, m, H-2), 1.33-1.31 (1H, m, H-17), 1.29-1.25 (2H, m, H-11), 1.18-1.14 (2H, m, H-12), 1.11-1.01 (2H, m, H-1), 0.92 (3H, d, *J*=6.5 Hz, H-28), 0.86 (3H, d, *J*=4.5 Hz, H-27), 0.84 (3H, d, *J*=7.5 Hz, H-21), 0.82 (3H, s, H-19), 0.79 (3H, d, *J*=8 Hz, H-26), 0.68 (3H,s, H-18)

^{13}C NMR ($CDCl_3$, CD_3COCD_3, 125MHz) : δ 140.56 (C-8), 137.70 (C-22), 128.43 (C-23), 120.13 (C-7), 70.19 (C-3), 55.27 (C-17), 55.17 (C-14), 45.05 (C-5), 41.50 (C-9), 41.38 (C-13), 39.78 (C-24), 39.04 (C-20), 38.93 (C-12), 35.71 (C-10), 35.33 (C-25), 33.10 (C-4), 31.15 (C-6), 31.07 (C-1), 30.71 (C-2), 27.42 (C-16), 22.18 (C-11), 20.23 (C-21), 20.06 (C-15), 18.74 (C-19), 18.38 (C-27), 17.96 (C-26), 17.79 (C-28), 11.21 (C-18)

HRMS-FAB m/z (rel.int.) : 398.06 $[M]^+$ $C_{28}H_{46}O$ requires 398.73



IX

3.3.7.5. Isolation of compound X

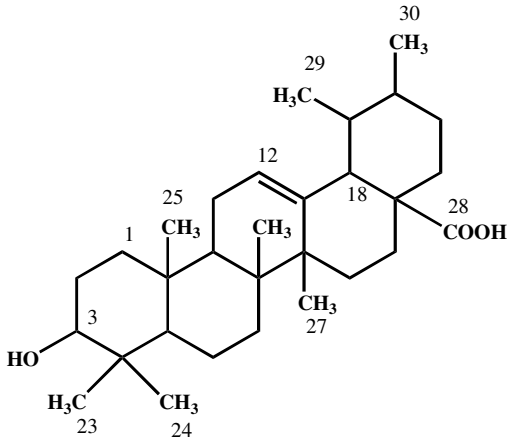
Compound **X** was obtained as off white powder (200 mg) and figure 3.2 summarized the isolation procedure. IR, ^{1}HNMR, ^{13}CNMR and mass spectral studies of this compound and on comparison to the literature values, confirmed it to be **ursolic acid** whose structure is shown below.

FT-IR (ν_{max}/cm^{-1}) : 3739, 3397, 1674, 1597, 1250, 1107, 957

^{1}H NMR ($CDCl_3$, 500 MHz) : δ 8.18 (s, 2 x –OH), 4.83-4.69 (1H, m, H-12), 3.02 (1H, dd, J_1= 9 Hz, J_2= 11.5 Hz, H-3), 2.19 (1H, d, *J*=4.5 Hz, H-18), 1.62-1.60 (19H, m, aliphatic protons), 1.21 (6H, s, H-23, 27), 0.98 (3H, s, H-25), 0.95 (3H, s, H-26), 0.94 (3H, s, H-24), 0.89 (3H, d, *J*=5.5 Hz, H-29), 0.87 (3H, d, *J*=6.5 Hz, H-30), 0.77 (3H, d, *J*= 6.5 Hz, H-25)

^{13}C NMR ($CDCl_3$, CD_3COCD_3 125 MHz) : δ 185.07 (C-28), 155.79 (C-1), 137.93 (C-13), 130.75 (C-12), 78.9 (C- 3), 51.28 (C-5, 18), 47.79 (C-9), 47.25 (C-17), 42.78 (C-14), 40.82 (C- 8), 37.99 (C-4), 36.69 (C- 19), 36.43 (C-20), 35.82 (C-22), 34.66 (C-10), 33.75 (C-7), 29.35 (C-21), 28.71 (C- 15), 27.51 (C-2), 26.52 (C-16), 25.13 (C-11), 21.82 (C-23), 21.71 (C-24), 21.61 (C-29), 17.90 (C-6), 16.38 (C-30), 16.27 (C-26), 15.20 (C-25), 11.10 (C-27)

HRESI-MS m/z (rel.int.) : 457.13 $[M+H]^+$ $C_{30}H_{48}O_3$ requires 456.70



X

3.3.7.6. Isolation of compound XI

Compound **XI** was obtained as dark yellow powder (160 mg) on column chromatographic separation of the fraction as depicted in figure 3.2. IR, ^{1}HNMR, ^{13}CNMR and mass spectral studies of this compound and on comparison to literature values confirmed the structure as **lapachol**, the structure of which is assigned as shown.

FT-IR (ν_{max}/cm^{-1}) : 3422, 1672, 1648, 1589, 1273

^{1}H NMR ($CDCl_3$, 500 MHz) : δ 8.24-7.70 (4H, m, Ar), 7.79 (1H, s, -OH), 4.99-4.95 (1H, m, C-12H), 3.78 (2H, d, *J*=7 Hz, C-11H), 1.61 (3H, s, C-15Me), 1.59 (3H, s, C-14Me)

^{13}C NMR ($CDCl_3$, CD_3OD, 125MHz) : δ 182.23 (C-4), 174.83 (C-1), 153.18-116.6 (C-2, 3, 5-10) for 8 carbons, 132.74 (C-13), 127.87 (C-12), 30.81 (C-11), 22.18 (C-14), 17.15 (C-15)

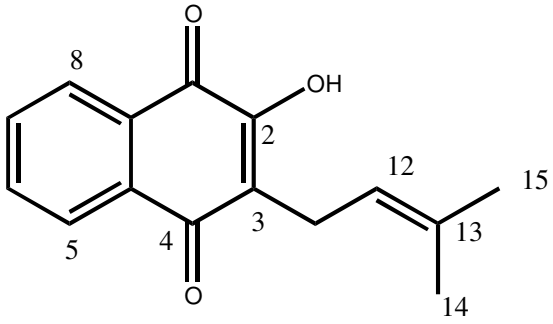
DEPT-135 ($CDCl_3$, CD_3OD, 125 MHz) : (i) CH, CH_3 : δ 133.97, 133.84, 127.01, 126.88, 103.73, 21.41, 17.78

(ii) CH_2 : δ 30.25

HRESI-MS m/z : 243.06 $[M+H]^+$ $C_{15}H_{14}O_3$ requires 242.27

(rel.int.)

m.p. (°C) : 141-142 (Lit. 140-141°C) (Rao *et al*., 1968)



XI

3.3.7.7. Isolation of compound XII

Compound **XII** was obtained as white powder (50 mg). It was obtained from the fraction as depicted in figure 3.2. Detailed analysis by IR, ^{1}HNMR, ^{13}CNMR and mass spectral studies and by comparing the values from the literature, the compound was confirmed as **pinoresinol**, whose structure is shown below.

FT-IR (ν_{max}/cm^{-1}) : 3482, 2921, 1741, 1521, 1458, 1028, 881

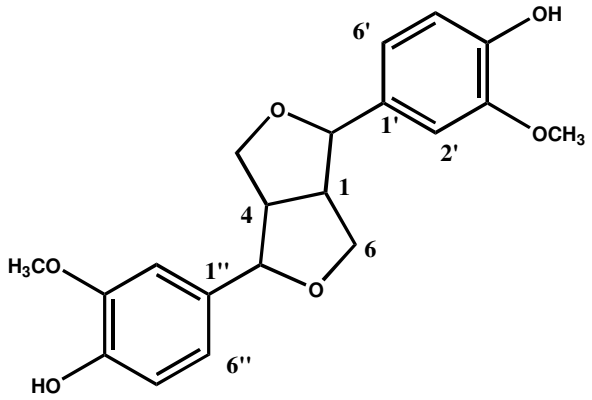
^{1}H NMR ($CDCl_3$, CD_3OD, 500 MHz) : δ 7.94 (2 x -OH, s), 7.61-7.56 (2H, m, H-2′ & H-2″), 7.49-7.46 (2H, m, 6′ & H 6″), 6.89-6.83 (2H, m, H-5′ & H-5″), 4.07 (4H, m, H-3 & H-6), 3.93(3H, s, -OCH_3), 3.87 (3H, s, -OCH_3), 3.72 (2H, m, H-2 & H-5), 3.35 (2H, m, H- 1 & H-4)

^{13}C NMR ($CDCl_3$, CD_3OD, 125 MHz) : δ 150.87 (C-3′ & C-3″), 147.02 (C-4′ & C-4″), 131.82 (C-1′ & C-1″), 124.13 (C- 6″), 121.71 (C-6′), 114.48 (C-5′), 112.50 (C-5″), 105.68 (C-2′ & C-2″), 95.99 (C- 2 & C-5), 72.79 (C-6 & C-3), 56.41 (2 x -OCH_3), 55.51 (C-1 & C-4)

DEPT-135 ($CDCl_3$, CD_3OD, 125 MHz) : (i) CH, CH_3 : δ 125.72 (2C), 115.98, 113.98, 106.73 (2C), 96.06 (2C), 58.02 (2C), 57.15 (2C)

(ii) CH_2 : δ 72.60 (2C)

HRESI-MS m/z (rel.int.) : 359.07 $[M+H]^+$ $C_{20}H_{22}O_6$ requires 358.38



XII

3.3.8. DPPH radical scavenging potential of isolated compounds

DPPH radical scavenging potential of isolated compounds was screened according to the procedure given in Chapter 2 section 2.3.7.1 of this thesis.

3.3.9. Inhibition of α-glucosidase and α-amylase enzymes

3.3.9.1. Inhibition of α-glucosidase enzyme

The inhibition potential of α-glucosidase enzyme was assayed by using varying concentrations of 50 µl of homogenized extract solutions (50–250 µg/ml) and isolated compounds such as β-sitosterol, 2-(4′-hydroxyphenyl) ethyl undecanoate, 2-(4′-hydroxyphenyl) ethyl pentadecanoate, 5α-ergostan-7, 22-dien-3β-ol, ursolic acid, lapachol, pinoresinol etc. and 100 µl of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (1.0 U/ ml) and incubated in 96-well plates at 25°C for 10 min. After

pre-incubation, 50 µl of 5 mM *p*-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixture was incubated at 25°C for 5 min. Before and after incubation, absorbance readings were recorded at 405 nm by a Synergy 4 Biotek multi plate reader (Biotek Instruments Inc., Highland Park, Vermont, USA) and compared to a control which had 50 µl of buffer solution in place of the extract (Apostolidis *et al*., 2007). Acarbose was used as the standard. The α-glucosidase inhibitory activity was expressed as inhibition % and was calculated using equation 2.2 and expressed in terms of IC_{50} values.

3.3.9.2. α-amylase inhibition assay

The inhibition potential of samples against α-amylase enzyme was carried out by the method of Apostolidis *et al*. (2007). Briefly, 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing α-amylase solution (0.5 mg/ml) and different concentrations of the stock solutions of extracts (200-1000 µg/ml) and isolated compounds were incubated at 25°C for 10 min. After pre-incubation, 500 µl of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) was added to each tube at timed intervals. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 1.0 ml of dinitrosalicylic acid color reagent. The test tubes were then incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding 10 ml distilled water and absorbance was measured at 540 nm using a Synergy 4 Biotek multiplate reader. Acarbose was used as the positive control. Percentage inhibition was calculated using equation 2.2.

3.3.10. Antiglycation assay

Inhibition of glycated end products was performed according to the method reported by Arom (2005) with slight modifications. About 500 µl of albumin (1 mg/ml final concentration) was incubated with 400 µl of glucose (500 mM) in the presence of 100 µl of samples at different concentrations of extracts (100–500 µg/ml) and isolated compounds such as β-sitosterol, 2-(4′-hydroxyphenyl) ethyl undecanoate, 2-(4′-hydroxyphenyl) ethyl pentadecanoate, 5α-ergostan-7, 22-dien-3β-ol, ursolic acid, lapachol, pinoresinol etc. The reaction was allowed to proceed at 60°C for 24 h and thereafter reaction was stopped by adding 10 µl of 100% TCA. Then the mixture was kept at 4°C for 10 min before subjected to centrifugation (Kubota, Tokyo, Japan) at 10,000 × *g*. The precipitate was redissolved in 500 µl alkaline phosphate buffer saline (PBS) (pH 10) and immediately quantified for relative amount of glycated BSA based on fluorescence intensity at 370 nm (excitation) and 440 nm (emission) using a Synergy 4 Biotek multiplate reader. Ascorbic acid was used as the positive control. Antiglycation activity of extracts was calculated using equation 2.2.

3.3.11. Antiinflammatory activity of 5α-ergostan-7, 22-dien-3β-ol

3.3.11.1. Protein denaturation inhibition assay

The assay was performed by following the method of Gambhire *et al*. (2009) with some modifications. The reaction mixture consists of 500 µl of BSA (5 mg/ml), 700 µl of phosphate buffered saline (PBS, 150 mM, pH 6.4) and 100 µl of varying concentrations of 5α-ergostan-7, 22-dien-3β-ol (25-251 nM), ASC (10-50 µg/ml) and MSC (10-50 µg/ml). Similar volume of double distilled water in place of samples served as control. After gentle mixing, denaturation was induced by incubating the reaction mixture at 37°C

in an incubator (Sanyo incubator, USA) for 15 min and then heated at 80°C in a water bath for 10 min. After cooling, the turbidity was measured at 660 nm using Synergy 4 Biotek multiplate reader. Diclofenac sodium at a concentration of 6-168 nM (10-50 µg/ml) was used as the positive control. The percentage of denaturation was calculated from control by using equation 2.2.

3.3.11.2. *In vivo* antiinflammatory activity

3.3.11.2.1. Animals

Adult female Sprague-Dawley rats (30 numbers, weighing 120±10 g) bred in the animal house of the Department of Biochemistry, University of Kerala were used for this study. They were kept in an environment with controlled temperature (24-26°C), humidity (55-60%) and photoperiod (12:12 h) light–dark cycle. A commercially balanced diet (Amrut Laboratory Animal Feeds, Maharashtra, India) and tap water were provided *ad libitum*. The animals received humane care, in compliance with the present institutional guidelines. All experiments were conducted as per the guidelines of the Animal Ethics Committee CPCSEA according to Government of India accepted principles for lab animals use and care under the number IAEC-KU-9/2012-13-BC-AH-PKA(1).

3.3.11.2.2. Carrageenan induced rat paw edema antiinflammatory activity measurement of 5α-ergostan-7, 22-dien-3β-ol

A concentration dependent study for the antiinflammatory activity, against carrageenan induced rat paw edema, was carried out in the range of 1-100 mg/kg body weight. Maximum edema inhibition was obtained at a minimal concentration of 5 mg/kg body weight. Antiinflammatory effect was evaluated in acute inflammatory model

induced by carrageenan in female Sprague-Dawley rats (Winter *et al*., 1962). For subsequent experiments, edema was induced on rat right hind paw by aponeurosis injection of 0.1 ml of 1% carrageenan in 0.9% saline. The experimental groups consisted of 6 groups (1-6) with five rats in each group (n=6). Group 1 received carrageenan alone; group 2 and 3, carrageenan with 100 mg/kg acetone and methanol extracts. Group 4 received 5 mg of compound and carrageenan, group 5 carrageenan + 10 mg of compound and group 6 received carrageenan and standard indomethacin (3 mg/kg). After one hour of pretreatment with extracts, 5α-ergostan-7, 22-dien-3β-ol and standard drug indomethacin given by oral incubation, the inflammatory agent lambda carrageenan (0.1 ml of 1% in normal saline) was injected to the rats into the plantar aponeurosis of the right hind paw of these rat producing acute inflammatory edema leading to marked increase in the volume of the limb. The paw volume was measured at hourly interval for 3 hours by using paw edema meter (Marsap Pvt. Ltd., USA). The percentage edema was calculated according to Winter *et al*. (1962).

$$\% \text{ edema inhibition} = [(Vc - Vt)/Vc] \times 100 \quad \text{……. Eqn 3.1}$$

where Vc and Vt are the average paw volume of control and test respectively

3.3.12. Antimicrobial activity studies on lapachol and ursolic acid

3.3.12.1. Test pathogens

Gram positive bacteria: *Staphylococcus aureus* MTCC 902, *Staphylococcus epidermis* MTCC 435; Gram negative bacteria: *Escherichia coli* MTCC 2622, *Klebsiella pneumoniae* MTCC 109, *Proteus mirabilis* MTCC 425 were used in the present study.; Medically important fungi: *Aspergillus flavus* MTCC 183, *Candida albicans* MTCC 277, *Cryptococcus gastricus* MTCC 1715 and *Trichophyton rubrum* MTCC 296 were used in

the present study. All the test microorganisms were purchased from Microbial Type Culture Collection Centre, IMTECH, Chandigarh, India. The test bacteria and fungi were maintained on nutrient agar slants and potato dextrose agar respectively and periodically sub cultured in every 15 days.

3.3.12.2. Antibacterial activity of compounds: Disc diffusion technique

Antibacterial activities of ursolic acid, lapachol and pinoresinol were determined by disc diffusion method (CLSI 2012a) against *S. aureus, E. coli, S. epidermis, P. mirabilis* and *K. pneumonia*. The test cultures maintained in nutrient agar slant at 4°C were sub-cultured in nutrient broth to obtain the working cultures approximately containing $1x10^6$ CFU/ml. The compounds lapachol, ursolic acid and pinoresinol (100 µg/ml) were incorporated in a 6 mm sterile disc. Mueller Hinton (MH) agar plates were swabbed with each bacterial strain and the test disks were placed along with the control disks. Ciprofloxacin disks (5 µg/disc) were used as positive control. Plates were incubated overnight at 37°C. Clear, distinct zone of inhibition was visualized surrounding the discs. The antibacterial activity of the test agents was determined by measuring the zone of inhibition expressed in millimeter.

3.3.12.3. Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration was determined according to the method described by Clinical and Laboratory Standards Institute (CLSI 2012 b), with some modifications. Two fold serial dilutions of the antibiotics and compounds were made with Mueller Hinton Broth (MHB) to give concentrations ranging from 1 to 1000 µg/ml. Hundred microliters of test bacterial suspension were inoculated in each well to give a final concentration of 1×10^5 CFU/ml. The tubes were incubated for 24 h at 37ºC. The

control tube did not have any antibiotics or test compounds, but contained the test bacteria and the solvent used to dissolve the antibiotics and compounds. The growth was observed both visually and by measuring optical density at 600 nm using Bio-rad ELISA reader Model 680 Bio-Rad (California, USA). Triplicate sets of tubes were maintained for each concentration of the test sample. Ciprofloxacin was used as a positive control. The lowest concentration of the test sample showing no visible growth was recorded as the MIC.

3.3.12.4. Antifungal assay: Disc diffusion technique

Compounds ursolic acid, lapachol and pinoresinol were screened for their antifungal activity against test fungi by disc diffusion method (CLSI 2009; CLSI 2010). The fungal cultures were grown on potato dextrose broth. The mycelia mat of fungi of 7 day old culture was suspended in normal saline solution and test inoculum was adjusted to 5 $\times 10^5$ CFU/ml. Inocula (0.1 ml) were applied on the surface of the potato dextrose agar plate and spread by using a cotton swab. Subsequently, filter paper discs (6 mm in diameter, Hi-media) containing 100 µg/ml of test compounds were placed on the agar plates and incubated at 35°C for 24-48 hours. Afterwards, the diameter of the inhibition zone was measured.

3.3.12.5. Minimum inhibitory concentration

MIC was determined using potato dextrose agar by the poisoned food technique against *A. flavus*, *T. rubrum, C. albicans* and *C. gastricus* (Rollas *et al*. 1993). A stock solution of 2000 µg/ml of the test compound was prepared, which was further diluted with methanol to give the required concentrations of 1000 to 1 µg/ml. One tube was used as solvent control. For *C. albicans,* the broth dilution method (CLSI 2012c) was adopted

using potato dextrose broth. Amphotericin B was used as standard antifungal agent. All experiments were in triplicate for each treatment against each fungus.

3.4. STATISTICAL ANALYSIS

The experimental results are expressed as the mean ± standard deviation (SD) of three parallel measurements. The results were subjected to one way analysis of variance performed by using the statistical program (SPSS), version 11.5 (SPSS, Chicago, IL, USA). Duncan's multiple range test was conducted for comparison of means at $P \leq 0.05$.

3.5. RESULTS AND DISCUSSION

3.5.1. Plant material and extraction

The roots of *S. colais* (2.5 kg) collected were washed, cut into small pieces and dried in an oven at 50°C. The powdered roots (1.25 kg) were extracted successively three times using acetone and methanol (6 L each) as solvents at room temperature. The crude extracts of acetone and methanol were combined individually and the solvent evaporated at 50°C to yield 23 g of acetone extract and 31 g of methanol extract.

3.5.2. GC/GC-MS analysis of roots of *Stereospermum colais*

Volatile oil (0.2%) obtained from hydrodistillation was analysed by GC/GC-MS. Nine components were identified and they contributed about 28% of total volatile composition. The constituents present in the essential oil are given in Table 3.1.

Table 3.1: Essential oil composition of roots of *S. colais*

No	Compound	Percentage
1	Ar-curcumene	1.23
2	n-Decanoic acid	2.28
3	Undecanoic acid	3.44
4	Cinnamyl tiglate	1.34
5	Tetradecanoic acid	3.44
6	Pentadecanoic acid	3.48
7	Widdrol hydroxy ether	3.13
8	**n-Hexadecanoic acid**	**7.93**
9	**2-methyl-z, z, 3, 13-octadecadienol**	**4.63**

It was found that the major constituents present in the root essential oil analysed here was n-hexadecanoid acid (7.93 %) and 2-methyl-z, z, 3, 13-octadecadienol (4.63 %).

3.5.3. Antioxidant properties of acetone and methanol extracts of *Stereospermum colais*

Successive acetone (ASC) and methanol (MSC) extracts with varying concentration were used for antioxidant and radical scavenging evaluation using different methods.

3.5.3.1. Xanthine oxidase inhibitory potential

Xanthine oxidase enzyme catalyses the oxidation of hypoxanthine to xanthine and generates superoxide and uric acid, which plays a pivotal role in gout (Chiang & Chen 1993). Molecular oxygen acts as an electron acceptor during the reoxidation of xanthine

oxidase and producing superoxide radical and hydrogen peroxide (Scheme 3.1) (Fridovich, 1970)

$$\text{Xanthine} + 2O_2 + H_2O \rightarrow \text{uric acid} + 2O_2^{\bullet -} + 2H^+$$

$$\text{Xanthine} + O_2 + H_2O \rightarrow \text{uric acid} + H_2O_2$$

Scheme 3.1

Pro-inflammatory cytokines in increased levels has been formed as a response to the elevated expression of xanthine oxidase and severe oxidative damage may occurs as a result of increased activities of xanthine oxidase and are involved in many pathological processes such as aging, cancer, atherosclerosis etc. Inhibitors of xanthine oxidase block the terminal step in uric acid biosynthesis and lower plasma uric acid concentration (Ishibuchi *et al*., 2001). Roots of *Stereospermum colais* are traditionally used as an antiinflammatory agent and in this study the extracts showed the potential to inhibit xanthine oxidase enzyme and thereby inhibits the production of superoxide radical. XO inhibitory activity of ASC and MSC were compared with standard allopurinol with an IC_{50} value of 8.3 µg/ml. ASC showed better activity with an IC_{50} value of 3.22 µg/ml, while MSC had an IC_{50} value of 22.19 µg/ml (Figure 3.3). Therefore, *S. colais* could be a promising candidate for human gout and ischemia by decreasing the concentration of uric acid and superoxide radical in human tissues.

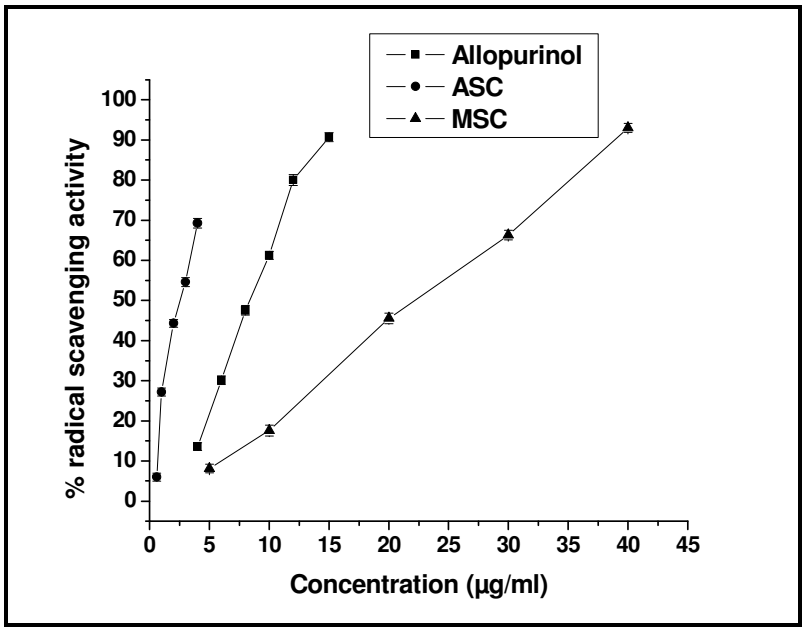


Figure 3.3: Xanthine oxidase radical scavenging activity of allopurinol, ASC and MSC

3.5.3.2. Antioxidant activity in linoleic acid emulsion system

In biological system, oxidative degradation of poly unsaturated fatty acids in the cell membrane (lipid peroxidation) generates a number of degradation products that may cause cell damage and cell membrane destruction as below (Scheme 3.2). In the present study, the inhibition of lipid peroxidation is measured using the linoleic acid-thiocyanate method where hydroperoxide is produced by linoleic acid oxidation in the medium (Yoshikawa *et al*., 1997).

$$LH \rightarrow L\bullet + H\bullet$$

$$L\bullet + O_2 \rightarrow LOO\bullet$$

$$LOO\bullet + LH \rightarrow LOOH + L\bullet$$

$$Radicals \rightarrow Non\ radical\ species$$

Scheme 3.2

Oxidation of linoleic acid produces hydroperoxides which decomposes into many secondary oxidation products. In the assay, the oxidized products on reaction with ferrous

chloride form ferric chloride and on further reaction with ammonium thiocyanate, formed the red colored ferric thiocyanate, which is measured. An antioxidant can prevent lipid peroxidation and slows down the formation of ferric thiocyanate. The linoleic acid emulsion system can be simulated with the biological lipid system or with fat emulsions (Prathapan *et al*., 2011).

Both ASC and MSC exhibited effective inhibition of lipid peroxidation at a concentration of 0.1 mg/ml. Acetone and methanol extract showed 66.59 and 54.89% inhibition of linoleic acid peroxidation at 24 h time interval while that of the positive control BHT was 81.82% at the same duration of time. The peroxidation inhibition of ASC, MSC and BHT was found to decline with time and reached 19.37, 11.21 and 33.19% at 120 h (Figure 3.4). Both extracts showed good antiperoxidative property at tested concentrations and showed its efficacy to prevent lipid peroxidation.

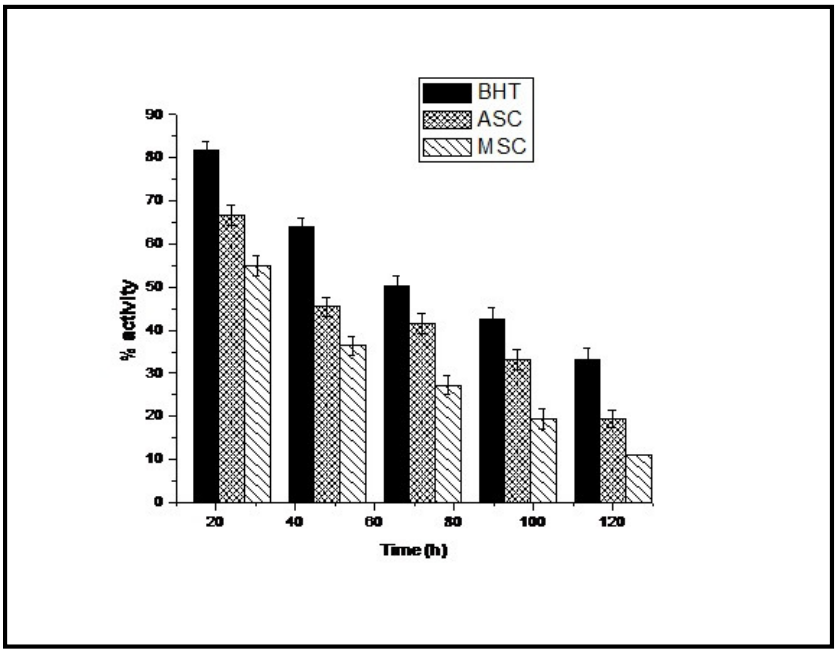


Figure 3.4: The inhibition of lipid peroxidation measured by linoleic acid emulsion thiocyanate method at a concentration of 0.1 mg/ml by ASC, MSC and BHT

3.5.3.3. Antioxidant activity of extracts of *Stereospermum colais* under accelerated storage of sunflower oil

Oxidative reactions in fats and fatty food stuffs deteriorates its quality and generates free radicals and reactive oxygen species which limit the shelf life of food stuffs and implicated in carcinogenesis, mutagenesis, inflammation, aging etc. (Pezzuto and Park, 2002). Recently synthetic antioxidants have been restricted as they may cause liver swelling and influenze liver enzyme activities (Siddhuraju and Becker, 2003). Secondary oxidation products mainly non-volatile carbonyls, formed during lipid oxidative degradation can be measured in terms of *p*-anisidine value. In the current study, *p*-anisidine react with the aldehydes in oil under acidic conditions resulting in the formation of a yellowish colored product and increased value of *p*-anisidine indicates the greater concentration of aldehydes and in consequence, a lower oxidative stability of the oil (Sultana *et al*., 2007). Refined, bleached and deodorized sunflower oil was used for the analysis because it is used in nutrition and is highly appreciated as a source of the essential linoleic acid. The stabilization effect is also more pronounced in sunflower oil due to its higher content of polyunsaturated fatty acids. The relative increase in *p*-anisidine value of sunflower oil (SFO) treatments stabilized with *Stereospermum colais* extracts during 15 days of storage at 65°C were shown in Table 3.2. SFO without any extracts (SFO-O) showed highest *p*-anisidine value (12.95 meq/kg), indicating high rate of oxidation. ASC (10.11 meq/kg) and MSC (12.01 meq/kg) showed relatively lesser values of *p*-anisidine reflecting an antioxidant activity. This study demonstrates that roots of *Stereospermum colais* can effectively inhibit the lipid oxidation of sunflower oil in thermal oxidation conditions.

Table 3.2: Antioxidant activity of extracts of *Stereospermum colais* under accelerated storage of sunflower oil (*p*-anisidine value)

Day	SFO-O (meq/kg)	SFO-A (meq/kg)	SFO-M (meq/kg)
0	4.2	4.2	4.2
3	5.36	4.5	5.19
6	7.04	6.83	6.92
9	9.63	7.31	8.97
12	10.79	8.55	9.73
15	12.95	10.11	12.01

SFO: Sunflower oil, SFO-O: Sunflower oil without any extracts, SFO-A: Sunflower oil with acetone extract, SFO-M: Sunflower oil with methanol extract

3.5.3.4. DPPH radical scavenging capacity

Both ASC and MSC were tested for antioxidant radical scavenging capacity using DPPH• (diphenyl picryl hydrazyl radical) and the results showed very high scavenging potential compared to standard gallic acid. Among the extracts, ASC showed 86.78% scavenging potential at a concentration of 10 µg/ml and MSC showed 80.89% at 50 µg/ml. The plot of DPPH• radical scavenging potential of extracts and standard gallic acid are depicted in figure 3.5. ASC showed better activity with an IC_{50} value of 3.69 µg/ml while MSC showed 27.89 µg/ml compared to standard gallic acid (IC_{50} 1.54 µg/ml). This can be measured by quantifying the decrease in absorbance at 517 nm. Increase in concentration increases the DPPH radical scavenging activity and further

increase in concentration showed less increment in activity, this showed that the sample attained their maximum activity with radicals present. The high radical scavenging capacity of acetone extract accords with the fact that the antioxidant phytoconstituents in acetone extract was higher than in methanol extract.

