PHYTOCHEMICAL INVESTIGATIONS ON 'BLACK GLUMED' NJAVARA (*Oryza sativa* L.), THE MEDICINAL RICE, AS COMPARED TO STAPLE VARIETIES AND EVALUATION OF THEIR ANTIOXIDANT, ANTI-INFLAMMATORY AND ANTICANCER EFFECTS

THESIS SUBMITTED TO COCHIN UNIVERISTY OF SCIENCE AND TECHNOLOGY IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN CHEMISTRY UNDER

THE FACULTY OF SCIENCE

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THIRUVANANTHAPURAM-695 019

DECEMBER 2011

DECLARATION

I hereby declare that the matter embodied in the thesis entitled, **"Phytochemical investigations on 'black glumed' Njavara (*Oryza sativa* L.), the medicinal rice, as compared to staple varieties and evaluation of their antioxidant, anti-inflammatory and anticancer effects"** are results of investigations carried out by me at the Organic Chemistry Section, Chemical Sciences and Technology Division of the National Institute for Interdisciplinary Science and Technology (CSIR), Thiruvananthapuram, under the supervision of Dr. A. Jayalekshmy and the same has not been submitted elsewhere for a degree.

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

19-12-2011

Thiruvanthapuram

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CERTIFICATE

This is to certify that the work embodied in the thesis entitled, **"Phytochemical investigations on 'black glumed' Njavara (*Oryza sativa* L.), the medicinal rice, as compared to staple varieties and evaluation of their antioxidant, anti-inflammatory and anticancer effects"** has been carried out by Ms. Smitha Mohanlal, under my supervision and the same has not been submitted elsewhere for a degree.

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ACKNOWLEDGEMENTS

I express my deep sense of gratitude to my research supervisor Dr. A. Jayalekshmy, for suggesting the research topic and for her guidance, constant support and encouragement that led to the successful completion of this work.

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 this work.

My sincere thanks are also due to:

- *Dr. Vijay Nair, Dr. Mangalam S. Nair, Dr. Luxmi Varma and Dr. K. V. Radhakrishnan, scientists of Organic Chemistry Section, for all their help and support.*
- *Dr. D. Ramaiah, Head, Chemical Sciences and Technology Division.*
- *Dr. T. Prasad Rao and Dr. A. Ajayaghosh, Chemical Sciences and Technology Division.*
- *Dr. C.H. Suresh, scientist of Computational Modeling and Simulation Section for computational studies.*
- *Dr. A. Helen (Kerala University), Dr. T. R. Santhoshkumar (RGCB, Trivandrum), for collaboration.*
- *Ms. Soumini Mathew for NMR analysis, Ms. S. Viji for HRMS analysis, Mrs. Prasannakumari L. for GC-MS analysis, Ms. M. J. Ajitha for computational studies, Mr. B. Adarsh and Mr. Aby Mathew and Mr. Preethanuj P. for 500 MHz NMR analysis.*
- *Ms. V. Shalini, Mr. Sathish Kumar M. and Mr. M. Ratheesh for all support and help during collaboration.*
- *M Sc. project students, Mr. Sreejith, Ms. Divya, Ms. Jeeja, Ms. Laxmi, Ms. Aishwarya, Ms. Ramya, Ms. Anju, Ms. Sharon for their help, support and affection.*

- *My colleagues Ms. T. Sreeja, Ms. P. S. Hema, Ms. Alan Sheeja, D. B, Ms. Priya Rani, A, Ms. Mini, V, Ms. Dhanya S., Mr. N. Adarsh, Mr. Sajin F., Ms. Sarika S., Ms. Rajsree K. P., Ms. Dhanya T. J., Ms. Shimi M. and all other members of Organic Chemistry Section for their valuable help and affection.*
- *I am thankful to library, administrative and technical staff of NIIST, Thiruvananthapuram for their help.*
- *All friends in other divisions of NIIST, Trivandrum for their help and support.*
- *Council of Scientific and Industrial Research (CSIR), Government of India, for fellowship and Kerala State Council for Science, Technology and Environment (KSCSTE) for financial assistance.*

I am most grateful to my junior, Ms. R. Parvathy for her sincere help, support, affection and unforgettable companionship throughout the research career.

I express my deep sense of gratitude to my teacher Mr. K. J. Jerly, for imparting me with the basics and knowledge in chemistry, and also his encouragement and support for pursuing research further.

Words are less though I am deeply indebted to my parents and brother for their immense, invaluable love. Without their understanding and patience it would have been difficult for me to complete my journey of research for PhD.

I express my deep love towards my better half 'Rudy', who constantly gave love, courage and confidence. I thank him for his guidance and admiration which motivated me throughout the difficult times in the development of this thesis.

Finally, I thank Almighty for everything.

Smitha Mohanlal

ABBREVIATIONS

A.D.	: Anno Domini
A°	:Armstrong
AFLPs	: Amplified fragment length polymorphisms
$AlCl_3$	: Aluminium chloride
ANR	: Anthocyanidin reductase
ANS	: Anthocyanidin synthase
$ArO^{\cdot}$	: Aroxyl radical
ArOH	: Flavonoid /Phenol
$ArOH^{+\cdot}$	: Flavonoid/Phenol cation radical
ARP	: Antiradical power
ATCC	:American type culture collection
ATP	: Adenosine triphosphate
B.C.	: Before Christ
B3LYP/6-31G*	: Becke 3-parameter level density functional theory
BDE	: Bond dissociation enthalpy
BF_3	: Boron trifluoride
br	: broad
C H O	: Carbon Hrdogen Oxygen
CAT	: catalase
CB-A	: Chain-breaking acceptor
CB-D	: Chain-breaking electron donor
$CCl_3^{\cdot}$	: trichloromethyl radical
CCl_3COOH	: Trichloroacetic acid
CD_3OCD_3	: deuterated acetone
CH_3CN	: Acetonitrile

CHI	: Chalcone isomerise
CHKR	: Chalcone polyketide reductase
$CHO^{\cdot}$	: Formyl radical
CHS	: Chalcone synthase
cm^{-1}	: Centimeter inverse
Co.	: Company
CO_2	: Carbon dioxide
CoA	: Co-enzyme A
COSY	: Correlation spectroscopy
COX	: Cyclooxygenase
COX-1	: Cyclooxygenase-1
COX-2	: Cyclooxygenase-2
cP	:Centipoise
CPT	: Camptothecin
CPT-II	: Camptosar
Cu^{2+}	: Cupric ion
d	: doublet
dd	: quartet
DEPT	:Distortionless enhancement by polarization transfer
DFR	: Dihydroflavonol-4-reductase
DFT	: Density functional theory
DMEM	: Dulbecco modified Eagle's minimal essential medium
DMSO	: Dimethyl sulphoxide
DNA	: Deoxyribonucleic acid
DPPH	: 2, 2-diphenyl-1-picrylhydrazyl
$DPPH^{\cdot}$	: 2, 2-dipheny-1-picryl hydrazil radical

DTT	: Dithiothreitol
e^-	: electron
EC_{50}	:Effective concentration for 50% activity
ESR	: Electron spin resonance
F3', 5'H	: Flavonoid 3', 5' hydroxylase
F3'H	: Flavanone 3'-hydroxylase
FAB	: Fast atom bombardment
FACS	: Fluorescence activated cell sorting
FBS	: Fetal bovine serum
Fe^{2+}	: Ferrous ion
Fe^{3+}	: Ferric ion
$FeCl_3$	: Ferric chloride
FGT	: Flavonoid glycosyltransferase
FHT	: Flavanone 3-β-hydroxylase
FID	: Flame Ionization Detector
FITC	: Fluorescein isothiocyanate
FLAP	: 5- Lipoxygenase activating protein
FLS	: Flavonol synthase
FNS	: Flavones synthase
FOMT	: Flavones O-methyltransferase
FTIR	: Fourier transform infrared
g	: gram
GAE	: Gallic acid equivalents
GC	: Gas chromatography
GCMS	: Gas chromatography mass spectrograph
GFP	:Green fluorescent protein
GPX	: Glutathione peroxidase

GSSG	: Glutathione disulphide
h	: hour
H^+	: Hydrogen ion
H_2O	: Water
H_2SO_4	: Sulphuric acid
H_3O^+	:Hydronium ion
HAT	: Hydrogen atom theory
Hb	: hemoglobin
HClO	: Hypochlorous acid
HCT 116	: Colon cancer cells
HMQC	: Heteronuclear multiple quantum coherence
hpBMCs	: Human peripheral blood mononuclear cells
HPETE	: Hydroperoxy eicosatetranoic acid
HPLC	: High performance liquid chromatography
HRMS	: High resolution mass spectrometer
Hz	: Hertz
i.d.	: inner diameter
IC_{50}	:Inhibition concentration for 50% activity
IFD	: Isoflavone dehydratase
IFS	: Isoflavone synthase
iNOS	: inducible Nitric oxide synthase
IP	: Ionization potential
IR	: Infra red
IR-64	: a rice variety
J	: coupling constant
JC1	: 5, 5', 6, 6'-tetrachloro-1,1',3, 3'-tetraethyl-benzamidazolocarbocyanin iodide

KBr	: potassium bromide
Kcal	: Kilocalorie
KCl	: Potassium chloride
Kg	: Kilogram
KJ	: Kilo Joule
$L^{\cdot}$	: Lipid radical
LAR	: Leucoanthocyanidin reductase
LH	: Unsaturated fatty acid
$LOO^{\cdot}$	: Lipid peroxy radical
LOOH	: Lipid hydroperoxide
LOX	: Lipoxygenase
LPS	: Lipopolysaccharide
LTB_4	: Leukotrienes B_4
Ltd.	: Limited
LTs	: Leukotrienes
m	: multiplet
m.p	:Melting point
m/z	: mass to charge ratio
M^+	: molecular ion
MCF-7	: Breast cancer cells
MDA	: malondialdehyde
MeOH	: Methanol
min	: minute(s)
ml	: millilitre
mm	: millimetre
MMP	: Mitochondrial membrane potential
Mn	: Manganese

$MnCl_2$	: Manganese chloride
mol	: mole
MPT	: Mitochondrial permeability transition
MS	: Mass spectral
n	: number
Na_2CO_3	: Sodium carbonate
$Na_2HPO_4.2H_2O$	: Sodium phosphate dibasic dihydrate
NADH	: Nicotinamide adenine dinucleotide
NADPH	: Nicotinamide adenine dinucleotide phosphate
$NaH_2PO_4.2H_2O$	: Sodium phosphate monobasic dihydrate
$NaNO_2$	: Sodium nitrite
NaOAc	: Sodium acetate
NaOH	: Sodium hydroxide
NaOMe	: Sodium methoxide
NB	: Njavara black
NBb	: Bran of Njavara Black
NBPGR	: National Bureau of Plant Genetic Resources
NBr	: Rice of Njavara Black
NBT	: Nitroblue tetrazolium dihydrochloride
NF-kB	: Nuclear factor kappaB
NIST	: National institute of standard and technology
NMR	: Nuclear magnetic resonance
NO	: Nitric Oxide
$NO^{\cdot}$	: Nitric oxide radical
NOS	: Nitric oxide synthase
NSAI	: Nonsteroidal anti-inflammatory
NSAID	: Nonsteroidal anti-inflammatory drugs

O_2	: Oxygen molecule
$O_2^{-\cdot}$	: Superoxide anion radical
O_3	: ozone
ODS	: Octa decyl silyl
OH	: Hydroxyl
$OH^{\cdot}$	: hydroxyl radical
p	: Probability
PA	: Proanthocyanidin
PCD	: Programmed cell death
PCM	: Polarizable continuum model
PDA	: Photodiode array
PE	: Phycoerythrin
PGD2	: Prostaglandin D2
PGF2	: Prostaglandin F2
$PGF_{2\alpha}$	: $Prostaglandin_{2\alpha}$
PGG_2	: Postaglandin G_2
PGH2	: Prostaglandin H2
PGHS	: Prostaglandin H synthase
PGI2	: Prostaglandin I2
pH	: Power of hydrogen
PLA_2	: Phospholipase A_2
PM	: Palakkadan Matta
PMb	: Bran of Palakkadan Matta
PMr	: Rice of Palakkadan Matta
PMS	: Phenazine methosulphate
PS	: Phosphatidyl serine
PTFE	: Polytetrafluoroethylene

PUFA	: Polyunsaturated fatty acids
q	: quartet
Q	: Quercetin
QE	: Quercetin equivalent
R & D	: Research and development
R^2	: Correlation coefficient
RA	: Rheumatoid arthritis
RAW 264.7	: cell line of mouse macrophages
RNS	: Reactive nitrogen species
$RO^{\cdot}$	: alkoxyl radicals
RO_2^{-}	: peroxyl anion
$ROO^{\cdot}$	: peroxyl radicals
ROOH	: Alkyl peroxide
ROS	: Reactive oxygen species
rpm	: revolution per minute
RPMI	: Roswell Park Memorial Institute medium
$RS^{\cdot}$	: thiyl radical
RT	: Retention time
s	: singlet
SD	: Standard deviation
SDS	: Sodium dodecyl sulphate
Se	: Selenium
SEM	: Standard error mean
SET	: Single electron transfer
sh	: shoulder
SJ	: Sujatha
SJb	: Bran of Sujatha

SJr	: Rice of Sujatha
SKOV3	: Ovarian cancer cells
SOD	: Superoxide dismutase
SOD-U	: enzyme concentration required to inhibit chromogen production by 50 % in 1 min
SPSS/PC+	: Statistical programme for social sciences for personal computer
T_1	: LPS+2 µg/ml of compound
T_2	: LPS+5 µg/ml of compound
T_3	: LPS+10 µg/ml of compound
TaOMT2	: Flavone O-methyltransferase
TBA	: Thiobarbituric acid
TCA	: Tricholoroacetic acid
TEGE	: Tricin-4'-O-(*erythro-β*-guaiacylglyceryl)ether
TFC	: Total Flavonoid Content
TLC	: Thin layer chromatography
TMRM	: Tetramethyl rhodamine methylester
TMS	: Tetramethyl silane
TNF-α	: Tumor necrosis factor-alpha
TPC	: Total phenolic content
TTGE	: Tricin-4'-O-(*threo*-β-guaiacylglycery)ether
TXA2	: Thromboxane A_2
UV-A	: Ultra violet between 320-400 nm (320-400nm)
UV-B	: Ultra violet between 290-320 nm(290-320nm)
UV-C	: Ultra violet below 290 (<290nm)
UV-vis	: Ultra violet-visible
UV-VIS-PDA	: Ultra violet-visible photo diode array
v/v/v	: volume by volume by volume

Vc	: Paw volume of control rats
Vitamin C	: Ascorbic acid
Vitamin E	: Tocopherol
Vt	: Paw volume of test rats
w/v	: weight by volume
w/v	: weight by volume
WHO	: World Health Organization
ZPE	: Zero point energy
α	: alpha
β	: beta
δ	: delta
λ_{max}	: maximum wavelength
μm	: micro metre
ν_{max}	: maximum frequency
%	: Percentage
% $[DPPH^{\cdot}]_{REM}$	:Percentage of DPPH radical remaining
$(K_3[Fe(CN)_6])$	: Potassium ferricyanide
$[DPPH^{\cdot}]_0$	: DPPH radical at zero time
$[DPPH^{\cdot}]_t$	: DPPH radical at any time
>C=O	: Carbonyl
°C	: degree celsius
μg	: micro gram
μl	: microlitre
μM	: micro molar
12-LOX	: 12-Lipoxygenase
^{13}C	: Carbon-13
15-LOX	: 15-Lipoxygenase

^{1}H	: Proton
$^{1}O_2$	: Singlet oxygen
$^{3}O_2$	: Triplet oxygen
$[\alpha]_D^{26}$	: Specific rotation
%	: Percentage
% $[DPPH^{\cdot}]_{REM}$	:Percentage of DPPH radical remaining
$(K_3[Fe(CN)_6])$	: Potassium ferricyanide
$[DPPH^{\cdot}]_0$	: DPPH radical at zero time
$[DPPH^{\cdot}]_t$	: DPPH radical at any time

CONTENTS

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Chapter 1

Introduction

1.1 Free radicals

Free radicals are known in chemistry since the beginning of the 20th century and were initially used to describe intermediate compounds in organic and inorganic chemistry and several chemical definitions were suggested for them. A free radical can be explained in simple terms as any species capable of independent existence that contains one or more unpaired electrons (an unpaired electron being one that singly occupies an orbital). It may be superoxide ($O_2^{-\bullet}$, an oxygen centred radical), thiyl ($RS^{\bullet}$, a sulphur-centred radical), trichloromethyl ($CCl_3^{\bullet}$, a carbon centred radical) or nitric oxide ($NO^{\bullet}$) radical in which the unpaired electron is delocalized between both atoms. Only in 1954 when the pioneering work of Daniel Gilbert and Rebecca Gersham was published[1] were these radicals suggested as important players in biological environments and responsible for deleterious processes in the cell. Soon after, in 1956, Herman Denham[2] suggested that these species might play a role in physiological events particularly, in the aging process.[3] His hypothesis, the free-radical theory of aging, inspired numerous studies and research efforts and contributed significantly to our knowledge of radicals and more specifically, oxygen-derived radicals and other oxygen derived, non-racial reactive species. These metabolites are now considered major players in biochemical reactions, cellular responses and in clinical outcome.[4,5] Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals.[6] This unpaired electron(s) usually gives a considerable degree of reactivity to the species. Free radicals are less stable than non-radical entities, although they are more reactive. Free radicals are formed from molecules *via* (i) the breakage of a chemical bond such that each fragment keeps one electron (ii) by cleavage of a radical to give another radical and also *via* (iii) redox reactions.[7]

Oxygen is an element indispensable for life. Antoine Lavoisier, a pioneer oxygen chemist, had pointed out, about 150 years ago that animals that respire are true

combustible bodies that burn and consume themselves.[8] Today we know that when cells use oxygen to generate energy, free radicals are created as a consequence of adenosine triphosphate (ATP) production by the mitochondria.[6] These by-products are generally reactive oxygen species (ROS) and reactive nitrogen species (RNS) that result from the cellular redox reactions. Thus, the biological combustion produces harmful intermediates called free radicals that are fundamental to any biochemical process and represent an essential part of aerobic life and our metabolism. They are continuously produced by the body's normal use of oxygen such as respiration and some cell-mediated immune functions. These species play a dual role - as both toxic and beneficial compounds.