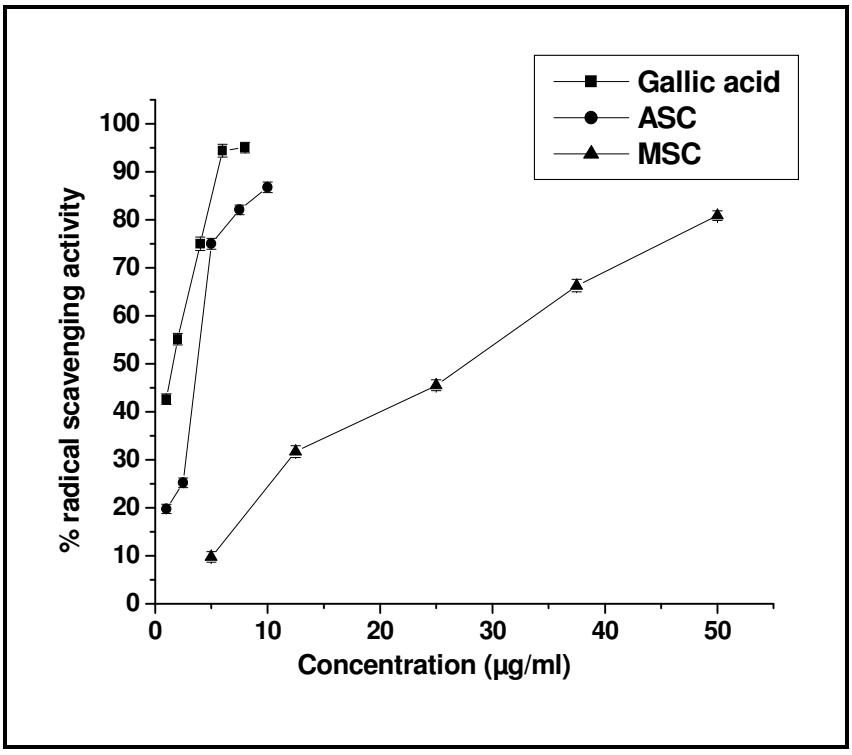
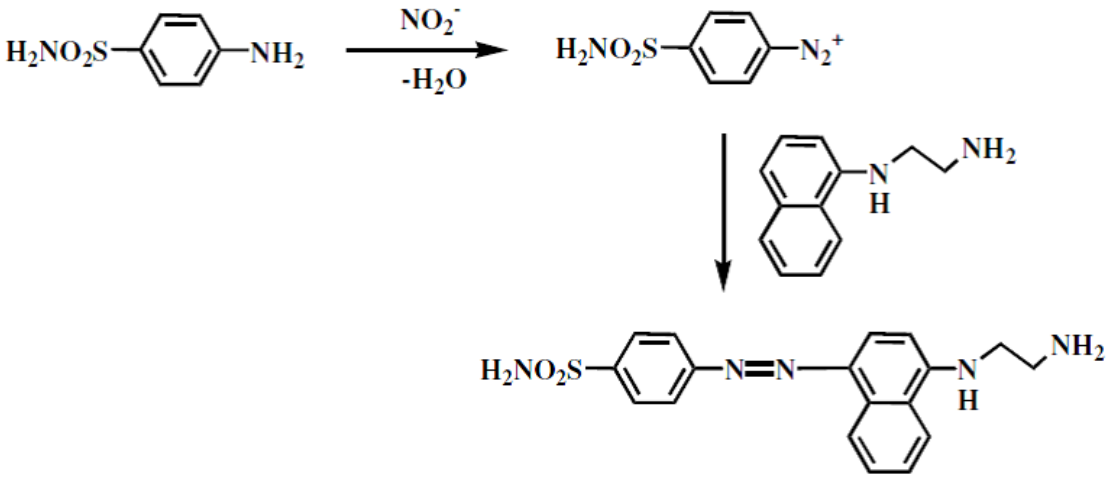


Figure 3.5: DPPH radical scavenging activity of ASC, MSC and gallic acid

3.5.3.5. Nitric oxide radical scavenging activity

Nitric oxide or reactive nitrogen species formed during the reaction with oxygen or superoxide has an important role in various inflammatory processes. RNS are considered as agents responsible for altering structural and functional behavior of many cellular components, toxic to tissues and contribute to vascular collapse. Nitric oxide is implicated for various cancers and inflammatory conditions including juvenile diabetes, multiple sclerosis etc. (Tylor *et al*., 1997). When it reacts with superoxide radical forming highly reactive peroxy nitrite radical (ONOO•) and toxicity of nitric oxide get elevated

greatly (Huie and Padmaja 1993). According to the assay principle, nitrite ions (NO_2^-) are produced as a result of interaction of O_2 with sodium nitro prusside in aqueous solution at physiological pH and estimated using Greiss reagent (Sulphanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride (NED) in phosphoric acid). Under acidic conditions and in presence of nitrite ions diazotization reaction occurs between sulphanilamide and NED yielding an azo compound (Scheme 3.3). Antioxidants can decrease the amount of nitrous acid by decreasing the formation of purple azo dye and will reflect in the extent of scavenging (Shetty Devika, 2013).



azo compound

Scheme 3.3

Both acetone and methanol extract showed a dose dependent nitric oxide scavenging potential (Figure 3.6) with IC_{50} values of 5.06 and 13.14 µg/ml respectively compared to standard curcumin (IC_{50} 43.73 µg/ml). Any antioxidant that can scavenge the *in situ* generated nitrite ions results in the inhibition of the production of the azo compound, resulting in the decreased absorbance of the reaction mixture at 546 nm. The extracts showed remarkable property to counteract the formation of nitric oxide by

preventing the effects of excessive NO formation in the human body (Prathapan *et al*., 2011).

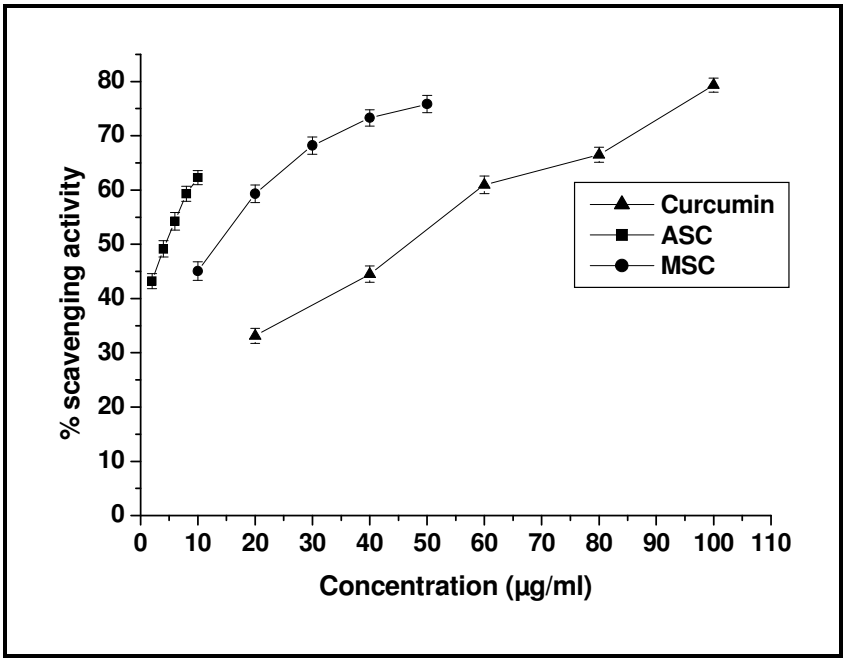


Figure 3.6: Nitric oxide radical scavenging activity of ASC, MSC and curcumin

3.5.3.6. Superoxide radical scavenging activity

Superoxide radical ($O_2^{\bullet -}$), the one electron reduced form of oxygen is normally formed first in cellular oxidation reactions and implicated in the initiation of oxidation reactions related to aging (Liu and Ng, 2000). Superoxide radicals also functions as a precursor of more reactive oxygen species and form deleterious oxidative species such as singlet oxygen species and hydroxyl radicals (Korycka-dahl and Richardson, 1978). The study follows the PMS-NADH-NBT system (Phenazonium methosulphate-Nicotinamide adenine dinucleotide disodium salt-Nitro blue tetrazolium chloride), the superoxide derived from dissolved oxygen, reduces NBT and absorbance was measured at a wavelength of 560 nm.

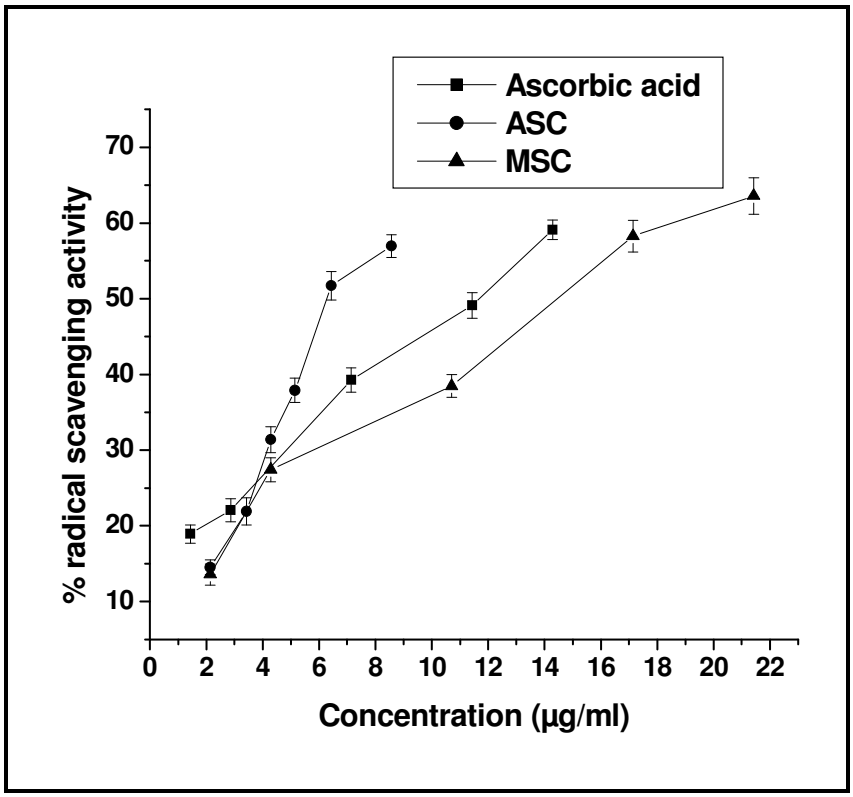


Figure 3.7: Superoxide radical scavenging activity of ASC, MSC and ascorbic acid

It can be seen that as concentration increases, superoxide radical scavenging potential also increases. Figure 3.7 shows the percentage scavenging of superoxide radical generation at concentration levels of 2-9 µg/ml ASC and 2-21 µg/ml MSC. ASC showed much higher scavenging potential (IC_{50} 6.27 µg/ml) compared to MSC (IC_{50} 14.46 µg/ml) whereas standard ascorbic acid showed an IC_{50} of 11.71 µg/ml. ASC showed a higher decrease in absorbance compared to MSC and standard ascorbic acid, indicating greater scavenging activity of superoxide radicals. The decrease in absorbance with extracts indicates the consumption of the generated superoxide anion in the reaction mixture and thereby decreasing NBT reduction. The results clearly indicate that the root extracts are superoxide radical scavengers in a dose dependent manner.

3.5.3.7. Total reducing power

The reduction of Fe^{3+} cyanide complex to its ferrous (Fe^{2+}) form occurs in presence of acetone and methanol extracts of varying concentration (2-50 µg/ml). The antioxidant action was exerted by the breakage of free radical chain through the donation of hydrogen atom by reductones (Lee *et al*., 2002). The reducing powers of the extracts were found to increase with increasing concentration (Figure 3.8) and this activity may contribute significantly to the antioxidant action.

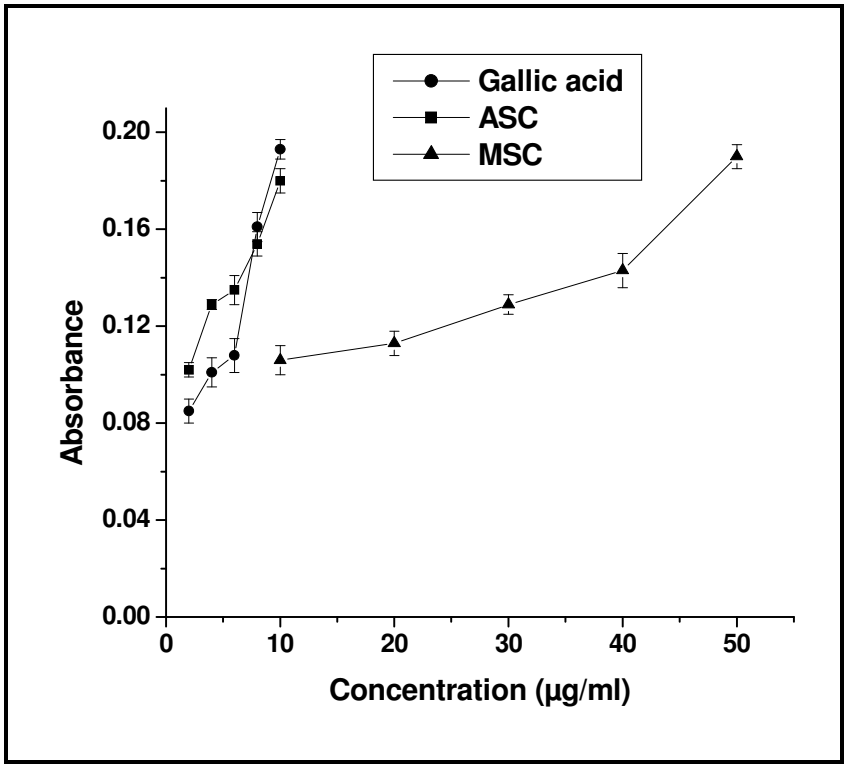
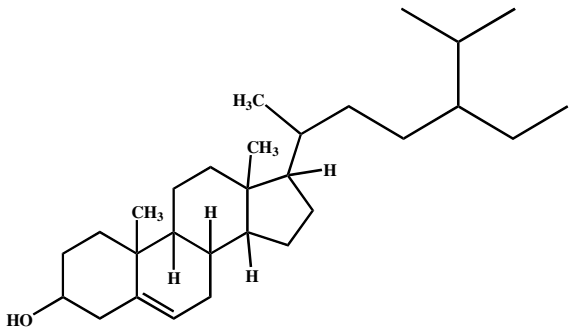


Figure 3.8: Total reducing power of ASC, MSC and gallic acid

Since absorbance increases with increase in concentration reducing power increases and the extracts acts as efficient electron donor capable of neutralizing free radicals and the property would enable free radicals to more stable products and terminated radical-initiated chain reactions. Among the extracts, ASC showed better reducing capacity compared to methanol extract.

3.5.4. Isolation and characterization of compounds from the acetone extract of *S. colais* roots

Since acetone extract posses very high radical scavenging and antioxidant potential, column chromatography of acetone extract had been carried out to screen the components present in the extract. In this regard, acetone extract (20 g) was subjected to gradient elution with 100-200 mesh silica gel column chromatography (CC) using the solvents hexane-ethyl acetate (100:0–0:100) to give 298 fractions of 70-80 ml which were grouped into 9 major fraction pools (1-9) based on similarities in TLC. Initial fraction pools were found to have very less polar components. All the components have been isolated for the first time from *S. colais* even though the compounds have been isolated previously from many *Stereospermum* species. From this, second fraction pool (F 41-56 & 71-80) was purified further by silica gel CC using 5% ethyl acetate-hexane (5:95) yielded 278 mg of compound **VI** and 151 mg of compound **VII.** The IR, ^{1}HNMR (Figure 3.9), ^{13}CNMR (Figure 3.10) and HRMS (Figure 3.11) spectral details of compound **VI** matched with that of β-sitosterol. Hence compound **VI** is assigned the structure of **β-sitosterol** as shown below. The compound was checked by co-spot with standard and forms a red colour TLC spot on reaction with iodine vapour. It is a common secondary metabolite found in most plants.



VI

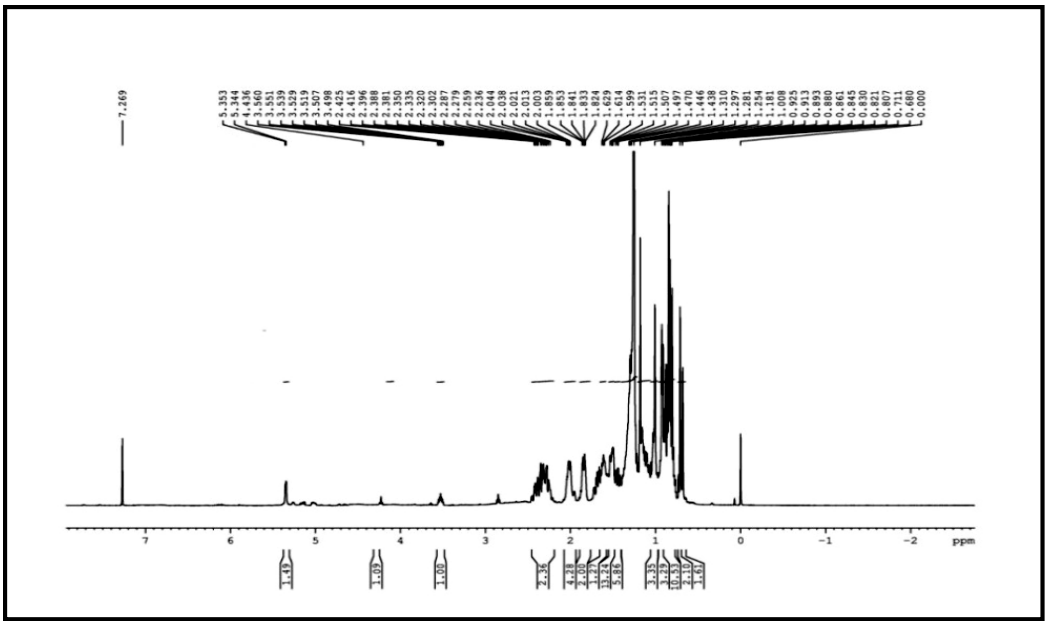


Figure 3.9: ^{1}H NMR spectrum of compound **VI**

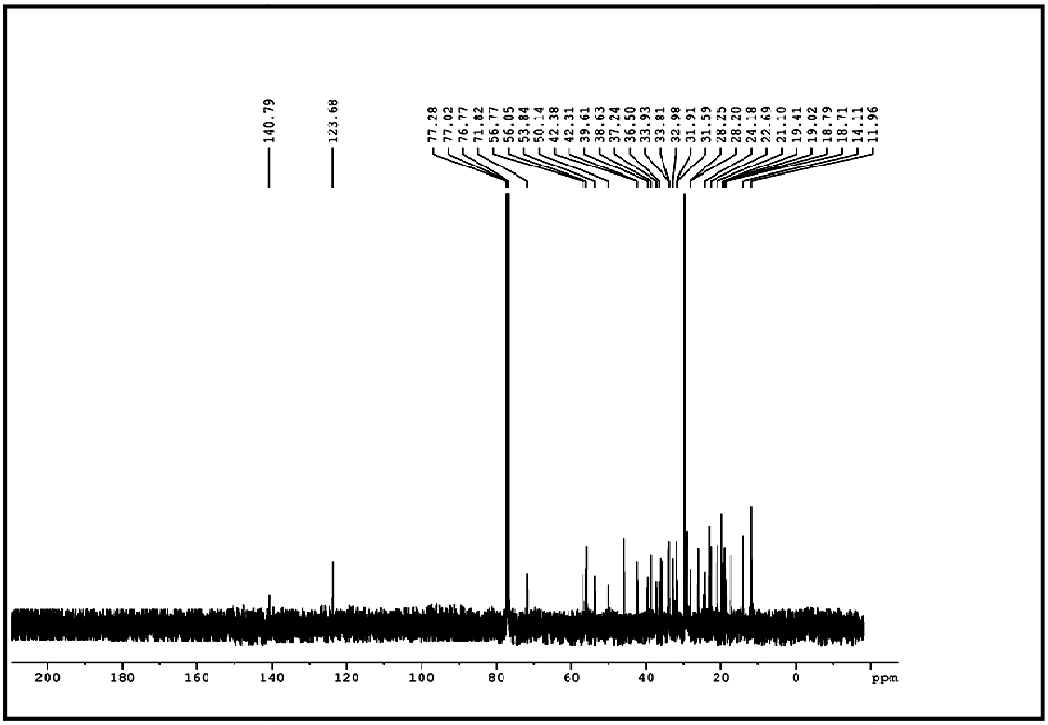


Figure 3.10: ^{13}C NMR spectrum of compound **VI**

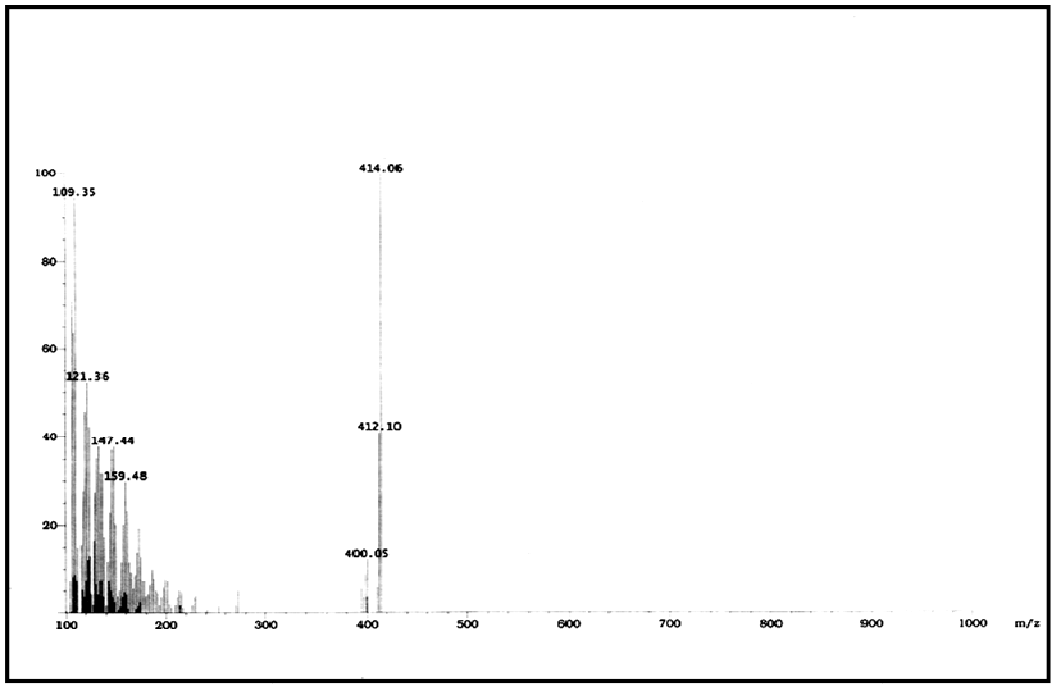
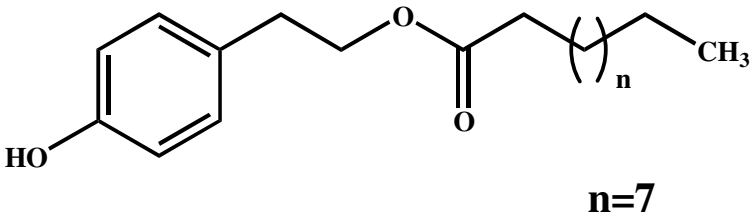


Figure 3.11: Mass spectrum of compound **VI**

Another major spot on second fraction pool was examined by various spectral methods viz. IR, ^{1}HNMR, ^{13}CNMR and HRMS. Compound **VII**, obtained as a white powder (151 mg) having molecular formula $C_{19}H_{30}O_3$ showed molecular ion peak at m/z 307.48. The IR spectrum revealed the presence of hydroxyl group at 2916 cm^{-1} and an ester carbonyl group at 1730 cm^{-1}. In ^{1}H nmr spectrum (Figure 3.12), the compound showed two two-proton doublets at δ 7.08 (2H, *J*=8 Hz) and 6.77 (2H, *J*=8 Hz) suggesting the presence of a 1, 4 disubstitued aromatic ring. Benzylic methylene and oxymethylene group of an ester were obtained as triplets at δ 2.86 (2H, J_1=J_2=7 Hz) and 4.23 (2H, J_1=J_2=7 Hz). A phenolic –OH group has been accounted as a singlet obtained at δ 5.35, which was exchanged with D_2O. The above data suggested the presence of a 4-hydroxy phenylethyl group, which substantiated the fragment obtained at m/z 121 in spectrum. Another triplet at δ 2.35 (2H, J_1=J_2=7.5 Hz) characterizes the presence of a methylene group adjacent to a carbonyl group. A broad singlet obtained at δ 1.25

integrating for 14 protons accounted for the presence of an aliphatic chain comprising seven methylenes. The spectrum further displayed a three proton triplet at δ 0.88 confirms the presence of a terminal methyl group of an aliphatic chain. ^{13}CNMR spectrum showed characteristic peaks at δ 173.88 and δ 154.20 suggesting the presence of a carbonyl carbon and a carbon at 4′ position (Figure 3.13). The DEPT NMR spectrum also substantiated the peaks (Figure 3.14). The $[M+H]^+$ ion peak was obtained at HRMS-FAB m/z 307.48 (Figure 3.15). From the above data and by comparing the values with those in the literature confirm the structure as undecanoic acid ester of 4-hydroxyphenyl ethanol or **2-(4′-hydroxyphenyl) ethyl undecanoate**, has been isolated previously from *S. personatum* (Sampath Kumar, 2005). The structure of compound **VII** is as shown below.



n=7

VII

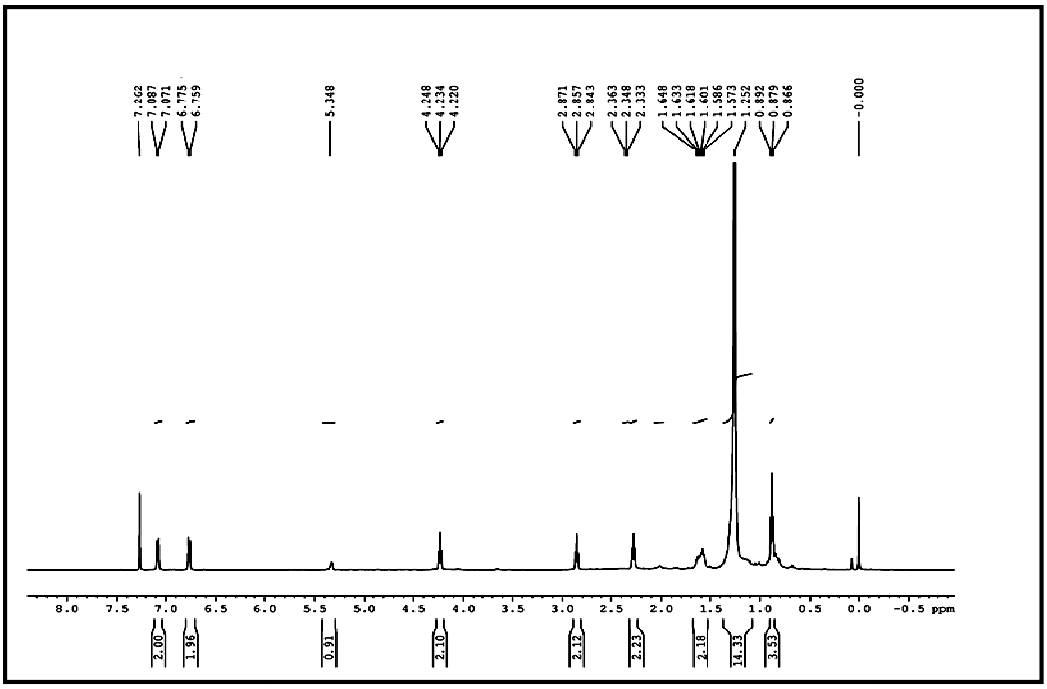


Figure 3.12: [1]H NMR spectrum of compound **VII**

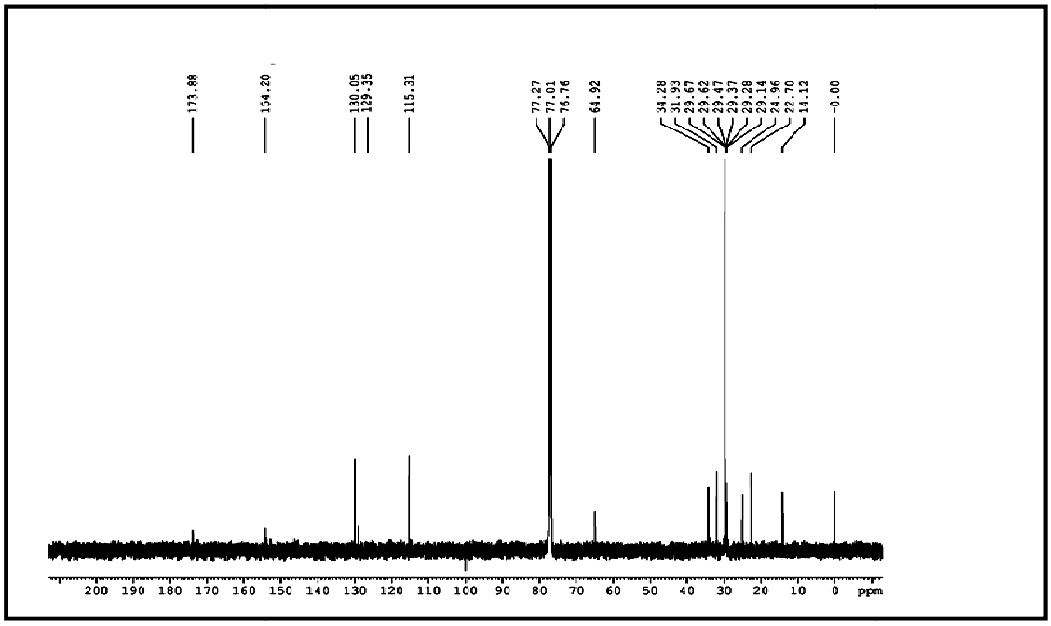


Figure 3.13: [13] C NMR spectrum of compound **VII**

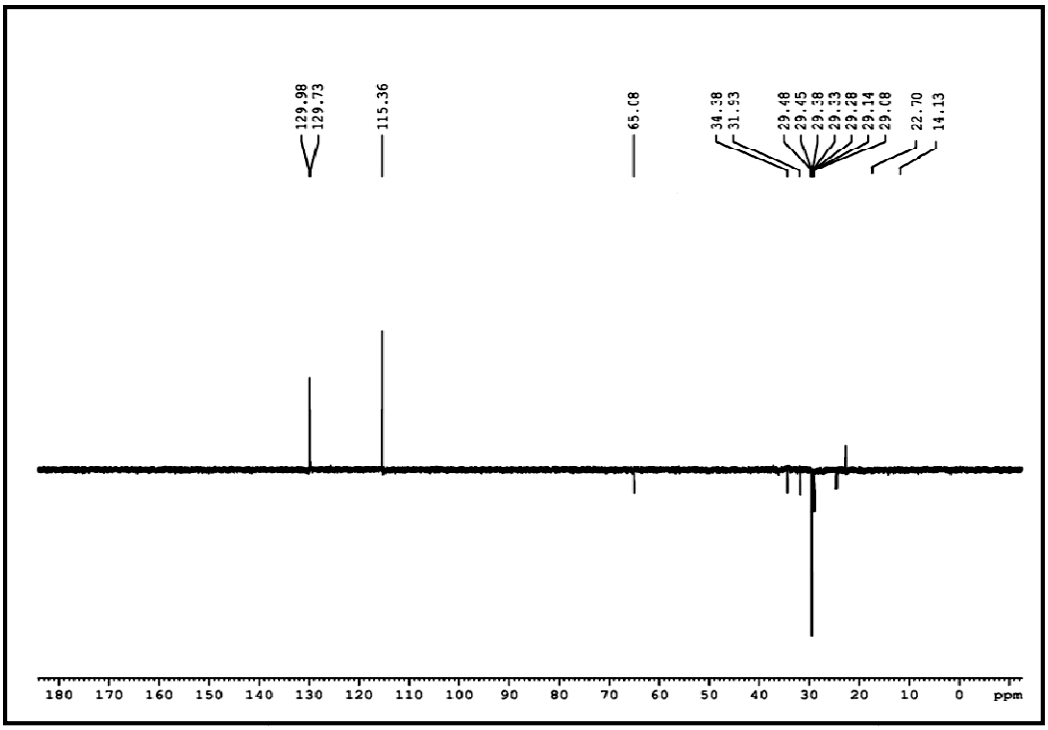


Figure 3.14: DEPT NMR spectrum of compound **VII**

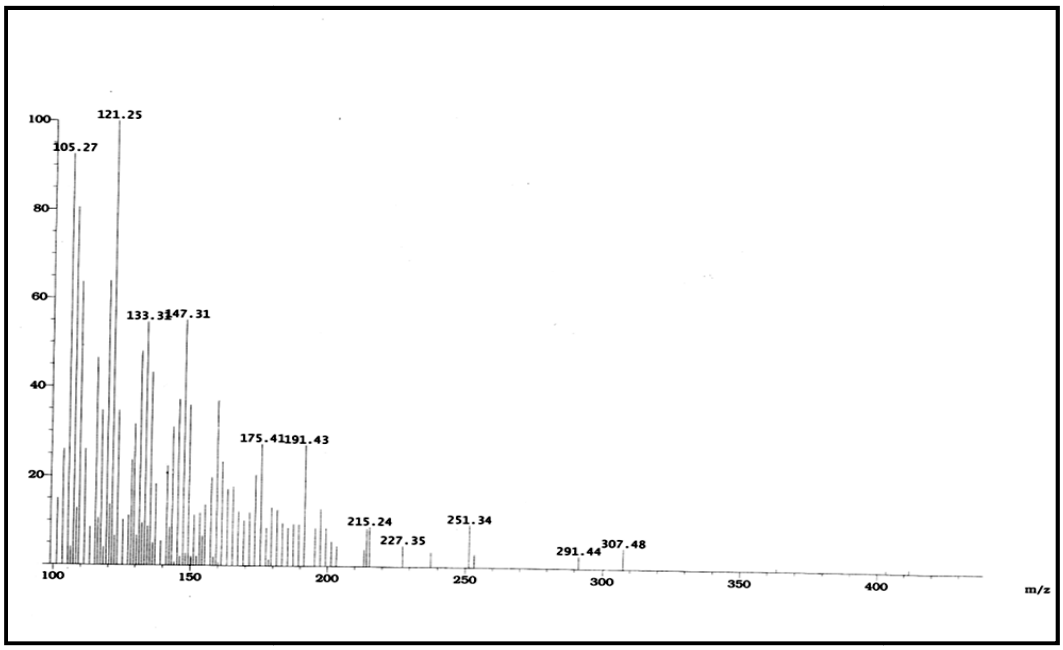
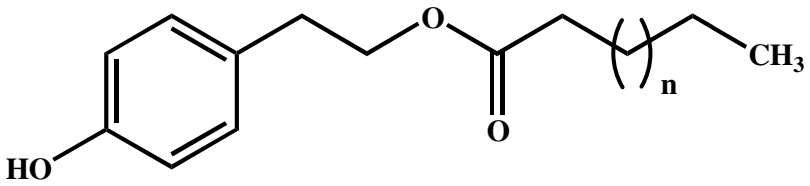


Figure 3.15: Mass spectrum of compound **VII**

The third fraction pool (50 mg, 57-70) on crystallization with hexane-dichloromethane mixture yielded a white solid (40 mg). IR spectrum showed peak corresponding to hydroxyl group at 2916 cm^{-1} and an ester carbonyl group at 1737 cm^{-1}. Proton nmr spectrum (Figure 3.16) showed two doublets at δ 7.08 (2H, *J*= 8.5 Hz) and δ 6.77 (2H, *J*= 8 Hz) indicates the presence of 1, 4-disubstitued aromatic ring and triplets at δ 2.86 (2H, J_1=J_2=7 Hz) and 4.23 (2H, J_1=J_2=7 Hz) characterizes the presence of a benzylic methylene and a oxymethylene group of an ester. Phenolic –OH group was obtained as a singlet at δ 4.81 and another triplet obtained at δ 2.35 (2H, J_1=J_2= 7.5 Hz) characterizes the presence of a methylene group adjacent to a carbonyl group. A broad singlet obtained at δ 1.26 integrating for 22 protons accounted for the presence of an aliphatic chain comprising eleven methylenes. The spectrum further displayed a three proton triplet at δ 0.88 confirms the presence of a terminal methyl group of an aliphatic chain. The ^{13}C NMR spectrum (Figure 3.17) confirms the presence of carbonyl carbon at δ 172.48 and carbon at 4′ position at δ 155.01. The other 21 carbon atoms were obtained at δ 128.96 (2C), 127.82, 114.48 (2C), 64.09, 32.02, 31.01, 28.64-21.75 (12C), 13.03. DEPT NMR spectrum also showed characteristic peaks (Figure 3.18). The molecular formula was deduced from its positive ion HRMS-FAB obtained at m/z 363.19 $[M+H]^+$ (Figure 3.19) as $C_{23}H_{38}O_3$. From the above data, the structure was confirmed as a new pentadecanoic acid ester of 4-hydroxyphenyl ethanol or **2-(4′-hydroxyphenyl) ethyl pentadecanoate.** This compound has been isolated and identified for the first time from natural sources.



n=11

VIII

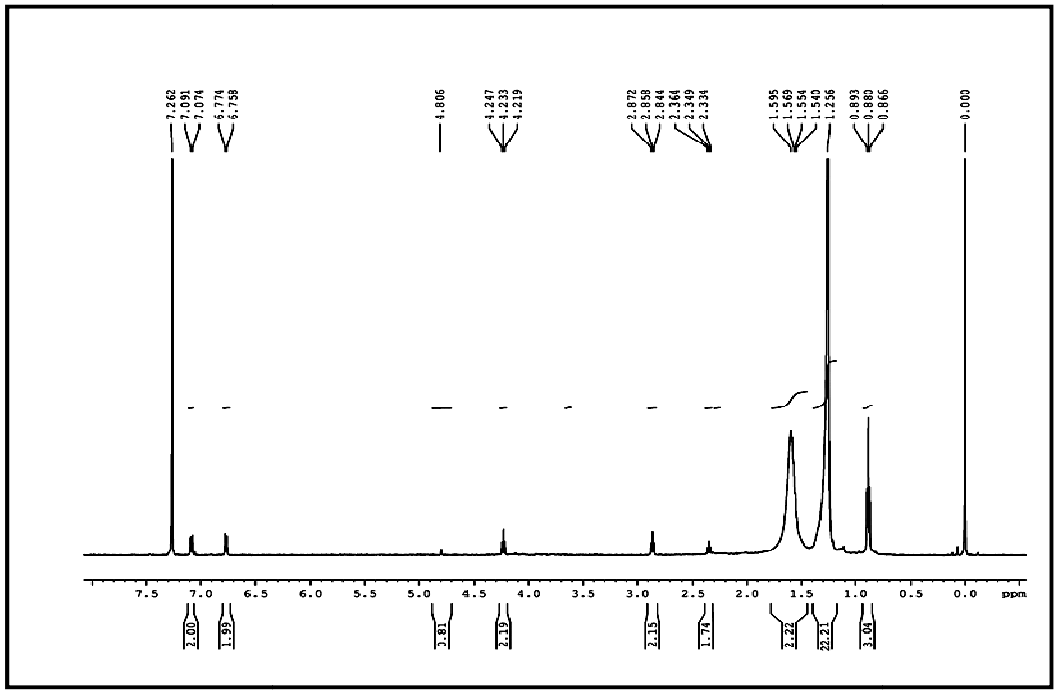


Figure 3.16: ^{1}H NMR spectrum of compound **VIII**

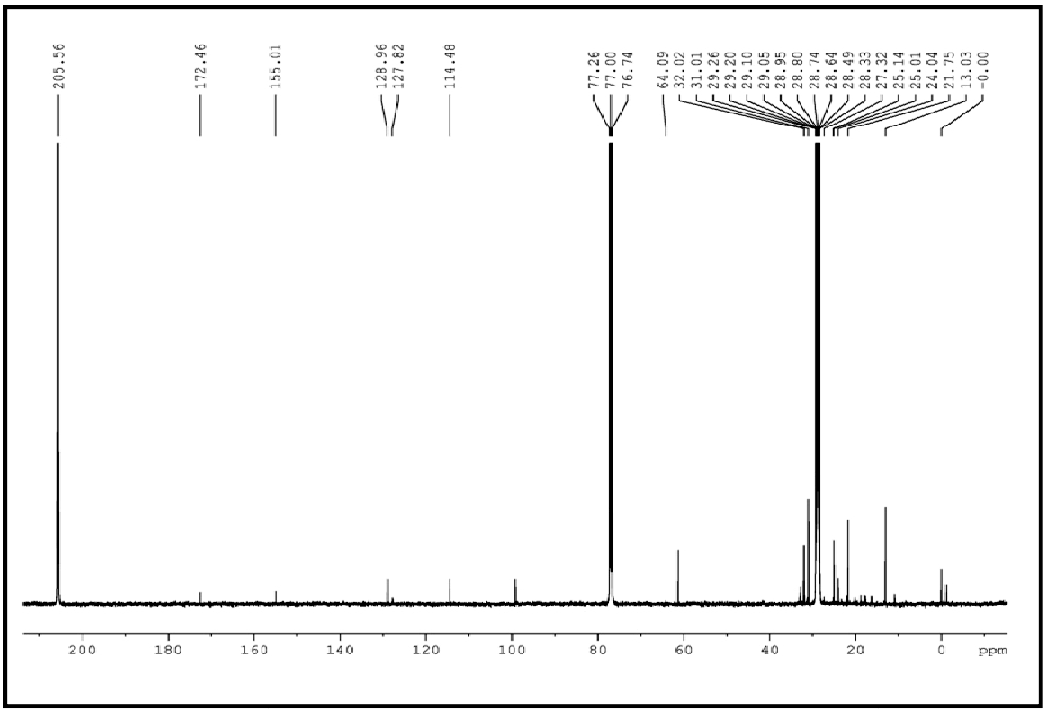


Figure 3.17: ^{13}C NMR spectrum of compound **VIII**

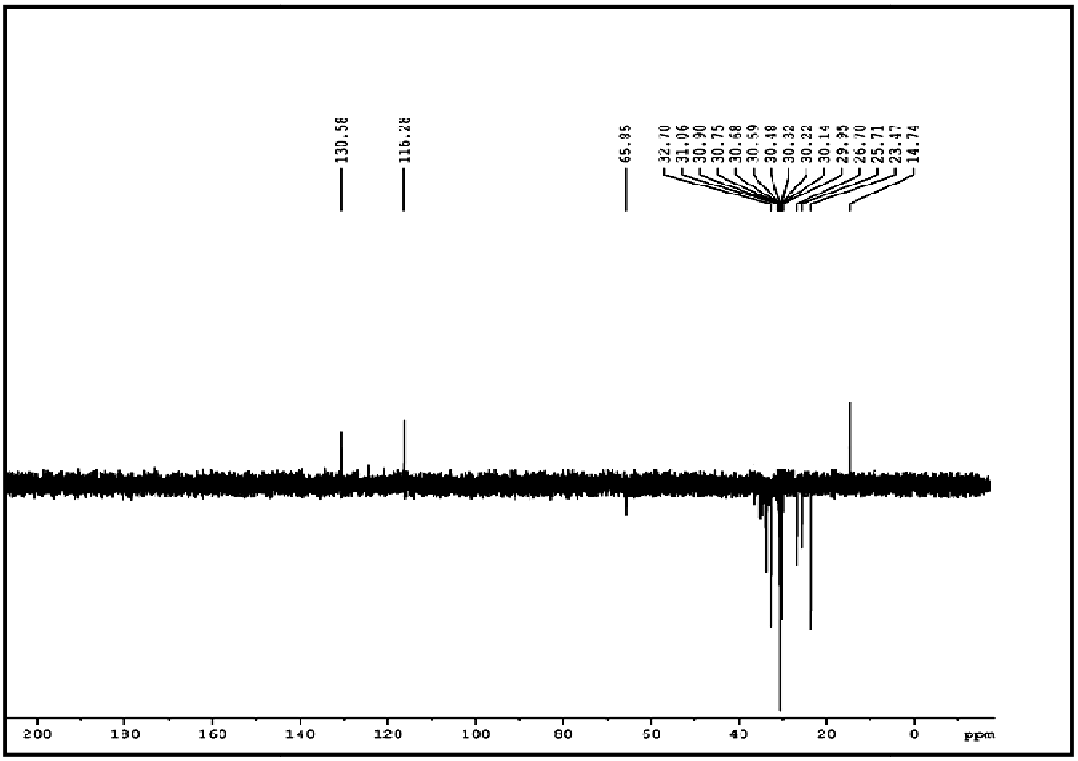


Figure 3.18: DEPT NMR spectrum of compound **VIII**

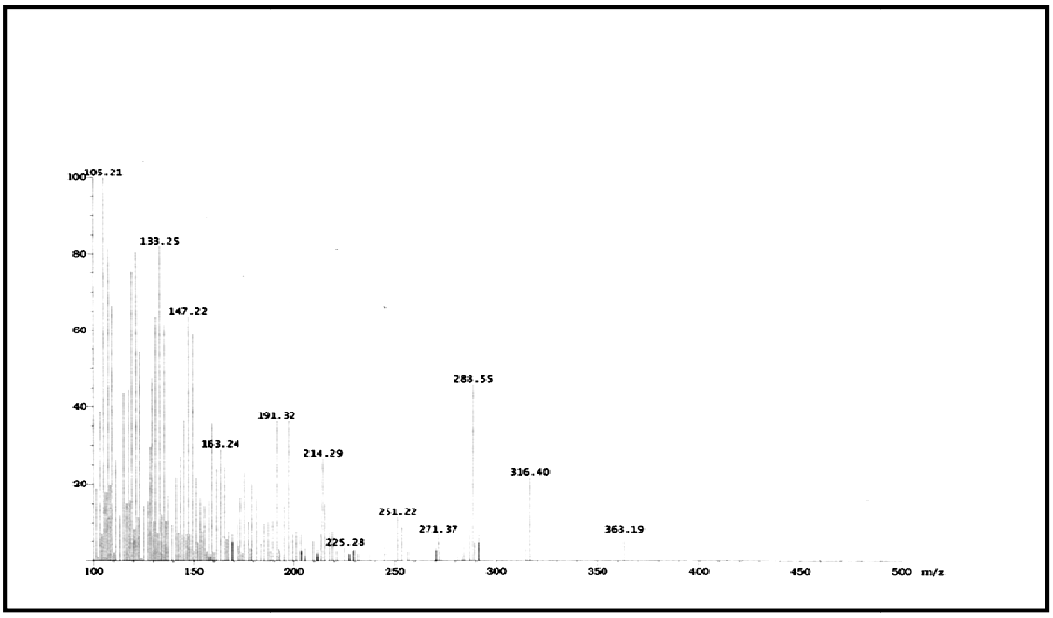
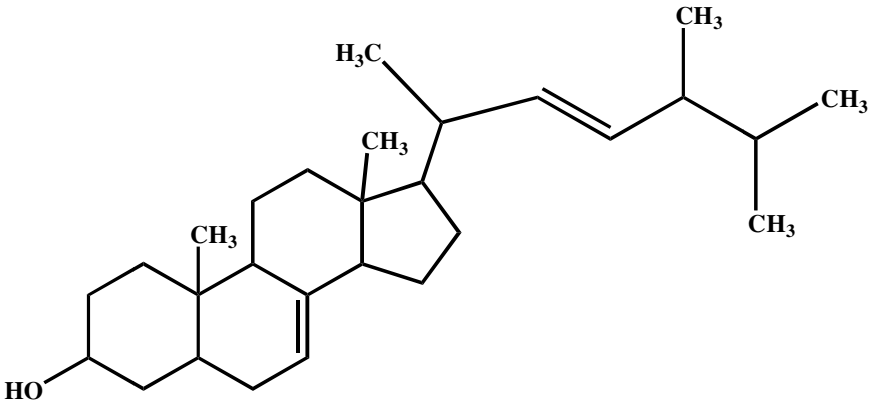


Figure 3.19: Mass spectrum of compound **VIII**

Fifth fraction pool (F 86-95; 75 mg) was purified by crystallization using hexane –dichloromethane solvent system yielded compound **IX** (70 mg) as a colourless amorphous powder in pure form. IR spectrum revealed peaks at 3282 cm^{-1} indicates –OH group and the peaks at 2933 and 2865 cm^{-1} indicates the presence of C=C group. Proton nmr spectrum (Figure 3.20) showed hydroxyl group at δ 5.92 and two olefinic protons at δ 5.01 and 5.15 (1H) as doublet of a doublet. Also showed two, three proton singlets at δ 0.82 and 0.68 indicates the presence of two methyl groups and doublets at δ 0.79, 0.84 and 0.86 (3H) indicates the presence of terminal methyl groups. Another methyl group was obtained at δ 0.92 (3H, *J*= 6.5 Hz) as a doublet. Furthermore, it showed 28 carbon signals including the characteristic peaks at δ 140.56, 137.70, 128.43 and 120.13 for olefinic four carbon atoms (Figure 3.21). The rest of the carbon atoms were obtained at δ 70.19, 55.27, 55.17, 45.05, 41.50, 41.38, 39.78, 39.04, 38.93, 35.71, 35.33, 33.10, 31.15, 31.07, 30.71, 27.42, 22.18, 20.23, 20.06, 18.74, 18.38, 17.96, 17.79 and 11.21. The

molecular formula was deducted as $C_{28}H_{46}O$ on the basis of HRMS-FAB at m/z 398.06 $[M]^+$ (Figure 3.22). These data as well as the spectral values obtained from literature (Ahmad *et al*., 2000) confirms compound **IX** as **5α-ergostan-7, 22-dien-3β-ol**.



IX

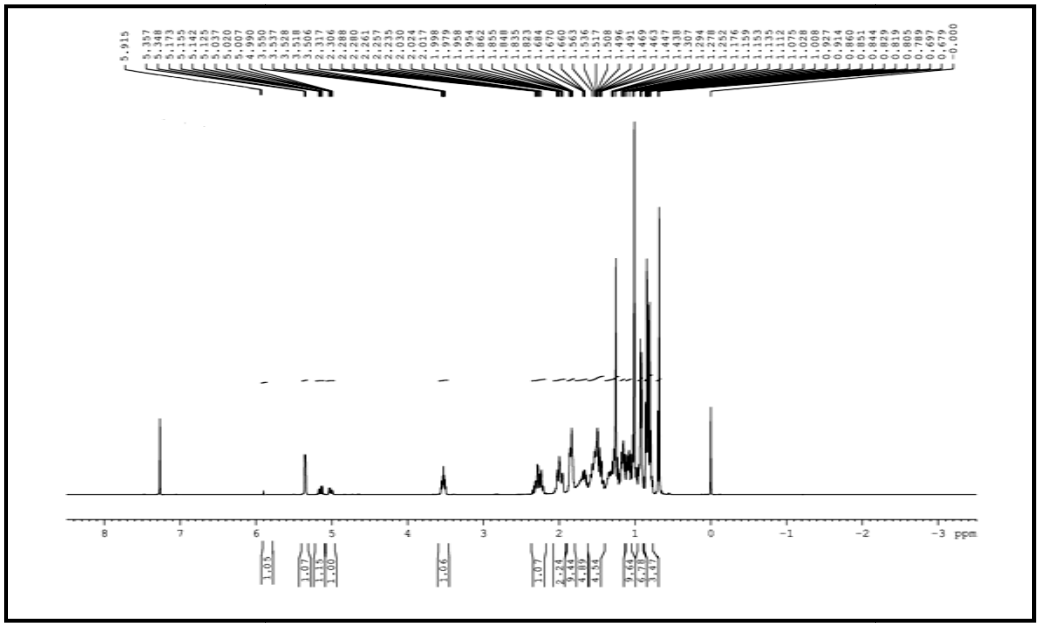


Figure 3.20: ^{1}H NMR spectrum of compound **IX**

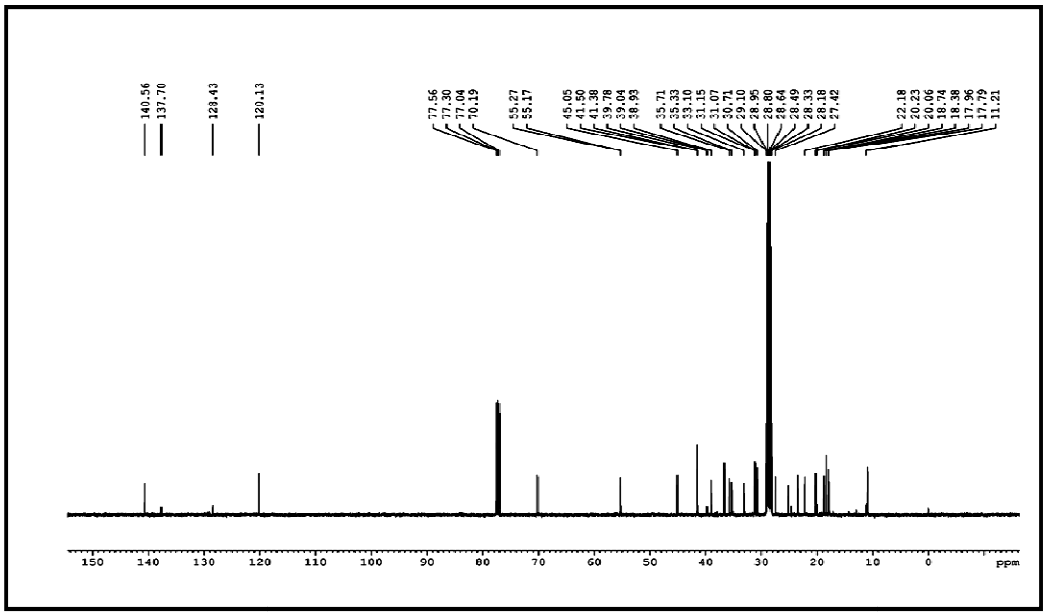


Figure 3.21: ^{13}C NMR spectrum of compound **IX**

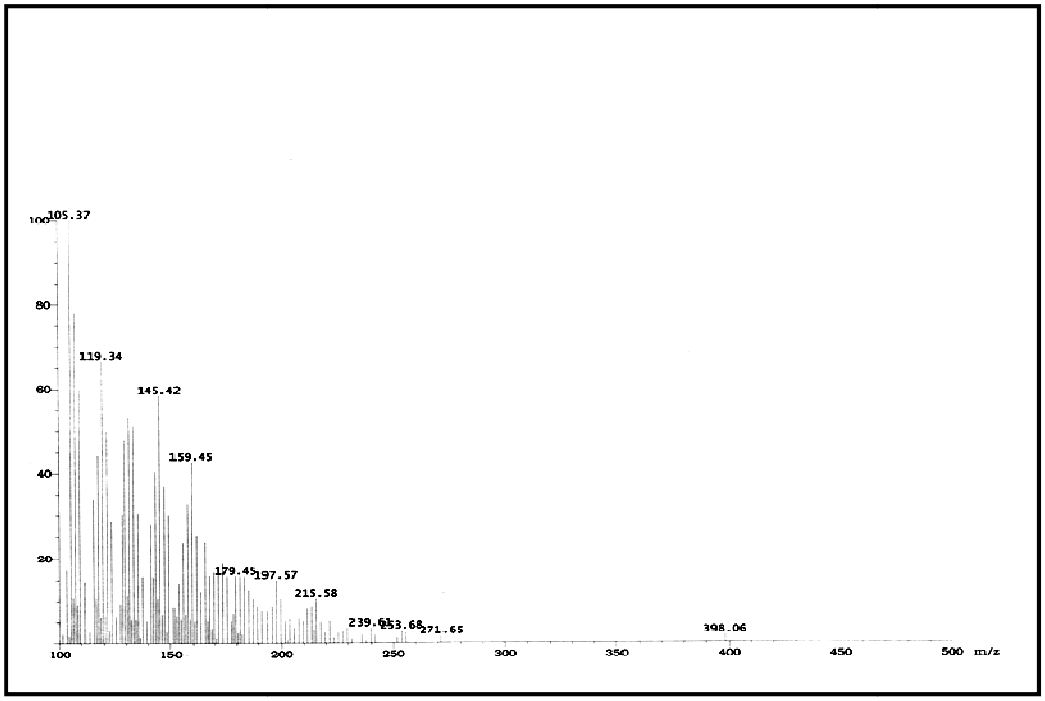
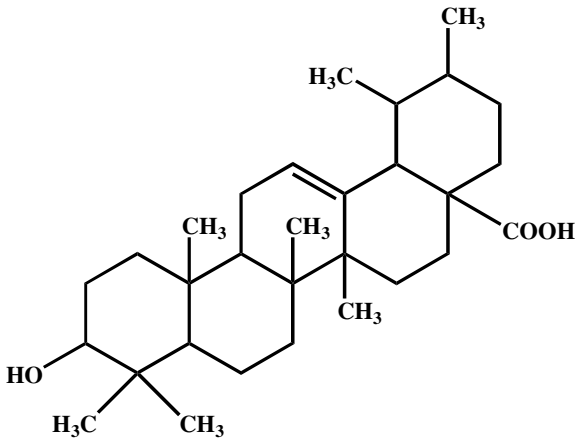


Figure 3.22: Mass spectrum of compound **IX**

The seventh fraction pool on column chromatography by silica gel (100-200 mesh) with hexane–ethyl acetate solvent system (85:15-70:30) yielded compound **X** (200

mg) and **XI** (160 mg). Compound **X** was obtained as an off white powder which shows IR absorption peak at 3739 cm^{-1} indicates the presence of hydroxyl group and other characteristic peaks were obtained at 3397, 1674, 1597, 1250, 1107 and 957 cm^{-1}. Proton nmr spectra (Figure 3.23) showed hydroxyl groups at δ 8.18, four singlet peaks at δ 1.21, 0.98, 0.95 and 0.94 indicates the presence of five methyl groups and the remaining methyl groups were obtained as two doublets at δ 0.89 (*J*= 5.5 Hz) and 0.87 (*J*= 6.5 Hz). Other protons were obtained as multiplets of 19 protons at δ 1.62-1.60. In ^{13}C NMR spectrum (Figure 3.24), a carbonyl carbon was obtained at δ 185.07 and olefinic carbon atoms were found at δ 137.93 and δ 130.75. The molecular formula of compound **X** was deducted as $C_{30}H_{48}O_3$ obtained from the molecular ion $[M+H]^+$ at m/z 457.13 (Figure 3.25) further confirmed that the compound **X** is **ursolic acid**, earlier isolated from *S. acuminatissimum* (Ramsay *et al*., 2012) as shown below.



X

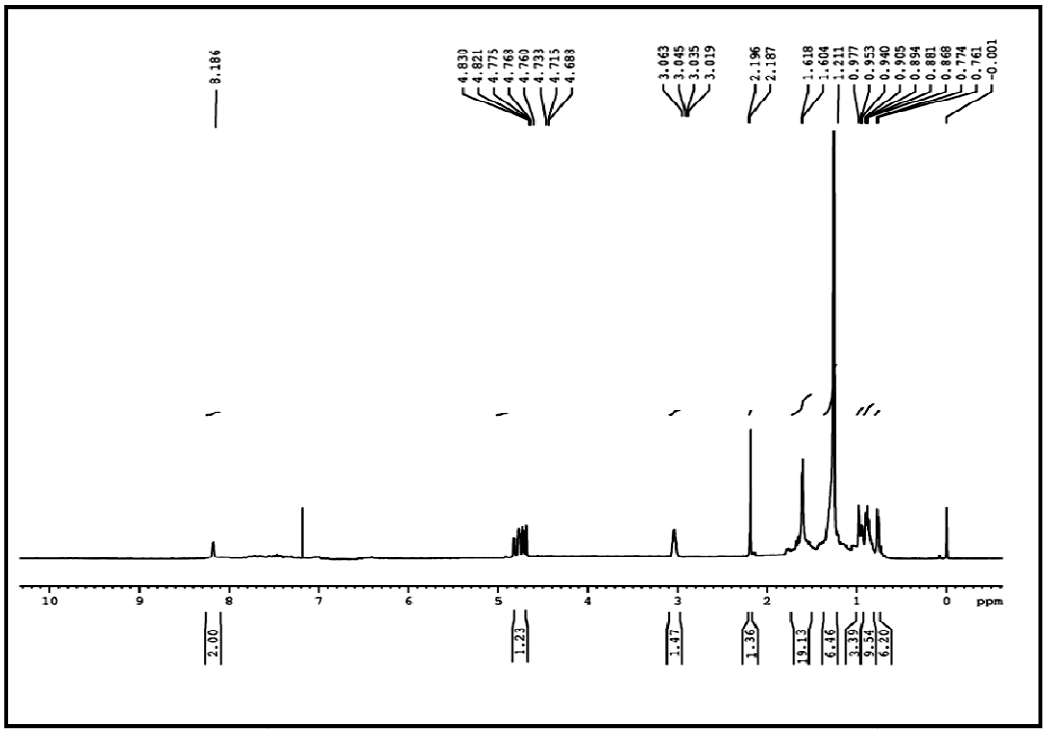


Figure 3.23: ^{1}H NMR spectrum of compound **X**

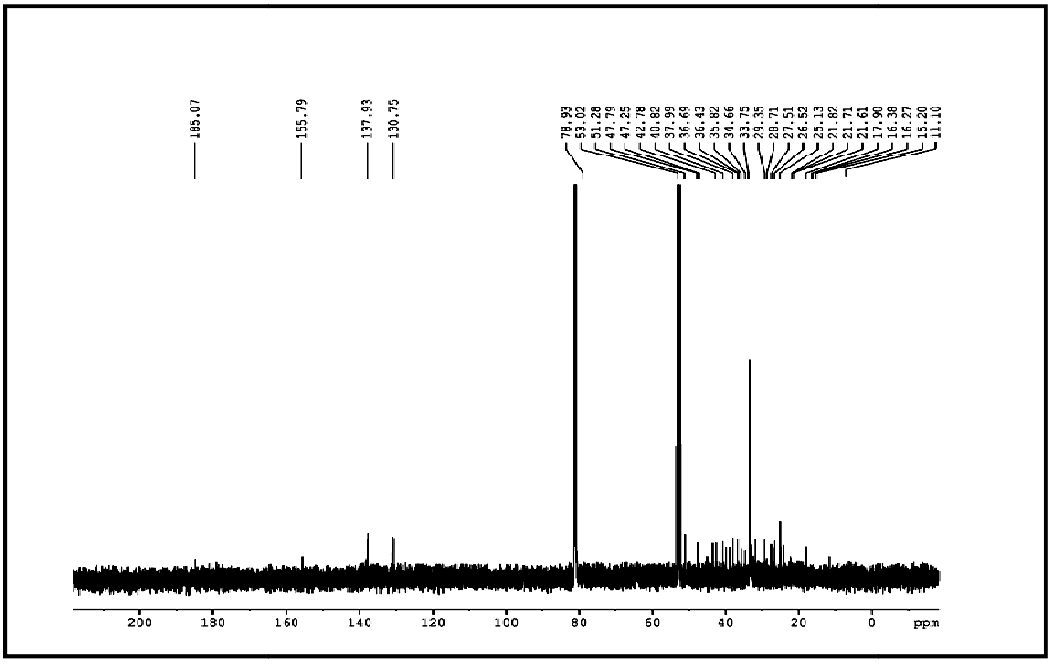


Figure 3.24: ^{13}C NMR spectrum of compound **X**

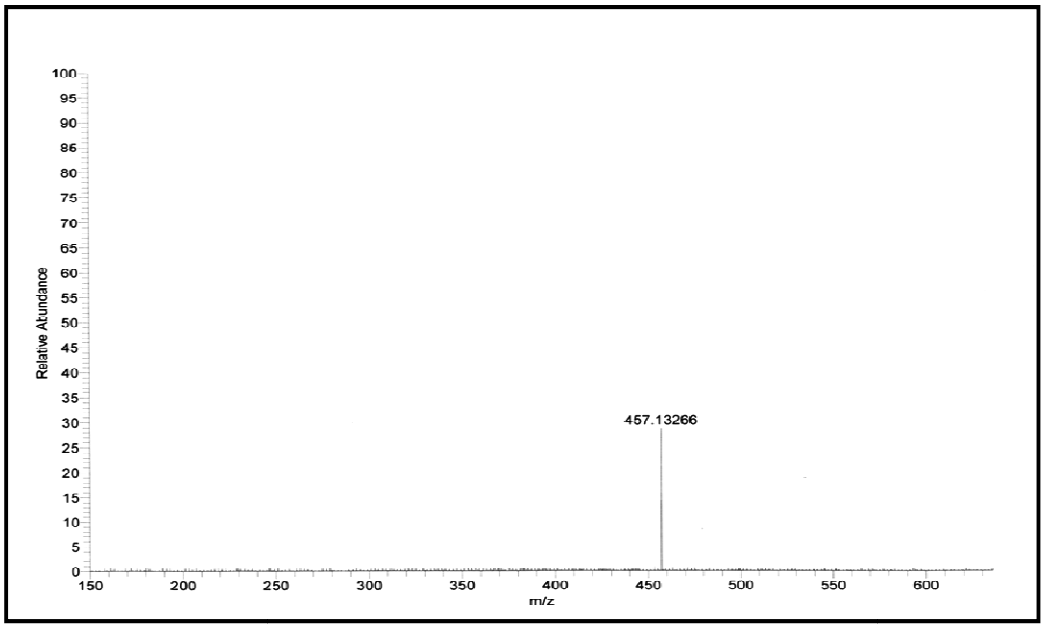
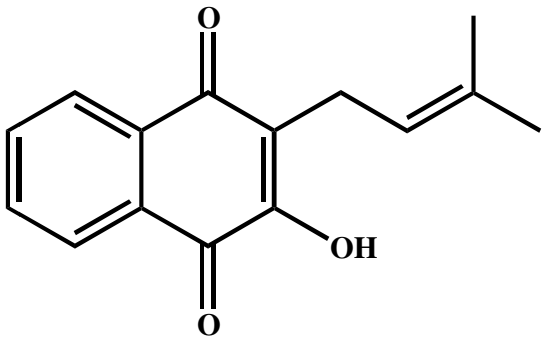


Figure 3.25: Mass spectrum of compound **X**

Compound **XI** was obtained as dark yellow powder (160 mg) which shows characteristic IR peaks at 1672 and 1648 cm^{-1} indicates the presence of two quinone carbonyl groups and a hydroxyl group was obtained at 3422 cm^{-1}. A C-O stretch was obtained at 1273 cm^{-1}. Proton nmr spectrum showed aromatic protons at δ 8.24-7.70 as a multiplet and a methylene group at δ 3.78 (2H, *J*=7 Hz) as a doublet. Further showed two singlet peaks at δ 1.61 and 1.59 (3H each) indicates the presence of two methyl groups. A hydroxyl group integrating for one proton was obtained at δ 7.79 as a singlet (Figure 3.26). Characteristic carbon signals obtained at δ 182.23 and 174.83 indicates the presence of quinone carbon and two olefinic carbon atoms were obtained at δ 127.87 and 132.74 (Figure 3.27). The DEPT-135 NMR spectrum (Figure 3.28) gave one -CH_2- group and two –CH_3 groups in the aliphatic region. The other –CH groups were present in the aromatic region of the spectrum. The molecular ion peak obtained at m/z 243.06 $[M+H]^+$ in HRESIMS spectrum (Figure 3.29) further confirmed that the compound **XI** is **lapachol**

($C_{15}H_{14}O_3$), as shown below and previously isolated from *S. suaveolens* showed marked anticancer activity against Walker 256 carcinosarcoma cells (Rao *et al*., 1968).