1.2 Major types of radicals in living organisms

Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are the terms collectively used for describing free radicals and other non-radical reactive derivatives which are also called oxidants. Radicals derived from oxygen represent the most important class of radical species generated in living systems.[9] These oxygen-derived pro-oxidants, in general are referred to as ROS that can be classified into two groups - radicals and nonradicals. The radical group contains molecules such as nitric oxide radical ($NO^{\bullet}$), superoxide ion radical ($O_2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$), peroxyl ($ROO^{\bullet}$) and alkoxyl radicals ($RO^{\bullet}$) and singlet oxygen ($^1O_2$). These species are radicals, because they contain at least one unpaired electron in the shells around the atomic nucleus and are capable of independent existence.[10,11] The occurrence of one unpaired electron results in high reactivity of these species by their tendency to donate or obtain another electron to attain stability. By definition, the oxygen molecule itself is a radical, due to its unique electronic configuration, as it contains two unpaired electrons in two different orbitals (Figure 1.1) and thus, is biradical.[10] The oxygen radical is not a reactive one due to the so-called spin restriction, which does not allow the donation or acceptance of another electron, before the rearrangement of the spin directions around the atom. The group of nonradical compounds contains a large variety of substances, some of which are extremely reactive although not radical by definition. Among these compounds produced in high concentrations in the living cells are hypochlorous acid

(HClO), hydrogen peroxide (H_2O_2), organic peroxides, aldehydes, ozone (O_3), and singlet oxygen ($^1O_2$), that can easily lead to free radical reactions in living organisms.

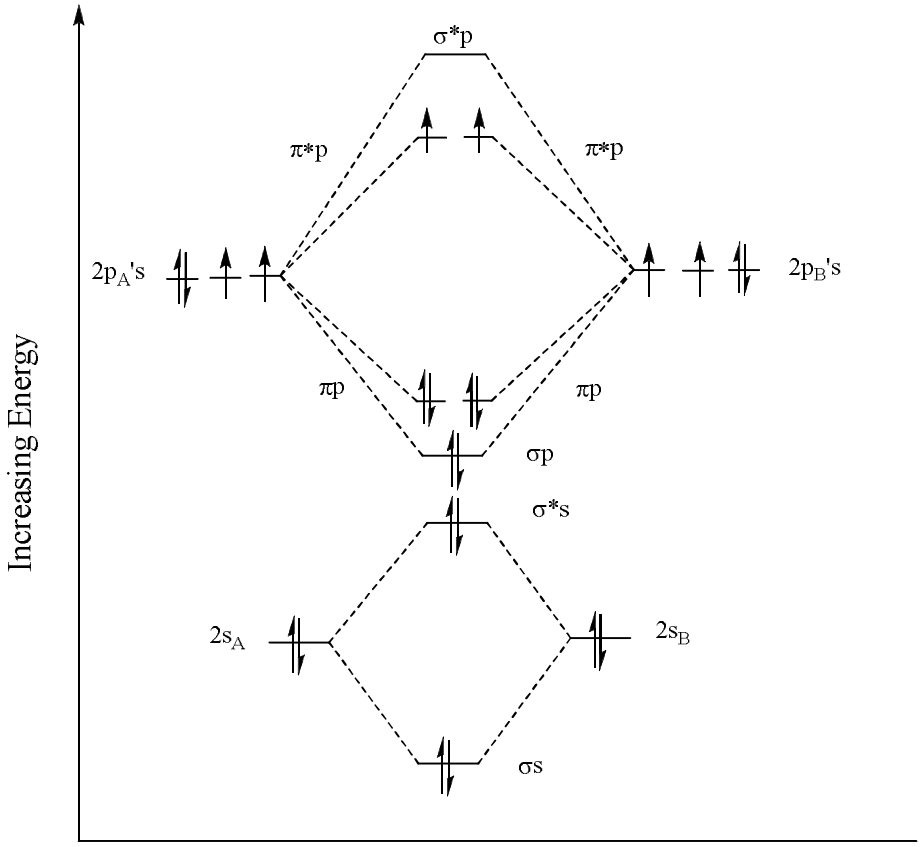


Figure 1.1: Molecular orbital diagram of oxygen molecule (O_2)

1.3 Sources of ROS and their role in biological systems

The cell is exposed to a large variety of ROS and RNS from two sources namely, exogenous and endogenous sources.[12]

1.3.1 Exogenous Sources

Exogenous sources include, first, exposure to O_2 molecule, which, although a nonreactive biradical, can independently cause oxidation and damage to proteins and enzymes.[10] Ozone which is not a radical like oxygen can damage lungs and can serve as a powerful oxidizing agent that can oxidize biological components directly.[13] Exposure of living organisms to ionizing and nonionizing irradiation constitutes another major exogenous source of ROS.[14] Even exposure to nonionizing irradiation such as UV-C (<

290 nm), UV-B (290–320 nm) and UV-A (320–400 nm) can indirectly produce a variety of ROS including $^1O_2$, H_2O_2 and $O_2^{-\bullet}$ radical; homolytic cleavage of H_2O_2 by UV radiation yields $OH^\bullet$ radicals. Air pollutants such as car exhaust, cigarette smoke and industrial contaminants encompassing many types of NO derivatives constitute major sources of ROS that attack and damage the organism either by direct interaction with skin or following inhalation into the lung.[15] Drugs are also a major source of ROS.[16] There are drugs, such as belomycin and adreamicine whose mechanism of activity is mediated via production of ROS. Narcotic drugs and anesthetizing gases are considered major contributors to the production of ROS.[17] A large variety of xenobiotics (eg, toxins, pesticides and herbicides such as paraquat) and chemicals (eg, mustard gas, alcohol)[18,19] produce ROS as a by-product of their metabolism *in vivo* (Table 1.1).

1.3.2 Endogenous sources

Although the exposure of the organism to ROS is extremely high from exogenous sources, the exposure to endogenous sources is much more important and extensive, because it is a continuous process during the life span of every cell in the organism (Table 1.1).[20] The reduction of oxygen to water in the mitochondria for ATP production occurs through the donation of four electrons to oxygen to produce water. During this process several major oxygen derivatives are formed.[21] In many cases there is a leakage of ROS from the mitochondria into the intracellular environment.[21] The mitochondrion serves as the major organelle responsible for ROS production and events that follow throughout the cell cycle.[22] The massive production of mitochondrial ROS is increased further in the aging cell where by the function of the mitochondrion is impaired and its membrane integrity gets damaged.[23] Enzymes comprise another endogenous source of ROS. While most enzymes produce ROS as a by-product of their activity, exemplified by the formation of superoxide radicals by xanthine oxidase, there are some enzymes designed to produce ROS, such as nitric oxide synthase that yields NO radicals, those that produce H_2O_2 and those responsible for hydroxylation.[24-26] White blood cells, including neutrophils, eosinophils, basophils and mononuclear cells (monocytes) and lymphocytes, with their mechanisms to combat bacteria and other invaders,[27,28] are major producers of endogenous ROS and other factors that act synergistically with ROS.[29,30] Nicotinamide adenine dinucleotide phosphate (NADPH)

serves as a donor of electrons to an activated enzyme complex in the plasma membrane. This NADPHoxidase complex utilizes electrons to produce superoxide radicals from the oxygen molecule. Following dismutation, the production of H_2O_2 leads to the formation of $OH^{\bullet}$ by the metal-mediated, Haber-Weiss reaction. The presence of the enzyme myeloperoxidase leads to the production of HClO by interaction between hydrogen peroxides and chlorides.[31,32]

Table 1.1: Sources of ROS

Reactive Oxygen Species (ROS)	
Exogenous Sources	**Endogenous sources**
γ irradiation	Cells (e.g., neutrophils)
UV irradiation	Direct-producing ROS Enzymes
Ultrasound	(e.g., NO synthase)
Food	Indirect-producing ROS enzymes
Drugs	(e.g., xanthin oxidase)
Pollutants	Metabolism (e.g., mitochondria)
Xenobiotics	Diseases (e.g., metal disorders, ischemic
Toxins	processes)

1.4 Beneficial effects of ROS

At low or moderate concentrations, ROS and RNS are necessary for the maturation process of cellular structures and can act as weapons for the host defense system. Beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in cellular responses to noxia, for example, in defense against infectious agents and in the function of a number of cellular signalling systems. At low/moderate concentrations, ROS invokes induction of a mitogenic response too.[33] Indeed, phagocytes (neutrophils, macrophages, monocytes) release free radicals to destroy invading pathogenic microbes as part of the body's defense mechanism against disease.[34] The importance of ROS production by the immune system is clearly exemplified by patients with granulomatous disease. These patients have defective membrane-bound NADPH oxidase system which makes them unable to produce the superoxide anion radical ($O_2^{-\bullet}$), thereby resulting in multiple and persistent

infection.[35,36] Other beneficial effects of ROS and RNS involve their physiological roles in the function of a number of cellular signalling systems.[33,6] Their production by non phagocytic NADPH oxidase isoforms play a key role in the regulation of intracellular signalling cascades in various types of nonphagocytic cells including fibroblasts, endothelial cells, vascular smooth muscle cells, cardiac myocytes and thyroid tissue. For example, nitric oxide ($NO^{\bullet}$) is an intercellular messenger for modulating blood flow, thrombosis and neural activity.[33] $NO^{\bullet}$ is also important for nonspecific host defense and for killing intracellular pathogens and tumours. In brief, ROS/RNS at low or moderate levels are vital to human health.

1.5 Deleterious effects of ROS

Free radicals and oxidants produced in excess can give rise to deleterious process which can seriously alter the cell membranes and other structures such as proteins, lipids, lipoproteins and deoxyribonucleic acid (DNA). For example, hydroxyl radical and peroxynitrite in excess can damage cell membranes and lipoproteins by a process called lipid peroxidation. This reaction leads to the formation of malondialdehyde (MDA) and conjugated diene compounds that are cytotoxic and mutagenic. Lipid peroxidation occurs by a radical chain reaction, i.e. once started, it spreads rapidly and affects a great number of lipid molecules.[37] Proteins may also be damaged by ROS/RNS, leading to structural changes and loss of enzyme activity.[37,38] Oxidative damage to DNA leads to the formation of different oxidative DNA lesions which can cause mutations.

1.6 Oxidative stress

Protective mechanisms present in our body, under normal physiological conditions, are sufficient only to cope up with the normal, and threshold level of free-radical generation. Any additional burden of free radicals , either from indigenous or exogenous sources, on the animal (human) physiological system, can tilt the balance between free radical (prooxidant) and anti-free radical (antioxidant) leading to oxidative stress.[39] Oxidative stress can thus arise when cells cannot adequately destroy the excess of free radicals formed. The oxidative stress, defined as the imbalance between oxidants and antioxidants, in favour of the former, potentially leading to damage has been

suggested to be the cause of aging and various human diseases depending upon the sensitivity and susceptibility of the organ.[40]

1.7 Pathogenesis of human diseases by ROS

Numerous pathologies and disease states serve as sources for the continuous production of ROS.[41] More than 200 clinical disorders have been described in the literature in which ROS were important in the initiation stage of the disease or produced during its course (Figure 1.2). ROS may be important initiators and mediators in many types of cancer, heart diseases, endothelial dysfunction, atherosclerosis, cardiovascular disorders, chronic inflammation, intestinal tract diseases, brain degenerative impairments, diabetes, eye diseases and ischemic and post-ischemic pathologies, such as damage to skin, heart, brain, kidney, liver and intestinal tract.[42-48]

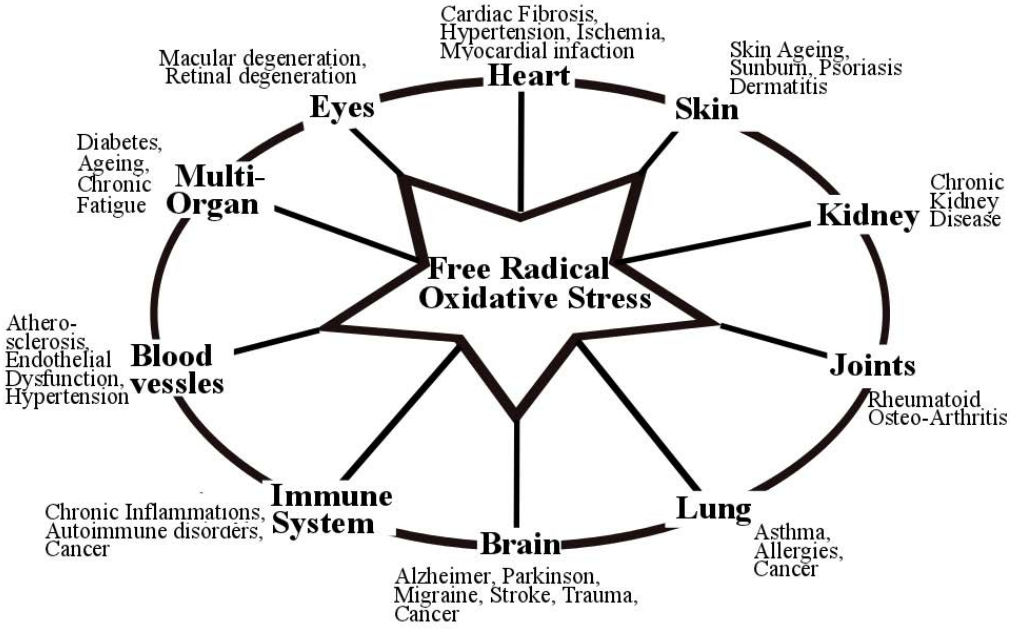


Figure 1.2: Pathogenesis of diseases in human by ROS

ROS is also produced under many normal conditions and play a role in the pathogenesis of the physiological condition. These are exemplified during the aging process where ROS production significantly increases as a result of impaired mitochondrial function and in the early stages of embryonic development.[49] Other

pathological disorders that are associated with impaired metal metabolism such as hemochromatosis, Wilson disease and thalassemia are known to increase the concentration of ROS significantly.[50-52]

1.7.1 Cancer

Carcinogenesis is a multistage disease process that has been classified into initiation, promotion and progression stages and each stage probably involves both genetic and epigenetic changes.[53] These observations have been substantiated experimentally by external administration of carcinogens.[54] Metabolic activation of carcinogen is a free radical-dependent reaction. DNA damage mediated by free radicals plays a critical role in carcinogenesis.[55,56] In biological systems, damaged DNA is repaired enzymatically and cells regain their normal functions. However, misrepair of DNA damage may result in mutations such as base substitution and deletion, leading to carcinogenesis.[57] Sequence specificity of DNA damage plays a key role in the mutagenic process. Endogenous DNA damage arises from a variety of intermediates of oxygen reduction and several free radicals have been reviewed to take part in this process by various mechanisms.[58] These reactive species have different redox potentials and redox potentials of these free radical species may play an important role in sequence-specific DNA damage.[59] Apart from redox-potential of free-radical species, oxidation potential of DNA bases also contributes to the determination of sequence specificity of DNA damage. Guanine is most easily oxidized among the four DNA bases, as its oxidation potential is lowest (1.29 V *vs* normal hydrogen electrode) among others (adenine 1.42 V, cytosine 1.6 V and thiamin 1.7 V).[60,61] Though the most common hydroxyl radical causes DNA damage with no marked site specificity, Kawanishi *et al.* (2001) have explained the mechanism of guanine-specific DNA damage by different free-radical entities and their role in carcinogenesis.[58] Apart from a variety of free radicals, non-radical oxidant like H_2O_2 also plays an important role in DNA damage. Hence, ROS plays a pivotal role in formation of cancer in human.

1.7.2 Inflammation

Inflammation involves a complex series of intra- and extracellular events. Cell-cell communication is critical and is accomplished by the release of numerous cell communicator substances (cytokines) from injured tissue and subsequent responser cells. Evidence, supporting a role for oxidants and radicals in this process, is

overwhelming. However, despite the beneficial effects of the inflammatory responses in destroying the invading organisms and generating chemotactic factors, the responses can also aggravate existing tissue damage. Thus, inflammation represents a normal response of injured tissue and despite the additional injury that may develop, is generally not pathologic because the production of reactive species is controlled and targeted at invading organisms and is reasonably well localized. However, when uncontrolled, initiated by an abnormal stimulus or occurring for prolonged duration of time, inflammation may become disease process. This appears to be the underlying basis of inflammation mediated diseases.[62,63]

1.8 Antioxidants

An antioxidant works by retarding the oxidation. Literally, antioxidant is defined as "a substance that opposes oxidation or inhibits reactions promoted by oxygen or peroxides". In biology, oxidation is often started by free radicals. The role of an antioxidant is to intercept a free radical before it can react with the substrate. The most important and widely accepted explanation of an antioxidant is that 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".[6] Antioxidants are enzymes or other substances such as vitamin E or β-carotene that is capable of counteracting the damaging effects of oxidation in animal tissues.[6] Antioxidants may exert their effects by different mechanisms, such as suppressing the formation of active species by reducing hydroperoxides ($ROO^{\bullet}$) and H_2O_2 and also by sequestering metal ions, scavenging active free radicals, repairing and/or clearing damage. Similarly, some antioxidants also induce the biosynthesis of other antioxidants or defence enzymes.