XI

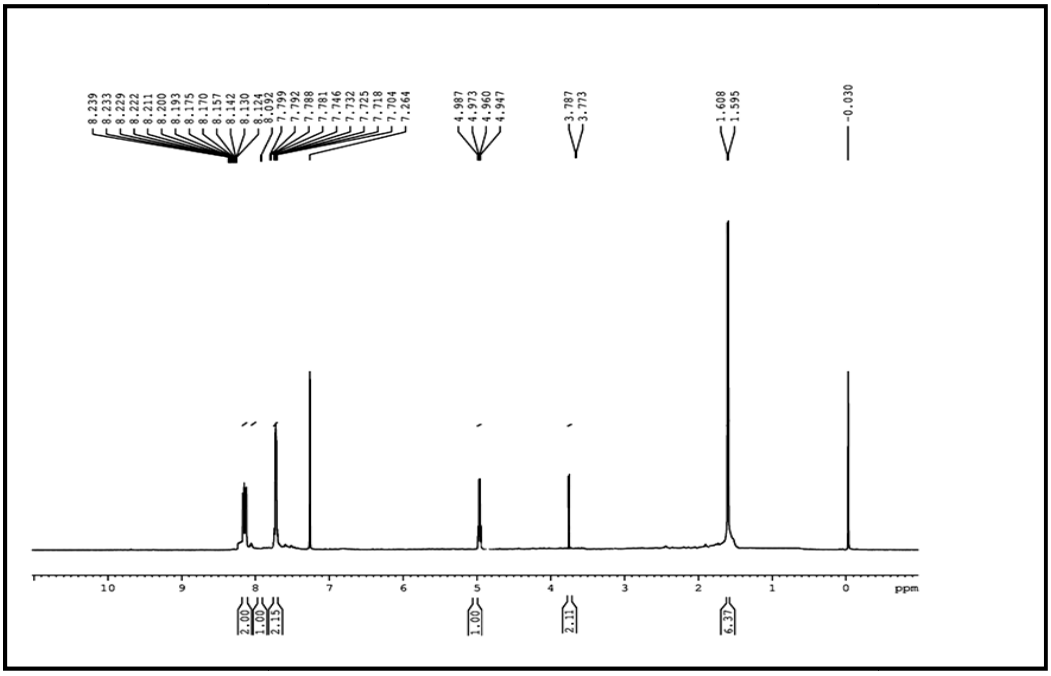


Figure 3.26: ^{1}H NMR spectrum of compound **XI**

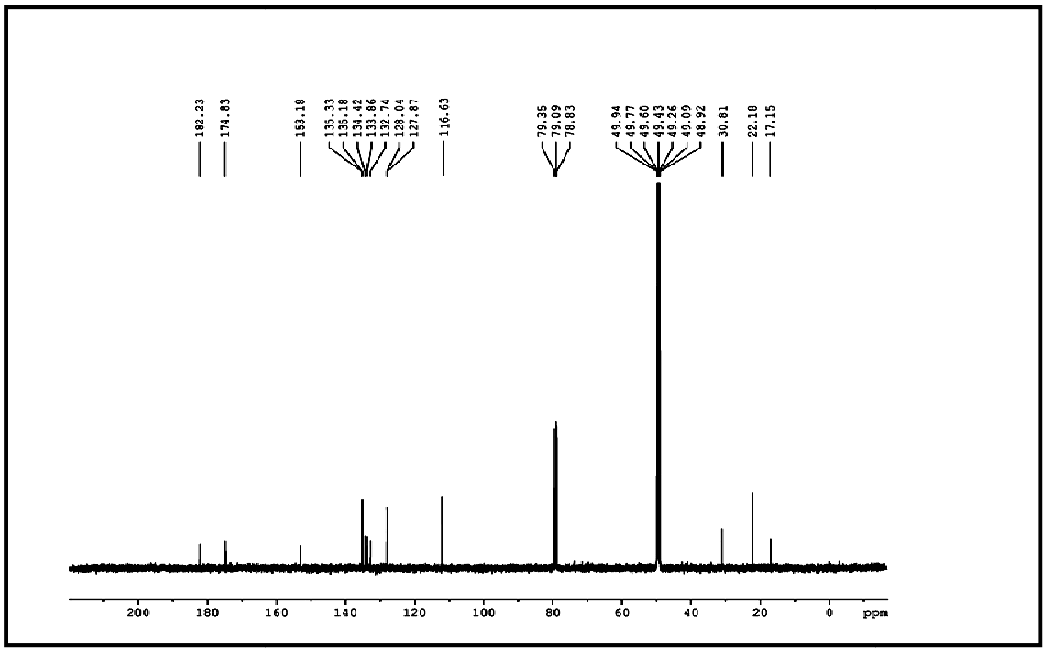


Figure 3.27: ^{13}C NMR spectrum of compound **XI**

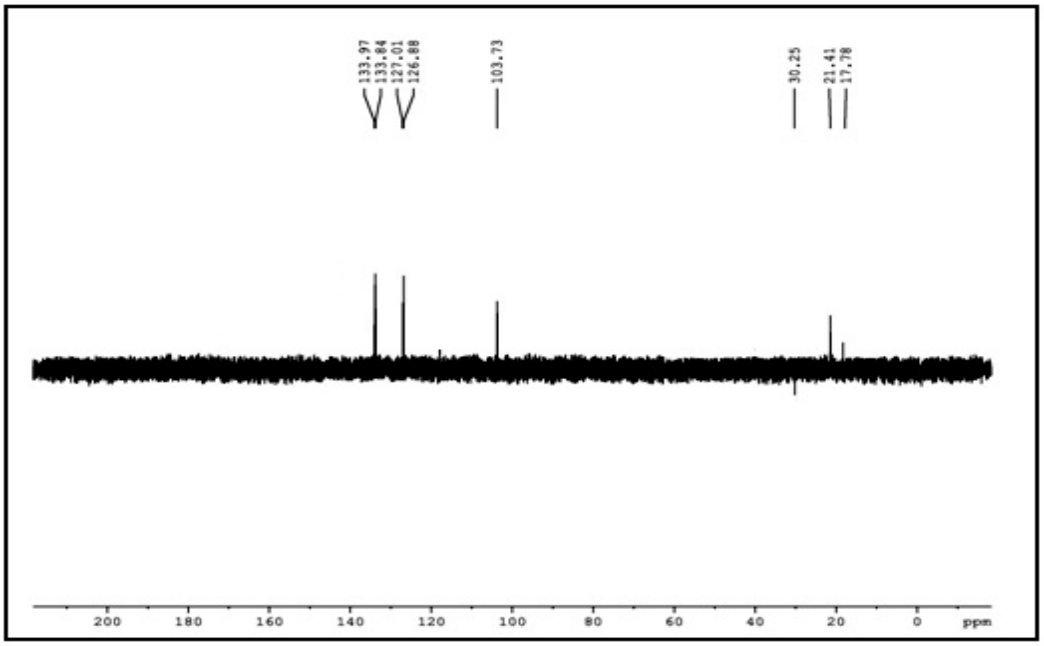


Figure 3.28: DEPT NMR spectrum of compound **XI**

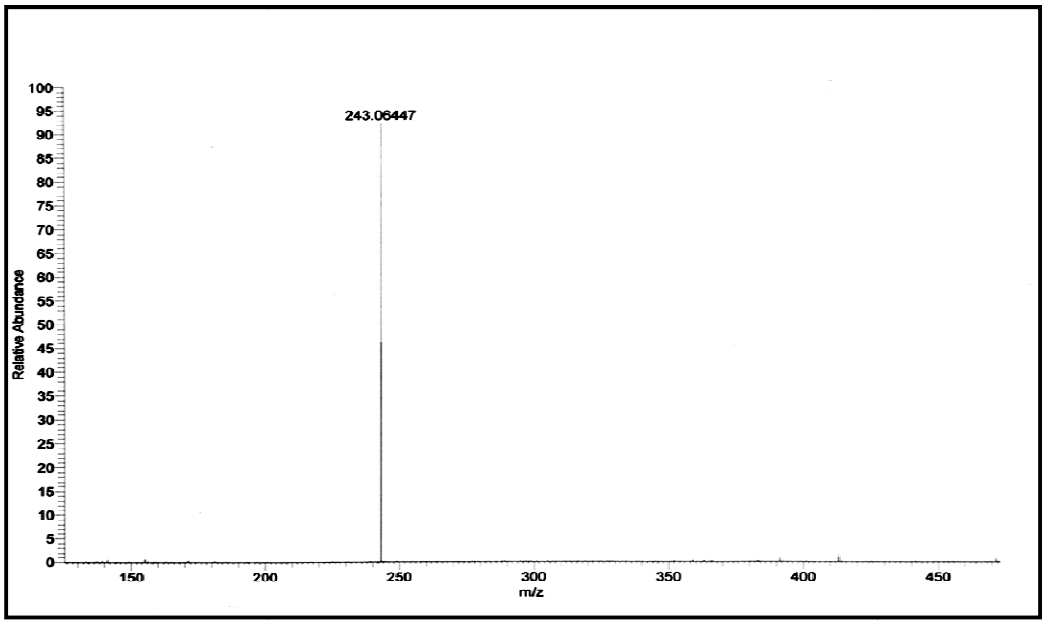
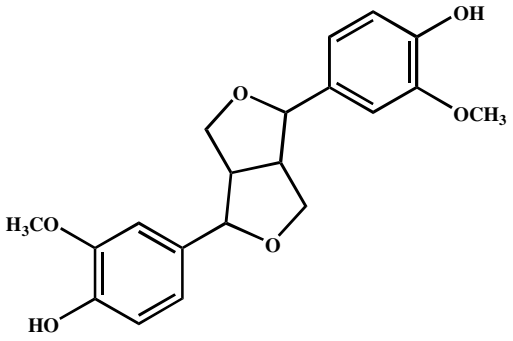


Figure 3.29: Mass spectrum of compound **XI**

The eighth fraction pool upon column chromatography (F 193-228, 1.31 g) on silica gel employing 50% ethyl acetate-hexane as eluant yielded compound **XII** (50 mg) as white powder. IR spectra of compound **XII** showed the presence of peaks at 3482cm^{-1} indicates the presence of hydroxyl groups and 1028 cm^{-1} indicates the presence of C-O group. Aromatic groups were obtained at 881 cm^{-1}. Proton nmr spectra (Figure 3.30) showed hydroxyl group at δ 7.94 as a singlet integrating for two hydroxyl protons and aromatic protons at δ 7.61-6.83 as multiplets integrated for six protons. Further two singlets at δ 3.93 and 3.87 characterize the presence of two methoxy groups. Further confirmation of the structure was gathered from ^{13}C spectrum which gave signals at δ 150.87 indicates the presence of carbon atoms bearing –OCH_3 groups in 3′ and 3″ positions. Carbon atoms at 4′ and 4″ positions were obtained at δ 147.02 and other carbon atoms were obtained at δ 131.82 (2C), 124.13, 121.71, 114.48, 112.50, 105.68 (2C), 95.99 (2C), 72.79 (2C), 56.41 (2 x OCH_3), 55.51 (2C) (Figure 3.31). DEPT-135 spectrum showed –CH_2 groups at δ 72.60 and –CH- and –CH_3 groups were obtained at δ 125.72,

115.98, 113.98, 106.73, 96.06, 58.02 and 57.15 (Figure 3.32). In the mass spectrum, **XII** was clearly obtained at 359.07 $[M+H]^+$ (Figure 3.33) confirmed the molecular formula as $C_{20}H_{22}O_6$ and the structure as **pinoresinol** as shown below as well as the comparison from literature values (Manuel Brenes *et al*., 2000).



XII

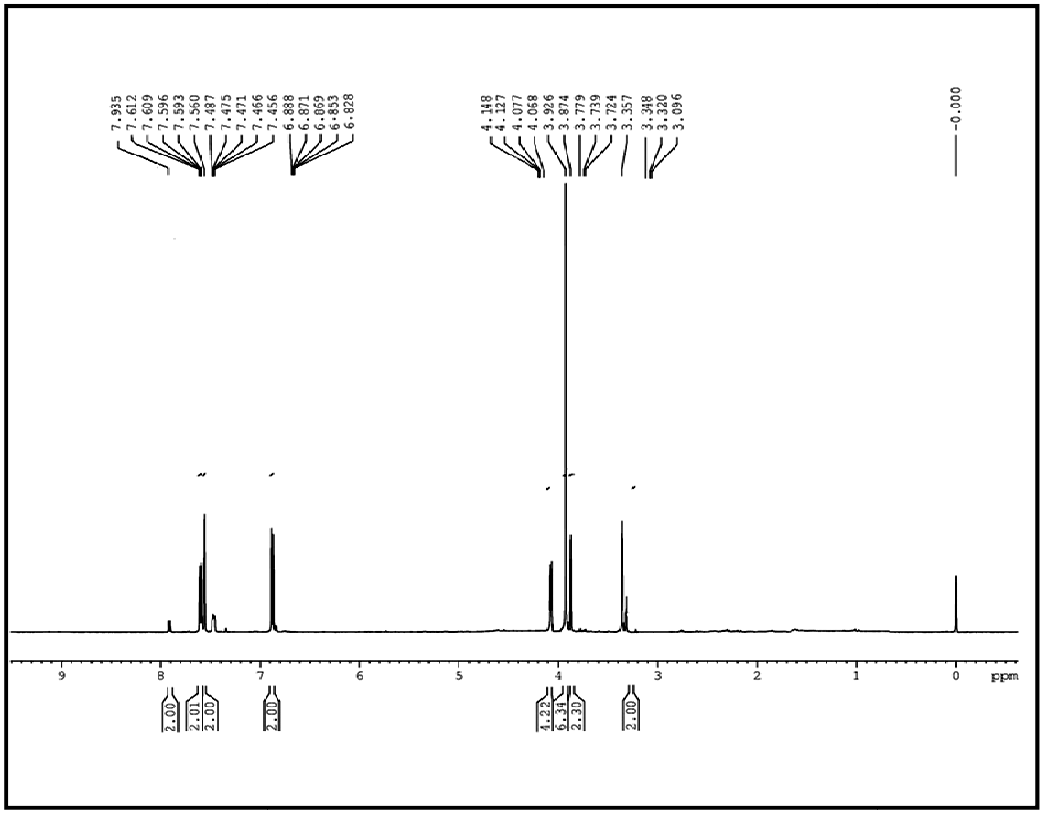


Figure 3.30: ^{1}H NMR spectrum of compound **XII**

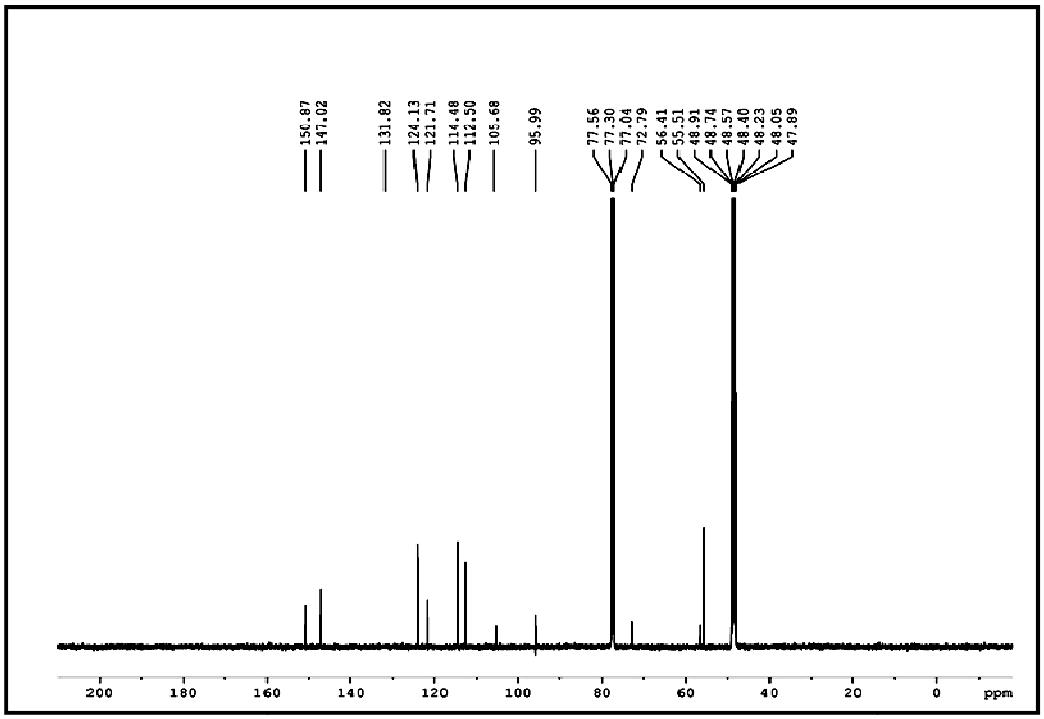


Figure 3.31: ^{13}C NMR spectrum of compound **XII**

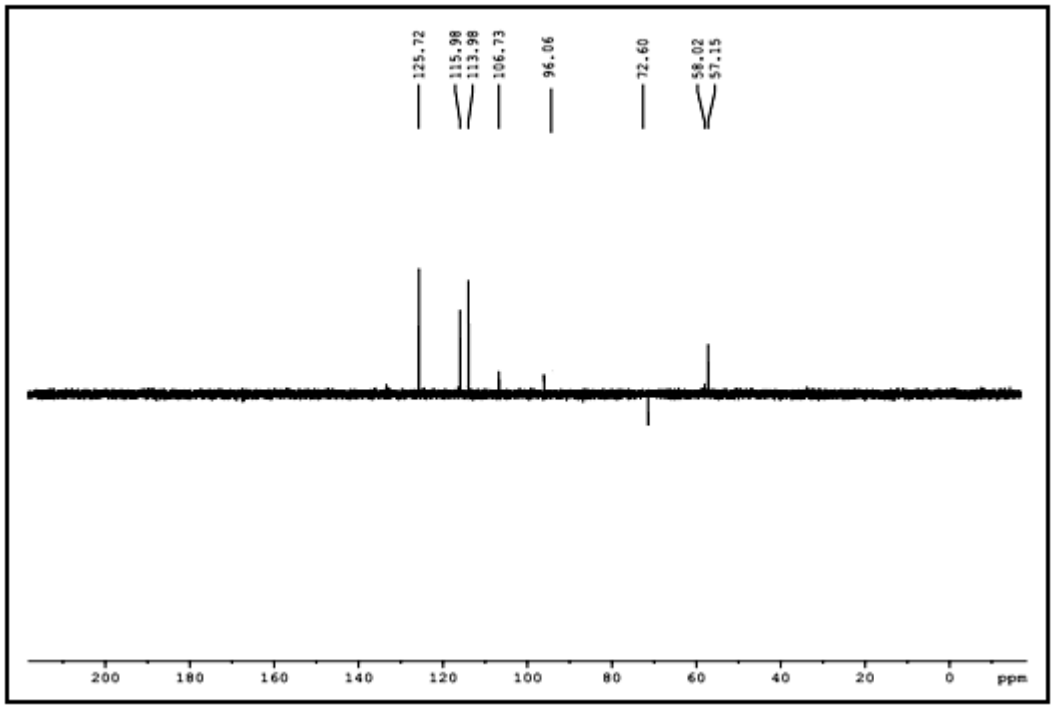


Figure 3.32: DEPT NMR spectrum of compound **XII**

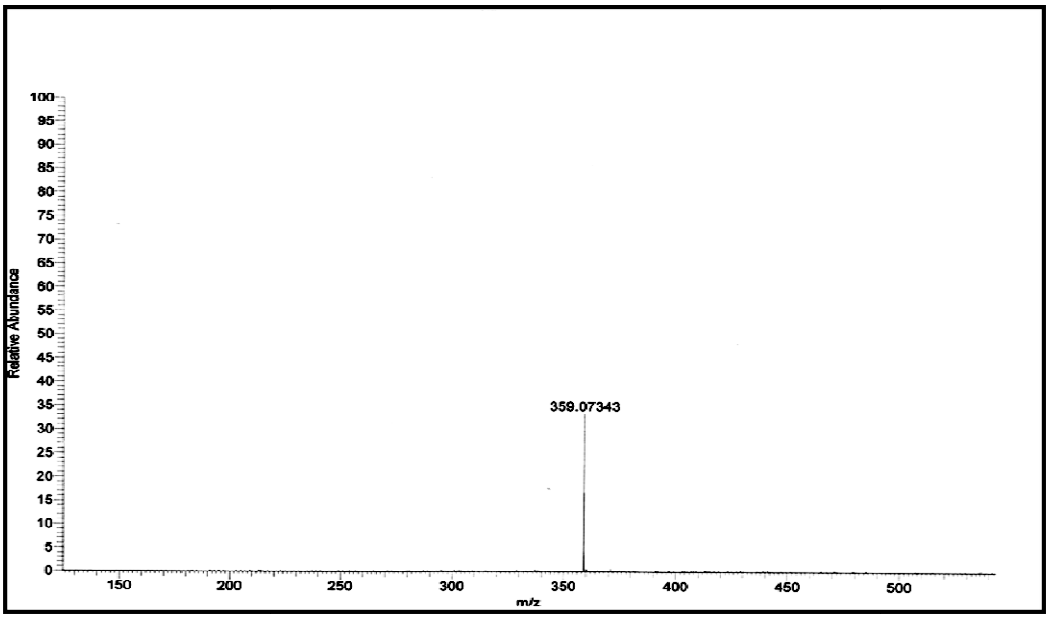


Figure 3.33: Mass spectrum of compound **XII**

3.5.5. DPPH radical scavenging potential of isolated compounds

Varying concentrations of β-sitosterol (**VI**), 2-(4′-hydroxyphenyl) ethyl undecanoate (**VII**), 2-(4′-hydroxyphenyl) ethyl pentadecanoate (**VIII**), 5α-ergostan-7, 22-dien-3β-ol (**IX**), ursolic acid (**X**), lapachol (**XI**) and pinoresinol (**XII**) were initially studied for antioxidant capacity using the diphenyl picryl hydrazyl (DPPH•) radical and pinoresinol showed remarkable scavenging activity with an IC_{50} value of 2.78 nM followed by ursolic acid (IC_{50} 39.02 nM) and lapachol (IC_{50} 62.81 nM) compared to standard gallic acid (IC_{50} 8.76 nM). β-sitosterol, 2-(4′-hydroxyphenyl) ethyl undecanoate, 2-(4′-hydroxyphenyl) ethyl pentadecanoate and 5α-ergostan-7, 22-dien-3β-ol did not show any DPPH radical scavenging capacity. The plot of DPPH radical scavenging capacity of ursolic acid, lapachol and pinoresinol along with standard gallic acid at different concentration is presented in figure 3.34. It is generally accepted that phenolic compounds greatly enhances the antioxidant capacity and its radical scavenging ability (Kefalas *et al*, 2003). Summarizing the results obtained, the radical scavenging

capacity is in the order pinoresinol> ursolic acid>lapachol. Thus, the higher free radical scavenging capacity of acetone extract of *S. colais* might be due to the synergistic effect of all the active compounds present in the extract.

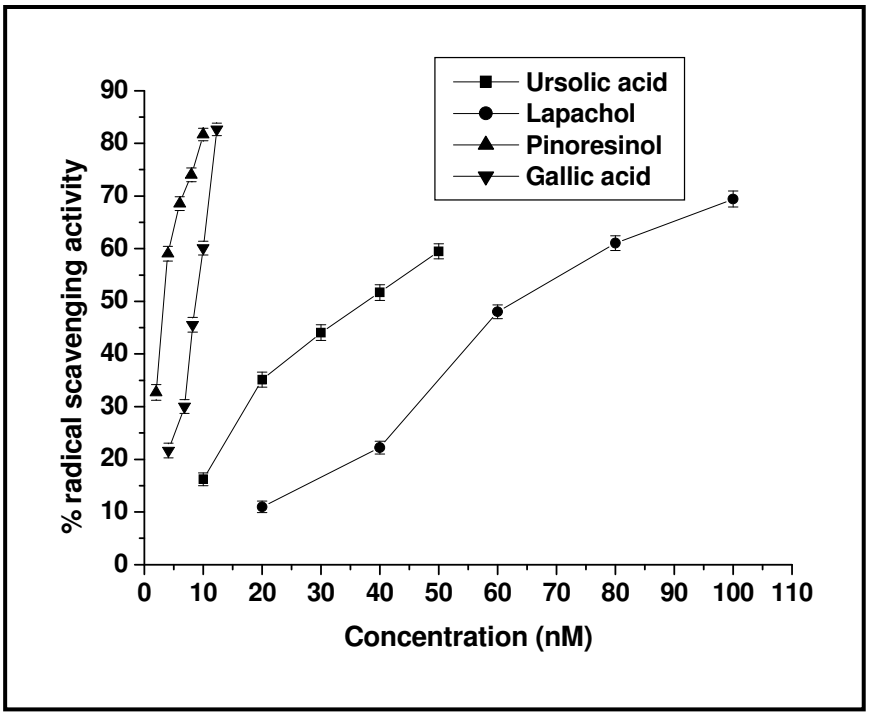


Figure 3.34: DPPH radical scavenging activity of pinoresinol, ursolic acid, lapachol and gallic acid

3.5.6. Inhibition of α-glucosidase and α-amylase enzymes by *S. colais*

Inhibition of α-glucosidase and α-amylase involved in carbohydrate metabolism is considered as one of the most important therapeutic approach for controlling post prandial hyperglycaemia (Ortris Andrade *et al*., 2007) which causes non-enzymatic glycosylation and resulting in the formation of chronic complications (Lopez Candales, 2001).

The treatment goal of diabetes patients is to maintain near normal levels of glycemic control by preventing the absorption of carbohydrates after food intake.

Digestive enzymes facilitated the breaking of oligosaccharides and disaccharides into monosaccharides. Synthetic inhibitors reduce the post prandial digestion and absorption of starch from intestine (Davis and Granner, 1996). Many natural resources have been investigated with respect to suppress glucose production from carbohydrates in the gut or glucose absorption from the intestine (Matsui *et al*., 2007). α-Glucosidase inhibitory activity was measured using five different concentrations of samples and the results showed dose dependent inhibition potential. Acetone extract showed inhibitory activity (IC_{50} 61.21 μg/ml). MSC failed to show the activity, not only in this concentration but also in higher concentrations. Among the isolated compounds, ursolic acid, lapachol and pinoresinol showcased promising inhibition potentials with IC_{50} values of 12.47, 11.07 and 45.64 nM respectively. The values were compared with standard acarbose which shows an IC_{50} value of 55.76 nM (36 μg/ml). The other compounds did not show any promising activity. The percentage inhibition of different concentrations of ursolic acid, lapachol and pinoresinol along with acarbose is shown in figure 3.35. From the figure, it is clear that lapachol showed highest activity with 91.1% at a concentration of 51.44 nM.

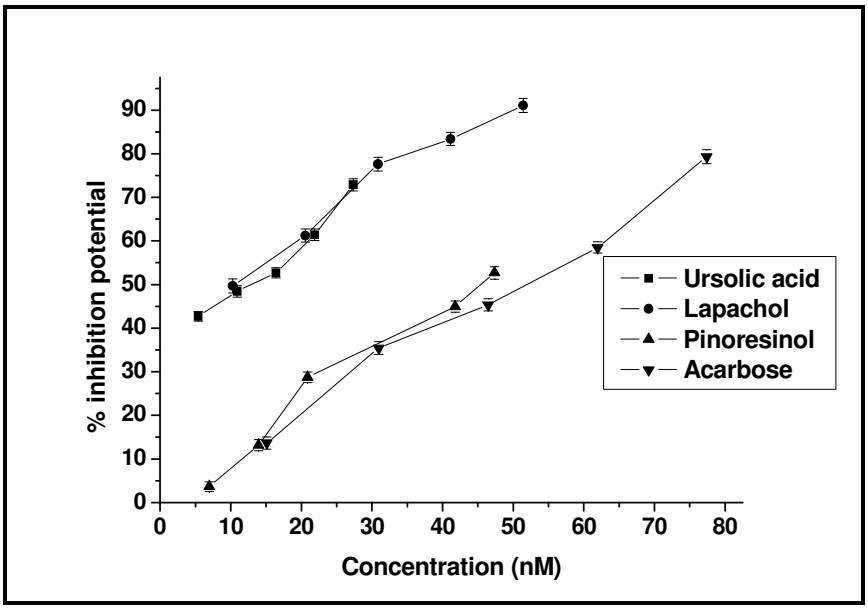


Figure 3.35: α-glucosidase inhibition potential of ursolic acid, lapachol, pinoresinol and acarbose

α-amylase enzyme involved in the digestion of starch can be considered as a scaffold for the treatment of disorders in carbohydrate uptake (Sales *et al*., 2012). α-amylase inhibitory activity was measured at various dosages. Compared with standard acarbose with IC_{50} 521.51 nM (336.69 µg/ml), ASC possess 681.08 µg/ml whereas ursolic acid, lapachol and pinoresinol possessed IC_{50} values of 1058.49, 1599.05 and 1472.51 nM respectively (Figure 3.36). The other isolated constituents failed to show the activity. According to Horii *et al*. (1986), excessive inhibition of α-amylase in the digestive tract could result in abnormal bacterial fermentation of undigested carbohydrates in the colon and therefore mild α-amylase inhibition activity is useful. So the compounds can be considered as better inhibitors of α-amylase enzyme. The inhibition of these enzymes is considered to be effective to control diabetes by diminishing the absorption of glucose decomposed from starch (Hara and Honda, 1990)

and therefore, effective and nontoxic inhibitors of α-amylase and α-glucosidase have long been sought.

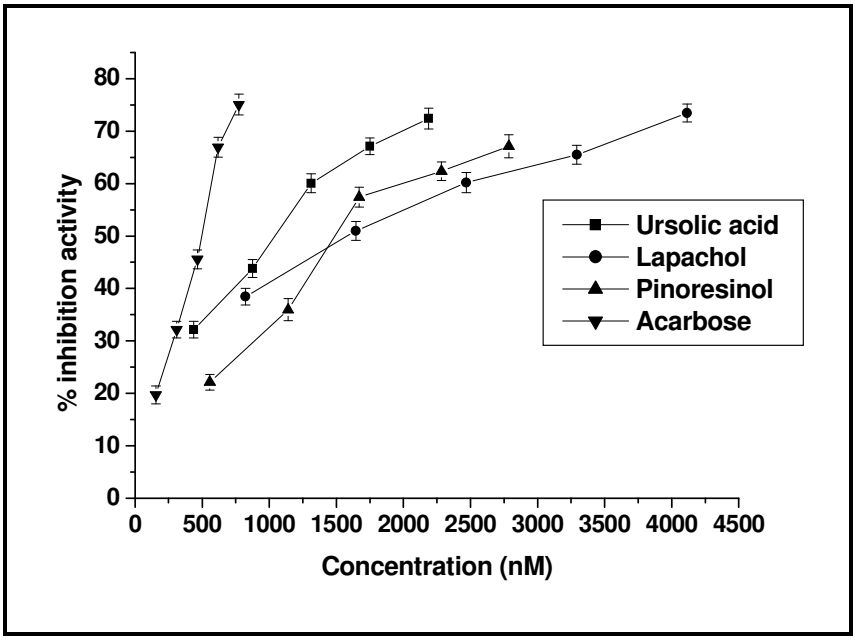


Figure 3.36: α-amylase inhibition activity of ursolic acid, lapachol, pinoresinol and acarbose

3.5.7. Antiglycation activity

Glycated end products inhibition potential was performed to test the ability to inhibit the formation of glycated BSA at 60°C without TCA precipitation. An elevation in protein glycation and subsequent production of advanced glycated end products (AGEs) contributed towards the pathogenesis of various complications (Elosta *et al*., 2012). As shown in figure 3.37, ursolic acid, lapachol and pinoresinol inhibited the formation of glycated BSA dose dependently. Increased glycation of proteins and accumulation of advanced glycation end products have been implicated in the pathogenesis of diabetic complications (Muhammed and Nessar, 2006). ASC showed an inhibition potential of IC_{50} 199.27 µg/ml compared to standard ascorbic acid (IC_{50} 201.01 nM (35.40 µg/ml)) whereas ursolic acid, lapachol and pinoresinol possessed high

antiglycation potential with IC_{50} values of 119.01, 130.29 and 125.62 nM respectively. The other isolated compounds did not show any activity. In this study, the compounds from *S. colais* found to inhibit the formation of fluorescent AGEs in the BSA/glucose system. The agents who inhibit the formation of AGEs professed to have therapeutic potentials in patients to have age related diseases (Arom 2005).

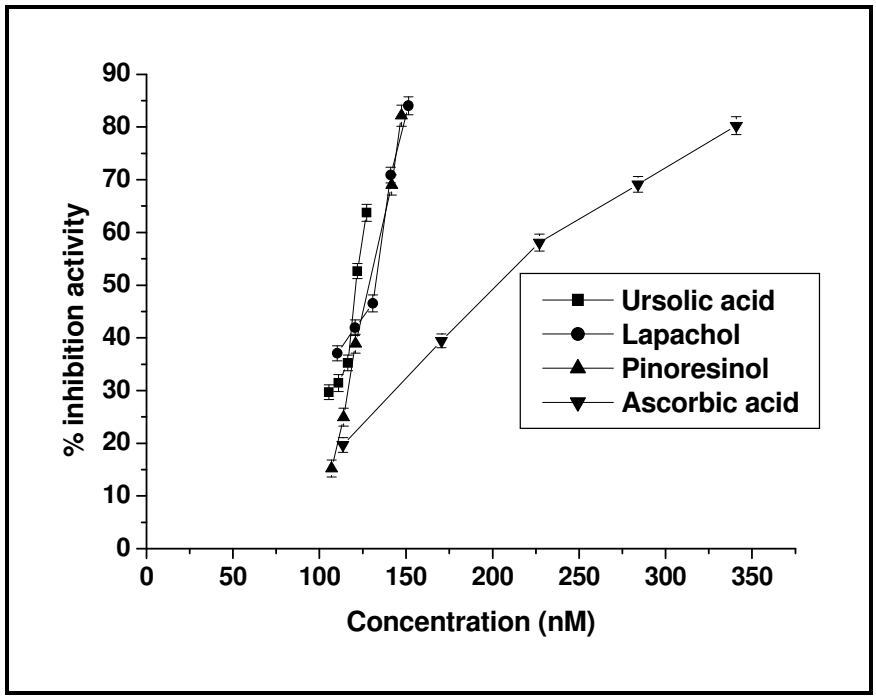


Figure 3.37: Antiglycation potential of ursolic acid, lapachol, pinoresinol and ascorbic acid

3.5.8. Antiinflammatory potential of 5α-ergostan-7, 22-dien-3β-ol, acetone and methanol extracts of *S. colais* roots

3.5.8.1. Evaluation of *in vitro* antiinflammatory activity via inhibition on protein denaturation assay

Inflammation involves a complex process of bodily response to injury and involves processes such as elevation in protein denaturation, vascular permeability, membrane alteration etc. (Umapathy *et al*., 2010). Chemical mediators produced from

migrating cells and injured tissues trigger the process and non-steroidal antiinflammatory drugs (NSAIDs) are commonly used for the prevention. But there are reports on the adverse effects of NSAIDs which produces gastric irritations ultimately leads to gastric ulcers (Tripathi, 2008). Now a days, interest on traditional medicines is of great importance and is being re-evaluated by extensive research on various plant species and its therapeutic potentials.

In the present study, the *in vitro* antiinflammatory effect was evaluated against denaturation of bovine serum albumin and the findings showed concentration dependent inhibition on protein denaturation by 5α-ergostan-7, 22-dien-3β-ol, ASC and MSC. Standard diclofenac sodium exhibited concentration dependent inhibition with an IC_{50} value of 73.49 nM (23.34 µg/ml) whereas 5α-ergostan-7, 22-dien-3β-ol showed an IC_{50} value of 71.37 nM. Acetone and methanol extracts showed 33.14 and 29.37 µg/ml respectively. The IC_{50} values become indicates that 5α-ergostan-7, 22-dien-3β-ol was more active than diclofenac sodium, being effective in lower concentrations. The activity may vary in the order 5α-ergostan-7, 22-dien-3β-ol > diclofenac sodium in a dose dependent manner (Figure 3.38). Denaturation of tissue protein is one of the well pronounced cause of inflammatory and arthritic diseases. The results supporting the ability of samples in inhibiting denaturation of protein and should be worthwhile for antiinflammatory drug development.

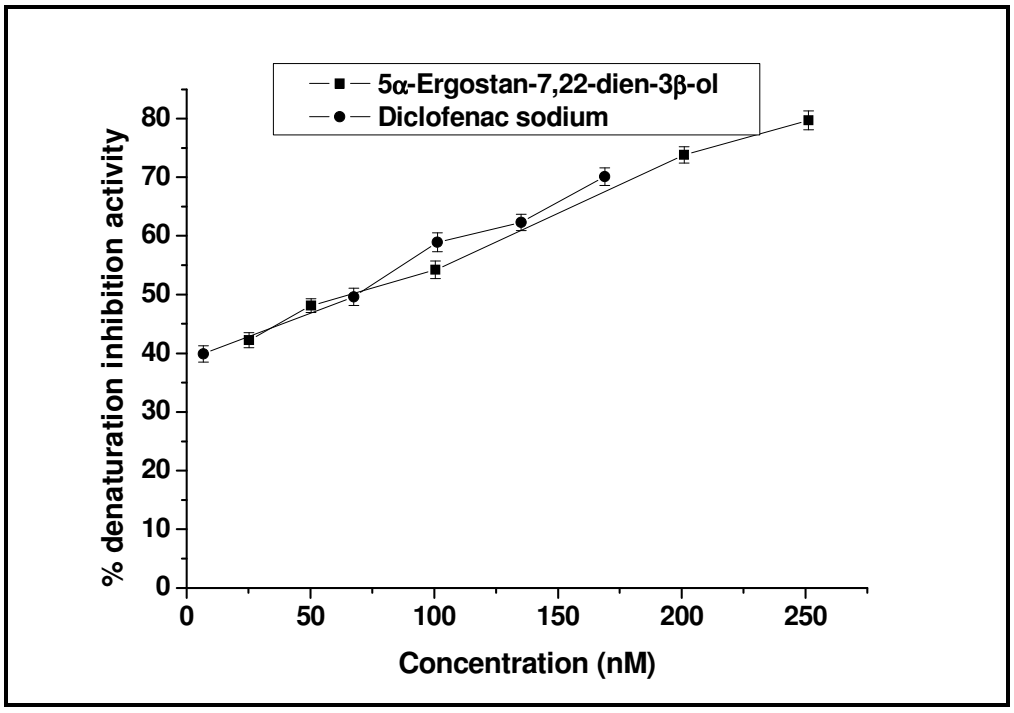


Figure 3.38: Inhibition on protein denaturation of 5α-ergostan-7, 22-dien-3β-ol and diclofenac sodium

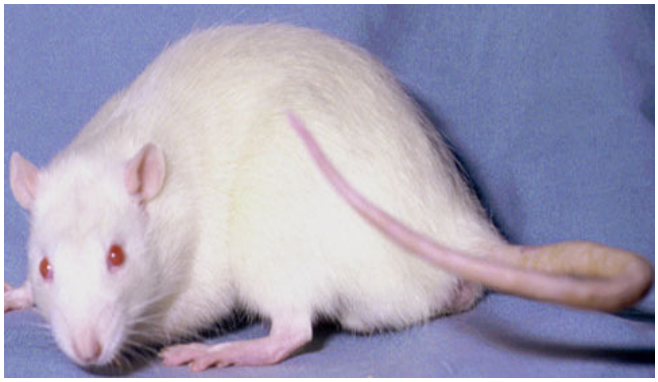
3.5.8.2. *In vivo* antiinflammatory activity

Carrageenan induced rat paw edema model is a very suitable working model for the determination of antiinflammatory effect since it is a strong chemical for the release of inflammatory mediators and also in determining the role of mediators involved in vascular changes associated with acute inflammation (Cuzzocrea *et al*., 2006; Mazzon *et al*., 2008). Since roots of *S. colais* are used traditionally against inflammation, both acetone and methanol extracts and compound 5α-ergostan-7, 22-dien-3β-ol was checked for its antiinflammatory activity in Sprague-Dawley rats (Figure 3.39a) in a dose and time dependent manner. The antiinflammatory activity of 5α-ergostan-7, 22-dien-3β-ol against acute edema (induced by carrageenan) was shown in figure 3.39b and 3.39c at a concentration of 5 mg/kg in the case of 5α-ergostan-7, 22-dien-3β-ol and 100 mg/kg in the case of ASC and MSC and the results are compared with that of standard

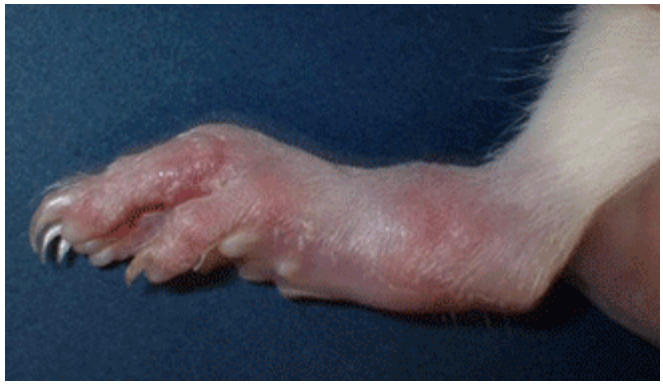
indomethacin (Table 3.3). After carrageenan injection at 3rd hour, 5α-ergostan-7, 22-dien-3β-ol showed 80% inhibition which reached 83% at the fifth hour compared to standard indomethacin which showed 40 and 68% inhibition at both times. ASC (100 mg/kg) showed 30 and 33% inhibition at 3rd and 5th hour whereas MSC (100 mg/kg) showed 50% inhibition at both times. According to Vinegar *et al*. (1969), development of paw edema after carrageenan injection is due to the release of prostaglandin like substances, serotonin etc. Significantly high activity of 5α-ergostan-7, 22-dien-3β-ol and moderate activity of extracts were due to the inhibition of mediators of inflammation.

In the current study, the edema inhibition by 5α-ergostan-7, 22-dien-3β-ol reached maximum and was more pronounced in the second stage suggesting the major mechanism of prostaglandin production by which the extract exert antiinflammatory effect. There are no previous reports on the antiinflammatory effect of *S. colais*. Aruna *et al* reported that oleanolic acid posses significant antiinflammatory activity in carrageenan induced inflammation in rats (Aruna and Shalini, 1995). Similarly, Ratheesh and Helen (2007) have reported the marked effect of *Ruta graveolens* Linn on carrageenan induced paw edema in male wistar rats.

a)



b)



c)

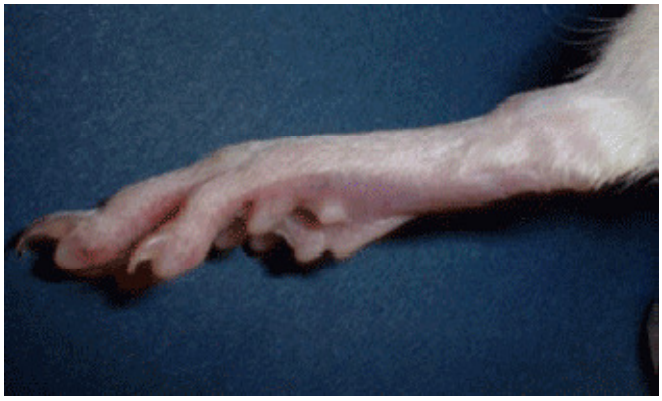


Figure 3.39: a) Sprague Dawley rats; b) Photograph showing rat paw edema after carrageenan injection; c) Photograph showing rat paw edema after treating with 5α-ergostan-7, 22-dien-3β-ol

Table 3.3: Antiinflammatory effect (*in vivo*) of 5α-ergostan-7, 22-dien-3β-ol, ASC, MSC and indomethacin at 3rd and 5th hour

Treatment group	Dose (mg/kg)	Inhibition (%)	
		3rd h	5th h
5α-ergostan-7, 22-dien-3β-ol	5	80 ± 1.01[a]	83 ± 1.56[b]
ASC	100	30 ± 0.83[d]	33 ± 0.95[c]
MSC	100	50 ± 0.99[e]	50 ± 1.05[f]
Indomethacin	3	40 ± 0.88[h]	68 ± 1.12[g]

[a-h]Mean ± SD of two analyses from three replicate (n = 6) determinations followed by different letters in a column are significantly different in the Duncan's test at $P \leq 0.05$

3.5.9. Antimicrobial activity

3.5.9.1. Antibacterial activity

Ursolic acid, lapachol and pinoresinol were tested for antibacterial activity against five bacterial stains like *S. aureus, E. coli, S. epidermis, P. mirabilis* and *K. pneumonia* using agar diffusion method. Agar disc diffusion values and MIC were determined and are shown in Table 3.4 and 3.5. The microorganism that presented highest sensitivity towards ursolic acid was *K. pneumonia* (16 µg/ml), followed by *S. epidermis* (32 µg/ml) (Figure 3.40). Lapachol also record significant activity against *K. pneumonia* (32 µg/ml). Interestingly ursolic acid recorded significantly good activity against test pathogens in low concentration. It appeared that effective MIC also represents the effective bactericidal concentration of the bacteria tested. The activity of the test compounds was lower than that of ciprofloxacin. Pinoresinol did not show any significant activity even at high concentrations. The highest antibacterial activity shown by ursolic acid against K. *pneumonia* (16 µg/ml) with a maximum zone of inhibition of 24 mm, which cause

destructive changes to human lungs via inflammation and hemorrhage with cell death (necrosis) that sometimes produces a thick, bloody, mucoid sputum.

Table 3.4: Antibacterial activity of compounds against test organisms

Test organisms	Zone of inhibition (dia. in mm)		
	Ursolic acid	Lapachol	Ciprofloxacin
S. aureus	21	17	27
E. coli	18	19	32
S. epidermis	18	20	29
P. mirabilis	22	22	31
K. pneumonia	24	16	28

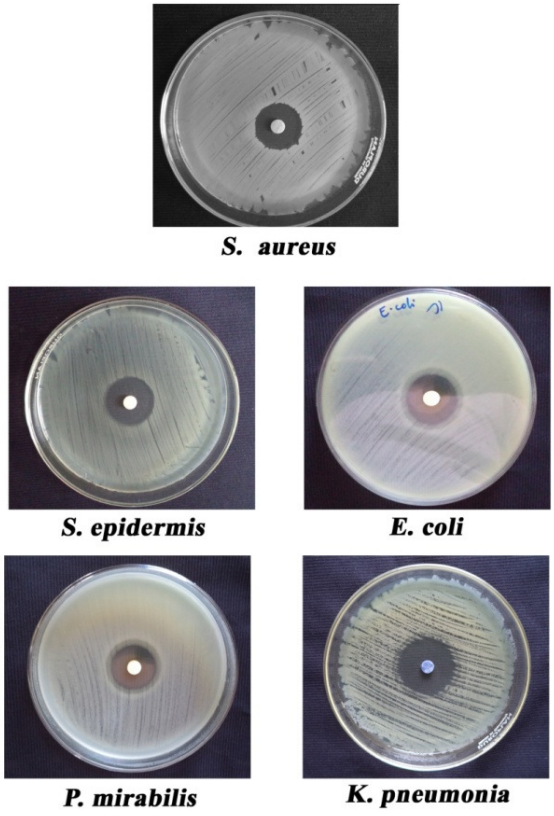


Figure 3.40: Antibacterial effect of ursolic acid against *S. aureus, S. epidermis*, *E. coli*, *P. mirabilis* and *K. pneumonia*

Table 3.5: MIC value of compounds against bacteria

Test organisms	MIC (µg/ml)		
	Ursolic acid	Lapachol	Ciprofloxacin
S. aureus	62	250	1
E. coli	62	64	0.5
S. epidermis	32	125	2
P. mirabilis	125	125	4
K. pneumonia	16	32	1

3.5.9.2. Antifungal activity

Antifungal activity against four fungi and corresponding MIC values are indicated in Table 3.6 and 3.7. Ursolic acid recorded significant activity against *T. rubrum* (4 µg/ml) with a maximum zone of inhibition of 32 mm (Figure 3.41). This activity is significantly better than the activity of standard antifungal agent amphotericin B. Pinoresinol did not show any significant activity. There are reports that the most common infectious agents in foot mycoses are *Trichophyton rubrum,* which cause athlete's foot, jock itch etc. The activity of ursolic acid against *T. rubrum* is better than the standard antifungal agent amphotericin B.

Table 3.6: Antifungal activity of test compounds against fungi

Test organisms	Zone of inhibition (dia. in mm)		
	Ursolic acid	Lapachol	Amphotericin B
A. flavus	12	10	17
C. albicans	14	11	22
C. gastricus	11	12	21
T. rubrum	32	19	24

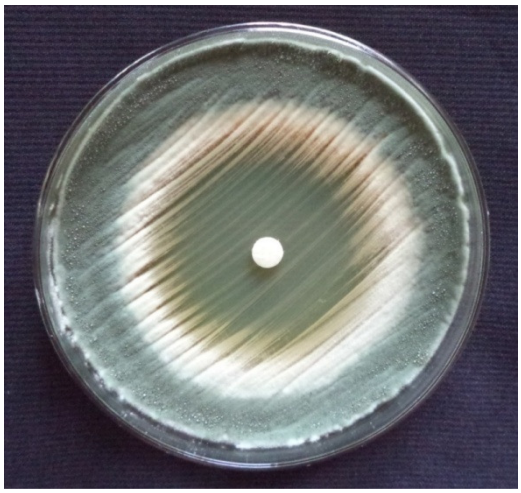


Figure 3.41: Antifungal effect of ursolic acid against *T. rubrum*

Table 3.7: MIC value of test compounds against fungi

Test organisms	MIC (µg/ml)		
	Ursolic acid	**Lapachol**	**Amphotericin B**
A. flavus	250	1000	16
C. albicans	64	500	2
C. gastricus	125	125	4
T. rubrum	4	125	8

REFERENCES

1. Ahmad, S.; Hussain, G.; Razaq, S. *Phytochemistry* **2000**, *15*, 1976.
2. Apostolidis, E.; Kwon, Y. I.; Shetty, K. *Innov. Food Sci. Emerg. Technol.* **2007**, *8*, 46-54.
3. Arom, J. *Naresuan Uni. J*. **2005**, *13*, 35-41.
4. Aruna, K.; Shalini, S. *J. Pharm. Pharmacol*. **1995**, *47*, 585-587.
5. Balakrishnan, N.; Panda, A. B.; Raj, N. R.; Shrivastava, A.; Prathani, R. *Asian J. Res. Chem*. **2009**, *2*, 148-150.

6. Chiang, H. C.; Chen, Y.Y. *J. Enz. Inhib.* **1993**, *7*, 225-235.

7. Chopra, R. N.; Nayar, S. l.; Chopra, I. C. Glossary of Indian Medicinal plants, **1956**, 234.

8. Clinical and Laboratory Standards Institute (CLSI). 1st edition. CLSI document M51-A., Villanova, PA, USA, **2010**.

9. Clinical and Laboratory Standards Institute (CLSI). 2nd edition. M44-A2, Wayne, PA, USA, **2009**.

10. Clinical and Laboratory Standards Institute (CLSI). 9th edition. CLSI documents M07-A9. West Valley Road, Suite 2500, Wayne, PA 19087, USA, **2012a**.

11. Clinical and Laboratory Standards Institute (CLSI). M27-S4., Wayne, PA, USA, **2012c**.

12. Clinical and Laboratory Standards Institute CLSI. 11th edition. CLSI documents M02-A11. West Valley Road, Suite 2500, Wayne, PA 19087, USA, **2012b**.

13. Cuzzocrea, S.; Nocentini, G.; Di paola, R.; Agostini, M.; Mazzon, E.; Ronchetti, S.; Crisafulli, C.; Esposito, E.; Caputi, A. P.; Riccardi, C. *J. Immunol*. **2006,** *177*, 631-641.

14. Davies, N.W. *J. Chromatogr. A*. **1990**, *503*, 1-24.

15. Davis, S. N.; Granner, D. K. The Pharmacological Basis of Therapeutics: Pergamon Press, New York, ISBN: 10:0071422803, **1996**, 1487-1517.

16. Duh, P. D.; Yen, W. J.; Du, P. C.; Yen, G. C. *J. Am. Oil Chem. Soc.* **1997**, *74*, 1059-1063.

17. Elosta, A.; Ghous, T.; Ahmed, N. *Curr. Diabetes Rev.* **2012**, *8*, 92-108.

18. Firestone, D. (ed.) AOCS, *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 4th edn., AOCS Press, Champaign, Additions and Revisions, Method Cd **1992**, 18-90.

19. Fridovich, I. *J. Biol. Chem.* **1970**, *245*, 4053-4057.

20. Gambhire, M.; Juvekar, A.; Wankhede, S. *Internet J. pharmacol*. **2009**, *7,* 1-6.

21. Hara, Y.; Honda, M. *Agric. Biol. Chem*. **1990**, *54*, 1939-1945.

22. Horii, S.; Fukase, H.; Matsuo, T.; Kameda, Y.; Asano, N.; Matsui, K. *J. Med. Chem*. **1986**, *29*, 1038-1046.

23. Huie, R. E.; Padmaja, S. *Free Radic. Res. Commun.* **1993**, *18*, 195-199.

24. Hutchinson J.; Dalziel J. in Flora of West Tropical Africa. F.N. Hepper (ed.), 2nd ed., (Millbank, London) **1963**, *2*, 386.

25. Ishibuchi, S.; Morimoto, H.; Oe, T.; Ikebe, T.; Inoue, H.; Fukunari, A.; Kamezawa, M.; Yamada, I.; Naka, Y. *Bioorg. Med. Chem. Lett*. **2001**, *11*, 879-882.

26. Jenning, W.G.; Shibamoto, T. Qualitative analysis of flavour and fragrance volatiles by glass capillary gas chromatography. Academic Press, New York. **1980**, 54.

27. Kamdem Soup, T. R.; Pascal, W.; Zulfiqar, A.; Ajmal, K.; Ogbole, O, O.; Bishnu P. M.; Ikhlas, A. K.; Ngadjui, T. B.; Iqbal Choudhary, M.; Atta-ur-Rahman. *Fitoterapia* **2012**, *83*, 204-208.

28. Kefalas, P.; Kallithraka, S.; Parejo, I.; Makris, D.P. *Food Sci. Technol. Int.* **2003**, *9*, 383-387.

29. Korycka-dahl, M.; Richardson, T. *J. Diary Sci*. **1978,** *61*, 400-407.

30. Lee, J. C.; Kim, H. R.; Kim, J.; Jang, Y.S. *J. Agric. Food Chem.* **2002**, **5***0*, 6490-6496.

31. Liu, F.; Ng, T. B. *Life Sci*. **2000**, *66*, 725-735.

32. Liu, F.; Ooi, V. F. C.; Chang, S. T. *Life Sci*. **1997**, *60*, 763-772.

33. Lopez-Candales, A. *J. do Medico* **2001**, *32*, 283-300.

34. Manuel Brenes; Francisco J. H.; Aranzazu Garcia; Jose J. R.; Pedro, G.; Rosario Z.; Antonio, G. *JAOCS* **2000**, *77*, 715-720.

35. Matsui, T.; Tanaka, T.; Tamura, S.; Toshima, A.; Miyata, Y.; Tanaka, K. *J. Agric. Food Chem*. **2007**, *55*, 99-105.

36. Mazzon, E.; Esposito, E.; Di Paola, R.; Muia, C.; Crisafulli, C.; Genovese, T.; Caminiti, R.; Meli, R.; Bramanti, P.; Cuzzocrea, S. C*lin. Exp. Immunol.* **2008,** *153*, 136-149.

37. Mohammad, R. H.; Khondaker, M. R.; Choudhury, M. H.; Mohammad, A. R. *Dhaka Univ. J. Pharm. Sci*. **2006,** *5*, 71-72.

38. Mohammad, R. H.; Khondaker, M. R.; Iskander, M. N.; Hasan, C. M.; Rashid, M. A. *Phytochem*istry **2006**, *67*, 2663-2665.

39. Muhammed Saeed, A.; Nessar, A. *J. Nutr.* **2006**, *136*, 796S-799S.

40. Ortiz-Andrade, R. R.; Garcıa-Jimenez, S.; Castillo-Espana, P.; Ramırez-Avila, G.; Villalobos-Molina, R.; Estrada-Soto. S. *J. Ethnopharmacol*. **2007**, *109*, 48-53.

41. Owen, P. L.; Johns, T. *J. Ethnopharmacol*. **1999**, *64*, 149-160.

42. Oyaizu, M. *Jpn. J. Nutr*. **1986**, *44*, 307-315.

43. Pezzuto, J. M.; Park, E. J. In: Encyclopedia of Pharmaceuticals Technology2nd Edition, Swarbrick, J.; Boylan, J.C.; Marcel Dekker (Eds:), Inc., New York, **2002**, *1*, 97-113.

44. Prathapan, A.; Mukesh Kumar Singh; Anusree, S. S; Soban Kumar, D. R.; Sundaresan, A.; Raghu, K. G. *J. Food Biochem.* **2011**, *35*, 1548-1554.

45. Ram, P. R.; Mehrotra, B. N. *A Compendium of Indian Medicinal Plants* (Drug Research Preparative: A CDRI series). New Delhi: Lucknow Publication and Information Directorate, **1993**, *1*, 390.

46. Ramsay, K. S.; Wafo, P.; Ali, Z.; Khan, A.; Oluyemisi, O. O.; Marasini, B. P.; Khan, I. A.; Bonaventure, N. T.; Choudhary, M. I.; Atta-ur-Rahman. *Fitoterapia* **2012**, *83*, 204-208.

47. Rao, K.V.; McBride T. J.; Oleson, J. *J. Cancer Res*. **1968**, *28*, 1952-1954.

48. Ratheesh, M.; Helen, A. *Afr. J. Biotechnol.* **2007**, *6*, 1209-1211.

49. Rollas,S.; Kalyoncuoglu,N.; Sur-Altiner,D.; Yegenglu,Y. *Pharmazie*, **1993**, *48*, 308-309.

50. Sales, P. M.; Souza, P. M.; Simeoni, L. A.; Silveira, D. *J. Pharm. Pharm. Sci.* **2012**, *15*, 141-183.

51. Sampath Kumar, U.; Aparna, P.; Jagadeeshwar Rao, R.; Prabhakar Rao, T.; Madhusudana Rao. J. *Phytochemistry* **2003**, *63*, 925-929.

52. Sampath Kumar, U.; Ashok K. T.; Venkat Reddy, S. ; Aparna, P.; Jagadeeshwar Rao, R.; Zehra Ali, A.; Madhusudana Rao, J. *J. Nat. Prod.* **2005**, *68,* 1615-1621.

53. Shetty Devika. *UJP* **2013**, *2*, 130-133.

54. Siddhuraju, P.; Becker, K. *J. Agric. Food Chem.* **2003**, *51*, 2144-2155.

55. Sultana, B.; Anwar, F.; Przybylski, R. *Food Chem.* **2007**, *104*, 1106-1114.

56. Tripathi, K. D. Essentials of medical pharmacology. New Delhi: Jaypee Brothers Medical Publishers Ltd.; **2008**, 189.

57. Tylor, B. S.; Kion, Y. M.; Wang, Q. I. Shapiro, R. A.; Billiar, T. R.; Geller, D. A. *Arch. Surg.* **1997**, *132*, 1177-1183.

58. Umapathy, E.; Ndebia, E. J.; Meeme, A.; Adam, B.; Menziwa, P.; Nkeh-Chungag, B. N.; Iputo, J. E. *J. Med. Plants Res.* **2010**, *4*, 789-795.

59. Vijaya Bharathi, R.; Kumudha Veni, B.; Jayashree; Suseela L.; Thirumal, M. *Int. J. Res. Pharm. Sci.* **2010**, *1*, 435-439.

60. Vinegar, R.; Schreiber, W.; Hugo, R. *J. Pharmacol. Exp. Ther.* **1969**, *166*, 96-103.

61. Warrier, P.K. Indian Medicinal Plants a Compendium of 500 species. Hyderabad: Orient Longman **1996**, *5*, 192-197.

62. Winter, C.A.; Risley, E.A.; Nuss, G.W. *Proc Soc. Exp. Biol. Med.* **1962**, *111*: 544-547.

63. Yoshikawa, T.; Naito, Y.; Kondo, M. Food and diseases. In M. Hiramatsu, T. Yoshikawa, & M. Inoue (Eds.), *Free radicals and diseases,* New York: Plenum press, 1997, 11-19.

Chapter 4

RP-HPLC PROFILING AND BIOLOGICAL EVALUATION OF THE RHIZOMES OF *Zingiber officinale*

This chapter deals with the characterization of chemical constituents of *Zingiber officinale*, a member of the genus *Zingiber* belonging to Zingiberaceae family, by the method of RP-HPLC-UV and screening of its biological potential.

4.1. INTRODUCTION

Figure 4.1: *Zingiber officinale* plant and rhizomes

The promising biological properties of *Zingiber officinale* (commonly known as ginger; Family: Zingiberaceae) have been realized worldwide. Ginger rhizomes were used in Ayurvedic system of medicine. The nutraceutical properties of ginger have long been of interest to the food processing and pharmaceutical industries. In Ayurveda, ginger is considered as a valuable medicine because of its action as rubefacient, antiasthmatic and stimulant to the gastrointestinal tract (Mascolo *et al*., 1989). Ginger rhizomes have been considered as a spice and are reputed to have medicinal properties

against digestive disorders, rheumatism, dropsy, neuralgia and diabetes (Afzal *et al*., 2001). Rhizomes have been employed in cuisine as a condiment in folk medicine as a carminative, diaphoretic, antispasmodic agent against intestinal colic and as an antiemetic agent (Yamahara *et al*., 1988; Phillips *et al*., 1993). The constituents of ginger are numerous and vary depending on the place of origin and whether the rhizomes are fresh or dry.

Presence of volatile oil components determines the odor of ginger and the yield varies from 1 to 3%. A number of volatile components have been identified and are mainly monoterpenoids (β-phellandrene, (+)-camphene, cineole, geraniol, curcumene, citral, terpineol, borneol) and sesquiterpenoids (α-zingiberene, β-sesquiphellandrene, β-bisabolene, (E, E)-α-farnesene, ar-curcumene, zingiberol). On drying, some oil components get converted into less odor-defining compounds (Langner *et al*., 1998; Evans, 2002). The plant possess a broad spectrum of pharmacological importance which includes its hypoglycaemic (Bhandari and Grover, 1998), antimicrobial (Mascolo *et al*., 1989), hypocholesterolemic (Bhandari and Grover, 1998; Fuhrman *et al*., 2000), antioxidant (Ahmed *et al*., 2000), antiinflammatory (Park *et al*., 1998), anticlotting, analgesic (Chrubasik *et al*., 2005) and cytotoxic activities (Katiyar *et al*., 1996). It has been widely used in traditional system of medicines all over the world, for a wide array of unrelated ailments that include arthritis, rheumatism, sprains, muscular aches, pains, sore throats, cramps, constipation, indigestion, vomiting, hypertension, dementia, fever and helminthiasis (Badreldin *et al*., 2008). From its origin in Southeast Asia and its spread to Europe, it has a long history of use as herbal medicine to treat a variety of

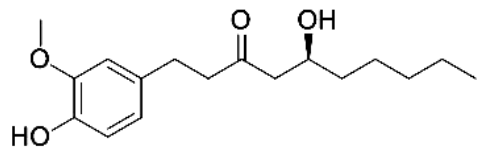
ailments including vomiting, pain, indigestion and cold induced syndromes (White, 2007; Wang and Wang, 2005).

The antihyperglycemic effect of ginger has been examined *in vitro* and *in vivo* models. Limited clinical studies have been conducted to investigate the potential beneficial effects of ginger in patients. After consuming 3 g of dry ginger powder in divided dose for 30 days, significant reduction in blood glucose, triglyceride, total cholesterol, LDL and VLDL were observed in diabetic patients (Andallu *et al*., 2003). In addition, ginger has been shown to modulate insulin release. Study on high-fat diet fed rats reported the protective effect of ginger against the development of metabolic syndrome, a condition predisposing to a high risk of type 2 diabetes, obesity and cardiovascular disorders. After treatment with an ethanolic extract of ginger at doses of 100, 200 and 400 mg/kg for 6 weeks, the marked rises in body weight, serum glucose, insulin, total cholesterol, LDL cholesterol, triglycerides, free fatty acid and phospholipids induced by high-fat diet was significantly reduced (Nammi *et al*., 2009). It was reported that oral administration of ethanolic extract of ginger (800 mg/kg) significantly reduced fasting blood glucose level after 1 hour treatment in STZ type 1 diabetic rat model. The effect peaked after 4 hours, with ginger producing a 24 to 53% reduction in blood glucose at doses ranging from 100 to 800 mg/kg (Ojewole, 2006). Ethanolic extract of ginger also reported antihyperglycaemic potential by diminishing serum total cholesterol and triglyceride levels (Uma *et al*., 2005).

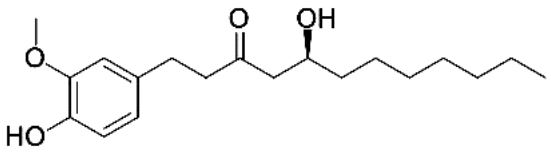
Pharmacological investigations have shown the chemopreventive and chemotherapeutic effects of ginger on a variety of cancer cell lines and on animal models (Chen *et al*., 2009). The species contains biologically active constituents including the

main pungent principles like gingerols and shogaols, which is reported to have many pharmacological potential including radical scavenging potential (Ali *et al*., 2008). The gingerols, a series of chemical homologs differentiated by the length of their unbranched alkyl chains, were identified as the major active components in the fresh rhizome (Govindarajan, 1982), with 6-gingerol (5-hydroxy-l-(4′-hydroxy-3′-methoxyphenyl) decan-3-one) being the most abundant. In addition, the shogaols, another homologous series and the dehydrated form of gingerols, result from the elimination of -OH group at C_5 and the consequent formation of a double bond between C_4 and C_5, are the predominant pungent constituents in dried ginger (Connell and Sutherland, 1969; Mustafa *et al*., 1993). 6-Gingerol has been found to possess various pharmacological and physiological effects including antiinflammatory, analgesic, antipyretic, gastroprotective, cardiotonic and antihepatotoxic activities (Bhattarai *et al*., 2001; Jolad *et al*., 2004). In addtion, another two compounds like 2-(4-hydroxy-3-methoxyphenyl) ethanol and 2-(4-hydroxy-3-methoxyphenyl) ethanoic acid with significant aldose reducatse property has been isolated from ginger (Kato *et al*., 2006). Iqbal *et al*. (2006) investigated the anthelmintic activity of crude powder and crude aqueous extracts of dried ginger (1–3 g/kg) in sheep naturally infected with mixed species of gastrointestinal nematodes. Both exhibited a dose and time dependent anthelmintic effect with respective maximum reduction of 25.6% and 66.6% in eggs per gram (EPG) of feces on day 10 post treatment. Ginger has gained considerable attention as a botanical dietary supplement in USA and Europe and especially for its use in the treatment of chronic inflammatory conditions (Shukla and Singh, 2007). Structures of some of the important compounds isolated from *Z. officinale* are given in chart 4.1.