1.8.1 Endogenous antioxidants

Antioxidants that are produced within the body for defence as a result of normal metabolic processes are called endogenous antioxidants. Catalase converts H_2O_2 to O_2 and H_2O while superoxide dismutase (SOD) converts the superoxide radical to H_2O_2 and O_2. Some of the antioxidant enzymes exist in several forms. For example, membrane, cytosolic and plasma forms of glutathione peroxidase have been isolated and SOD has membrane, cytosolic and extracellular forms. The levels and locations of these

antioxidants must be tightly regulated for cell survival. The antioxidant enzymes SOD, glutathione peroxidase (GPX) and catalase (CAT) work within the cells to remove most superoxides and peroxides, before they react with metal ions to form more reactive free radicals. Peroxidative chain reactions initiated by free radicals that escaped the antioxidant defenses are terminated by chain-breaking water or lipid soluble antioxidants.[64] Hence, there is a vast network of intracellular and extracellular antioxidants with diverse roles within each area of defence.

1.8.2 Exogenous antioxidants

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

1.9 Natural products as drugs

Natural products are chemical entities produced by living organisms. Sources of natural products are terrestrial plants, microorganisms, vertebrates, invertebrates and marine organisms etc. Nature is unravelled in its ability to craft small organic molecules endowed with structural and biological complexities. All living organisms are made up of these complex organic molecules known as primary and secondry metabolites. Of these, secondary metabolites are complex organic natural products which are not directly necessary for host survival. Secondary metabolites may be alkaloids, terpenoids, flavonoids, coumarins, steroids, peptides, glycosides, phenolics etc.[65] They are typically produced by organisms such as bacteria, plants or various marine intermediates and are usually used as “chemical warfare” to protect parent organisms from predators or as a means of attack. To efficiently fulfil this role, the natural

products have been optimised in a very long natural selection process for optimal interactions with biological macromolecules. Natural products are therefore an excellent source of validated substructures for the design of new drugs. In early 1800s, an emerging fascination with such molecules, later known as natural products or secondry metabolites, gave rise to the field of organic chemistry of living things.

The Greek physician Galen (AD 129–200) devised the first pharmacopoeia describing the appearance, properties and use of many plants of his time.[66] In the early 1500s, the bark of cinchona tree (*Cinchona officinalis*) known as Indian fever bark was one of the first medicinal plants to find appreciative consumers in Europe. Natural products chemistry actually began with the work of F. W. Serturner, who first isolated morphine from opium poppy (*Papaver somniferum*) in 1815 that had been used for over 5000 years.[67] In 1860, a German chemist Carl Koler isolated cocaine and found its biological activity as a local anaesthetic in eye surgery. Later, scientists observed that cocaine paralysed nerve endings responsible for transmitting pain. As a local anaesthetic, it revolutionized several surgical and dental procedures. Tube curare in the West Amazon is from *Chrondrodendron tomentosum*; curare in modern medicine is made from this and named as tubocurarine.[66] Alkaloid rich aromatic oil extracted from jaborandi tree (*Pilocarpus jaborandi*) is a constituent of an alkaloid pilocarpine which acts against the blinding disease, glaucoma. American Indians used pineapple (*Ananas comosos*) poultices to reduce inflammation in wounds and stomach ache. In 1891, bromelain, an enzyme was isolated from the fresh juice of pineapple that broke down proteins and was found to break down blood clots.[66] Many such similar developments followed. Other pharmaceuticals that have their origin in botanicals include atropine, hyoscine, digoxin, colchicine, reserpine and emetine. [66]

The above discoveries initiated an era wherein drugs from plants could be purified, studied and administered in precise dosages that did not vary with the source or age of the material. The basis of development of currently accepted modern medicine or allopathy remains rooted in traditional medicine and therapies from Natural products, especially plants. The emergence of today's pharmaceutical industry, in large part, has been based on natural products. Drugs such as digoxin, taxol, artemisinin and scores more have been developed from phytochemicals.[68] Not only have many medical breakthroughs been based on compounds of natural origin, but these also represent a

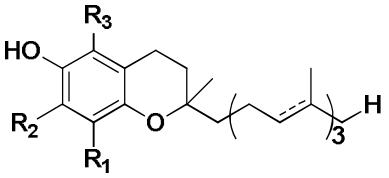
large share of the drug market. In 1999, close to 50% of the 20 best-selling drugs were derived from natural products and their sales amounted to approximately $16 billion.[69] According to a survey by the National Cancer Institute, 61% of the 877 small molecules, which are new chemical entities introduced as drugs worldwide from 1981 to 2002, were inspired by natural products.[69]

1.9.1 Natural products as antioxidants

Terrestrial plants provide a rich source of natural antioxidants.[70] These include tocopherols, vitamin C, carotenoids and phenolic compounds. Plant phenolics are thought to protect the plants against tissue injuries as they oxidize and combine with proteins and other components. In addition, phenolic compounds in plants may serve as defence systems against herbivory.[71] By-products of photosynthesis may also produce high levels of oxygen, free radicals and reactive oxygen species (ROS) in profusion; thus, plants use a myriad of antioxidant compounds to deal with these, in order to survive. Many of these compounds have basic molecular similarities in that most of them have at least one aromatic ring and a hydroxyl group. These include phenolic acids, flavonoids and isoflavones, gallate esters (hydrolysable tannins), lignans, coumarins, stilbenes, flavonones and oligomeric proanthocyanidins.

1.9.1.1 Tocopherols

Tocopherols and tocotrienols are grouped as chromanols; within each of these two classes, there are four isomers (α, β, γ, δ), making a total of eight tocophehrols (Figure 1.3).



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

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

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

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

Figure 1.3: Structure of tocopherols

Tocopherols can act as antioxidant by two primary mechanisms: (i) a chain–breaking electron donor (CB-D) mechanism and (ii) a chain-breaking acceptor (CB-A) mechanism.[72] The second major mechanism, CB-A, includes singlet oxygen scavenging

or quenching. CB-D antioxidants compete with the unsaturated fatty acid (LH) for the lipid peroxy radical ($LOO^{\bullet}$) and as a result, minimise the formation of the lipid radical ($L^{\bullet}$) that occurs when LH reacts with $LOO^{\bullet}$; this competition ultimately slows the propagation stage of autoxidation. Tocopherols can effectively compete with LH for the $LOO^{\bullet}$ by readily transferring a hydrogen atom to the $LOO^{\bullet}$ to produce the more stable LOOH.[73] In the case of tocopherols, a hydrogen transfer occurs, resulting in a semiquinone intermediate (Figure 1.4) which converts to tocopheryl quinone (Figure 1.4) as the final stable end product.

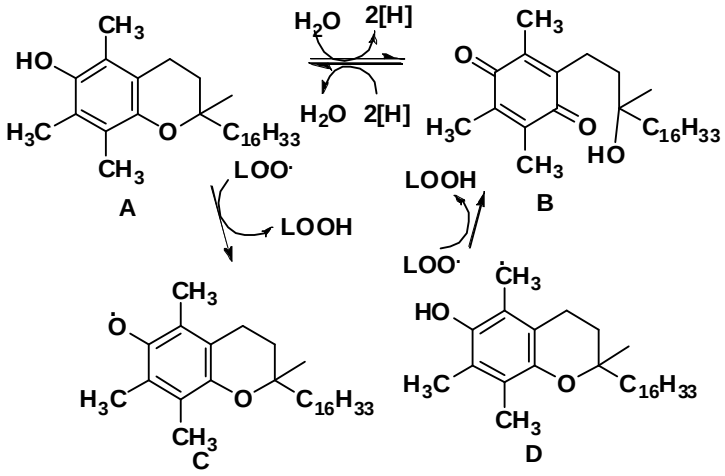


Figure 1.4: *α*-tocopherol oxidation to (B) *α*-tocopheryl quinone through semiquinone intermediates (C, D)

The tocopheryl quinone and possible semiquinones can react with the $L^{\bullet}$ to produce LH, thus slowing the chain propagation stage. This type of mechanism is defined as the CB-A mechanism and in this mechanism tocopheryl quinone competes with oxygen (triplet) for the $L^{\bullet}$.[73] As the reactions between carbon-centered radicals ($L^{\bullet}$) and oxygen ($^3O_2$) are favoured over those of the quinone oxygen and $L^{\bullet}$, it is likely that this type of antioxidant activity occurs primarily in biological systems or other low oxygen pressure systems.[73]

Tocopherols can also act on singlet oxygen ($^1O_2$) as a means to control oxidation processes. The oxygen- scavenging ability of tocopherols occurs through two primary methods (i) singlet oxygen quenching and (ii) irreversible reaction with singlet oxygen to form a variety of products. The quenching mechanism is more predominant[74] and is dependent on the rate constants of the reaction between $^1O_2$ and tocopherol *vs* the rate constant of $^1O_2$ being quenched to $^3O_2$.[75]

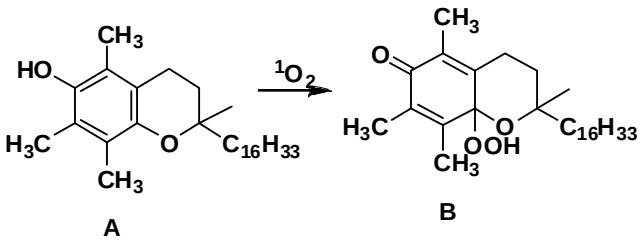


Figure 1.5: Singlet oxidation of (A) α-tocopherol to (B) hydroperoxydienone as given by Clough *et al.*[74]

However, it is not very clear how tocopherols and $^1O_2$ react. Clough *et al.* (1979) proposed that the irreversible reaction of singlet oxygen with tocopherol results in the formation of hydroperoxydienone (Figure 1.5) as the major end product.[74] Grams *et al.* (1972) found a variety of products and proposed that the 1, 4-cycloaddition of oxygen to form the endoperoxides (Figure 1.6) was the first stage of $^1O_2$ reaction with tocopherol.[76] Clough *et al.* (1979) also agreed that endoperoxides could be intermediates for the hydroperoxydienone compounds found in their study.[74]

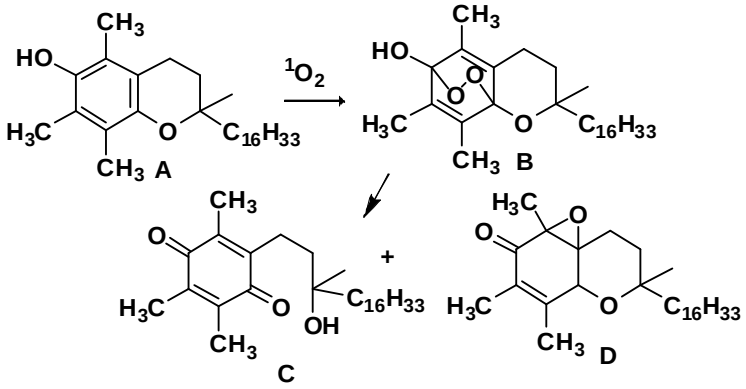


Figure 1.6: Singlet oxidation of (A) α-tocopherol to (C) α-tocopheryl quinone and (D) quinone epoxide through (B) α-tocopherol endoperoxide as presented by Gram *et al.* (1972).[76]

Vitamin E acts as a primary antioxidant, whereas vitamin C reductively regenerates oxidized viramin E. Finckh and Kunert (1985) found that peroxidative cell damage was highly controlled by an antioxidative system of vitamins C and E.[77] The lipophilic antioxidants α-tocopherol and ascorbyl palmitate were more effective in an

oil-in-water emulsion system than in bulk oil, whereas the opposite was found for the hydrophilic antioxidants, Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid, a hydrophilic carboxylic acid derivative of α-tocopherol) and ascorbic acid. Vitamin E has been proposed for the prevention against colon, prostate and breast cancers, some cardiovascular diseases, ischemia, cataract, arthritis and certain neurological disorders. The dietary sources of vitamin E are vegetable oils, wheat germ oil, whole grains, nuts, cereals, fruits, eggs, poultry, meat. [78]

1.9.1.2 Vitamin C

Vitamin C, also known as ascorbic acid, is a water-soluble vitamin (Figure 1.7). It is essential for collagen, carnitine and neurotransmitters biosynthesis.[79] Health benefits of vitamin C are as antioxidant, anti-atherogenic, anti-carcinogenic and as an immunomodulator. The positive effect of vitamin C resides in reducing the incidence of stomach cancer and in preventing lung and colorectal cancer. Vitamin C works synergistically with vitamin E to quench free radicals and also regenerates the reduced form of vitamin E. Natural sources of vitamin C are acidic fruits such as lemon, orange, green vegetables, gooseberry, tomatoes etc.[80]

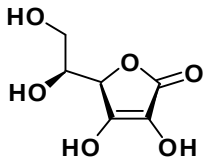


Figure 1.7: Structure of Vitamin C

1.9.1.3 Carotenes

Like tocopherols, carotenes (in particular β–carotene) are also effective $^1O_2$ quenchers. β-Carotene is a fat soluble member of the carotenoids which are considered pro-vitamins because they can be converted to active vitamin A. β -Carotene is present in many fruits, grains, oil and vegetables.[81]

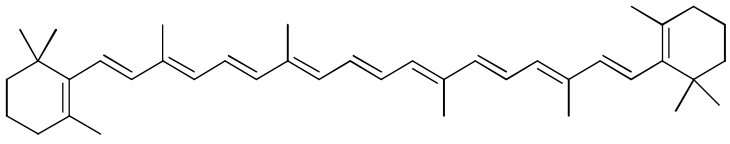


Figure 1.8: Structure of β–carotene

Foote *et al.* (1968, 1971)[82,83] and Farmilo and Wilkenson[84] found that the quenching of $^1O_2$ by *β*–carotene was due to an energy transfer from $^1O_2$ to *β*–carotene as shown in equation 1.1. Foote *et al*. (1970) also found that the rate of quenching was dependent on the number of conjugated double bonds.[85] The presence of nine or more double bonds in the carotene structure greatly enhanced the quenching ability (Figure 1.8). Carotenoids with seven or fewer double bonds were not as effective due to the inability of the conjugated chain to delocalize the unpaired electrons gained from the $^1O_2$.[85] A radical trapping mechanism has also been proposed.[86,87] This mechanism relies on the delocalization of the unpaired electrons of the peroxy and free radical species over the carotenoid conjugated polyene system.[86,87]

$$^1O_2 + \beta - \text{carotene} \longrightarrow {}^3O_2 + 3\ \beta - \text{carotene}^* \text{ (excited state)} \qquad \text{-----(1.1)}$$

1.9.1.4 Phenolic Acids

Phenolic compounds constitute one of the most widespread and diverse groups of secondary plant metabolites. Phenolic compounds are widely distributed in cereals and legumes. Ferulic, caffeic, protocatechuic, *p*-hydroxybenzoic, vanillic, syringic and *p*-coumaric acids are the most common phenolic acids found in cereals and legumes.[88] Figure 1.9 shows the structures of these phenolic caids. Phenolic antioxidants are primary antioxidants which act as free radical terminators. The position and the degree of hydroxylation are of primary importance in determining antioxidant activity.[89] The *o*-dipheols, such as caffeic acid, hydroxytyrosol and oleuropein exhibit strong antioxidant activity compared to less sterically hindered phenolic acids such as tyrosol.[90] In addition, phenolic acids that are hydroxyl derivatives of cinamic acid such as caffeic acid, ferulic, sinapic and *p*-coumaric acids, are more active antioxidants than hydroxy derivatives of benzoic acid (Figure 1.9), i.e., *p*-hydroxybenzoic, vanillic, syringic and 3,4-dihydroxy benzoic acid.[91]

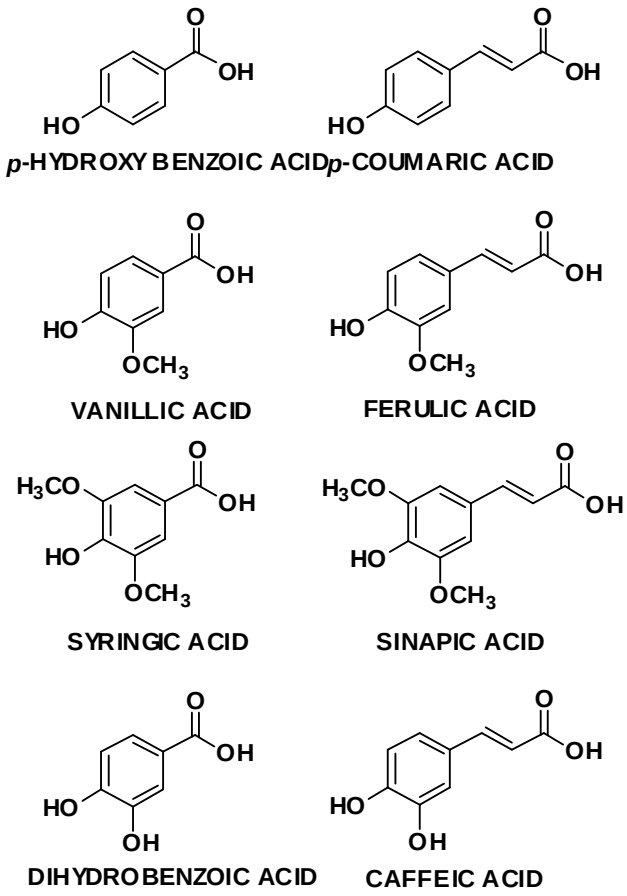


Figure 1.9: Structure of phenolic acids

Phenolic antioxidants act to inhibit lipid oxidation by trapping the peroxy radical. This can be accomplished in one of the two ways. In the first mechanism, the peroxy radical (LOO•) abstracts a ($H^{\bullet}$) from the antioxidant (ArOH) to yield an aroxyl radical (ArO•) and the hydroperoxide (LOOH) (equation 1.2). In the second mechanism, a peroxy and an aroxyl radical react by radical-radical coupling to form a non-radical products (equation 1.3).[90] The aroxyl radicals formed from the oxidation of an antioxidant can further react and can, in some instances, contribute to the production of free radicals.[90]

$$LOO^{\bullet} + ArOH \longrightarrow LOOH + ArO^{\bullet} \qquad \text{-------(1.2)}$$

$$LOO^{\bullet} + ArO^{\bullet} \longrightarrow \text{Non-radical products} \qquad \text{-------(1.3)}$$

$ArO^{\bullet} + LOOH \longrightarrow LOO^{\bullet} + ArOH$ -------(1.4)

$2\ ArO^{\bullet} \longrightarrow$ Non-radical products -------(1.5)

$ArO^{\bullet} + LH \longrightarrow ArOH + L^{\bullet}$ -------(1.6)

Chimi *et al.* (1991) reported that for sterically hindered phenols, the rates of reactions 1.3 and 1.5, which produce non-radical products, exceed the rates of reaction 1.4 and 1.6, which produce free radicals, resulting in an overall inhibition of lipid oxidation.[90] Lack of hindrance favours reactions 1.4 and 1.6, which produce free radicals, thus decreasing the overall antioxidant activity of the phenol. Dziedzic and Hudson (1984)[92] evaluated a series of hydroxy aromatic acids (phenolic acids), their esters and lactones for antioxidant activity and tried to correlate with their structures. Based on the results of their studies, several structure-activity conclusions were drawn. High antioxidant activity was related to the molecule containing at least, two neighboring phenolic hydroxyl groups; three such groups were even more desirable. In addition, a carbonyl group such as in an aromatic acid, an ester or a lactone, enhanced activity. Activity also increased when the carbonyl group was separated from the aromatic ring. Cinnamic acid derivatives were more effective than corresponding benzoic acids and the most effective were the phenyl acetic and phenylpropionic acids. Steric hindrance of the phenolic hydroxyls by a neighboring inert group such as methoxyl group, enhanced activity.