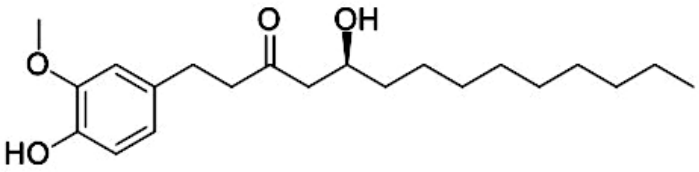
Chart 4.1: Phytochemical constituents isolated from *Zingiber officinale*



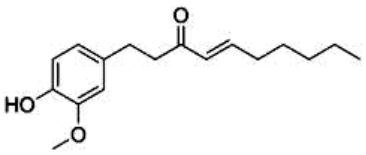
6-gingerol



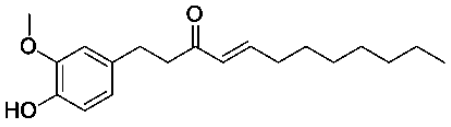
8-gingerol



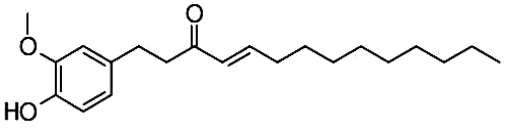
10-gingerol



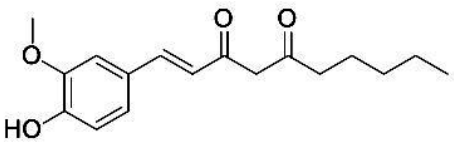
6-shoagol



8-shoagol

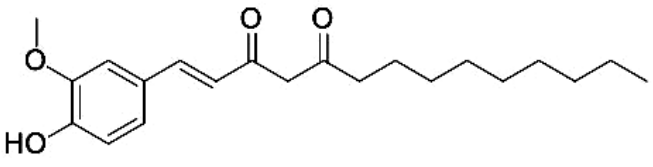


10-shoagol

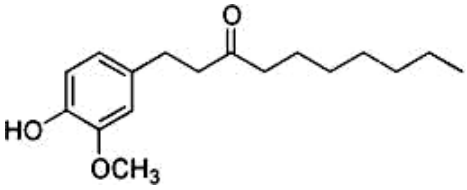


Dehydro-6-gingerdione

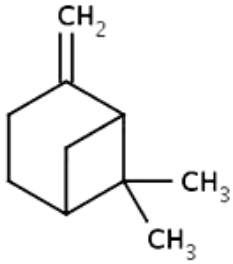
Chart 4.1. continued……



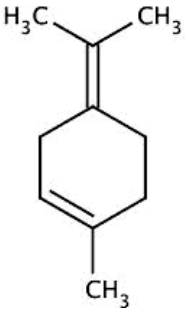
Dehydro-10-gingerdione



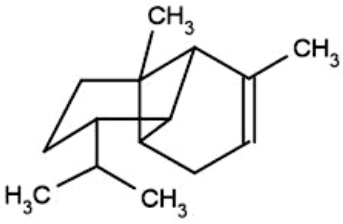
6-paradol



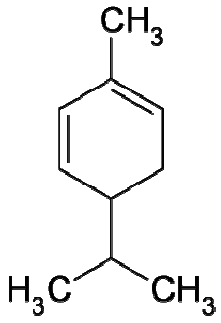
β-pinene



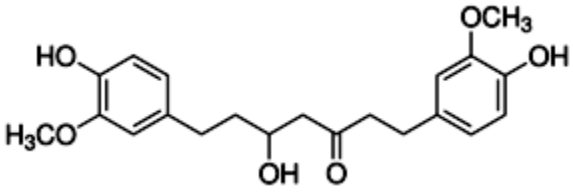
Terpinolene



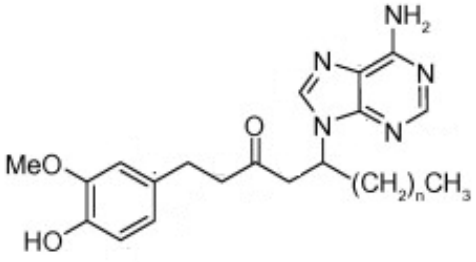
α-copaene



α – phellandrene

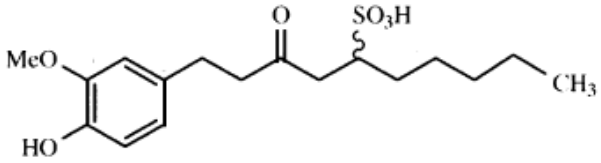


Hexahydrocurcumin

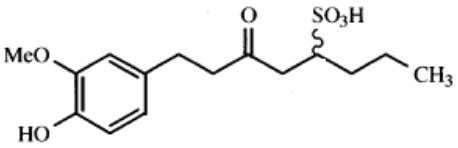


n = 4, 6-zingerine
n = 6, 8-zingerine
n = 8, 10-zingerine

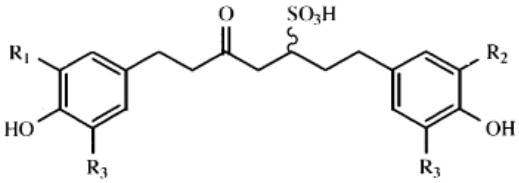
Chart 4.1. continued……



6-gingesulfonic acid



4-gingesulfonic acid



	R_1	R_2	R_3
Shogasulfonic acid A	**OMe**	**OMe**	**H**
Shogasulfonic acid B	**OMe**	**OH**	**H**
Shogasulfonic acid C	**OH**	**OH**	**H**
Shogasulfonic acid D	**OMe**	**OMe**	**OH**

4.2. AIM AND SCOPE OF THE PRESENT INVESTIGATION

It is clear from above reports that ginger (Figure 4.1.) has been evaluated for its activities against various disorders but information about its hypoglycaemic, antiinflammatory and obesity is very limited. Therefore it was our interest to studying these effects in detailed manner and an HPLC analysis was done to characterize the active constituents.

4.3. MATERIALS AND METHODS

4.3.1. Collection of plant material

Fresh ginger rhizomes (2.5 kg) were collected from NIIST campus, Thiruvananthapuram, Kerala, India and were identified and authenticated by Dr. H. Biju

(Taxonomist, JNTBGRI, Palode, Thiruvananthapuram, Kerala). A voucher specimen (No. 019/2010) has been deposited in the herbarium for further use. The rhizomes were cleaned, cut into small pieces, dried at 50°C in a hot air oven for 8 h and powdered.

4.3.2. Reagents used

N, N, N′, N′-tetramethyl-*p*-phenylenediamine (TMPD), Hematin, Tris-HCl, dinitrosalicylic acid, arachidonic acid, 2′, 7′-dichlorodihydrofluoresceindiacetate (DCFH-DA), 6-gingerol, indomethacin and secondary antimouse immunoglobulin (IgG; Fab specific)-fluorescein isothiocyanate (FITC) antibody produced in goat were purchased from Sigma-Aldrich (St. Louis, MO, USA). The COX inhibitor assay screening kit was obtained from Cayman (Ann Arbor, MI, USA). Dulbecco's modified Eagle's media (DMEM), bovine serum albumin, streptomycin-ampicillin-amphotericin B mix, insulin, dexamethasone, isobutyl methylxanthine (IBMX), roziglitazone, MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), low density lipoprotein (LDL), angiotensin converting enzyme (ACE) of rabbit lung, hippuryl-L-histidyl-L-leucine (HHL), gallic acid, thiobarbituric acid (TBA), ascorbic acid, captopril, monoclonal antiGlut antibody etc. were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fetal bovine serum (FBS) was purchased from Gibco- BRL (Auckland, NZ) and horse serum was purchased from PAN Biotech (Aidenbach, Germany). 2-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino-2-deoxy-D-glucose (2-NBDG) was purchased from Molecular Probe (Invitrogen Life Technologies, Carlsbad, CA, USA). Anhydrous sodium phosphate (monobasic & dibasic), trichloroacetic acid, sodium chloride, hydrogen peroxide etc. were purchased from Sisco Research Laboratories (Mumbai, India). Solvents for high performance liquid chromatography (HPLC) were purchased from

Merck (Mumbai, India). All other chemicals and solvents used were of standard analytical grade.

4.3.3. Cell culture and treatment

Mouse myoblast (C2C12), rat skeletal muscle (L6) and mouse pre-adipocyte (3T3L1) cells were purchased from National Centre for Cell Science (NCCS, Pune). Cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin. To maintain the pH of media at 7.2, 20 mM $NaHCO_3$ was added and cells were maintained at 37°C in a 5% CO_2 incubator with humidified atmosphere.

Cells were sub cultured and maintained at log phase of growth throughout the study. After reaching more than 80% confluent state, spent media was removed and cells washed with phosphate buffered saline (0.2 M) two times. Then the cells were detached from the plate surface using trypsin (0.25%)-EDTA (0.1%). Trypsin was inactivated with serum and centrifuged to attain cell pellet after which it was suspended in serum containing media. Based on the assay, cells were plated in 24 or 96 well plates at a concentration of $5x10^4$ or $1x10^4$ cells per well respectively. These plates were grown to required cell density in the above mentioned growth conditions.

4.3.4. PROXIMATE COMPOSITION ANALYSIS

Proximate composition analysis viz, moisture, protein, sugar, fat, crude fibre, ash content etc. have been carried out for dry ginger according to standard methods (AOAC, 2000).

4.3.4.1. Estimation of moisture content

Moisture content of dry ginger was determined by oven dry method. About 10 g of the dry ginger powder was accurately weighed in a pre weighed petri dish and placed in hot air oven and dry for 2 hours at 100 ± 2°C. The dish with sample was cooled in a desiccator and weight was noted. Heated again at 100 ± 2°C in oven for 30 minutes, cooled and weighed. This process of heating for 30 minutes was repeated till the difference in weight between two successive weighing was less than 1 mg. From the loss of weight during drying, amount of moisture was calculated.

4.3.4.2. Estimation of protein - Kjeldahls method

Dried ginger powder (2 g), 0.5 g digestion mixture (2.5 g SeO_2 + 100 g K_2SO_4 + 20 g $CuSO_4$) and 20 ml conc. H_2SO_4 were taken in a Kjeldahl flask and heated on a mantle (in slanting position) around 70^{o}C until colour of the solution changed to blue green. The solution had been made up to 25 ml under cold conditions using distilled water. This solution was used for the protein estimation. A round bottom flask half filled with water was connected to the apparatus and heated on a mantle. 20 ml 2% boric acid and 1 ml mixed indicator (10 ml bromocresol green + 2 ml methyl red) was taken in a conical flask and placed under condenser. 5 ml sample with 20 ml 40% NaOH and a little water were added to distillation tube through funnel. When water boils inside the round bottom flask, steam produced was passed into distillation tube. Ammonia evolved in distillation tube was trapped in boric acid and the colour of boric acid changed to blue. For maximum ammonia the process continued for 20 minutes. Titrate with standard HCl (0.01 N) till blue colour of the solution disappears. Percentage of nitrogen and then the percentage of protein were calculated using equations 4.1 and 4.2.

$$\% \text{ nitrogen} = \frac{V_{HCl} \times N_{HCl} \times 14 \times \text{volume of made up solution} \times 100}{\text{Volume of sample taken} \times \text{weight of sample} \times 1000} \quad \ldots\ldots\ldots \text{ Eqn 4.1}$$

% protein = % nitrogen x 6.25 …… Eqn 4.2

4.3.4.3. Total fat content

Dry ginger powder (5 g) was weighed out into a soxhlet apparatus and hot extracted with hexane at a temperature of about 40°C. The extract obtained was collected, filtered and the solvent was evaporated under vacuum using a rotary evaporator in a pre weighed RB. The extract was dried at high vacuum using a rotary evaporator and the weight of the extract was noted which determines the total fat content.

4.3.4.4. Determination of crude fibre

Defatted ginger powder (2 g) was refluxed with 200 ml 2.5% conc. H_2SO_4 in a round bottom flask. The solution was filtered and the residue obtained was again refluxed with 2.5% NaOH solution. It is then filtered through a previously weighed sintered crucible and dried in an air oven and weighed.

4.3.4.5. Estimation of ash content

About 3 g dry ginger powder was accurately weighed into a pre weighed clean crucible. The crucible heated to the point of charring of the sample on a hot plate. The crucible with carbon residue obtained as a result of ignition, was placed in a muffle furnace at 650°C until the carbon residue disappears. Allowed to cool and then weighed. From the difference in weight obtained, the ash content was determined.

4.3.5. Extraction procedure

Coarsely powdered dry ginger rhizomes (500 g) were subjected to successive cold extraction at room temperature (27°C) with hexane, ethyl acetate, methanol, 70% methanol-water and water (1 L each) as solvents. The solvents were evaporated using a rotary evaporator under reduced pressure and yield was noted for hexane (HG), ethyl

acetate (EAG), methanol (MG), 70% methanol-water (MWG) and water (WG) extracts. Various concentrations of sample stock solution (5 mg/ml) of extracts were used for biological and chemical assays.

4.3.6. Antioxidant and radical scavenging assays

4.3.6.1. Total phenolic content: Folin-Ciocalteu's reagent assay

Total phenolic content (TPC) of the successive extracts was analysed by employing the literature methods involving Folin-Ciocalteu reagent (Singleton and Rossi, 1965). To 1 ml of appropriately diluted extracts taken in stoppered test tubes, 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 reagent blank. Using gallic acid as standard, the total phenolic content was expressed as % TPC using equation 4.3.

$$\% \text{ TPC} = \frac{\text{Observed concentration}}{\text{Actual concentration}} \times 100 \qquad \ldots\ldots\ldots \text{ Eqn 4.3}$$

4.3.6.2. DPPH radical scavenging activity

Various concentrations of successive extracts of ginger were prepared and the assay has been done according to the procedure given in Chapter 2 section 2.3.7.1 of this thesis.

4.3.6.3. Superoxide radical scavenging activity

As per procedure given in Chapter 3 section 3.3.6.6 of this thesis

4.3.6.4. Total reducing power

As per procedure given in Chapter 3 section 3.3.6.7 of this thesis

4.3.7. α-Glucosidase and α-amylase inhibition assay

α-glucosidase and α-amylase inhibition assays were carried out according to the procedure of Apostolidis *et al.* as given in Chapter 3 section 3.3.9.1 and 3.3.9.2 of this thesis.

4.3.8. Glycated end products inhibition (Antiglycation) assay

As per procedure given in Chapter 3 section 3.3.10 of this thesis.

4.3.9. Inhibition of human LDL oxidation

Malondialdehyde formation by the oxidation of low density lipoprotein (LDL) was measured by reaction with TBA according to the method of Kotamballi *et al*. (2002) with slight modification. Various concentrations of EAG were taken in test tubes; 250 µl LDL (50 µg/ml) and 50 µl copper sulphate (2 mM) was added and the volume was made up to 1.5 ml in all test tubes with sodium phosphate buffer (pH 7.4). All tubes were incubated at 37°C. Aliquots of 0.5 ml from each tube were drawn at 2 h intervals and 250 µl of TBA (1% in 50 mM NaOH) was added followed by 250 µl of TCA (2.8%). Samples were incubated at 95°C for 45 min. A pink chromogen formed was extracted by centrifugation after the tubes had cooled to room temperature. TBA reactive species in the pink chromogen was detected by fluorescence at 515 nm excitation and 553 nm emission using multiplate reader (Synergy-4, Biotek, USA). Ascorbic acid was used as the standard. Data expressed in terms of malondialdehyde (MDA) equivalent, which was estimated by comparison with the standard ascorbic acid up on oxidation. Using the amount of MDA, the percentage inhibition was calculated using equation 4.4,

$$\text{Inhibition (\%)} = \frac{\text{oxidation in control} - \text{oxidation in sample}}{\text{oxidation in control}} \times 100 \quad \ldots\ldots\ldots\ldots\text{Eqn 4.4}$$

4.3.10. Determination of Angiotensin-1 Converting Enzyme (ACE) inhibition activity

Different concentrations (0.2-1 µg/ml) of EAG (50 µl), was added to 100 µl of 5 mM hippuryl-L-histidyl-L-leucine (HHL) and then preincubated for 5 min at 37°C. EAG and HHL were prepared in 50 mM Hepes buffer (pH 8.3). The reaction was then initiated by adding 150 µl of 0.1 U/ml ACE prepared in the same buffer and incubated for 1 h at 37°C. The enzyme reaction was stopped by the addition of 250 µl of 0.5 N HCl. The released hippuric acid (HA) was extracted by the addition of 1 ml of ethyl acetate, shaken well and centrifuged the solution at 3000 rpm for 10 min. From this, organic layer (750 µl) was taken and evaporated at 90°C for 15 min. The released hippuric acid was redissolved in 1 ml of distilled water and the absorbance was measured at 228 nm (Blanca *et al*., 2003). Captopril was used as the standard. The percentage inhibition was calculated by equation 2.2. Percentage radical scavenging activity was plotted against the corresponding extract concentration to get the IC_{50} value.

4.3.11. Antiinflammatory activity: *In vitro* evaluation of COX inhibitory activity

Enzymatic activity of cyclooxygenase (COX) was measured according to the method of Copeland *et al*. (1994) with slight modifications using a chromogenic assay based on the oxidation of N, N, N′, N′-tetramethyl-*p*-phenylenediamine (TMPD) during the reduction of prostaglandin G_2 (PGG_2) to prostaglandin H_2 (PGH_2). In this assay, various concentrations of EAG (30-200 µg/ml) was mixed with Tris-HCl buffer (100 mM, pH 8.0), hematin (15 mM), sodium salt of ethylenediamine tetraacetic acid (3 mM) and COX enzyme (100 mg). The mixture was preincubated at 25°C for 15 min and then the reaction was initiated by the addition of arachidonic acid and TMPD, in total volume

of 1 ml. The enzymatic activity was determined by estimating the rate of TMPD oxidation for the first 25 sec of the reaction by following the increase in absorbance at 603 nm. Indomethacin was used as the standard. A low rate of non-enzymatic oxidation observed in the absence of COX was subtracted from the experimental value while calculating the percentage inhibition (Equation 2.2). The IC_{50} value was calculated from the concentration inhibition response curve.

4.3.12. Cytotoxicity assay

The subtoxic concentration of EAG against L6, C2C12 and 3T3L1 cell lines was determined by MTT assay (Wilson *et al*., 2000). Prior to the assay, cell viability was confirmed by counting with trypan blue stain. Cells were seeded at log phase in 24 well plates with a count of $5x10^4$ cells per well. After 16 h of incubation or after attaining 80% confluency, growth medium was removed and fresh medium added with extracts at different concentrations (10, 50, 100 and 200 μgml^{-1}) or dimethyl sulfoxide (DMSO) as control and incubated for 24 h. The volume of DMSO per sample was restricted to 0.1% to avoid solvent induced cytotoxicity. After incubation, medium was removed and cells washed with freshly prepared phosphate-buffered saline. MTT (500 μgml^{-1}) working concentration was prepared in complete medium and 500 µl was added per well. After an incubation period of 4 h, the medium was removed and the purple formazan crystals were dissolved in DMSO containing 10% sodium dodecyl sulfate. All samples were treated in triplicate and the absorbance was measured at 570 nm with multi-plate reader. Values were expressed as the percentage of a normal control.

4.3.13. Determination of intracellular reactive oxygen species

Cytoprotective effect against the oxidative stress induced by H_2O_2 was measured by determining the intracellular content of reactive oxygen species (ROS). Intracellular ROS levels were measured by employing DCFH-DA, an oxidant sensing fluorescent probe cleaved intracellularly by non-specific esterase and forms highly fluorescent 2,7-dichlorofluorescein (DCF) upon oxidation by ROS (Rastogi *et al*., 2010), which were analyzed using flow cytometry (FACS Aria II, BD Bioscience, San Jose, CA, USA). C2C12 cells were pretreated with varying concentrations of EAG (10, 20, 40, 80 and 100 µg/ml) and standard ascorbic acid (25 µg/ml) for 1 hour and were incubated with 50 µM H_2O_2 for 15 minutes. Cells were washed and incubated with DCFH-DA at 37°C for 20 minutes and fluorescent intensity in the cells were analysed by FACS Aria II.

4.3.14. Glucose uptake assay by 2-NBDG

Glucose uptake assay was analyzed by measuring the uptake of glucose in undifferentiated and differentiated L6 rat skeletal muscle cells (Chen *et al*., 2010).

4.3.14.1. With un-differentiated L6

Cells were cultured in DMEM with 10% fetal bovine serum and maintained at 37°C in a humidified 5% CO_2 environment. Cells were plated at $1x10^4$ cells/well in 96 well plates and assay started after overnight incubation. After removing culture medium from each well and the wells were replaced with 100 µl of extract containing media at 0.5, 1, 5, 10 and 50 µg/ml concentrations for 1 h pre-incubation. Media was removed and cells washed twice with pre-cooled Kreb's Ringer Buffer (KRB). The fluorescent analogue of glucose, 2-NBDG (100 µM) was given and incubated for 20 min prior to cell isolation.

4.3.14.2. With differentiated L6

Cells were grown to above 80% confluency and media was switched to DMEM with 5% horse serum. After 5 days of incubation, cells were treated with extract and then with 2-NBDG (100 µM) as mentioned above. The 2-NBDG uptake was stopped by removing the incubation medium and washed the cells with pre cooled KRB.

Un-differentiated and differentiated cells in each well were subsequently resuspended in 200 µl pre cold fresh growth medium. For each measurement, data from 10000 single cell events was collected using FACS. Insulin (100 U) and roziglitazone (100 nM) treated wells were taken as control. Roziglitazone is an antidiabetic drug that works as an insulin sensitizer by binding to the Peroxisome Proliferator Activated Receptors (PPAR) in fat cells and making the cells more responsive to insulin.

4.3.15. Detection of Glut 4 in cell surface using antiGlut 4 antibody

Immuno cytochemical staining method was used for the analysis of translocation of Glut 4 proteins with respect to extract (Maldie, 2008). Briefly, cells were seeded in black walled glass bottom 96 well plates. After an initial incubation of cells in DMEM with 10% FBS, media was switched to DMEM with 5% Horse Serum (HS) for 5 days. A change in cell morphology and attachment was visible which denotes the transformation to myotubes. Media was removed and replaced with those containing EAG at 0.5-50 µg/ml concentrations. Cells treated with insulin (100 U) alone and in combination with roziglitazone (100 nM) served as standards. After 1 h incubation, cells were washed thrice with KRB (pH 7.2) and primary antibody (monoclonal antiGlut 4 antibody) given for another 1 h. The secondary goat antimouse immunoglobulin G (IgG-FITC conjugate) was incubated in dark conditions and gentle shaking for 1 h. After 3-4 washes with KRB,

plates were sealed and taken out for confocal imaging. Change in fluorescence was compared with untreated control cells.

4.3.15.1. Image acquisition by confocal microscopy

Images were taken with confocal microscopy (Pathway 855, BD Bioscience, USA) equipped with FITC range excitation (495 nm) and emission (520 nm) filters to detect antiGlut 4 antibody signal. Mode of acquisition was set to automatic focusing and captured using macro setup. Images were analysed by Image Data Explorer (BD-IDE) from BD Bioscience.

4.3.16. Adipocyte differentiation assay

Inhibition on differentiation of 3T3L1 preadipocyte to mature adipocyte by EAG was carried out based on reported protocol (Kim *et al*., 2007). Briefly, 3T3L1 preadipocyte cells were maintained in DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 mg/ml amphotericin B at 37ºC in 5% CO_2 incubator. Confluent cells were treated with differentiation media, abbreviated as MDI media hereafter is a formulated culture media containing 100 U insulin, 100 nM dexamethasone, 0.5 mM isobutyl methyl xanthine (IBMX) and 10% FBS to differentiate pre-adipocytes to adipocytes. Such MDI treated cells were incubated with/without EAG at 0.5-50 µg/ml concentration for 48 h. Roziglitazone (100 nM) served as positive control. Cells were maintained in post-differentiation DMEM containing 100 U insulin, 100 ng/ml dexamethasone in 10% FBS and the media was replaced in every 2 days. In control cells, normal media was replaced in every 24 h. Differentiation, as measured by the appearance of lipid droplets was completed at day 10. Within 30 min, cells were analysed cytometrically (FACS Aria II).

4.3.17. Quantitative determination of major constituents of ginger using HPLC-UV method

4.3.17.1. Sample preparation

Methanolic stock solution of successive ginger extracts (5 mg/ml) and standard 6-gingerol at a concentration of 1 mg/ml was used for HPLC analysis.

4.3.17.2. Chromatographic conditions

The analytical HPLC was performed on a Waters liquid chromatography equipped with rheodyne injector and Waters 2487 UV detector using a C-18 column (150 x 4.6 mm, 5.0 μm). The mobile phase consisted of solvent A, 1% acetic acid in water and B, acetonitrile (40:60). The flow rate was 1.5 ml/min. Retention time of the constituents was so close and better resolution was obtained at a wavelength of 280 nm. The constituent peaks in extracts were identified by the comparison of retention time values of individual components (ISO 13685). All solvents and mobile phases used were of HPLC grade and water was purified on a Millipore (Milli Q system).

4.4. STATISTICAL ANALYSIS

The experimental results are expressed as the mean ± standard deviation (SD) of three parallel measurements. The results were subjected to one way analysis of variance performed by using the statistical program (SPSS), version 11.5 (SPSS, Chicago, IL, USA). Duncan's multiple range test was conducted for comparison of means at $P \leq 0.05$.

4.5. RESULTS AND DISCUSSION

4.5.1. Collection of plant material and proximate composition

Rhizomes of *Z. officinale* (1.5 kg) collected from NIIST campus were cleaned, dried and coarsely powdered. About 100 g of powdered rhizomes was subjected to

proximate composition analysis (moisture content, protein content, total sugar, total fat, crude fibre, ash content, starch content, carbohydrate content etc.) and percentage composition was calculated (Table 4.1).

Table 4.1: Proximate composition of dry ginger

Sl. No.	Proximate composition	Percentage
1	Moisture content	5.0
2	Protein content	10.59
3	Total fat	8.24
4	Ash content	5.77
5	Total carbohydrate content (starch, crude fibre, sugar)	70.4

4.5.2. Extraction

Powdered rhizomes (500 g) were extracted successively with hexane, ethyl acetate, methanol, 70% methanol-water and water at room temperature. Solvents were evaporated using a rotary evaporator to get 4.28%, 1.32%, 4.65%, 4.82% and 5.68% of hexane (HG), ethyl acetate (EAG), methanol (MG), 70% methanol-water (MWG) and water (WG) extracts respectively. Varying concentrations of extracts were used for chemical and biochemical assays.

4.5.3. Antioxidant assays

4.5.3.1. Total phenolic content

The antioxidant capacity of rhizomes of ginger was determined in terms of its phenolic constituents. Total phenolic content of ginger was estimated using the Folin-Ciocalteu reagent 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 reproducible. The phenolic constituents present in the reaction medium reacts with yellow colored Folin-Ciocalteu reagent containing heteropolyphosphotungstates and molybdates undergo reduction reaction and get converted into a blue species possibly $(PMoW_{11}O_{40})^{4-}$ and the absorbance was measured at 760 nm (Huang *et al*., 2005). Based on this assay, the amount of total phenolics was 1.98 ± 0.6 g gallic acid equivalents / 100 g of dry rhizomes in hexane extract, 24.2 g ± 1.1 g gallic acid equivalents/100 g of dry rhizomes in ethyl acetate extract, 17.33 g ± 0.8 g gallic acid equivalents/100 g of dry rhizomes in methanol extract, 17.4 g ± 0.9 g gallic acid equivalents/100 g of dry rhizomes in 70% methanol-water extract and 19.06 g ± 1.2 g gallic acid equivalents/100 g of dry rhizomes in water extract. Thus it is clear that the concentration of phenolic content was higher in the ethyl acetate extract compared to other extracts. This may also be due to the fact that hexane, methanol, 70% methanol-water and water extracts contains some starch, glycosides or some other non polar constituents.

4.5.3.2. DPPH radical scavenging activity

The results showed a concentration dependent increase in DPPH radical scavenging activity (Figure 4.2). From the IC_{50} values, it was revealed that EAG shows highest DPPH radical scavenging activity (4.69 µg/ml) followed by MWG (7.49 µg/ml), WG (25.01 µg/ml), MG (34.71 µg/ml) and HG (58.85 µg/ml) on comparison with

standard gallic acid (1.54 µg/ml). Lower IC_{50} value of EAG indicates its greater radical scavenging activity. The increase in radical scavenging activity may be attributed to the enrichment of phenolic antioxidants.

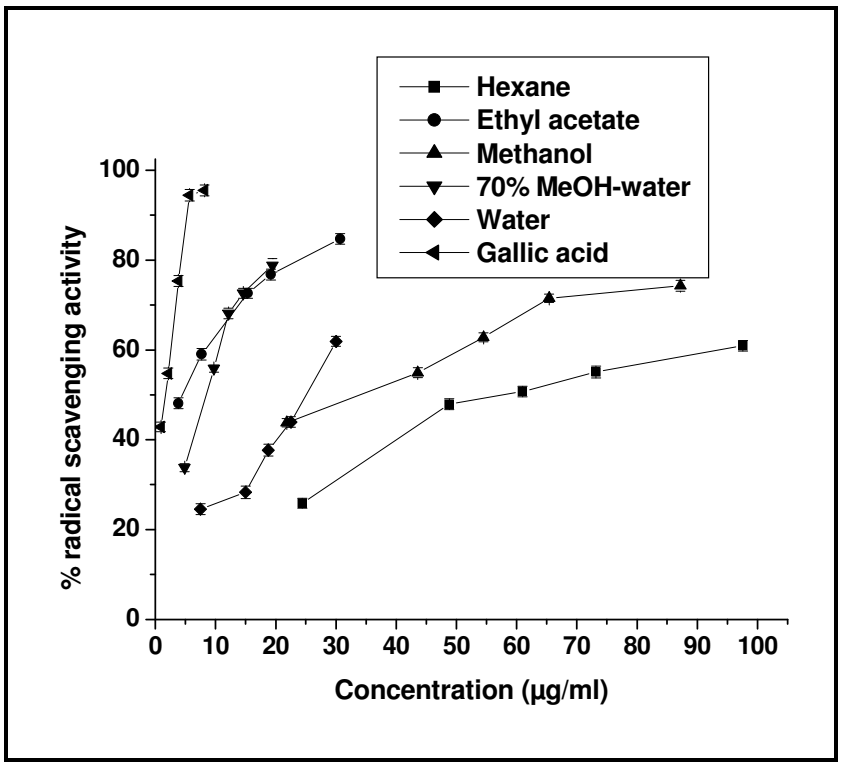


Figure 4.2: DPPH radical scavenging potential of hexane, ethyl acetate, methanol, 70% methanol-water and water extracts of *Z. officinale* compared with standard gallic acid

4.5.3.3. Superoxide radical scavenging capacity

In the present investigation, the successive extracts were also tested for its superoxide radical scavenging capacity and they showed much lower activity compared to standard ascorbic acid (IC_{50} 11.71 µg/ml). Among the extracts, EAG showed scavenging potential with an IC_{50} value of 117.63 µg/ml compared to HG (IC_{50} 945.81 µg/ml), MG ((IC_{50} 421.83 µg/ml), MWG (IC_{50} 171.69 µg/ml) and WG (IC_{50} 269.27 µg/ml). EAG showed a scavenging power for superoxide radical with 75% scavenging

capacity at a concentration of 307 µg/ml. The concentration dependent activity was shown in figure 4.3. .

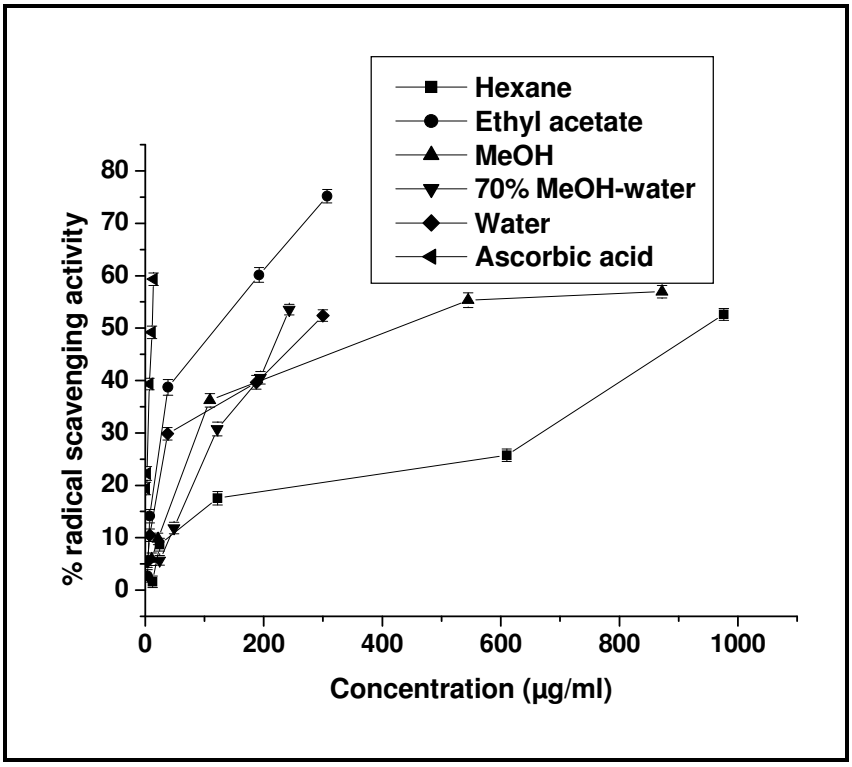


Figure 4.3: Superoxide radical scavenging potential of hexane, ethyl acetate, methanol, 70% methanol-water and water extracts of *Z. officinale* compared with standard ascorbic acid

4.5.3.4. Total reducing power

The reducing powers of successive extracts were assessed based on their ability to reduce Fe (III) to Fe (II) and the results are shown in figure 4.4. An increased absorbance indicates a higher reducing power compared with standard gallic acid. All the extracts showed reducing capacities indicated that they can donate electrons and can react with free radicals to form more stable products and thereby terminating free radical chain reaction (Phanipa *et al*., 2010).