1.9.1.5 Flavonoids

Flavonoids are a group of plant phenols characterized by the carbon skeleton C_6-C_3-C_6. The basic structure of these compounds consists of 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.[93] The basic flavonoid structure is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings (C_6-C_3-C_6), which are labeled A, B, and C (Figure 1.10).

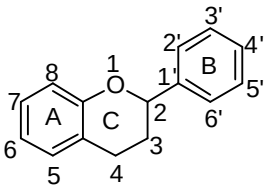


Figure 1.10: Basic flavonoid structure

The different classes of flavonoids differ in the level of oxidation and pattern of substitution in the C ring while individual compounds within a class differ in the pattern of substitution of the A and B rings. Among the many classes of flavonoids, those of particular interest are flavones, flavanones, isoflavones, flavonols, flavanonols, flavan-3-ols and anthocyanidins. The structures of important subclasses of flavonoids are shown in Figure 1.11. Flavones and flavonols are found in almost every plant, particularly in the leaves and petals, with flavonols occurring more frequently than flavones.[94, 95]

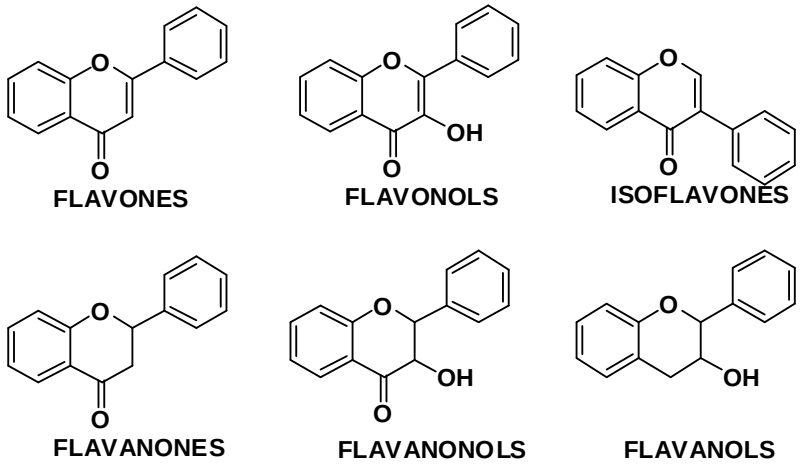


Figure 1.11: Typical chemical structures of the flavonoid family

1.9.1.5.1 Biosynthesis of flavonoids

All flavonoids derive their 15-carbon skeletons from two basic metabolites, malonyl-CoA and *p*-coumaroyl-CoA.[96] Basically, flavonoids are derivatives of 1, 3-diphenylpropan-1-one (C_6-C_3-C_6). The crucial biosynthetic reaction is the condensation of three molecules of malonyl-CoA with one molecule *p*-coumaroyl-CoA to a chalcone intermediate. Chalcones and dihydrochalcones are classes of flavonoids that consist of

two phenolic groups which are connected by an open three carbon bridge. Derived from the chalcone structure, a flavonoid-class containing three rings, the flavanones, can be formed. Here, the three-carbon bridge is part of an additional heterocyclic six-membered ring that involves one of the phenolic groups on the adjacent ring. Based on these flavanones, all other flavonoid-classes are generated, including isoflavones, flavanols, anthocyanidins, flavonols and flavones (Figure 1.12).[96]

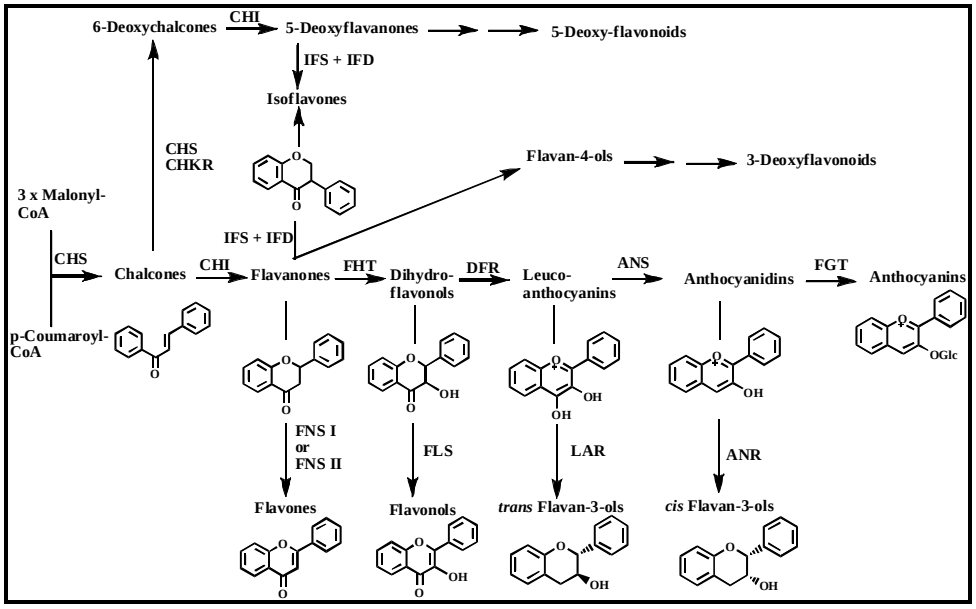


Figure 1.12: Scheme of bioynthesis of flavonoid pathway. Enzymes are abbreviated as follows: CHS, chalcone synthase; CHKR, chalcone polyketide reductase; CHI, chalcone isomerase; FHT, flavanone 3-β-hydroxylase; DFR, dihydroflavonol-4-reductase; ANS, anthocyanidin synthase; FGT, flavonoid glycosyltransferase; FNS, flavone synthase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; IFS, isoflavone synthase; IFD, isoflavone dehydratase

1.9.1.5.2 Antioxidant activity of flavonoids and their structure activity relationship

Flavonoids occur as glycosides which, during hydrolysis with acid and heat, produce an aglycone and a sugar moiety.[97] Many of the flavonoids and related compounds have strong antioxidant characteristics. According to Halliwell and Gutteridge (2007), mechanisms of antioxidant action can include (1) suppressing

reactive oxygen species formation either by inhibition of enzymes or chelating trace elements involved in free radical production; (2) scavenging reactive oxygen species and are free radical chain breakers; and (3) upregulating or protecting antioxidant defences.[6]

Hudson and Lewis[98] studied the antioxidant activity of a series of polyhydroxy flavonoids and related their activity to their structures (Figure 1.13). The results of their study led to several conclusions relative to the structural features which affect the antioxidant activity of the flavonoids. It was noted that primarily, the molecule needed multiple hydroxyl groups: a 3', 4'–dihydroxy configuration gave strong antioxidant activity and additionally the molecule must have a 4-carbonyl group for activity.

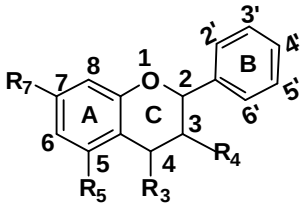


Figure 1.13: General chemical structure of flavonoid compounds.

The presence of 3-hydroxy group opposite to 5-hydroxy group, makes the molecule effective. They projected that there was cooperation between the 4-carbonyl and the 3- or 5- hydroxyl groups which acted to chelate the copper ion (Figure 1.14). Flavones act as ligands with the cupric (Cu^{2+}) ion whereas the flavanones complex with copper ions through their enediol or enolic groups (Figure 1.14).[98] This mechanism of chelation of metal ions (copper ions) has found to operate for controlling lipid oxidation.

Das and Pereira studied the antioxidant activity of flavonoids and related the observed antioxidant activity to their structure.[99] They found that the potency of flavonoids with polyhydroxylated substitutions was affected by the location of the hydroxyl group on the B ring (Figure 1.13). Hydroxyl substitution in the *ortho* positon in the B ring gave less antioxidant activity; however, hydroxyl substitution in the *ortho* position accompanied by an additional hydroxyl group in the *para* position enhanced activity. Strong antioxidant activity was found in compounds having hydroxylation in the *para* position in the B ring. In addition, it was found that a double bond between the

2 and 3 positions on the C ring contributed to antioxidant activity (Figure 1.13). Das and Pereira (1990)[99] also found that the aglycones were stronger antioxidant than their corresponding glucosides, possibly due to the lack of a free 3-hydroxyl substitution in the C ring.[99]

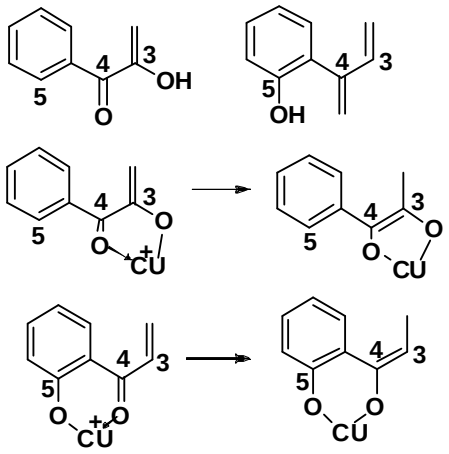


Figure 1.14: Formation of metal chelates with flavones and flavonones. [98]

Shahidi and Wanasundara[100] noted that the position and degree of hydroxylation was important in the antioxidant activity of flavonoids. They stated that *o*-dihydroxylation of the B ring contributed to the antioxidant activity of the compound and a *p*-quinol structure on the B ring produces greater activity than the *o*-quinol. However, they also noted that *p*- and *m*- hydroxylation of the B ring do not occur in nature. In short, criteria for effective radical-scavenging are: (i) the ortho-dihydroxyl structure in the B-ring, (ii) the 2, 3-double bond, which allows p electron delocalization from the B-ring and (iii) the 3- and 5-OH with 4-oxo function in the A- and C-rings.

Apart from antioxidant activity, flavonoids play remarkable spectrum of biological activties; antiallergic, anti-inflammatory, antimutagenic, anticarcinogenic and modulation of enzymatic activities.[101]

1.9.1.6 Lignans

Lignans are widely found in all parts of plants and include compounds with great chemical diversity. By definition, lignans are dimers of phenlypropanoid (C6-C3) units linked by the central carbons of their side chains.[102] According to their oxygenation, four major groups of linear lignans are found, namely, lignan or butane derivatives lignanolides or butanolide derivatives, monoepoxylignans or tetrahydrofuran

derivatives and bisepoxylignans or derivatives of 3,7-dioxabicyclo-(3,3,0)-octane. Cyclized lignans or cyclolignans occur as tetrahydronaphthalene or naphthalene derivatives.[103] Lignan-type compounds, isolated from plants and oilseeds, have shown good antioxidant properties.[104]

1.9.2 Anticancer compounds from plants

Cancer is a complex disease that involves uncontrolled multiplication and spread (metastasis) of abnormal form of body's own cells. As per WHO 13% of world deaths, that is, about 7.6 million deaths accounted in 2005 are because of cancer, and this percentage is expected to increase in coming years.[105] Plant derived compounds have played an important role in treatment of cancers and some of the most promising and better drugs have come up from plant sources like Taxol, Camptothecin, Combrestatin, Epipodophyllotoxin and Vinca alkaloids (vinblastine, vincristine). These drugs have also been the major source of new drug candidates for the treatment of cancers.The chemistry and properties of few of them are discussed below.

1.9.2.1 Podophyllotoxin

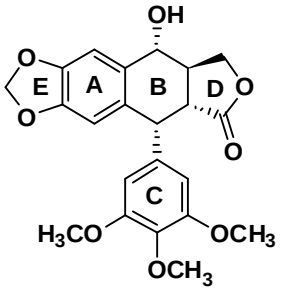


Figure 1.13: Structure of Podophyllotoxin

Podophyllotoxin is a well known naturally occurring aryltetralin lignan (Figure 1.13). It was first isolated by Podwyssotzki in 1880 from the North American plant *Podophyllum peltatum* (May Apple).[106] Podophyllotoxin is effective in the treatment of Wilms' tumours, various genital tumours in non-Hodgkin, other lymphomas and lung cancer.[107,108] Chemically, podophyllotoxin is an aryltetralin lignan having a lactone ring. The structure activity relationship studies reveal that only the A and E rings of this compound is essential for its activity and the D-ring in lactone form enhances the

activity. Also, introduction of bulky groups at the C-4 position in ring C enhances the activity. Epipodophyllotoxin, etoposide and tenetoposide are synthetic analogues, having less toxic side effects than podophyllotoxin. Two of the semisynthetic derivatives of podophyllotoxin viz. etoposide and teniposide are currently used in frontline cancer chemotherapy against different types of cancers.[109]

1.9.2.2 Taxol

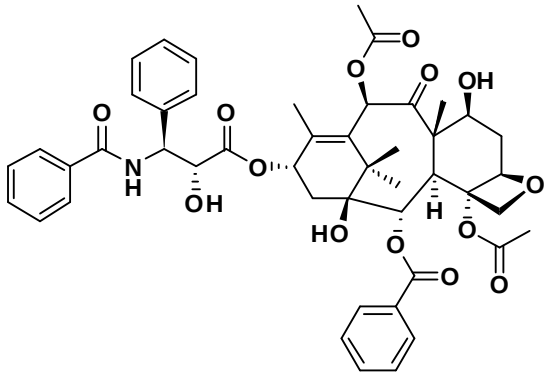


Figure 1.14: Structure of Taxol

Taxol (generic name paclitaxel, trade name Taxol) is a complex polyoxygenated diterpenoid isolated from the pacific yew (Figure 1.14), *Taxus brevifolia.*[110] Taxol has a basic [9.3.1.0] pentadecane, tetracyclic ring system. It has a N-benzoyl-*β*-phenylisoserine side chain attached at the C-13 hydroxyl through an ester linkage. This side chain is essentially required in taxol for anticancer activity and so is the C-2'-hydroxyl. Taxol is used for the treatment of refractory ovarian cancer, metastatic breast and lung cancer and Kaposi's sarcoma. Taxol has a unique mode of action.[111] It acts as a microtubulin stabilizing agent. Tubulin polymerizes to microtubulin which in turn reverts back to tubulin. In a normal case, this process is in equilibrium. Taxol makes a microtubulin bundle larger in size than the normal bundle size required for the process of cell multiplication. Due to this, a defective polymerization occurs and the cells have unnatural bundles of microtubules with the absence of the mitotic spindle. The cancerous cells lack a check point to detect the absence of a spindle and attempt to continue the cell cycle which eventually leads to cell death. Taxol is also referred to as a "spindle poison" because of this reason.[112] A major drawback of Taxol is that it has poor bio-availability due to its poor solubility in water. Taxol is a drug tolerated by its

recipients better than any other anticancer drugs used today. Taxotere (docetaxel), one of its semisynthetic derivatives, is now known as a better anticancer drug than Taxol. Many derivatives of Taxol like Taxotere and Isotaxel having more advantages such as better potency, greater solubility and lesser side effects have been developed.[112]

1.9.2.3 Camptothecin

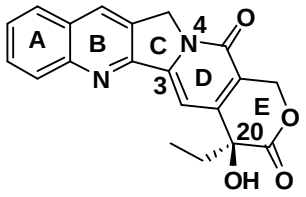


Figure 1.15: Structure of Camptothecin

Camptothecin was first extracted from the stem wood of the Chinese ornamental tree *Camptotheca acuminate.*[113,114] It belongs to quinolinoalkaloid group which consists of a pentacyclic ring structure that includes a pyrrole (3,4β) quinoline moiety and one asymmetric centre within the α-hydroxy lactone ring with 20(S) configuration (ring E). The planar pentacyclic ring structure (rings A–E) was suggested to be one of the most important structural features of this type of compounds (Figure 1.15). The stereochemistry at C-20 of CPT is very crucial for its activity, as 20(S) hydroxyl is active while the corresponding 20(R) hydroxyl compound is inactive.[115] The discovery of Camptothecin by Wall and Wani (1993) as an anticancer drug in the early sixties added an entirely new dimension to the field of chemotherapy.[115] The molecule became so important and at present the first generation analogues of Camptothecin, Hycamtin (topotecan) and Camptosar (CPT – II, irinotecan) are used for the treatment of ovarian and colon cancers. One of the major drawbacks observed in the use of CPT analogues in clinical studies was a marked loss of therapeutic activity due to their intrinsic instabilities resulting from the rapid hydrolysis of the lactone ring in the body. Apart from the above drawback, it is a potent cytotoxic agent. It shows anticancer activity mainly for solid tumours. It shows anticancer activity mainly against ovarian, colon and pancreatic cancer cells.

Analogues of Camptothecin showed anticancer activity in breast, liver and prostate cancers etc. Camptothecin inhibits DNA topoisomerase 1[116,117] thereby preventing DNA replication. The development of synthetic and semisynthetic strategies has facilitated the study of the CPT mechanism, as well as the identification of analogues with improved properties.The most successful derivatives of CPT have been obtained due to modifications of rings A and B. To date, the only CPT analogues approved for clinical use[118,119] are topotecan and irinotecan. All the anlogues of CPT have proved as potent cytotoxic agents by inhibiting cellular DNA topoisomerase 1 by a mechanism similar to CPT with comparable or better activity. Continued studies on the camptothecin-DNA topoisomerase 1 interaction in addition to its detailed mechanism of action may suggest new directions in the synthesis of new Camptothecins.

1.9.3 Anti-inflammatory agents from terrestrial plants

Plant derived preparations have been used for ages to obtain effective pain relief and herbal medications are becoming increasingly popular because of their relatively few side effects. Since ancient times our ancestors have used phytochemicals found in plants to curtail the inflammatory process. For example, the bark of the willow tree was used as an analgesic and antipyretic medication even 2400 years ago, by the Greeks and Romans.[120] The discovery of aspirin in 1899 was based on this observation. A few commonly used natural, anti-inflammatory agents and their mechanism of action are given below.

1.9.3.1 White willow bark

Bark from the white willow (*Salix alba*) tree is one of the oldest herbal remedies for pain and inflammation. It has been used by the ancient Egyptian, Roman, Greek and Indian civilizations as an analgesic and antipyretic agent.[120] The mechanism of action of white willow bark is similar to that of aspirin in that it is also a nonselective inhibitor of COX-1 and COX-2, thus reducing the release of inflammatory prostaglandins.[121] Salicin from white willow bark is converted to salicylic acid by the liver and is considered to have fewer side effects than aspirin.[121]

1.9.3.2 Curcumin from turmeric

Curcumin is a naturally occurring yellow pigment derived from turmeric (*Curcuma longa* L), a flowering plant in the ginger family.[122] Turmeric has traditionally been used for ages, as a coloring and flavoring spice in food products.[123] Curcumin has

long been used in both Ayurveda and Chinese medicine as an anti-inflammatory agent, a treatment for digestive disorders and to enhance wound healing. Several clinical trials have demonstrated curcumin's antioxidant, anti-inflammatory and antineoplastic effects.[122-125] Curcumin is known to inhibit inflammation by suppressing nuclear factor kappaB (NF-kB), restricting various activators of NF-kB as well as stemming its expression.[126,127] In addition, it regulates the activity of several enzymes and cytokines by inhibiting both COX-1 and -2.[126] Most of the studies are performed in experimental animals[128] but given the centuries of use of turmeric as well as its recently demonstrated activity in the NF-kB, COX-1 and COX-2 inflammatory pathways, it may be considered a viable natural alternative to nonsteroidal anti-inflammatory(NSAI) agents for the treatment of inflammation. Curcumin's therapeutic effects are considered comparable to pharmaceutical nonsteroidal medications such as phenylbutazone, but with a major difference in that this compound is relatively nontoxic and free of side effects.[129, 126]

1.9.3.3 *Uncaria tomentosa* (Cat's Claw)

Uncaria tomentosa and *U. guianensis* are Peruvian herbs with small clawlike thorns (hence the vernacular name, cat's claw).[130-131] Traditionally, a decoction of the bark of the cat's claw is used to treat arthritis, bursitis and intestinal disorders.[120,132,133] The active ingredients appear to be polyphenols (flavonoids, proanthocyanidins and tannins), alkaloids and sterols.[120,134] Studies indicate that this Peruvian herb induces a generalized reduction in proinflammatory mediators .[120,135] This herb has been shown to prevent the activation of the transcriptional factor NF-kB and it directly inhibits Tumour necrosis factor- alpha (TNFα) production by up to 65 to 85%.[120,135] It inhibits the expression of inducible genes associated with inflammation, specifically negating the expression of inducible nitric oxide synthase and hence attenuates nitrous oxide production .[135]

1.10 Ayurveda

Ayurveda remains one of the most ancient but still prevailing traditions practised extensively in India, Sri Lanka and other countries. This system of using natural resources for betterment of health was developed through experimentation and experiences of day-to-day life style of Indian people and has a sound philosophical basis. The origin of Ayurveda is prehistoric, but its concepts and approaches have been

perfected between 2500 and 500 B.C. in India.[136] Ayurveda is a Sanskrit word meaning "the complete knowledge for long life". In Sanskrit, words *ayus*, meaning "longevity", and *veda*, meaning "related to knowledge" or "science". The earliest literature on Indian medical practice appeared during the Vedic period in India. The *Susruta Samhita* and the *Charaka Samhita* were influential treatises on traditional medicine during this era. Over the centuries, ayurvedic practitioners developed a number of medicinal preparations and surgical procedures for the treatment of various ailments. The *Charaka Samhita* (900 B.C.) is considered as the first recorded treatise, devoted to the concepts and practice of Ayurveda; its primary focus was therapeutics.[137] *Charaka Samhita* an ancient text in Ayurveda classified the plant drugs into 50 groups based on their Sanskrit name. This work listed 341 plants and plant products for use in medicine. The next landmark in Ayurvedic literature was the *Susruta Samhita* (600 B.C.), which placed special emphasis on surgery. It described 395 medicinal plants, 57 drugs of animal origin and 64 minerals and metals as therapeutic agents. Sushruta, the father of surgery, lived and practiced surgery in Varanasi, India, approximately 2,500 years ago.

Another important authority in Ayurveda was Vagbhatta, who practised around 700 A.D. His work *Ashtanga Hridaya* is considered unrivalled for the principles and practice of medicine. The *Madhava Nidana* (800–900 A.D.) was the next important milestone; it is the most famous Ayurvedic work on the diagnosis of diseases. The last celebrated writer on traditional Indian medicine was Bhava Mishra of Magadha, whose treatise *Bhava Prakasha,* written around 1550 A.D., is held in high esteem by modern Ayurvedic practitioners for its descriptions of approximately 470 medicinal plants. Other than these monumental treatises, more than 70 *Nighantu Granthas* (pharmacy lexicons) were written, mostly between the seventh and sixteenth century. *Raj Nighantu* by Narhari Pandit and *Madanpala Nighantu* by Madanpala are considered masterpieces on medicinal plants.[138] Thus, Ayurveda, from its origin, is a scientifically organized discipline. Ayurvedic texts are much respected in neighbouring countries and were translated into Greek (300 B.C.), Tibetan and Chinese (300 A.D.) and several other Asian languages.[139]

Considerable research on pharmacognosy, chemistry, pharmacology and clinical therapeutics has been carried out on these ayurvedic medicinal plants. Numerous molecules have resulted from the experience of Ayurvedic practice. The first *Ayurvedic*

herb which attracted international attention was '*Rauwolfia serpentina*' when it was found that its constituent alkaloid, reserpine, had the twin effect of lowering high blood pressure and can act as a tranquilizer.[140] Other examples include rauwolfia alkaloids for hypertension, psoralens in vitiligo, holarrhena alkaloids in amoebiasis, guggulsterons as hypolipidemic agents, *Mucuna pruriens* for Parkinson's disease, piperidines as bioavailability enhancers, baccosides in mental retention, picrosides in hepatic protection, phyllanthins as antivirals, curcumin in inflammation, withanolides, and many other steroidal lactones and glycosides as immunomodulators.[141] Hence, newer and less studied indigenous plants utilised in Ayurveda, are being investigated for more potential 'leads' and drugs.

1.11 Njavara

Although rice varieties with medicinal properties occur in different parts of the world,[142] none is used for medicinal applications as extensively as Njavara (*Oryza sativa* L., var. 'njavara'), being used in Ayurveda. Descriptions of the medicinal and nutritive qualities of Njavara rice can be found in various ancient treatises of Ayurveda such as *Ashtanga Hrudayam* (Vagbhatta, *circa* 400–500 A.D.) as cited by Murthy (2001).[143] This, short duration variety is currently cultivated and utilized only in Kerala state. In Ayurveda, there is reference of a rice variety as "Shashtikovreehista shreshtaha" i.e., "red rice reaped in 60 days is greatest or superior". The traditional Ayurveda scholars of Kerala, called 'Vaidyas' developed several health-conducive treatment systems in the past using Njavara grains. Black glumed Njavara has been used in Ayurveda from the age of Charaka i.e. BC.600. "Njavara" was recommended by saints to keep juvenile and to have long life. Njavara is a rice variety endemic to Kerala, mainly grown in the northern parts of Kerala (Figure 1.16). Due to many reasons this species is on the verge of extinction.

Figure 1.16: Njavara rice plant

1.11.1 Properties of Njavara

Njavara is a unique medicinal rice variety with extra short duration, grown only in Kerala, from time immemorial. Njavara as a special cereal, have the properties to rectify the basic ills affecting our circulatory, respiratory, digestive systems and has the properties to rectify various disorders of nervous system. This is the only cultivar traditionally used effectively in the Ayurvedic system of medicine in certain specific treatments like Panchakarma in 'Njavara Kizhi' and 'Njavara Theppu'.[144]

1.11.1.1 Njavara Kizhi

It is a part of "Pindasweda" and in this method of healing; the body is forced to perspire using Njavara and other herbal medicines. In Panchakarma, Njavara Kizhi is an integral part for keeping the balance between "vatha", "pitha" and "Kapha". In "Pindasweda" the Njavara rice is boiled in "Kurunthottikazhayam", which is a decoction of *Sida* (*Sida cordifolia*) root and milk. It is taken in cloth bags (Kizhis) and is used for massaging.

1.11.1.2 Njavara Theppu

A paste of boiled Njavara rice with light warmth is applied on the body. It is recommended for patients unable to bear the Njavara Kizhi. Here the rice is boiled in "Kurunthottikazhayam".

These treatment protocols are suggested in Ayurveda, in conditions of arthritis, paralysis and neurological complaints. The "Shashtikathailam" extracted from the bran of the rice is used for curing neural diseases and also used to cure body pain and eye disorders [www.njavara.com]. Njavara has applications in many contexts such as in the treatment for degeneration of muscles, tuberculosis, children with anaemia, women during lactation, certain types of ulcers, skin diseases etc. The rice with little sourness and sweetness is used for increasing immunity during monsoon especially during the

month of “Karkkidakam”(July-August) in the form of “Marunnu kanji’(medicinal porridge). Njavara is a health food for people of all ages. It is revered as a sacred grain and is used in temples for ceremonies.

Edaphic, hydrological and atmospheric factors play an important role in quality and yield of Njavara rice and medicinal property may vary with environmental conditions. This may be due to the variation in the biosynthesis of active principles behind the medicinal property.

1.11.2 Types of Njavara

Two types of Njavara are recognized based on glume color differences, the black and golden yellow glumed.

‘Black glumed’ **‘Golden yellow glumed’**

Figure 1.17: Types of Njavara

1.11.2.1 Black glumed

Black glumed type has black-shaded grains and the seed is red (Figure 1.17). It matures in about 60-90 days and reaches more than 1m height. This type is generally resistant to diseases and is highly resistant to drought conditions. Black glumed type, an ecotype of “Njavara” rice is best for circulatory, respiratory as well as digestive problems. It is also used in Panchakarma for treating paralysis. The qualitative and quantitative medicinal properties vary with the habitat of this type. This is preferred in northern districts of Kerala. The Biochemical parameters like total free amino acids are higher for the black glumed Njavara. This plant type has a special capacity to absorb Manganese (Mn) and translocate it to the grain.[144]

1.11.2.2 Golden yellow glumed

Grains of yellow type are golden yellow and the seed colour is red (Figure 1.17). It requires 60-90 days to mature depending on the season and the land for cultivation. The plant will reach about 1m height. Generally this variety is grown in the second cropping season. The crop is susceptible to lodging and diseases upon maturity. It is susceptible to drought. The Biochemical parameters like total soluble sugars showed that it was higher for the yellow glumed. The Golden yellow glumed plant was superior in yield but did not have the Mn preference. This variety is characterized by good seed bearing and high threshability.[144]

1.12 Literature survey

Njavara-based healthcare practices and their health benefits are now gaining wider attention. Despite its distinctness among traditional rice strains as a medicinal plant, only recently did Njavara become the focus of research interest. The medicinal value of Njavara is being recognised increasingly which can be understood from the fact that recently a website on Njavara[144] has been launched by the agriculturists, scientists and producers of Njavara in Kerala. Consequently, research on, different aspects of Njavara has progressed and is still on its way for substantiating its unique property compared to other native varieties. In the beginning, Menon and Potty (1998) had reported on the agronomic evalution of Njavara.[145] Later, Sreejayan *et al.*(2005) evaluated Njavara morphologically and subsequently studied genomic DNA of Njavara and other rice varieties with molecular marker-assisted characterization of germ plasm and reported Njavara as a distinct gene pool.[146] Their results showed three distinct varietal types of Njavara. Though it represented a composite of varietal types, its gene pool, as a group, is distinct from that of other rice varieties, including the traditional ones that have been grown with Njavara in Kerala since ancient times. According to these researchers, Njavara may represent either an ancient gene pool that remained unadulterated or a distinct lineage that had probably undergone independent divergence after its separation from the ancestral gene pool.[146] Further, studies by Deepa *et al.* (2009) have also confirmed Njavara rice to be a distinct, unadulterated gene pool and found molecular markers responsible for properties such as higher protein content and starch characteristics (for example distinct pasting and thermal properties) of Njavara.[147] Very recently, Sreejayan *et al.*(2011) have reported the genetic diversity and population

genetic characteristics of Njavara germplasm, depicted by a combination of morphological traits and amplified fragment length polymorphisms (AFLPs).[148] Studies on nutritional attributes by Deepa *et al.*(2008) have shown that dehusked Njavara rice consisted of 73% carbohydrates, 9.5% protein, 2.5% fat, 1.4% ash and 1628 kJ per 100 g of energy.[149] Physicochemical properties and nutritive components of dehusked rice of Njavara were also evaluated and compared with two commonly consumed non-medicinal rice varieties – Jyothi (red coloured) and IR 64 (brown coloured). The carbohydrates, fats, apparent amylose equivalent, fatty acid profile and triglycerides of Njavara were comparable to Jyothi and IR 64. However, Njavara rice had 16.5% higher protein and contained higher amounts of thiamine (27–32%), riboflavin (4–25%) and niacin (2–36%) compared to the other two rice varieties.[149] The total dietary fibre content in Njavara was found to be 34–44% higher than that of Jyothi and IR 64. Significantly higher levels of phosphorus, potassium, magnesium, sodium and calcium levels were found in Njavara rice, compared to the other two varieties. The cooking time of dehusked Jyothi and IR 64 varieties were found to be 30 min, while Njavara needed longer time to cook (38 min). The cooked rice of Njavara was slimy in nature, probably due to the presence of non-starch polysaccharides.[149] Simi and Abraham (2008) have reported Njavara rice starch for its morphological, physicochemical and thermal properties and were found to be different from the native 'Chamba' variety of rice.[150] Njavara rice starch has bigger granule and has a high (85°C) gelatinization temperature and shows high thermal stability. The swelling power, solubility, water absorption capacity and enthalpy of gelatinization of Njavara starch were found to be high compared to the native rice starch. The 6% (w/v) Njavara rice starch gel had 87.45% clarity and its pasting properties such as peak viscosity (957 cP), break down viscosity (324 cP) and set back values (421 cP) were also higher. It also had better freeze thaw stability, gel strength and high springiness against shear stress. Other properties like hardness, gumminess, adhesiveness, cohesiveness and chewiness of the gel are slightly higher than native rice starch. Their study concluded that the physicochemical, thermal, rheological and textural characteristics of Njavara starch to be unique compared to the native 'Chamba' rice starch. Recently Deepa *et al.* (2010) have also studied the *in vitro* starch digestibility and glycemic indices of the medicinal rice and found Njavara rice as easily digestible.[151]

1.13 Objectives and organisation of present work

Literature search on Njavara, as discussed above, shows that available scientific reports are on agronomic aspects, genetic characteristics, proximate composition, lipid profile and starch characteristics. There are no detailed reports on the antioxidant activity, phytochemical investigations or bioactivity of the compounds of authentic samples of Njavara grown in Kerala. This is a major gap in the knowledge base on Njavara. Hence, the present study on phytochemical investigations on Njavara for bioactive compounds was undertaken so as to explore the basis of its traditional use in Ayurveda, with the help of modern science.