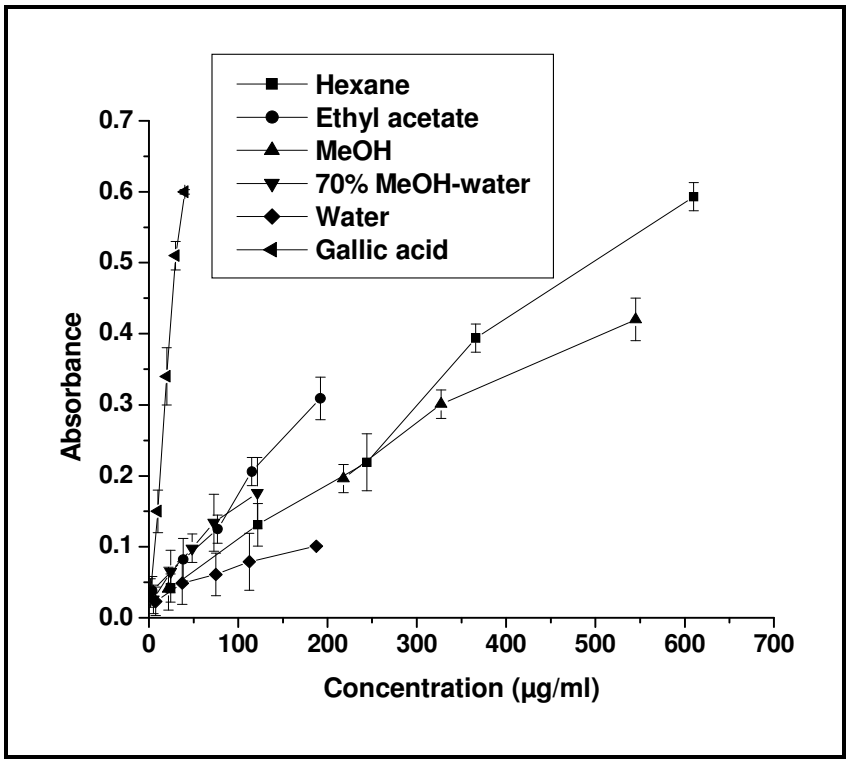


Figure 4.4: Total reducing power of hexane, ethyl acetate, methanol, 70% methanol-water and water extracts of *Z. officinale* compared with standard gallic acid

Since EAG showed the highest antioxidant and radical scavenging potentials, further biological studies were conducted for EAG only.

4.5.4. α-glucosidase and α-amylase inhibition assay

The results showed a concentration dependent inhibition of EAG against α-glucosidase and α-amylase enzymes and compared with standard acarbose. EAG showed an IC_{50} value of 180.13 µg/ml compared to acarbose (IC_{50} 36.83 µg/ml) against α-glucosidase (Figure 4.5).

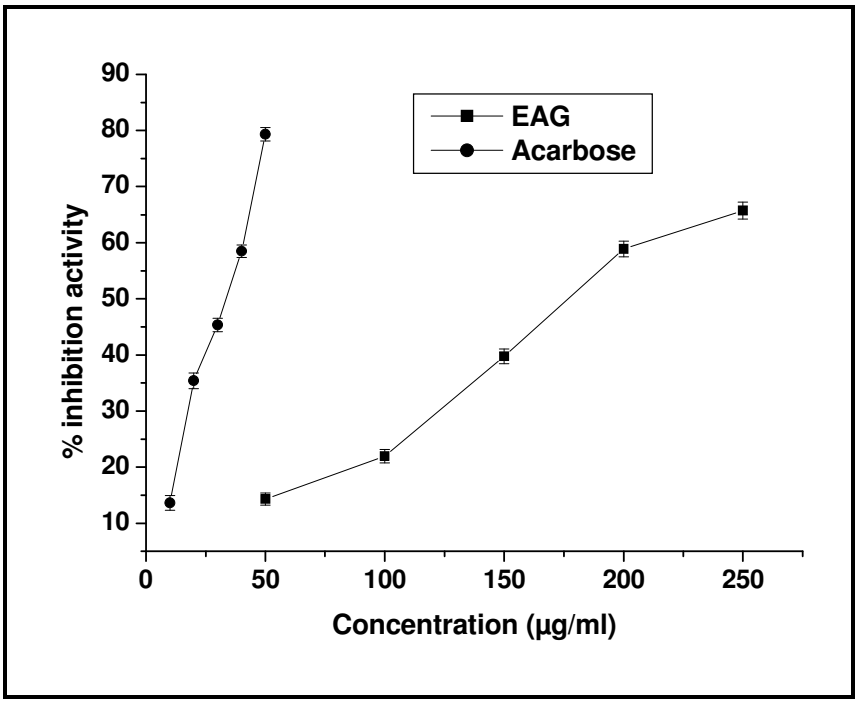


Figure 4.5: α-glucosidase inhibition potential of EAG

α-amylase inhibition activity was measured at different concentrations (500, 750, 1000 and 1250 µg/ml). In this case, EAG showed inhibition potential with an IC_{50} value of 980.21 µg/ml whereas acarbose showed an IC_{50} value of 336.69 µg/ml (Figure 4.6). Previous reports are there that enzyme inhibition was mediated by phenolics which have the potential to contribute to the management of type 2 diabetes (McCue and Shetty, 2004). According to Horii *et al*. (1986), mild α-amylase inhibition is beneficial since excessive inhibition could result in the abnormal bacterial fermentation of undigested carbohydrates in the colon. EAG gave mild α-amylase inhibition activity compared with α-glucosidase. Therefore, EAG may be considered as a better candidate for the inhibition of enzymes and the higher value of inhibition may be related to the phenolic constituents, gingerol and shoagol present in the ethyl acetate extract.

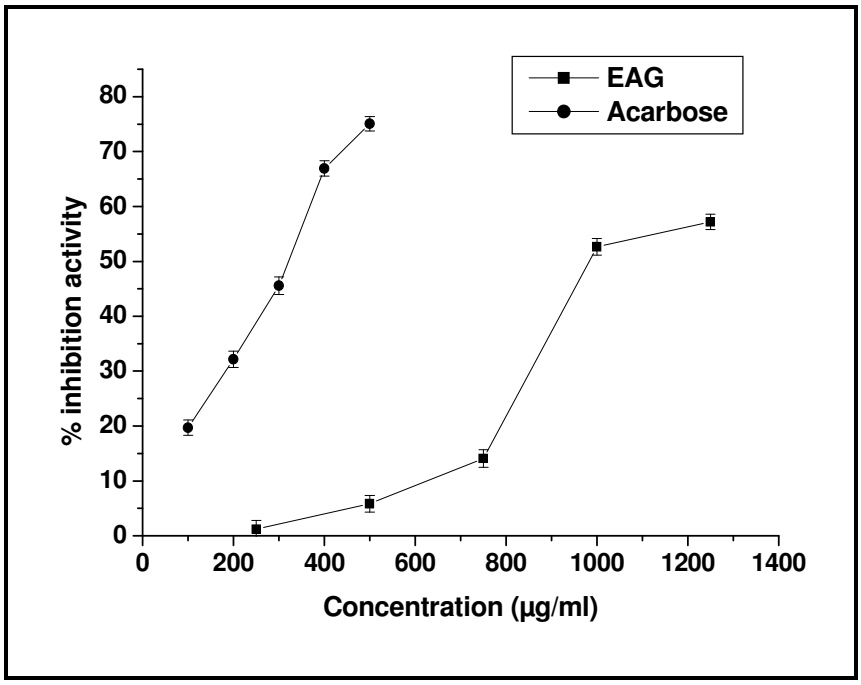


Figure 4.6: α-amylase inhibition potential of EAG

4.5.5. Antiglycation activity

Diabetes pathogenesis mainly involves the formation of Advanced Glycated End products (AGEs). Natural compounds with antioxidative property are proposed to prevent AGEs formation (Arom, 2005) and found to have therapeutic potential in patients with diabetes and age related diseases. EAG showed inhibition potential (IC_{50} 290.84 µg/ml) to glycation reactions at *in vitro* conditions compared to standard ascorbic acid (IC_{50} 84.50 µg/ml) as shown in figure 4.7.

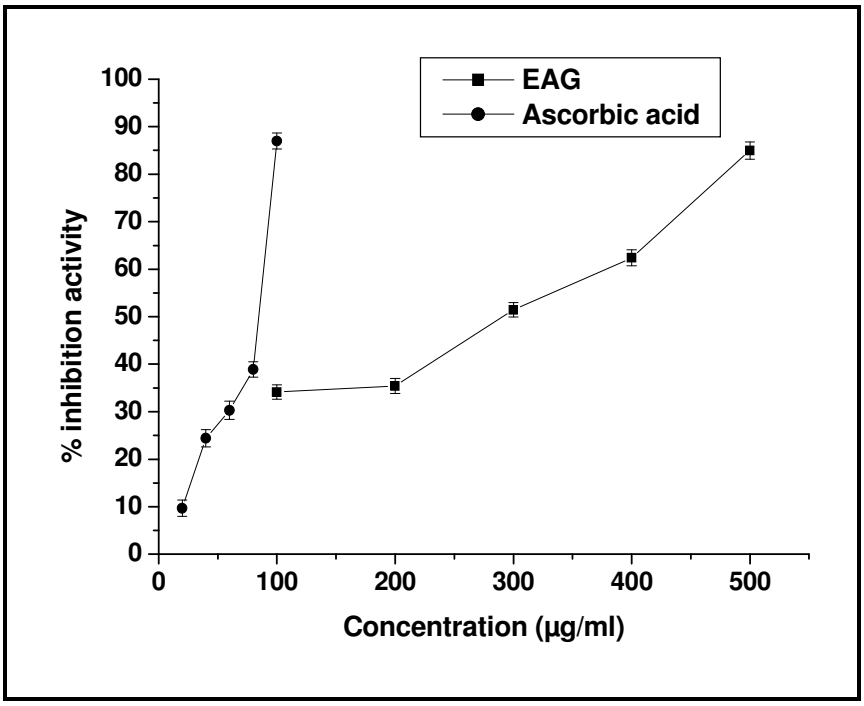


Figure 4.7: Antiglycation potential of EAG

4.5.6. Inhibition of low density lipoprotein oxidation

Oxidation of LDL has been implicated as one of the major reason for atherosclerosis and coronary artery diseases in humans (Wiztum and Steinverg, 1991). Oxidized LDL has many atherogenic effects in vascular tissue those can oxidize LDL and this oxidized LDL is processed by a scavenger receptor of macrophages, leading to cholesterol ester accumulation. This lipid - laden macrophages become foam cells which create fatty streaks in blood vessels (Prathapan *et al*., 2012). Malondialdehyde formed as a major product of lipid peroxidation in biological system is very active and is used to study LDL oxidation by means of derivatizing with TBA at high temperature and acidic conditions (Halliwell, 1990).

LDL oxidation inhibition assay showed that EAG was able to prevent lipid peroxidation effectively and was proportional to applied concentration. After 2 h incubation, EAG (IC_{50} 23.63 µg/ml) exhibited 23.33%, 62.49%, 82.77%, 83.73% and

91.47% of activity at 10, 30, 50, 100 and 150 µg/ml respectively compared to standard ascorbic acid (IC_{50} 18.17 µg/ml). The concentration dependent inhibition of LDL oxidation by EAG is shown in figure 4.8.

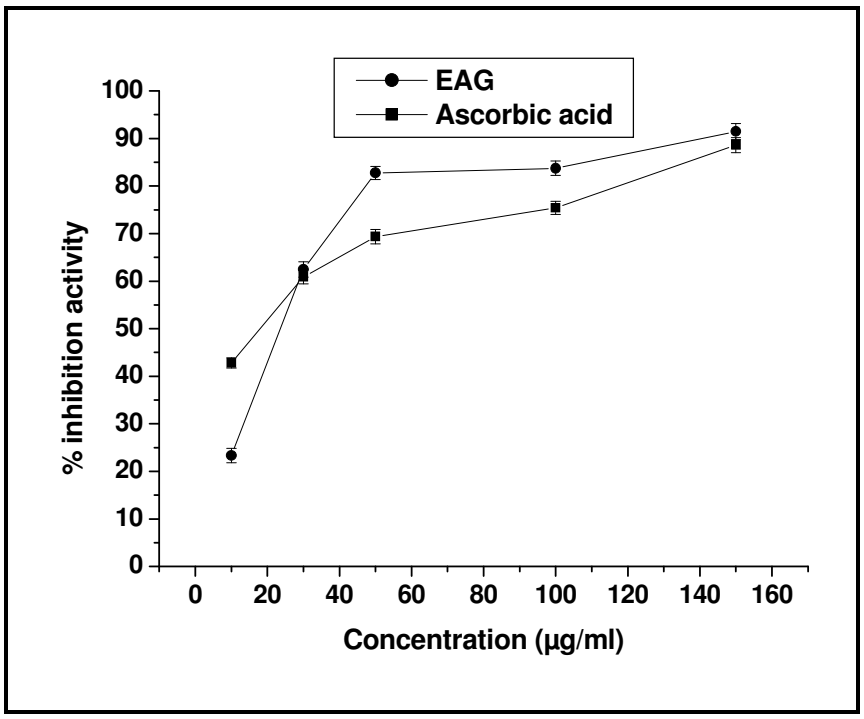


Figure 4.8: LDL oxidation inhibition potential of EAG

4.5.7. ACE inhibition activity of EAG

It has been well demonstrated that angiotensin converting enzyme (ACE) plays an important role in the regulation of blood pressure (Skeggs *et al*., 1956). It enhances blood pressure by converting the inactive angiotensin I to potent vasoconstrictor angiotensin II. Treatment in hypertension reduces the risk of coronary heart disease and therefore inhibition of ACE-I activity is considered as a useful therapeutic approach since it reduces the activity of angiotensin II (Collins *et al*., 1990). EAG was assayed for ACE inhibition activity and exhibited dose dependent ACE inhibition activities with 15.21%, 30.43%, 34.78%, 78% and 83.71% at 0.2, 0.4, 0.6, 0.8 and 1 µg/ml (Figure

4.9). EAG posses an IC_{50} value of 0.67 µg/ml compared to captopril (IC_{50} 0.69 µg/ml) against ACE inhibition.

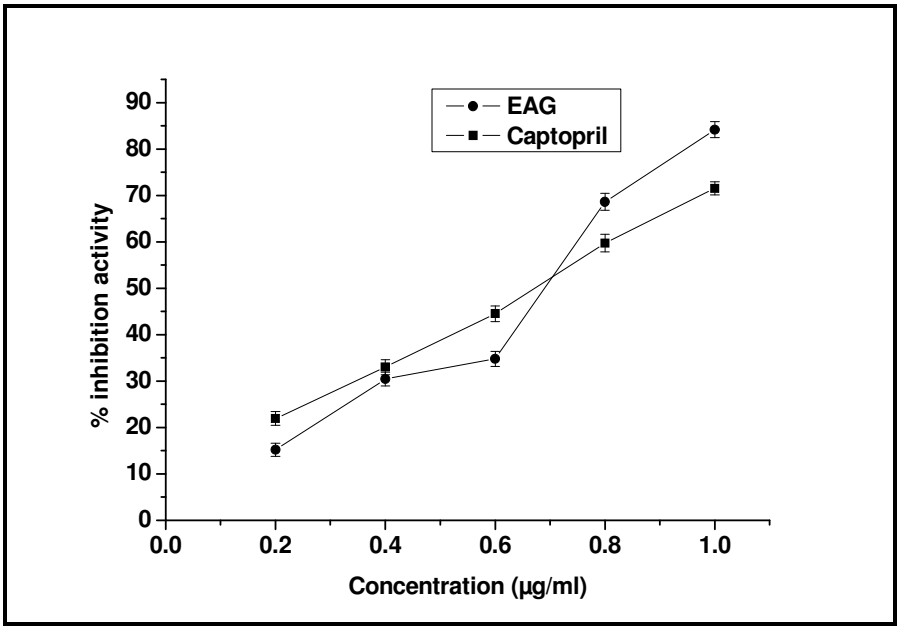


Figure 4.9: ACE inhibition potential of EAG

4.5.8. COX inhibition potential

Dose dependent inhibition of cyclooxygenase (COX) enzyme by different concentration of EAG was measured, which showed an IC_{50} value of 145.04 µg/ml compared with standard indomethacin (IC_{50} 10.2 µg/ml) (Figure 4.10). Cyclooxygenase enzyme was considered as the key marker enzyme for the diseases with impaired arachidonic metabolism. The conversion of arachidonic acid into prostaglandins was catalyzed by COX enzymes and play significant roles in health and in disease in the gastrointestinal tract and in the renal, skeletal and ocular systems (Fitzpatrick and Soberman, 2001). The traditional uses of ginger may be due to its efficiency to suppress inflammatory response, to reduce oxidative stress and their inhibitory activity on COX enzymes.

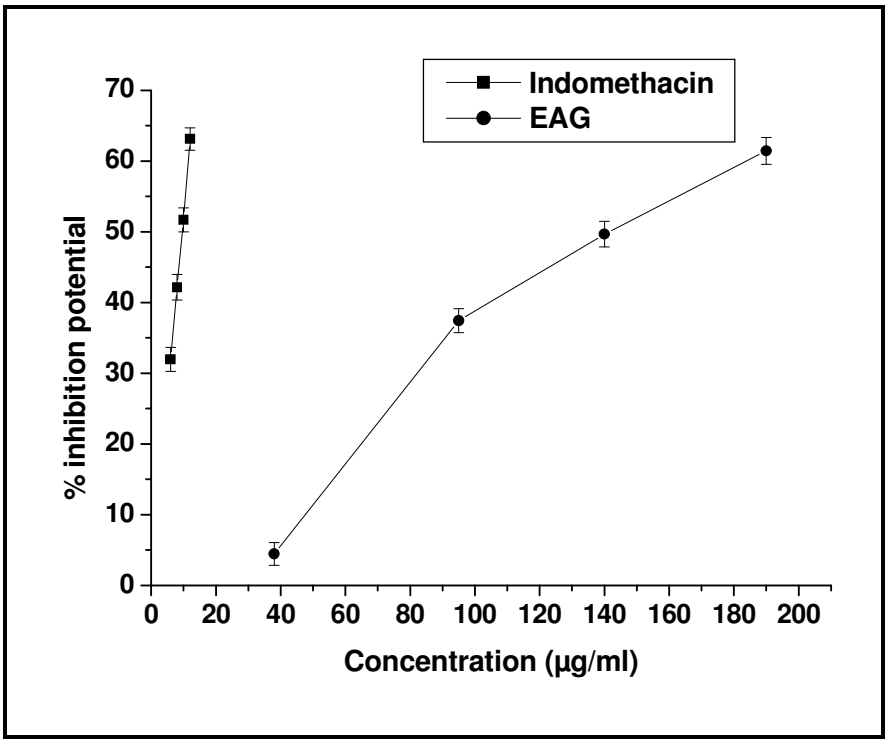


Figure 4.10: COX inhibition potential of EAG

4.5.9. Cytotoxic effect of ginger

Cytotoxic effect of EAG was checked against L6, C2C12 and 3T3L1 cell lines at 10, 50, 100 and 200 µg/ml concentrations. Results showed that the extract was toxic to cell lines at a concentration above 50 µg/ml upon incubation time of 24 h (Figure 4.11). Based on the results, bio activity studies with EAG were confined within 50 µg/ml concentration limits.

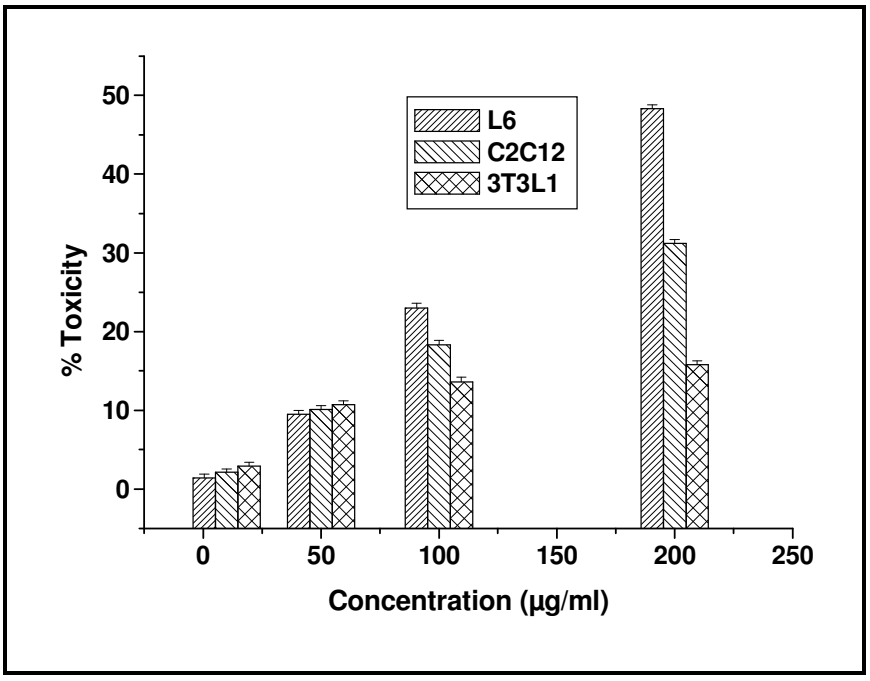


Figure 4.11: Cytotoxic effect of EAG

4.5.10. Protective effect of EAG against oxidative stress in C2C12 cell lines

Enhanced oxidative stress is a widely accepted candidate in the development and progression of diabetes and its complications (Ceriello, 2000). Vascular cells produce hydrogen peroxide during inflammation and induce oxidative stress which may contribute to endothelial dysfunction and vascular diseases (Coyle and Kader, 2007). Increased formation of reactive oxygen species (ROS) occurs in diabetes for reasons which may be possibly related to an increase in glucose level concentration in plasma and tissue.

Protective effect of EAG against H_2O_2 induced oxidative stress in C2C12 cell lines was evaluated by determining intracellular content of ROS. H_2O_2 treated cells showed increased production of ROS which was significantly reduced by the treatment with EAG (10-100 µg/ml) in a dose dependent manner as shown in figure 4.12 (average mean fluorescence from triplicates is expressed per each group of cells). Figure 4.13

shows the dot plot and histogram demonstrating the amount of fluorescence in EAG (100 µg/ml) treated cells. This could be explained as EAG inhibits C2C12 cells from intracellular oxidative damage. At 100 µg/ml concentration, EAG exhibited maximum ROS inhibition (18.2%) compared to H_2O_2 treated C2C12 cell whereas ascorbic acid which is used as the positive control showed a reduction of 9.7% at 25 µg/ml. No evidence of any increase or decrease in 2, 7-dichlorofluorescein fluorescence was observed in cells incubated with extract alone. Clinical observations demonstrate oxidative stress to be an important mechanism in obesity associated metabolic syndrome, in development of diabetes and its complications, damage to all molecular targets, DNA, proteins etc.

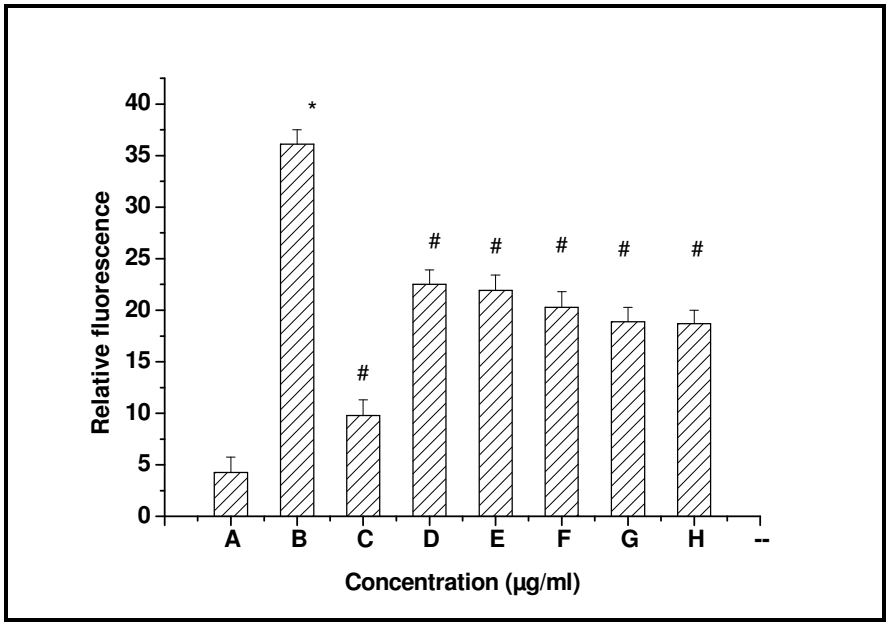


Figure 4.12: Evaluation of oxidative stress in C2C12 cell lines by flow cytometry
A, blank cells (without any treatment); B, control cells (treated with H_2O_2); C, cells treated with H_2O_2 and ascorbic acid (25 µg/ml); D-H, cells treated with H_2O_2 & EAG at various concentrations (10, 20, 40, 80 and 100 µg/ml)

*Significant difference from blank cells ($P \leq 0.05$)
Significant difference from H_2O_2 treated control cells ($P \leq 0.05$)

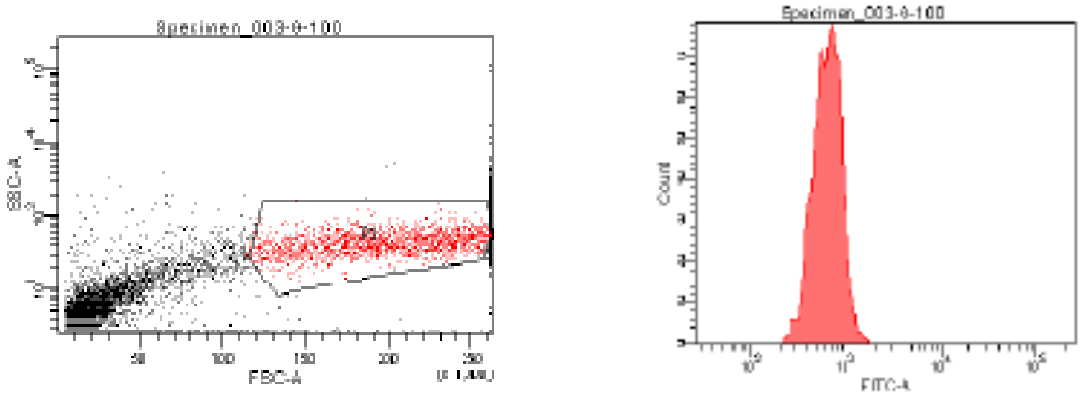


Figure 4.13: Percentage population of cells with fluorescence by flow cytometry

4.5.11. Hypoglycemic effect of ginger

4.5.11.1. EAG stimulates 2-NBDG uptake in L6 myoblast and myotubes

To evaluate whether EAG can stimulate the glucose absorption in muscle cells, 2-NBDG uptake assay was carried out. The assay was planned parallel in two sets with undifferentiated (myoblast) and differentiated (myotubes) L6 cell lines. Previous studies on expression of Glucose Transporters (GLUTs) in L6 cell lines have proved its translocation to cell surface upon differentiation to myotubes (Mitsumoto and Klip, 1992) Cells were pre-treated for 1 h with EAG at 0.5-50 µg/ml concentration. Results indicated a dose dependent increase in uptake of 2-NBDG in both myoblasts and myotubes (Figure 4.14). Change in glucose uptake showed considerable variation between undifferentiated and differentiated cells. Compared to control, both cell types showed increase in 2-NBDG fluorescence. Differentiated L6 cells or myotubes showed significantly high uptake of 2-NBDG, but was not in a concentration dependent manner. At a concentration of 50 µg/ml, myoblast cells showed 25.4% of increase in glucose uptake. Differentiated L6 cells treated with extract at 5 µg/ml concentration showed 57.4% increase in glucose uptake and were higher than roziglitazone treated cells under similar condition. Above 5

µg/ml concentration, uptake was reduced drastically which can be due to toxicity induced by EAG leading to altered metabolism.

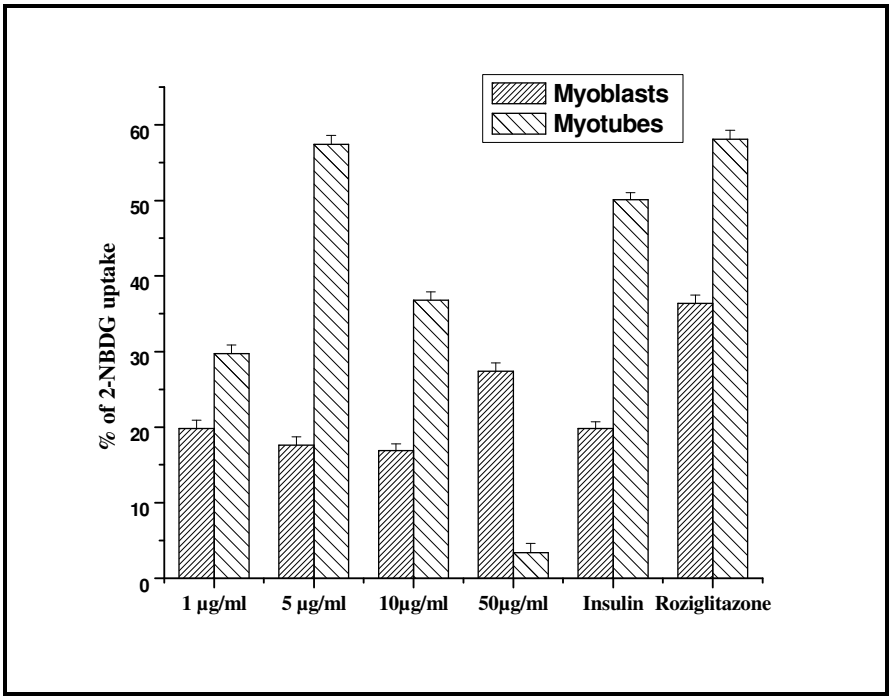


Figure 4.14: Dose dependent uptake of 2-NBDG in L6 myoblasts and myotubes

4.5.12. EAG stimulates Glut 4 expression in L6 myotubes cell surface

Expression of Glut 4 proteins was analysed using antibody based tagging in cultured myotubes. Confocal imaging and analysis showed that cells treated with EAG (5 µg/ml) expressed high fluorescence than control cells. Analysis of captured image by Image Data Explorer (IDE) showed that fluorescence intensity of extract treated cells was nearly similar to that of insulin (100 U) and roziglitazone (100 nM) + insulin (100 U) treated wells. Roziglitazone is a well known insulin sensitizing drug. It was concluded that the extract was able to induce surface expression of Glucose Transporter 4 (Glut 4), which is internally compartmentalized in myoblasts (Figure 4.15).

The results point out the possibility of action of EAG through insulin receptors or as insulin sensitizers. This is also supported by earlier studies by Henriksen *et al*. (1990)

which shows a 2-5 fold increase in glucose uptake by rat muscle cells in response to insulin. In the present study, increase in glucose uptake in EAG treated myotubes was 2.25 fold.

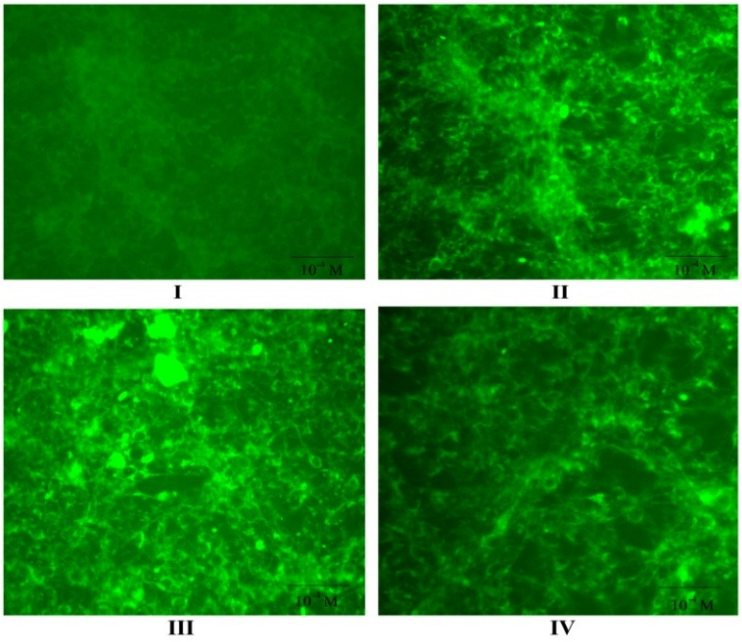


Figure 4.15: Glut-4 expression in L6 myotubes cell surfaces. Change in fluorescence in (I) untreated control cells; (II) cells treated with insulin (100 U); (III) cells treated with roziglitazone (100 nmol) + insulin (100 U) and (IV) cells treated with EAG (5 $\mu g ml^{-1}$)

4.5.13. Inhibitory effect on adipocyte differentiation

As mentioned in materials and methods section, confluent 3T3L1 preadipocytes incubated in presence of 100 nM insulin, 100 nM dexamethasone and 0.5 mM IBMX in 10% FBS/DMEM initiated differentiation. To investigate the potential effect and to arrange the optimal dose of EAG, various doses of active extract (0.5, 1, 5, 10 and 50 µg/ml) were treated with cells during MDI-induced differentiation. After 10^{th} day, cells were trypsinised out and analysed by flow cytometer. Around 10000 events were recorded and analysed based on difference in size (measured by Side Scatter Component or SSC) and granularity (measured by Forward Scatter Component or FSC) which gave a

separation between undifferentiated and differentiated cells with lipid droplets. Results showed that EAG was able to inhibit lipid accumulation in cytoplasm in a dose-dependent pattern. Compound was treated only up to 50 µg/ml, above which it alters cell morphology and viability as evident by MTT assay.