The study was planned to investigate the bioactive compounds in Njavara compared to staple varieties and their bioactivity to substantiate the medicinal properties. Details of the materials and methods used in this study are included in Chapter 2. Results of the study on chemical indices, antioxidant activity and anti-inflammatory activity (*in vivo*) of Njavara black rice bran and rice in comparison with non-medicinal varieties like Sujatha and Palakkadan Matta rice bran and rice are given in Chapter 3. The phytochemical investigation and quantification of Njavara extracts in comparison with staple varieties are detailed in Chapter 4. The last chapter (Chapter 5) is divided in three sections (A, B and C). Section A comprises the antioxidant activity by *in vitro* assays like DPPH, superoxide anion radical and hydrogen peroxide scavenging activity of the compounds. Also, theoretical studies using DFT were carried out based on DPPH radical scavenging activity for understanding the radical stability and mechanism of antioxidant activity. Section B comprises the anti-inflammatory activity of the identified compounds namely tricin and two flavonolignans in both *in vivo* and *in vitro* models. Section C describes the cytotoxicity of the rare flavonolignans, tricin 4'-O-(*erythro-β*-guaiacylglyceryl) ether and tricin 4'-O-(*threo-β*-guaiacylglyceryl) ether towards multiple cancer cells belonging to colon, ovarian and breast tumours. Finally, summary and conclusions are also enclosed.

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Chapter 2

Materials and Methods

2.1 Plant material

Authentic 'Njavara black' (NB) samples were collected directly from a certified farm namely, the 'ECO FARM' Karukamanikalam at Chittoor, Palakkad, Kerala. Plant specimens were verified and confirmed by Dr. Maya C. Nair, Department of Botany, Government Victoria College, Palakkad- 678 001, Kerala, India as identical with the specimen sample IC 539968 deposited at National Bureau of Plant Genetic Resources, (NBPGR), New Delhi, India, against collection (voucher) No.MS004/05. Samples of staple varieties 'Sujatha' (SJ) and 'Palakkadan Matta' (PM) were also collected from the same farmer, for comparison. Freshly milled bran samples were stabilized by heating 100 g lots of bran, spread on a petri dish, at 100°C for 30 min in air oven (Sri Rudran Instruments Co., Chennai, India).[1]

2.2 Extraction

100 g lots of stabilised rice bran of 'Njavara black' (NBb), 'Sujatha'(SJb) and 'Palakkadan Matta'(PMb) and rice of 'Njavara black' (NBr), 'Sujatha'(SJr) and 'Palakkadan Matta'(PMr) were defatted using 800 ml of petroleum ether solvent for about 16 h in a Soxhlet extractor. The solvent from the extract was evaporated at 30°C by using rotary evaporator (Laborota 4000-Heidolph, Germany). The petroleum ether extract residue of NBb (8.61 g), SJb (6.79 g) and PMb (5.52 g) rice bran and NBr (1.80 g), SJr (1.57 g) and PMr (1.98 g) rice were made up to a definite volume in chloroform solvent and stored in refrigerator until further analysis for oryzanol content. The residual rice bran and rice were further extracted with 800 ml of methanol as described above. Methanolic extract residue of NBb (6.71 g), SJb (3.91g) and PMb (5.59 g) rice bran and NBr (1.41 g), SJr (1.60 g) and PMr (1.53 g) rice were made up to a definite volume in methanol solvent and stored in refrigerator until different assays for antioxidant activity and chemical indices were carried out.

2.3 Determination of chemical indices and antioxidant activity

2.3.1 Chemicals

2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$), nitroblue tetrazolium dihydrochloride (NBT), phenazine methosulphate (PMS), nicotinamide adenine dinucleotide (NADH), sodium phosphate monobasic dihydrate ($NaH_2PO_4.2H_2O$), sodium phosphate dibasic dihydrate ($Na_2HPO_4.2H_2O$), gallic acid, (+)-catechin, vanillin and quercetin were procured from Sigma-Aldrich Co.,USA. Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), aluminium chloride ($AlCl_3$), sodium hydroxide (NaOH), potassium ferricyanide ($K_3[Fe(CN)_6]$) and ferric chloride ($FeCl_3$), hydrogen peroxide (H_2O_2), trichloroacetic acid (CCl_3COOH), sodium hydroxide (NaOH), methanol and sulphuric acid (H_2SO_4) were purchased from Merck (India).

2.3.2 Determination of total phenolic content (TPC)

The total phenolic content of the extracts were determined spectrophotometrically. About 0.5 ml of Folin-Ciocalteu reagent, previously diluted with water (1:2), was added to 1.0 ml extract of different concentrations (200-1000 µg/ml) and mixed thoroughly. To the mixture, 1 ml saturated sodium carbonate was added and made up to 10 ml with distilled water. The mixture was allowed to stand at (30 ± 1ºC) for 45 min. Contents were centrifuged if a precipitate was noticed. The absorbance of the supernatant was read at 760 nmusing spectrophotometer (Shimadzu UV-1601 UV-VIS).[2] The total phenolic content was assessed by plotting the gallic acid calibration curve (20 to100 µg/ml) and expressed as mg of gallic acid equivalents (GAE) per g of dry weight of sample (bran or rice). The equation of the gallic acid calibration curve was Y = 0.0097 X + 0.0120 (where X was concentration of gallic acid in µg/ml and Y was measured absorbance) and the correlation coefficient was R^2 = 0.9982.

2.3.3 Determination of total flavonoid content (TFC)

Total flavonoid content was determined by colorimetric method[3] employing quercetin as the standard. To appropriately diluted samples (1 ml), distilled water was added to make up to 5 ml and 0.3 ml of 5% (w/v) $NaNO_2$ was added to it and placed for 5 min, followed by reaction with 0.3 ml of 10% (w/v) $AlCl_3$ to form a flavonoid-

aluminium complex. At the sixth min, 2 ml of 1M NaOH was added and the total volume was made up to 10 ml with distilled water. The solution was mixed well again and the absorbance was measured against a reagent blank, at 510 nm. Blank was prepared by adding all reagents except $AlCl_3$. Distilled water was added in place of $AlCl_3$ in the blank. The TFC was assessed by plotting the quercetin calibration curve (20 to100 µg/ml) and expressed as mg of quercetin equivalents (QE) per g of dry weight of sample (bran or rice). The equation of the quercetin calibration curve was Y = 0.0064 X + 0.0200 (where X was concentration of quercetin in µg/ml and Y was measured absorbance) and the correlation coefficient was $R^2 = 0.9997$.

2.3.4 Determination of total proanthocyanidin content (TPAC)

This assay was performed with vanillin and H_2SO_4 according to the method described by Sun ***et al.*** (1998) 100 µl of 1% (w/v) vanillin in MeOH and 100 µl of 9 M H_2SO_4 in MeOH were added to 40 µl of the sample solution dissolved in MeOH.[4] The mixture was allowed to stand for 15 min at (30±1ºC) and the absorbance was measured at 500 nm. The interference from the compounds such as anthocyanins, was eliminated by running a blank determination of the proanthocyanidin content. The total proanthocyanidin content was assessed by plotting the (+)-catechin calibration curves (20 to100 µg/ml) and expressed as mg of (+)-catechin equivalents (GAE) per g of dry weight of sample (bran or rice). The equation of the (+)-catechin calibration curve was Y = 0.0190 X + 1.4300 (where X was concentration of (+)-catechin in µg/ml and Y was measured absorbance), and the correlation coefficient was $R^2 = 0.9995$.

2.3.5 DPPH radical scavenging assay

$DPPH^{\bullet}$ solution of 0.1 mM was prepared in methanol. Different concentrations (50-1000 µg/ml) of the extracts, pure compounds and standard were prepared and for the scavenging experiments, the sample volume was fixed to be 0.5 ml. 5 ml of $DPPH^{\bullet}$ solution was added to each test tube and shaken well. The test tubes were kept at ambient temperature (30 ± 1ºC) in the absence of light for 30 min. A control was prepared with 0.5 ml MeOH and $DPPH^{\bullet}$ solution (5 ml). Absorbance was read after 30 min at 517 nm.[5] Percentage of radical scavenging was calculated using the formula:

$$\% \text{ of radical scavenging activity} = [(A_0-A_t)/A_0] \times 100 \qquad \text{------(2.1)}$$

where A_0 and A_t are the absorbance of control and sample respectively. IC_{50} values (concentration of extract required to scavenge 50% of free radicals) were calculated from the regression equation, derived from the graph plotted with concentration of the samples against percentage inhibition of the free radical formed in the assay system.[6] Quercetin was used as standard.

The antioxidant capacity of compounds was expressed in EC_{50} values. The remaining concentration of ($DPPH^{\bullet}$) in the reaction medium was calculated using a calibration curve, determined by linear regression:

$$A_{517nm} = 0.01326 + 14.07831(DPPH^{\bullet}) \quad \text{-----(2.2)}$$

The percentage of remaining $DPPH^{\bullet}$ ($\%DPPH^{\bullet}_{REM}$) was calculated using the following equation:

$$\%\,(DPPH^{\bullet})_{REM} = ([DPPH^{\bullet}]_t / [DPPH^{\bullet}]_o) \times 100 \quad \text{-----(2.3)}$$

where $[DPPH^{\bullet}]_t$ is the radical concentration at any time (t) and $[DPPH^{\bullet}]_0$ is the concentration at zero time. The $\%\,[DPPH^{\bullet}]_{REM}$ was found to be proportional to the antioxidant concentration that caused a decrease in the initial $[DPPH^{\bullet}]$ concentration by 50%, defined as EC_{50} and was calculated graphically. The antiradical power (ARP) of the extract was calculated as:[5]

$ARP = 1/EC_{50}$

2.3.6 Superoxide anion radical ($O_2^{-\bullet}$) scavenging assay

The extracts of Njavara and staple varieties, pure compounds and standard (quercetin) were evaluated for the efficacy to scavenge superoxide anion ($O_2^{-\bullet}$) radical also. The PMS-NADH–NBT system was giving consistent results and was followed in this study.[7] About 1 ml of nitro blue tetrazolium (NBT) solution (156 μM/L of NBT in 100 mM/L phosphate buffer, pH 7.4) and 1 ml NADH solution (468 μM/L of NADH in 100 mM/L phosphate buffer, pH 7.4) was added to 0.1ml of sample solution of extract (with concentrations in the range 50–1000 μg/ml). The reaction was started by adding 100 μl of phenazine methosulphate (PMS) solution (60 μM/L of PMS in 100 mM/L phosphate buffer, pH 7.4) to the mixture, incubated at 25ºC for 5 min and the absorbance was measured at 560 nm against blank. Decrease in absorbance of the

reaction mixture indicated increased scavenging activity. The percentage inhibition of superoxide anion was calculated using the following formula:

$$\% \text{ Inhibition} = [(A_0-A_t)/A_0] \times 100 \quad \text{-----(2.4)}$$

where A_0 and A_t are the absorbance of control and sample respectively. IC_{50} values were calculated from the regression equation, derived from the graph plotted with concentration of the samples and against percentage inhibition of the free radical formed in the assay system. Quercetin was used as standard.

2.3.7 Hydrogen peroxide (H_2O_2) scavenging assay

About 0.5 ml aliquots of the extract, pure compounds, standards (with concentrations 50-1000 µg/ml) were dissolved in 3.4 ml of 0.1 M phosphate buffer (pH 7.4) and mixed with 600 µl of 43 mM solution of hydrogen peroxide. The absorbance value at 230 nm of the reaction mixture was recorded after 10 min. For each concentration, a separate 'sample blank' was used for background subtraction.[8] The % inhibition was calculated according to the equation**:**

$$\% \text{ Inhibition} = [(A_0-A_t)/A_0] \times 100 \quad \text{-----(2.5)}$$

where A_0 and A_t are the absorbance of control and sample respectively. IC_{50} values were calculated from the regression equation, derived from the graph plotted with concentration of the samples against percentage inhibition of the free radical formed in the assay system. Quercetin was used as standard.

2.3.8 Reducing power assay

Total reduction capability of extracts was estimated by the method of Oyaizu (1986).[9] 1 ml aliquots of extract and standard (quercetin) having concentrations in the range of (200-1000 µg/ml) was pipetted and 2.5 ml phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%) were added. The mixture was incubated at 50ºC for 20 min followed by addition of 2.5 ml of (10%) trichloroacetic acid. It was centrifuged at 3000 g for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Higher absorbance of the mixture indicates greater reducing power.

2.4 Isolation and quantification of compounds

2.4.1 Chemicals

HPLC grade acetonitrile, dichloromethane, acetic acid, methanol, n-butyl alcohol, water, silica gel for column chromatography, boron trifluoride (BF_3) etherate, sodium methoxide, sodium acetate, aluminium chloride, magnesium ribbon, ferric chloride, silica gel GF_{254} TLC aluminium sheets and deuterated solvents for NMR, were purchased from Merck (India). Sephadex LH-20 (particle size 25-100 µm) was purchased from Pharmacia Fine chemicals (Pharmacia Fine Chemicals AB Uppsala, Sweden). Standard compounds of oryzanol and 24-methylene cycloartanyl ferulate were a gift from M/s. Tsuno Rice Fine Chemicals, Wakayama, Japan.

2.4.2 Analysis and quantification of petroleum ether extract by HPLC

The petroleum ether residue of NBb, SJb, PMb, NBr, SJr and PMr obtained after Soxhlet extraction as explained in section 2.2 was anlaysed and quantified for oryzanols using analytical HPLC. The analysis was performed using a Shimadzu high performance liquid chromatography (HPLC) system consisting of an SCL-10Avp system controller, two LC-8A solvent delivery pumps, Rheodyne injector, an SPD-M20A ultra violet-visible photo diode array (UV-VIS-PDA) detector, and equipped with Multi-PDA LC Solution (software). A YMC-Pack R&D ODS analytical column (YMC Co., Ltd. Japan) of 250 mm x 4.6 mm i.d., 5µm was used in the reverse phase with the solvent system of acetonitrile, dichloromethane, and acetic acid (42:3:5, v/v/v): methanol, n-butyl alcohol, water (45:1:4, v/v/v) in the ratio of 75:25 (v/v), at a flow rate of 1ml/min. The UV detector was set at 325 nm. All extracts were filtered through a 0.2 µm pore size syringe-driven polytetrafluoroethylene (PTFE) membrane filter (Millipore Corporation, Billerica, MA) before HPLC analysis. The petroleum ether extract of NBb, SJb, PMb, NBr, SJr and PMr were analysed. All samples were diluted with the mobile phase in 0.5 mg/ml concentration and analysed by injecting 20 µl. Peak identification was based on comparison of retention time (RT) values with authentic standard of 24-methylene cycloartanyl ferulate and mixture of oryzanol standards. The compounds (**I**, **II**, **III**, **IV**, **V**) corresponding to the peaks (with the same number) were further confirmed by mass spectral (MS) analysis in FAB/HRMS at 5000 resolution using JMS 600H (JEOL) mass spectrometer. For mass spectral analysis, the analytical HPLC was repeated ten times to collect eluting solvent corresponding to each

peak, pooled and concentrated in rotary evaporator. The different peaks were quantified based on the peak area of 24-methylene cycloartanyl ferulate alone (since the molar extinction coefficient (ε) is comparable for all the oryzanols), the major oryzanol component in rice bran [10] and rice.

2.4.3 Isolation of compounds from diethyl ether extract

About 250 g of bran was collected and stabilized as explained in section 2.1.The bran was extracted with 2 L of petroleum ether followed by 2 L of methanol with Soxhlet extractor as explained in section 2.2. The methanolic residue of NBb (16.77 g) obtained was suspended in 200 ml water and partitioned with (5x100) ml of diethyl ether. The diethyl ether extract was concentrated and the dried residue of NBb (2.30 g) was obtained. About 2 g of diethyl ether soluble residue was chromatographed over silica gel open column using petroleum ether:ethyl acetate gradient to collect A_1-A_{118} fractions. The TLC plates were kept in an air oven for 15 min at 100 °C prior to use. The spots were visualized under UV lamp or in an iodine chamber. Similar fractions were pooled, based on silica gel GF_{254} tlc, to get B_1-B_{12} fractions and subjected to DPPH activity. Fraction B_3 (500 mg) which was $DPPH^{\bullet}$ inactive was subjected to spectroscopic characterisation and was esterified for GCMS analysis. Fraction B_{11} (300 mg), active towards $DPPH^{\bullet}$, was further chromatographed on a Sephadex LH-20 column by elution with MeOH to give C_1-C_{20} fractions. The C_{18} fraction gave a yellow powder which was purified by re-crystallization with MeOH to give pure compound **VI** (24 mg). C_5-C_6 were subjected to preparative HPLC on a 250 mm x 20 mm i.d., 5µm, YMS-Pack R&D ODS column (YMC Co., Ltd. Japan) with (28:72) CH_3CN:H_2O at a flow rate of 20 ml/min at 330 nm (λ_{max}) giving impure compounds **VII** and **VIII,** [11] which were then recrystallised in HPLC grade methanol to afford **VII** (3.1 mg) and **VIII** (3 mg), respectively. ^1H (500MHz), ^{13}C (125 MHz), DEPT-135, ^1H- ^1H correlation spectroscopy (COSY) and heteronuclear multiple quantum coherence (HMQC) of the compounds, were recorded in were recorded in CD_3COCD_3 using a Bruker AMX 500 spectrometer (Bruker Avance II 500) with TMS as the internal standard. Chemical shifts are given in scale with TMS as internal reference. Abbreviations used in ^1H NMR are: s - singlet, d - doublet, dd - doublet of a doublet, q - quartet and m - multiplet. Melting points were recorded on Aldrich Meltemp-II apparatus. Mass spectrum was recorded under FAB/HRMS at 5000 resolution using JMS 600H (JEOL) mass spectrometer. IR

spectra were recorded using Spectrum one FT-IR One spectrometer (Perkin Elmer). Melting points were recorded on Aldrich Meltemp-II apparatus. Specific rotations were recorded on Rudolph Research Analytical Autopol I automatic polarimeter.