Cells co-treated with EAG (50 µg/ml) showed 21.5% cells with high SSC-FSC value. Compared to MDI treated cells, ginger extract was able to reduce 43.5% lipid content at a concentration of 50 µg/ml (Figure 4.16). Percentage of reduction was steady in cells treated with extract from 5 µg/ml onwards. Roziglitazone, a well known anti diabetic drug also possess side effect of inducing obesity in human body. In the study, roziglitazone (100 nM) was used as standard which enhanced lipid accumulation to 56% compared to pre-adipocytes. The results support the cardio protection and lipid lowering effect of *Z. officinale* in human body and a suitable substitute to roziglitazone.

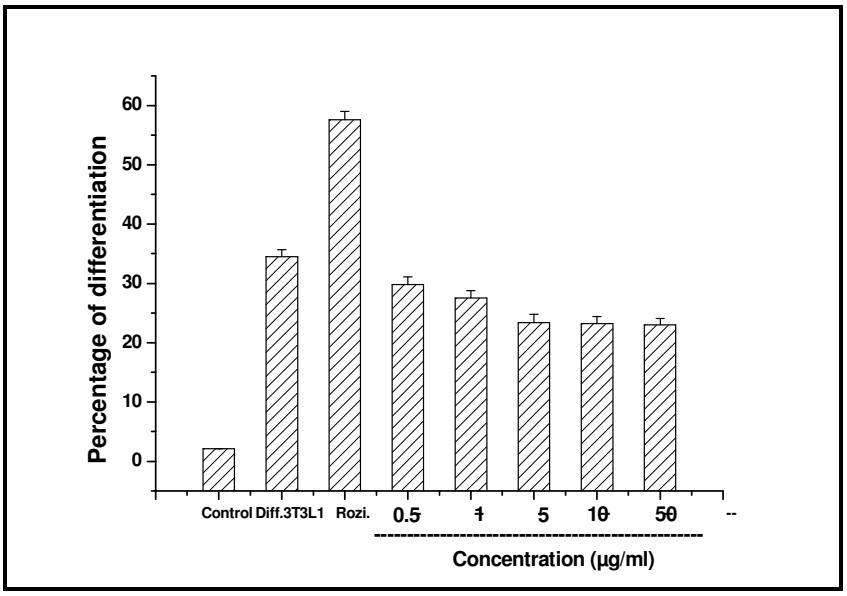


Figure 4.16: Adipocyte differentiation potential of EAG

4.5.14. Identification of constituents of successive extracts of ginger using RP-HPLC method

The pungent constituents of ginger, gingerols and shoagols were determined by RP-HPLC method using isocratic system within a reasonable period of time. An isocratic mobile system (1% acetic acid in water and acetonitrile (40:60)) was used to obtain a complete gingerol profile and the chromatogram was obtained using a UV detector. With the optimized chromatographic conditions, 6-gingerol has been found to be the major component in EAG obtained at a retention time of 3.06 min (Figure 4.17) compared with standard gingerol (Figure 4.18) whereas 8 & 10 gingerols were obtained at 4.45 and 7.49 min. Based upon the chromatogram profile shown in figure 4.17; 6, 8 and 10 shoagols were observed at retention time of 5.03, 8.68 and 16.78 min. Due to the insoluble nature of 70% methanol-water and water extracts, gingerol and shoagol quantification was not done in these extracts. The total pungency was calculated and found to be comparable in ethyl acetate and hexane extracts. The reduced antioxidant activity observed in hexane extract might be due to its dilution with other inactive or retarding constituents as is shown by its higher yield. The total pungency calculated for each extracts is given in Table 4.2.

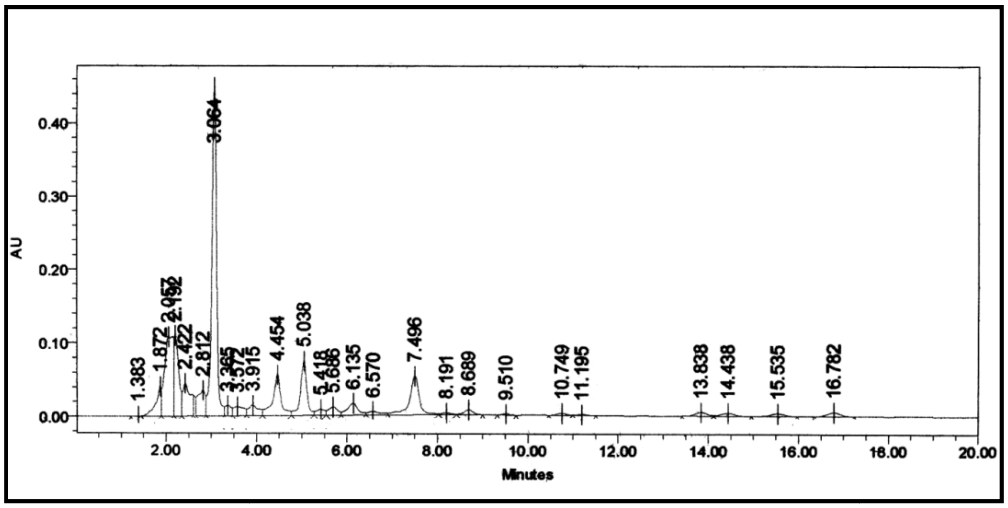


Figure 4.17: HPLC chromatogram of EAG

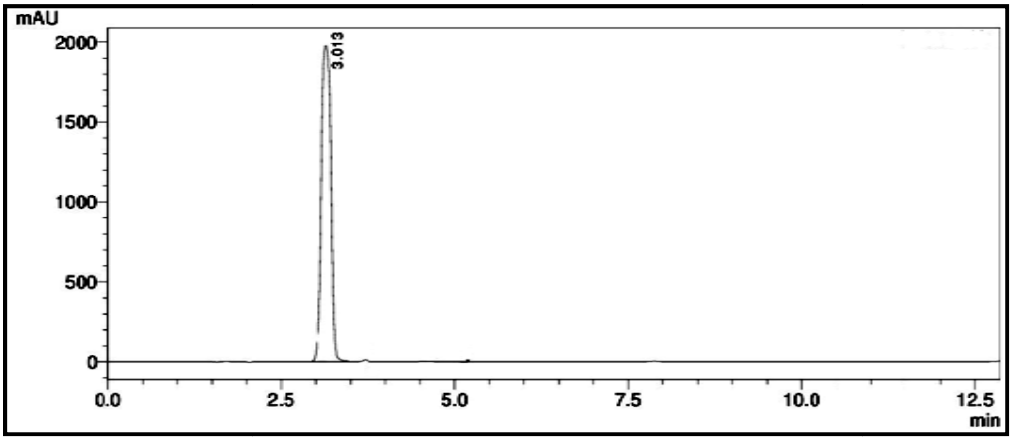


Figure 4.18: HPLC chromatogram of 6-gingerol

Table 4.2: Percentage composition of gingerols and shoagols in successive extracts of ginger

Sl. No.	Extract	Yield (%)	Total gingerols (%)	Total shogaols (%)	Total pungency (%)
1	Hexane	3.87	15.58	0.52	16.10
2	Ethyl acetate	1.93	17.22	0.72	17.94
3	Methanol	3.75	4.41	1.86	6.27
4	70% Methanol -water	4.82	-	-	-
5	Water	5.68	-	-	-

The results of the present study showed that gingerols are the major bioactive compounds present in the ethyl acetate extract of ginger and is responsible for the biological potential of this extract.

REFERENCES

1. Afzal, M.; Al Hadidi, D.; Menon, M.; Pesek, J.; Dhami, M.S. *Drug Metab. Drug Interact.* **2001**, *18*, 159-190.
2. Ahmed, R. S.; Seth, V.; Banerjee, B. D. *Indian J. Exp. Biol*. **2000**, *38*, 604-606.
3. Ali, B. H.; Blunden, G.; Tanira, M. O.; Nemmar, A. *Food Chem. Toxicol.* **2008**, *46*, 409-420.
4. Andallu, B.; Radhika, B.; Suryakantham, V. *Plant Foods Hum. Nutr.* **2003**, *58*, 1-7.
5. Arom, J. *Naresuan Univ. J.* **2005**, *13*, 35-41.

6. Association of Official Analytical Chemists (AOAC). Official Methods of Analysis of AOAC International, 17th ed.; AOAC International: Gaithersburg, MD, USA, **2000**.

7. Badreldin, H. A.; Gerald, B.; Musbah, O.; Abderrahim, N. *Food Chem. Toxicol.* **2008**, *46*, 409-420.

8. Bhandari, U.; Grover, J. K. *Int. J. Diabetes* **1998**, *6*, 95-96.

9. Bhattarai, S.; Tran, V. H.; Duke, C. C. *J. Pharm. Sci.* **2001**, *90*, 1658-1664.

10. Blanca, H. L.; Pedro, J. M. A.; Encarnacion, P. *J. Agric. Food Chem.* **2003**, *51*, 4175-4179.

11. Ceriello, A. *Metabolism* **2000**, *49*, 27-29.

12. Chen, C. Y.; Li, Y. W.; Kuo, S. Y. *Molecules*, **2009**, *14*, 959-969.

13. Chen, Q. C.; Zhang, W.Y.; Jin, W.; Lee, I. S.; Min, B. S.; Jung, H. J.; Na, M.; Lee, S.; Bae, K. *Planta Med.* **2010**, *76*, 79-81.

14. Chrubasik, S.; Pittler, M. H.; Roufogalis, B. D. *Phytomedicine* **2005**, *12*, 684-701.

15. Collins, R.; Peto, R.; MacMahon, S.; Hebert, P.; Fiebach, N. H.; Eberlein, K. A.; Godwin, J.; Qizilbash, N.; Taylor, J. O.; Hennekens, C. H. *Lancet* **1990**, *335*, 827-838.

16. Connell, D.W.; Sutherland, M. D. *Aust. J. Chem.* **1969**, *22*, 1033-1043.

17. Copeland, R. A.; Williams, J. M.; Giannaras, J.; Nurnberg, S.; Covington, M.; Pinto, D.; Pick, S.; Tizaskos, J. M. *Proc. Natl. Acad. Sci*. USA **1994**, *91*, 11202-11206.

18. Coyle, C. H.; Kader, K. N. *ASAIO J*. **2007**, *53*, 17-22.

19. Evans, W. C. Ginger. Trease and Evans Pharmacognosy, 15th ed. W.B. Saunders, Edinburgh, **2002**, 277-280.

20. Fitzpatrick, F. A.; Soberman, R. *J. Clin. Invest*. **2001**, *107*, 1347-1351.

21. Fuhrman, B.; Rosenblat, M.; Hayek, T.; Coleman, R.; Aviram, M. *J. Nutr*. **2000**, *130*, 1124-1131.

22. Govindarajan, V. S. *Crit. Rev. Food Sci.* **1982**, *17*, 1-96.

23. Halliwell, B. *Free Rad. Res. Commun.* **1990**, *9,* 1-32.

24. Henriksen, E. J.; Bourey, R. E.; Rodnick, K. J.; Koranyi, L.; Permutt, M. A.; Holloszy, J. O. *Am. J. Physiol.* **1990**, *259*, E593-E598.

25. Horii, S.; Fukase, H.; Matsuo, T.; Kameda, Y.; Asano, N.; Matsui, K. *J. Med. Chem.* **1986**, *29*:1038-1046.

26. Huang, D. J.; Ou, B. X.; Prior, R. L. *J. Agric. Food Chem*. **2005**, *53*, 1841-1856.

27. Iqbal, Z.; Lateef, M.; Akhtar, M. S.; Ghayur, M. N.; Gilani, A. H. *J. Ethnopharmacol*. **2006**, *106*, 285-287.

28. ISO/FDIS 13685 International Standard Final Draft.

29. Jolad, S. D.; Lantz, R. C.; Solyom, A. M.; Chen, G. J.; Bates, R. B.; Timmermann, B. N. *Phytochemistry* **2004**, *65*, 1937-1954.

30. Katiyar, S. K.; Agarwal, R.; Mukhtar, H. *Cancer Res*. **1996**, *56*, 1023-1030.

31. Kato, A.; Higuchi, Y.; Goto, H.; Kizu, H.; Okamoto, T.; Asano, N.; Hollinshead, J.; Nash, R.J.; Adachi, I. *J. Agric. Food Chem*. **2006**, *54*, 6640-6644.

32. Kim, J. K.; Kim, Y.; Na, K. M.; Surh, Y. J. Kim, T. Y. *Free Radical Res*. **2007**, *41*, 603-614.

33. Kotamballi, N.; Chidambara, M.; Ravendra, P. S.; Jayaprakasha, K. G. *J. Agric. Food Chem.* **2002**, *50*, 5909–5914.

34. Langner, E., Greifenberg, S., Gruenwald, J. *Adv. Ther*. **1998**, *15*, 25-44.

35. Maldie, D. Adipose tissue protocols, in *Methods in Molecular Biology*, 5th edition, ed. by Ailhaud G. Humana Press, New Jersey, **2008**, 54-56.

36. Mascolo, N.; Jain, R.; Jain, S. C.; Capasso, F. *J. Ethnopharmacol*. **1989**, *27*, 129-140.

37. Mc Cue, P.; Shetty, K. *Asia Pac. J. Clin. Nutr*. **2004**, *13*, 101-106.

38. Mitsumoto, Y.; Klip, A. *J. Biol. Chem.* 1992, **267**, 4957-4962.

39. Mustafa, T.; Srivastava, K. C.; Jensen, K.B. *J. Drug Dev*. **1993**, *6*, 25-39.

40. Nammi, S.; Sreemantula, S.; Roufogalis, B. D. *Basic Clinic. Pharmacol. Toxicol.* **2009**, *104*, 366-373.

41. Ojewole, J. A. O. *Phytother. Res.* **2006**, *20*, 764-772.

42. Park, K. K.; Chun, K. S.; Lee, J. M.; Lee, S. S.; Surh, Y. J. *Cancer Lett*. **1998**, *129*, 139-144.

43. Phanipa, P.; Soottawat, B.; Wonnop, V.; Sittiruk, R. *LWT-Food Sci. Technol*. **2010**, *43*, 86-97.

44. Phillips, S.; Ruggier, R.; Hutchinson, S. E. *Anaesthesia* **1993**, *48*, 715-717.

45. Prathapan, A.; Suresh V. N.; Mini. S.; Raghu, K. G. *Eur. Rev. Med. Pharmcol. Sci.* **2012***, 16,* 57-65.

46. Rastogi, R. P.; Singh, S. P.; Hader, D. P.; Sinha, R. P. *Biochem. Biophys. Res. Commun*. **2010**, *397*, 603-607.

47. Shukla, Y.; Singh, M. *Food Chem. Toxicol*. **2007**, *45*, 683-690.

48. Singleton, V. L.; Rossi, J. A. *Am. J. Eno. Viti*. **1965**, *16*, 144-158.

49. Skeggs, L. T.; Kahn, J. R.; Shumway, N. P. *J. Exp. Med.* **1956**, *103*, 295-299.

50. Uma, B.; Raman, K.; Pillai, K. K. *J. Ethnopharmacol.* **2005**, *97*, 227-228.

51. Wang, W. H.; Wang, Z. M. *Zhongguo Zhongyao Zazhi*, **2005**, *30*, 1569-1573.

52. White, B. *Am. Fam. Physician*, **2007**, *75*, 1689-1691.

53. Wilson, A. P. Cytotoxicity and viability assays, in *Animal Cell Culture: A Practical Approach*, 2nd edition, ed. by Masters, JRW. Oxford University Press, Oxford, **2000**, *1*, 175-219.

54. Wiztum, J. L.; Steinverg D. *J. Clin. Invest*. **1991**, *88*, 1985-1972.

55. Yamahara, J.; Mochizuki, M.; Rong, H. Q.; Matsuda, H.; Fujimura, H. *J. Ethnopharmacol.* **1988,** *23*, 299-304.

Chapter 5

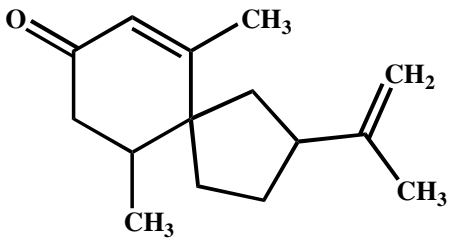
SUMMARY AND CONCLUSION

The importance of natural products for medicine and health has been tremendous throughout the evolution and people are always in association with plants for his food, shelter, medicine etc. The use of natural ingredients in drugs, cosmetics and food industries are increasing and these include extracts from various medicinal and aromatic plants. The current growing trend of dependence on medicinal plants is due to several reasons, including escalating faith in traditional systems of medicines, natural and herbal remedies and also as promoting a habit of going back to nature. Kerala, with its rich diversity and age old practice of Ayurveda can significantly contribute to traditional systems of medicine to combat many diseases. The development of new types of diseases and evolution of new drug resistant strains of pathogens forced the chemists and biologists to search for new drug molecules among the natural resources. This thesis involves the study of three medicinal plants belonging to three different important families viz, *Cyperus rotundus* (Cyperaceae), *Stereospermum colais* (Bignoniaceae) as well as the well known medicinal plant *Zingiber officinale* (Zingiberaceae) as the third.

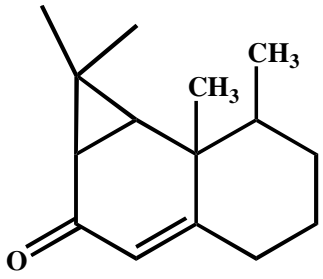
The first chapter gives an overview of biologically active natural products with special reference to antioxidant, antidiabetic, antiinflammatory and antimicrobial molecules from terrestrial sources. The overwhelming effect of free radicals and reactive oxygen species (ROS) in degenerative diseases and conditions like atherosclerosis,

cancer, ageing etc. and the importance of naturally occurring antioxidants for the control of such free radicals are also portrayed in the first chapter.

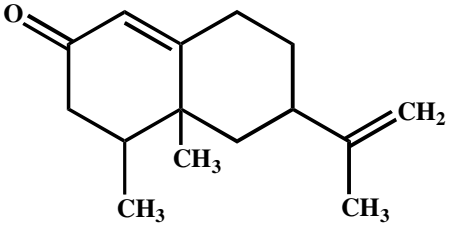
Chapter 2 of the thesis deals with the isolation of phytochemical constituents of the medicinal plant *Cyperus rotundus* and its antioxidant and radical scavenging potential. Here a brief outline of the genus *Cyperus* is given along with detailed survey of literature on the phytochemistry and biological potential of *C. rotundus*. Three sesquiterpenoids with identical molecular weight (solavetivone (**I**), aristolone (**II**) and nootkatone (**III**)) and two flavonoids (quercetin (**IV**) and amentoflavone (**V**)) have been isolated from the acetone extract. Solavetivone has been reported for the first time from the species.



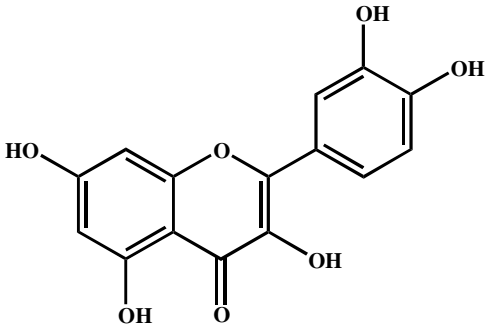
(**I**)



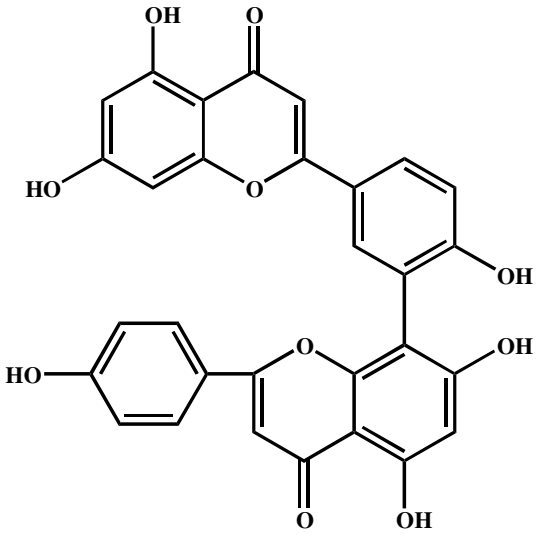
(**II**)



(**III**)



(**IV**)

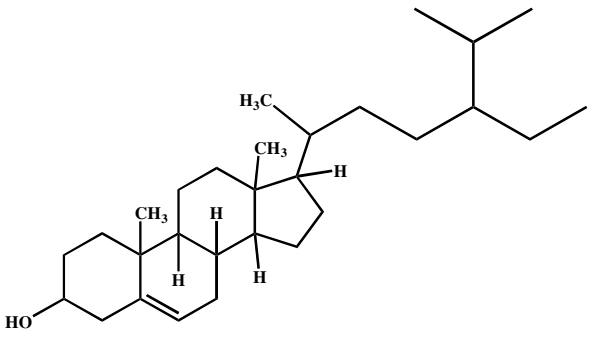


(V)

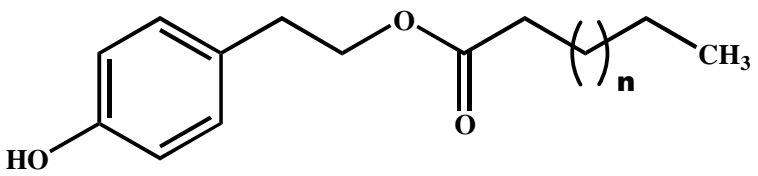
HPTLC and reverse phase HPLC methods were used for the quantification of sesquiterpenoids isolated from the acetone extract of the rhizomes of *C. rotundus*. The chromatographic methods were validated in terms of their linearity, correlation coefficient, precision, accuracy, LOD, LOQ etc. Among the sesquiterpenoids isolated, nootkatone present in highest amount (0.5%) in plant material. The components showed good linearity with a correlation coefficient value of r = 0.98 in a relatively wide concentration range. The evaluation of LOD and LOQ suggested the sensitivity of the method. Screening of total antioxidant assay by phosphomolybdenum reagent and DPPH radical scavenging potential of solavetivone, aristolone and nootkatone showed good activity and nootkatone possessed higher antioxidant and radical scavenging potential compared to standard ascorbic acid and gallic acid respectively. The studies revealed the presence of biologically active sesquiterpenoids in the rhizomes of *C. rotundus*.

Chapter 3 of the thesis describes the studies on the roots of *Stereospermum colais*, a Bignoniaceae plant belonging to the genus *Stereospermum* which is used extensively in

Ayurveda. Here a brief outline of the genus *Stereospermum* is portrayed along with a literature survey of *S. colais*. The antioxidant activity studies on the acetone (ASC) and methanol (MSC) extracts of *S. colais* was analyzed by various *in vitro* models. The extracts were effective in scavenging certain free radicals and reactive oxygen species. Acetone extract showed promising xanthine oxidase inhibition potential compared to standard allopurinol. The lipid peroxidative inhibition potential was also shown by acetone extract comparable to the standard BHT and found to decline with time while methanol extract showed moderate activity. In thermal oxidation conditions also, acetone extract effectively inhibit the lipid oxidation of sunflower oil. Acetone extract also showed good radical scavenging properties in DPPH radical scavenging assay and superoxide radical scavenging assay. It showed nitric oxide scavenging activity and good reducing power. Since acetone extract possess high antioxidant potential, column chromatographic method was done to isolate the active components. Column chromatographic fractionation of acetone extract using varying polarities of hexane-ethyl acetate solvent system yielded 7 compounds viz, β-sitosterol (**VI**), 2-(4′-hydroxyphenyl) ethyl undecanoate (**VII**), 2-(4′-hydroxyphenyl) ethyl pentadecanoate (**VIII**), 5α-ergostan-7, 22-dien-3β-ol (**IX**), ursolic acid (**X**), lapachol (**XI**) and pinoresinol (**XII**). All the compounds were identified for the first time from the species.

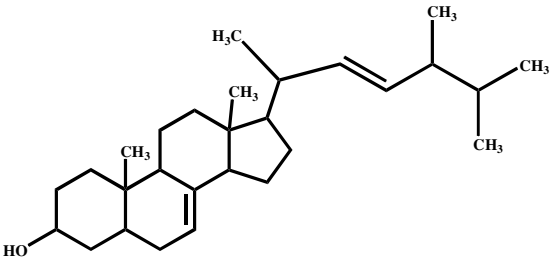


(VI)

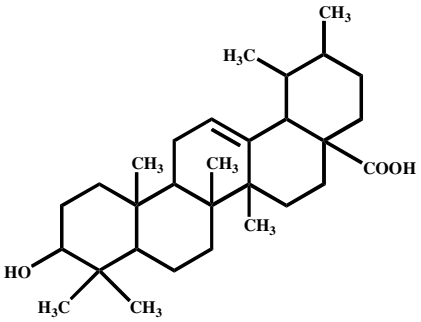


n = 7 (**VII**)

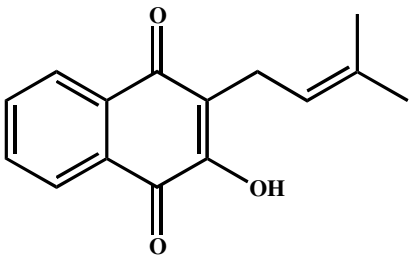
n = 11 (**VIII**)



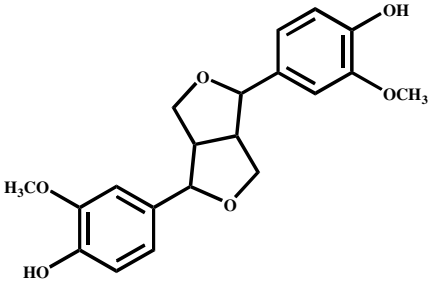
(**IX**)



(**X**)



(**XI**)



(**XII**)

The compounds were screened for its DPPH radical scavenging potential and pinoresinol showed the good antioxidant potential compared to standard gallic acid. Ursolic acid and lapachol also showed activity moderate to standard compound and other compounds failed to show the activity. This study portrayed the presence of antioxidant molecules in *S. colais*. Ursolic acid, lapachol and pinoresinol were also screened for the inhibition of α-glucosidase and α-amylase enzymes. The results showed that the compounds posses significant inhibition potential against enzymes. Inhibition of

advanced glycated end products also showed significant potency of the molecules against diabetes and other metabolic disorders. The order of activity based on these assays is ursolic acid>lapachol>pinoresinol.

Results of the *in vitro* protein denaturation inhibition potential showed that 5α-ergostan-7, 22-dien-3β-ol, ASC and MSC substantially inhibited protein denaturation. 5α-ergostan-7, 22-dien-3β-ol showed promising activity compared to standard diclofenac sodium even at low concentrations. During the *in vivo* assay to check antiinflammatory effect, the isolated compound 5α-ergostan-7, 22-dien-3β-ol as well as the acetone and methanol extracts of *S. colais* significantly inhibited carrageenan induced paw edema in rats with 83%, 33% and 50% inhibition respectively at 5^{th} hour. Evaluation of antibacterial and antifungal effects of ursolic acid and lapachol showed a significant clearance zone by ursolic acid against *Klebsiella pneumonia* and *Trichophyton rubrum*.

Chapter 4 describes the biological potential of rhizomes of *Zingiber officinale*. A detailed description of the biological potential of *Z. officinale* is given in the introduction part of chapter 4. The rhizomes were sequentially extracted with solvents like hexane, ethyl acetate, methanol, 70% methanol-water and water. The antioxidant potential of sequential extracts estimated using various *in vitro* models viz., total phenolic content, DPPH radical scavenging activity, superoxide radical scavenging activity and total reducing power are also discussed in detail in chapter 2.

The results showed that ethyl acetate extract of ginger (EAG) possessed antioxidant activity as is evident from the results of various *in vitro* assays compared to other extracts. DPPH radical scavenging activity showed that ethyl acetate extract of ginger possessed highest activity compared to standard gallic acid. EAG exhibited

comparably higher super oxide scavenging potential than catechin, which served as positive control. The extract possessed good reducing power and contains phenolic constituents. Thus it could be summarized from the results of various assays in chapter 4 that ethyl acetate extract of the rhizomes of *Z. officinale* possessed potent biologically active molecules. Owing to the presence of more phenolic constituents in ethyl acetate extract, detailed biological studies including hypoglycaemic effect, inhibition on adipocyte differentiation etc. was done for EAG only. Inhibition of α-glucosidase and α-amylase enzymes and glycated end products were also carried out for EAG and showed potent activity. Inhibition of low density lipoprotein oxidation, angiotensin converting enzyme activity, cyclooxygenase (COX) enzyme etc. was carried out and EAG showed promising results compared to standards.

Cytotoxicity of EAG was checked against L6, C2C12 and 3T3L1 cell lines within 10-100 µg/ml concentration. From the results, it was confirmed that EAG was safe to selected cell lines within 50 µg/ml when incubated for a time period of 24 hr. EAG was able to ameliorate H_2O_2 induced oxidative stress in C2C12 cell lines at a concentration of 100 µg/ml when administered for 1 hr prior to stress induction. The change in ROS production in cells was evaluated by DCFDA fluorescence and measured using flow cytometer.

A reverse phase HPLC method was employed to find out the active pungent constituents, gingerols and shoagols present in the successive extracts of ginger. Ethyl acetate extract of ginger possessed highest amount of pungent constituents and 6-gingerol was found to be the major constituent resolved at a retention time of 3.06 as it is visible from the chromatogram, which is responsible for the biological potential of the extract.

Owing to the insoluble nature of 70% methanol-water and water extracts, quantification was not done in these extracts. Hence the use of ginger appears to be safe and its effect seems to be mighty and amazing in most of the biological studies.

In conclusion, medicinal plants *Cyperus rotundus* and *Stereospermum colais* have been analysed for their phytochemical constituents. Also, the positive results obtained from biological activity studies such as antioxidant, antiinflammatory and antimicrobial activity on the isolated compounds/extracts add on to the medicinal properties of these plants. Apart from that, ethyl acetate extract of *Zingiber officinale* (ginger) rhizomes has been shown to have very good biological potential including glucose lowering and adipocyte differentiation inhibitory effect.

LIST OF PUBLICATIONS IN SCI JOURNALS

1. **M. Priya Rani**, K. P. Padmakumari, B. Sankarikutty, O. Lijo Cherian, V. M. Nisha, K. G. Raghu. Inhibitory potential of ginger extracts against enzymes linked to type 2 diabetes, inflammation and induced oxidative stress. **International Journal of Food Sciences and Nutrition**, 2011, *62*, 106-110.
2. **M. Priya Rani**, Mahesh S. Krishna, K. P. Padmakumari, K. G. Raghu, A. Sundaresan. *Zingiber officinale* extract exhibits antidiabetic potential via modulating glucose uptake, protein glycation and inhibiting adipocyte differentiation: an *in vitro* study. **Journal of the Science of Food and Agriculture**, 2012, *92*, 1948-1955.
3. **M. Priya Rani**, K. P. Padmakumari. HPTLC and reverse phase HPLC methods for the simultaneous quantification and in vitro screening of antioxidant potential of isolated sesquiterpenoids from the rhizomes of *Cyperus rotundus*. **Journal of Chromatography B**, 2012, *904*, 22-28.
4. **M. Priya Rani**, K. P. Padmakumari. *In vitro* studies to assess the antidiabetic, antiperoxidative, and radical scavenging potential of *Stereospermum colais.* **Pharmaceutical Biology**, 2012, *50*, 1254-1260.
5. **M. Priya Rani,** K. G. Raghu, Mangalam S. Nair, K. P. Padmakumari. Isolation and Identification of α-glucosidase and protein glycation inhibitors from *Stereospermum colais*. Accepted in **Applied Biochemistry and Biotechnology** (DOI: 10.1007/s12010-014-0898-y).

PRESENTATIONS

Oral presentation

1. **M. Priya Rani**, K. G. Raghu, K. P. Padmakumari. Chromatographic methods for the simultaneous quantification and screening of antioxidant potential of sesquiterpenoids from the rhizomes of *Cyperus rotundus*. Oral presentation at International Conference on Bioactive Phytochemicals and Therapeutics-2013. Annamalai University, 5-7 April, **2013.**

Posters presented

1. **M. Priya Rani**, Indu Sasidharan, K. G. Raghu, K. P. Padmakumari Amma and A. Sundaresan. Inhibitory potential of ginger extracts against enzymes linked to type 2 diabetes and inflammation. Poster presented at the 97th Indian Science Congress held in Thiruvananthapuram, Kerala, January 3-7, **2010.**
2. **M**. **Priya Rani**, K. P. Padmakumari. *In vitro* studies to assess the antidiabetic, antiperoxidative and radical scavenging potential of *Stereospermum colais.* Poster presented at the 6th CRSI-RSC Symposium in Chemistry held in CSIR-NIIST, Thiruvananthapuram, Kerala, February 2-5, **2012.**
3. Attended National workshop on Mass Spectrometry, Inter University Instrumentation Centre, M. G. University, Kottayam, Kerala, September 5-6, **2013.**