This typical method of isolation of compounds from Njavara bran was followed for further isolation of pure compounds for different assays like antioxidant, anti-inflammatory and anticancer activity. In total 1-2 kg of bran was collected from the same farm for carrying out rest of the studies.

2.4.3.1 GCMS analysis of fraction B_3

About 20 mg of dried reisdue of B_3 fraction was dissolved in 2 ml of toluene in a stoppered test tube. About 2 ml of dry methanol and two drops of BF_3-etherate was added to the mixture. The mixture was boiled in steam bath for 2 min. When the reaction was complete, the mixture was cooled and water (5 ml) was added. The esters were extracted using diethyl ether (10 ml) and concentrated.[12] The residue obtained was stored in hexane and was analysed for GCMS analysis. Esters were analysed in a Hewlett Packard 5890 Gas chromatograph. The column used was fused silica capillary column (50 m x 0.2 mm id, film thickness 0.25 μm), coated with cross linked methyl silicone. The oven temperature was 80-200ºC at the rate of 2ºC/min. Flame Ionization Detector (FID) was used as detector at 300ºC and injection temperature was 250ºC. GC-MS analysis was carried out using Shimadzu, GC-MS, QP5050A mass spectrometer with the same column and GC conditions. Helium was used as the carrier gas and ionization voltage used was 70eV. Identification of the components was achieved 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.[13-16]

2.4.3.2 UV-Visible spectroscopy of flavonoids

After the measurement of the spectrum of the flavonoids in methanol, for recording sodium methoxide spectrum, 3 drops of sodium methoxide were added to the cuvette and after mixing, the spectrum was recorded and the sample discarded. For $AlCl_3$/HCl spectrum, to the fresh flavonoid solution, six drops of $AlCl_3$ (5g dry $AlCl_3$/100 ml methanol) and three drops of HCl were added to fresh flavonoid solution, mixed and spectrum recorded. Powdered sodium acetate was added to fresh flavonoid solution and mixed thoroughly to record the sodium acetate spectrum. Two drops

sodium hydroxide solution added to fresh flavonoid and mixed to record NaOH spectrum.[17]

2.4.4 Quantification of compounds in diethyl ether extract by HPLC-PDA analysis

Isolated compounds were quantitated in NBb, SJb and PMb by analytical, reverse phase HPLC. 250 g bran of PMb and SJb were subjected to Soxhlet extraction with petroleum ether and methanol as explained in section 2.2. Methanolic extract residue of PMb (13.98 g) and SJb (9.78 g) were extracted with diethyl ether with same methodology applied to NBb in section 2.4.3. The diethyl ether extract was concentrated and the dried residue of PMb (2.11 g) and SJb (976 mg) were made up to a definite volume in methanol solvent and stored in refrigerator. All samples (NBb, PMb and SJb) were diluted with the mobile phase in 0.5 mg/ml concentration and were filtered through a 0.2 µm pore size syringe- driven filter (Millipore Corporation, Billerica, MA) before injection. A 20-µl aliquot of sample solution was separated using a Shimadzu HPLC system consisting of an SCL-10Avp system controller, two LC-8A solvent delivery units, an SPD-M20A UV-vis photo diode array (PDA) detector, and equipped with Multi-PDA LC Solution (software) on a 250 mm x 4.6 mm i.d., 5µm, YMC-Pack R&D ODS analytical column (YMC Co., Ltd. Japan). The mobile phase consisted of acetonitrile and water (28:72) at a flow rate of 1 ml/min. The separated compounds were quantified using detector response at 330 nm.

2.5 Anti-inflammatory assays

In vitro and *in vivo* anti-inflammatory assays were determined in collaboration with Department of Biochemistry, University of Kerala, Thiruvananthapuram.

2.5.1 Chemicals

Histopaque, Dithiothreitol (DTT), hemoglobin (Hb), RPMI (Roswell Park Memorial Institute medium) 1640 were purchased from Sigma chemicals Co, USA. Tween 20, linoleic acid, haemoglobin, GSH, arachidonic acid, thiobarbituric acid (TBA), tricholoroacetic acid (TCA), sodium phosphate monobasic dihydrate ($NaH_2PO_4.2H_2O$), sodium phosphate dibasic dihydrate ($Na_2HPO_4.2H_2O$), phenazine methosulphate (PMS), nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium dihydrochloride (NBT), glacial acetic acid, n-butanol, potassium chloride (KCl), sodium dodecyl sulfate (SDS), L-arginine, manganese chloride ($MnCl_2$),

nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin, copper sulphate, sodium potassium tartarate, Tris HCl, sodium carbonate and Folins phenol reagent from SRL chemicals, Mumbai, India. The inflammatory agent used for the study was Type IV Lamda carrageenan from Spectrochem Pvt. Ltd. India.

2.5.2 Animals

Female rats (Sprague Dawley strain) (120-200 g) bred in the Biochemistry department animal house were used for this study. They were kept in an environment with controlled temperature (24–26^{0}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 [IA EC-KU-12/08-09-BC-AH (13)] according to Government of India accepted principles for laboratory animals' use and care.

2.5.3 Isolation of monocytes

Human peripheral blood mononuclear cells (hPBMCs) were used as the *in vitro* experimental model. For isolation of monocytes, blood was collected from healthy individuals and the method of isolation was by Radhika *et al.* (2007).[18] Isolation of monocytes was carried out in highly sterile condition i.e., in laminar air chamber. The blood for the isolation of monocytes was collected in heparin coated tubes. The cells were isolated by layering 3 ml blood over 3 ml histopaque solution in a 15 ml tarson tube. The tubes were centrifuged at 2500 rpm for 30 min. After centrifugation four layers were formed. The outer layer was yellow colored plasma, beneath that there was a ring of mononuclear cells; the next two layer was histopaque solution and sedimented red cells. The monocytes were pipetted out into a separate tubes and PBS was added and centrifuged at 2500 rpm for 30 min. This was done twice. The supernatant was removed and resuspended in RPMI 1640 media. This was then centrifuged at 2500 rpm for 30 min. Removed the supernatant and resuspended the cells in fresh medium.

2.5.4 Culture of monocytes

Monocytes after isolation were seeded onto 35 mm collagen coated culture plates. After giving 4 h for attachment, the medium and unattached cells were removed and supplemented with fresh medium. The cells were maintained at 95% air and 5%

CO_2 on a Sanyo CO_2 incubator maintained at 37^0C. The medium was removed and supplemented with fresh medium after 24 h and subjected to further treatment. The cells were divided five groups for each compound with six cells in each group. Groups 3-5 were treated with three different concentrations (2, 5 and 10 μg/ml) of each compound (**VI**, **VII** and **VIII)** 1 h prior to LPS priming. After 1 h groups 2-5 were primed with LPS. Group 1 was treated as normal control and the duration of exposure to various agents was 24 h.

2.5.5 Assay of inflammatory mediators

For the biochemical evaluation of inflammatory enzymes like cyclooxygenase (COX), 5-lypoxygenase (5-LOX) and nitric oxide synthase (NOS), the lysed cellular fraction of each group (1-5), by performing three freeze thaw cycles using liquid nitrogen was used as the enzyme source. Subsequently media were collected to measure the activity of superoxide dismutase (SOD) and level of malondialdehyde (MDA).

2.5.5.1 Assay of 5-lipoxygenase (5-LOX)

The assay of lipoxygenase was measured by Axelrod *et al.* (1981)[19] Reagents required: 0.2 M sodium phosphate buffer (pH-6.5), 0.2M Tris HCl (pH-7.4) and Sodium linoleate. 70 mg of linoleic acid and equal weight of Tween 20 was dissolved in 4 ml oxygen free water, mixed back and forth with a pipette avoiding air bubbles. Sufficient amount of 0.5 N NaOH was added to yield a clear solution (0.55 ml) and made up to 25 ml using oxygen free water. This was divided into 0.5 ml portion and flushed with nitrogen gas before closing and it is kept frozen until needed. The reaction was carried out in a quartz cuvette at 25^0C with 1 cm light path. The assay mixture contains 2.75 ml Tris HCl buffer (pH-7.4), 0.1 ml sodium linoleate and 50 μl of the enzyme source (cells (monocytes) of each group is the enzyme source). The increase in OD was measured at 234 nm.

2.5.5.2 Assay of cyclooxygenase (COX)

The assay of cycloxygenase was done by TBA method.[20] Reagents required: 100 mM Tris HCl (pH-8), 5 mM GSH, 5 mM Hemoglobin, 200 μM Arachidonic acid, 10% TCA in HCl and 1% Thiobarbituric acid. The assay mixture contains 1 ml Tris HCl, 50 μl hemoglobin and 50 μl enzyme (cultured cells (monocytes) of each group is the enzyme source). The reaction was started by the addition of the 100 μl arachidonic acid and incubated at 37^0C for 20 min. The reaction was terminated by the addition of 10%

TCA in HCl mixed well and 200 µl thiobarbituric acid was added and condensed, heated in a boiling water bath for 20 min, cooled and centrifuged at 1000 rpm for 3 min. The supernatant was measured at 532 nm for COX activity.

2.5.5.3 Assay of superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was determined by the method described by Kakkar *et al.* (1984)[21] The reagents required: Sodium pyrophosphate buffer: 0.052mM (pH-8.3), Tris HCl buffer: 0.025 M (pH-7.4), Phenazine methoulphate: 186 µM, Nitroblue tetrazolium (NBT): 300 µM, NADH: 780 µM, Glacial acetic acid and n-butanol. The assay mixture contained 1.2 ml sodium pyrophosphate buffer, 0.1 ml phenazine methosulphate, 0.3 ml nitroblue tetrazolium, appropriately diluted enzyme preparation (0.2 ml cultured media (cultured monocytes) of each group) and water in a total volume of 3 ml. The reaction was started by the addition of 0.2 ml NADH. After incubation at 30^0C for 90 s, the reaction was stopped by the addition of 1 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml of n-butanol. The mixture was allowed to stand for 10 min. Centrifuged and butanol layer taken out. Colour intensity of chromogen in the butanol was measured at 560 nm against butanol. The colour developed was stable up to 48 h. A system devoid of enzyme served as control. One unit of enzyme activity is defined as the enzyme concentration required to inhibit the OD at 560 nm of chromogen produced by 50% in one minute under the assay condition and expressed as specific activity in units/mg protein.

2.5.5.4 Assay of malondialdehyde (MDA)

Malondialdehyde was estimated by the method of Ohkawa *et al* (1979).[22] Reagents required for experiment are 1.15% KCl, 8.1% SDS, 20% Acetic acid (pH-3.5), 0.8% Thiobarbituric acid and n-butanol – pyridine reagent (15:1). 0.2 ml enzyme source (cultured media (cultured monocytes) of each group) was taken and mixed with 0.2 ml 8.1% SDS, 1.5 ml acetic acid (pH-3.5), 1.5 ml of 0.8% thiobarbituric acid. The volume was made up to 4 ml by adding distilled water. Heated in a boiling water bath at 95^0C for 1 h, capped with glass ball. After incubation it was cooled under tap water. Added 1 ml of distilled water and 5 ml of butanol- pyridine reagent and shaken vigorously. Then centrifuged at 4000 rpm for 10 min. The organic layer was taken and absorbance was read at 532 nm against blank.

2.5.5.5 Assay of nitric oxide synthase (NOS)

Nitric oxide synthase was determined by the method described by Salter *et al.* (1997)[23] The freeze fractured cells were used as the enzyme source (cultured media (cultured monocytes) of each group). The assay system contained substrate, 0.1ml L-arginine, 0.1ml $MnCl_2$, 0.1ml DTT, 0.1ml NADPH, 0.1ml tetrahydrobiopterin, 0.1ml oxygenated haemoglobin (Hb) and 0.1ml enzyme preparation. Increase in absorbance was recorded at 401nm.

2.5.5.6 Estimation of protein

Protein was estimated by Lowry's method.[24] The reagents required: Alkaline copper reagent: Reagent A: 1% copper sulphate+2%sodium potassium tartarate(1:1), Reagent B: sodium carbonate (2% in 0.1N NaOH) : Added A and B in the ratio 1:50 and Folins phenol reagent (1%). Different volumes of sample and standard BSA 1mg/ml were taken and made upto 500µl. To this added 2.5ml alkaline copper reagent and kept for 15 min. After adding 250 µl of Folins reagent, the solution was allowed to stand for 30 min before measuring the optical density at 650nm.

2.5.6 *In vivo* anti-inflammatory activity

2.5.6.1 *In vivo* anti-inflammatory activity of methanolic extracts

A dose response study for the anti-inflammatory activity, against carrageenan induced rat paw edema, was carried out in the range of 1-100 mg/kg bodyweight. From this, a dose of 5 mg/kg bodyweight was found to be the minimal concentration for maximum edema inhibition in case of NBb and NBr. The dose of 5 mg/kg was selected for all the extracts for further comparison with staple varieties. For subsequent experiments, the rats were divided into nine groups (1-9) with six rats in each group (n = 6). Group 1 received saline. Acute inflammation was produced by the sub-plantar administration of 0.1 ml of carrageenan (1% in normal saline) from group 2-9 into the right hind paw of the rats. The animals were pre-treated intraperitoneally (i.p.) in groups 3-9, with methanolic extracts of NBb, SJb, PMb, NBr, SJr, PMr (dose 5 mg/kg) and diclofenac (dose 20 mg/kg) in saline respectively, 30 min before the administration of carrageenan. The volume of each paw was measured by means of a plethysmograph [25] at 0, 3rd and 5th after carrageenan injection. The percentage of paw oedema was calculated according to Winter C.A *et al.* (1962).[26]

Percentage inhibition = [(Vc-Vt)/Vc] x 100 -----(2.6)

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

2.5.6.2 *In vivo* anti-inflammatory activity of compounds

For the anti-inflammatory activity of compounds, the rats were divided into six groups (1-6) with six rats in each group (n = 6). The first group received saline. Second group to fifth group were administered with compounds **VI**, **VII** and **VIII** in saline (2 mg/kg, respectively). Acute inflammation was produced by the subplantar administration of 0.1 ml of carrageenan (1% in normal saline) from group two to six into the right hind paw of the rats. The animals were pre-treated intra-peritoneally to the rats in Groups two to five, with the compound **VI**, **VII**, **VIII** and diclofenac (Voveran) (Novartis India Ltd.) respectively, 30 min before the administration of carrageenan. The percentage of paw oedema was calculated according to above discussed method in section 2.5.6.1.

2.6 Cytotoxicity and apoptosis measurement

Anticancer studies were determined in collaboration with Department of Integrated cancer research programme, Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, Kerala, India.

2.6.1 Cell culture materials

The compounds **VII** and **VIII** were dissolved in DMSO to get 50 mg/ml stock solutions and stored at – 20 ^{o}C. Further dilutions were made in complete medium. Hoechst 33342 and Alex Fluor 488 conjugated Annexin V reagent and the mitochondrial membrane potential specific fluorescent dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzamidazolocarbocyanin Iodide (JC1) and Tetramethyl rhodamine methyl ester (TMRM) were procured from Molecular Probes (USA). Dulbecco modified Eagle's minimal essential medium (DMEM), fetal bovine serum (FBS) and trypsin were purchased from Invitrogen (USA). The human colon adenocarcinoma cell line HCT 116, Ovarian Cancer cell line SKOV3 and Breast cancer cell line MCF-7 were obtained from ATCC, USA. SKOV3 and MCF-7 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum. HCT 116 cells were maintained in Mc.Coys Medium supplemented with 10% FBS.

2.6.2 Chromatin condensation

The compounds **VII** and **VIII** were dissolved in DMSO to get 1 mg/ml stock solutions and stored at –20^{o}C. Further dilutions were made in complete medium. HCT 116 cells were maintained in Mc. Coy's medium supplemented with 10% FBS, SKOV3 and MCF-7 cells were maintained in (DMEM) medium supplemented with 10% FBS. For analyzing chromatin condensation by Hoechst 33342 staining, the cells were grown on 96 well plates, after indicated treatment, were stained with 0.5 µg/ml of Hoechst 33342 for 10 min and viewed under UV filter sets using Nikon fluorescent microscope (TE2000E). Apoptotic nucleus with condensed chromatin was scored by two investigators, in percentage, from 200 to 300 cells/sample.

2.6.3 Mitochondrial membrane potential loss

The lipophilic and weakly acidic dye that acts as a voltage sensitive probe, JC-1 was used to analyze mitochondrial membrane potential. The cells after treatment were re-suspended in serum free Opti-MEM containing 25 nm of JC-1 for 30 min. The cells were washed with fresh Opti-MEM and immediately observed under fluorescent microscope using long pass GFP filter, as per published protocol.[27] For qauntitating mitochondrial membrane potential, another membrane potential specific dye TMRM was employed. After treatment with compounds **VII** and **VIII**, cells were trypsinized and centrifuged at 3000 rpm for 5 min and once again washed with Opti-MEM medium. Cells were then incubated for 10 min at 37ºC after adding Opti-MEM medium containing TMRM dye at 50 nM and analyzed by FACS Aria1 in PE (Phycoerythrin) channel.

2.6.4 Annexin V staining

The cells were treated with 40 µg/ml of the compound **VII** or 30 µg/ml of compound **VIII** for 24 h and 48 h. The dying cells and attached cells were collected by trypsinisation and washed with Annexin binding buffer two times. Then the cells were stained with Annexin V reagent in the binding buffer for 30 min as per the instruction from the manufacturer. The cells were analyzed by FACS Aria II. The green fluorescence from at least 10, 000 cells were collected.

2.7. Statistical analysis

Values were represented as mean ± standard deviation (SD) and standard error mean (SEM) from two analyses of three replications (n = 6). Analysis of variance was

performed by using the statistical program software (SPSS/ PC+), version 11.0 (SPSS, Chicago, IL, USA). Duncan's multiple range tests was conducted for comparison of means at $P < 0.05$.

2.8 Computational details for density functional theory (DFT) studies

The structures of compound **VI**, **VII**, **VIII** and standard (quercetin) were optimized using the B3LYP/6-31G* level density functional theory.[28,29] For all the radical systems, the unrestricted UB3LYP/6-31G* method is used. All the calculations were performed using Gaussian03 suite of programs.[30] Total enthalpy at 298^o K was taken to obtain the bond dissociation enthalpy (BDE) for the homolytic cleavage of O-H bonds to generate the radicals.[31] The ionization potential (IP) is calculated from the zero point energy (ZPE) corrected electronic energy difference between the parent molecule and its radical cation. The effect of solvation on IP is taken into account by single point energy calculation using self consistent reaction field polarizable continuum model (PCM) on the optimized geometries.[32,33] The solvent used for PCM calculation is methanol (dielectric constant = 32.63).

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Chapter 3

Chemical Indices, Antioxidant and Anti-inflammatory Activity of Njavara Extracts as Compared to Staple Varieties

The petroleum ether extract residue of NBb (8.61 g), SJb (6.79 g), PMb (5.52 g) rice bran and NBr (1.80 g), SJr (1.57 g), PMr (1.98 g) rice and methanolic extract residue of NBb (6.71 g), SJb (3.91g), PMb (5.59 g) rice bran and NBr (1.41 g), SJr (1.60 g), PMr (1.53 g) rice, obtained from 100 g lots of stabilized bran and rice as explained in Chapter 2 under section 2.2, were evaluated by DPPH radical scavenging assay over a range of concentrations. The petroleum ether extract of NBb showed <1.0% scavenging activity at 1000 µg/ml whereas similar extracts of other samples including NBr, did not show any measurable activity at the same concentration. On the other hand at 200 µg/ml, the methanolic extract of NBb rice bran showed 93.0% activity whereas SJb and PMb showed 65.56% and 41.50% respectively. Corresponding activity of rice extracts were: 34.64% (NBr), 9.07% (SJr) and 7.03% (PMr). Hence, the methanolic extracts of rice bran and rice were studied further by *in vitro* antioxidant assays. Njavara is extensively used in the treatment of rheumatoid arthritis in which inflammation is a major problem; the methanolic extracts were also evaluated for *in vivo* anti-inflammatory activity.

3.1 Total phenolic, flavonoid and proanthocyanidin content (TPC, TFC and TPAC)

The methanolic extracts of rice bran and rice were also subjected to evaluation of chemical indices like total phenolic, flavonoid and proanthocyanidin content. Table 3.1 shows the values of above indices. Total phenolic content in the extract expressed as gallic acid, is highest in NBb followed by SJb > PMb > NBr > SJr > PMr. Total flavonoid content, expressed in terms of quercetin, is found to be highest in NBb, followed by PMb > SJb > NBr > PMr > SJr. Total proanthocyanidin content, determined by vanillin assay and expressed as catechin equivalent, is highest in NBb, followed by PMb > SJb > NBr ≈ PMr > SJr.

Plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators. Therefore, it is beneficial to determine their total amount in the food samples. Flavonoids, as one of the most diverse and widespread group of natural compounds, are likely to be the most important natural phenolics.[1] Proanthocyanidins (PA) are oligomeric and polymeric flavans or flavan-3-ols. Recently catechins and low molecular weight PA have received considerable attention owing to their different biological activities, in particular their effects on arteriosclerosis and their oxygen free radical scavenging ability.[2] Chotimarkorn ***et al.*** (2008)[3] had reported that the total phenolic and flavonoid content in a staple variety CV. Khao Pathum Thani 60, was 3.2 mg/g and 0.06 mg/g respectively whereas in NBb, we find that the corresponding values are 27.15 mg/g and 4.495 mg/g. Kong and Lee (2010)[4] have reported the total phenolic content in black rice of two cultivars (Heugjinjubyeo and Heugkwangbyeo) as 2.73 mg/g and 1.73 mg/g where as NBr showed 2.28 mg/g which is comparable. Similarly, in the same report, total flavonoid content of the above two cultivars of black rice, were 0.32 mg/g and 0.15 mg/g respectively and NBr showed 0.31 mg/g of flavonoid content. Laokuldilok ***et al***. (2011)[5] have reported the total phenolic contents in red, normal and black rice bran varieties as 1.53, 2.1 and (2.7-3.2) mg/g respectively which are significantly low compared to NBb. Ragace ***et al***. (2006)[6] have reported total phenolic content of other whole cereals like wheat, barley, millet and rye as 0.562, 0.879, 1.387, and 1.026 respectively and these are significantly low compared to NBr and NBb. Thus, it can be seen that NBb is a rich source of phenols, flavonoids and proanthocyanidins compared to bran of staple varieties and corresponding rice samples, based on literature reports as well as our own results in this study (Table 3.1).

3.2 DPPH radical scavenging activity

DPPH is a stable free radical and accepts one electron or hydrogen radical to become a diamagnetic molecule.[7] The reduction of DPPH radicals, as induced by antioxidants, can be followed by measuring the decrease in absorbance of the system at 517 nm. Due to the simplicity and reliability of this method, DPPH is generally used as a substrate to evaluate radical scavenging activity of plant extracts and antioxidant compounds.

TABLE 3.1: Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and Total Proanthocyanidin Content (TPAC) of Njavara Black bran (NBb), Sujatha bran (SJb), Palakkadan Matta bran (PMb), Njavara Black rice (NBr), Sujatha rice (SJr) and Palakkadan Matta rice (PMr)

Sample	TPC (mg of gallic acid/g dry weight of sample)	TFC (mg of quercetin/g dry weight of sample)	TPAC (mg of catechin/g dry weight of sample)
NBb	27.16 ± 0.06^{a}	4.50 ± 0.16^{a}	0.98 ± 0.03^{a}
SJb	5.27 ± 0.06^{b}	0.82 ± 0.01^{c}	0.22 ± 0.00^{c}
PMb	3.11 ± 0.03^{c}	1.04 ± 0.03^{b}	0.37 ± 0.03^{b}
NBr	2.28 ± 0.10^{d}	0.31 ± 0.02^{d}	0.07 ± 0.00^{d}
SJr	1.93 ± 0.09^{e}	0.03 ± 0.00^{f}	0.05 ± 0.00^{d}
PMr	0.56 ± 0.03^{f}	0.14 ± 0.01^{e}	0.07 ± 0.00^{d}

[a-c]Mean ± Standard Deviation (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 $P<0.05$.

Figure 3.1 illustrates the concentration dependent scavenging effect of sample extracts in DPPH system.The scavenging effect of NBb is highest followed by SJb > PMb > NBr > SJr > PMr and is significantly different. Quercetin was used as antioxidant standard. The IC_{50} values are as follows: SJr (1700 µg/ml) > PMr (1680 µg/ml) > NBr (287.67µg/ml) > PMb (268.67µg/ml) > SJb (166.73 µg/ml) > NBb (84.66 µg/ml) > quercetin (10 µg/ml). At 200-1000 µg/ml NBb showed a comparable activity as that of standard quercetin compared to the staple varieties.

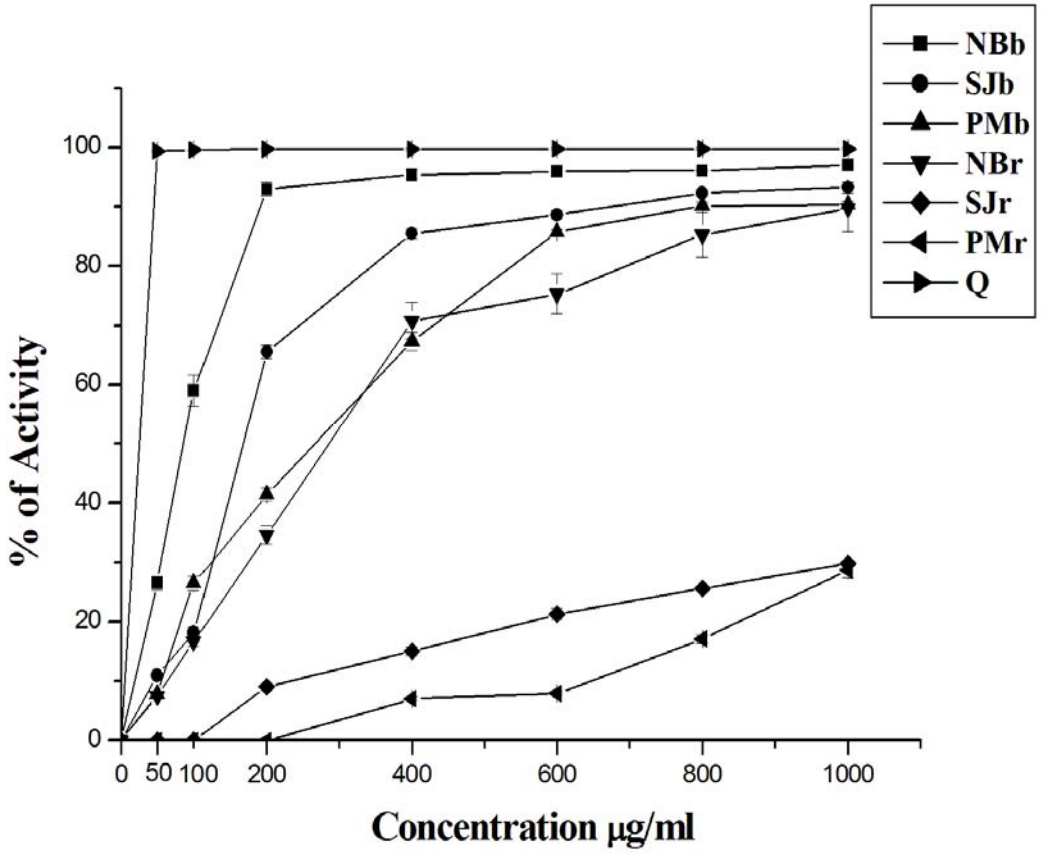

Figure 3.1: DPPH radical scavenging activity of different concentrations of methanolic extracts of NBb: Njavara black bran; SJb: Sujatha bran; PMb: Palakkadan Matta bran; NBr: Njavara black rice; SJr: Sujatha rice; PMr: palakkadan Matta rice; Q: Quercetin. Values are expressed as the mean±SD of 3 x 2 (n = 6) determinations at all concentrations (50-1000 µg/ml).

Lower IC_{50} value indicates higher activity. Results confirm that NBb is more efficient as a radical scavenger than SJb, PMb, NBr, SJr and PMr. Muntana and Prasong (2010)[8] have reported on the DPPH activity of bran extracts of 21 Thai rice varieties. According to them, the order of EC_{50} is red > black > white varieties. The numerical values are not compared here with Njavara results as there is difference in methodology especially, reagent concentration. In another study by Laokuldilok *et al.* (2011)[5] black varieties from Hongkong and Lundburg are compared for antioxidant activity with aromatic red variety (Japonica) and normal white variety. In this study, they report that black > red > white variety with respect to DPPH activity. These literature reports also support our observation that red variety is better than white types, in terms of radical scavenging effect. Choi *et al.*(2007) have reported that pigmented red sorghum (92%) and black rice (87%) showed relatively higher activity than non-pigmented samples like white rice, millet, barley etc.[9] Miller *et al.* (2000)[10] have reported higher activity for highly pigmented berries, raisins, dates and prunes. These results are consistent with our results as Njavara belong to pigmented variety. Laloo and Sahu (2011) have reported activity (methodology being almost comparable) of the medicinal plants (*Cinnamomum wightii, Ochrocarpus longifolius and Mesua ferrea* known in Ayurveda) used for anti-inflammatory purposes with IC_{50} value in the range of 108-300 µg/ml, whereas NBb have shown lower IC_{50} value of 84.66 µg/ml compared to these medicinal plants.[11] Hence, the DPPH radical scavenging activity of NBb is higher compared to these medicinal plants.

3.3 Superoxide anion radical ($O_2^{-\cdot}$) scavenging activity

Superoxide anion radical is a biologically important active species since; it decomposes to form deleterious oxidative species such as singlet oxygen and hydroxyl radicals.[12] In the PMS/NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction, reduces NBT, which absorbs at 560 nm (λ_{max}). The decrease of absorbance in the presence of antioxidants indicates the scavenging of superoxide in the reaction mixture. Figure 3.2 shows % inhibition of superoxide radical generation, at concentration levels of (200-1000 µg/ml) of methanolic extract of NBb and NBr in comparison with SJb, PMb, SJr and PMr.

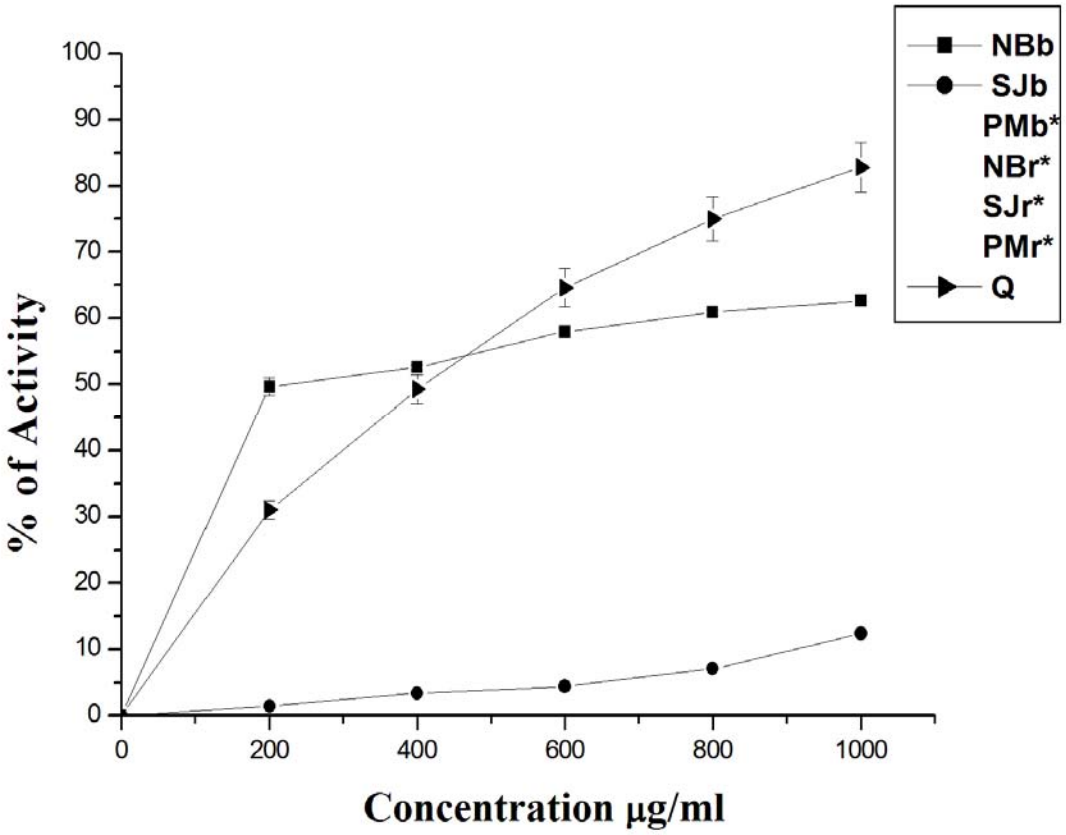

Figure 3.2: Superoxide anion scavenging activity of different concentrations of methanolic extracts of NBb: Njavara black bran; SJb: Sujatha bran; PMb: Palakkadan matta bran; NBr: Njavara black rice; SJr: Sujatha rice; PMr: Palakkadan matta rice; Q: Quercetin. Values are expressed as the mean±SD of 3 x 2 (n = 6) determinations at all concentrations (200-1000 µg/ml).*nd = not detected.

It can be seen that, as concentration increased superoxide scavenging activity also increased, with higher values for NBb than SJb; whereas PMb, NBr, SJr and PMr did not show any activity in this range. At lower concentrations of 200 and 400 µg/ml NBb showed higher activity compared with standard quercetin. The IC_{50} value of NBb was found to be 203.16 µg/ml whereas for standard the value was 410 µg/ml (quercetin). NBb showed a higher decrease in absorbance compared to staple varieties and quercetin, indicating greater scavenging activity for superoxide radicals. Although reports are available on superoxide anion scavenging activity of rice bran extracts,[13] the values cannot be compared with our results due to difference in methodologies followed. Laloo and Sahu (2011) have reported activity of the medicinal plant ***Mesua ferrea*** used in Ayurveda for anti-inflammatory purposes with IC_{50} value in the range of 273.66 µg/ml, whereas NBb have shown lower IC_{50} value of 203.16 µg/ml, indicating higher activity for NBb extract.[11]

3.4 Hydrogen peroxide (H_2O_2) scavenging activity

Hydrogen peroxide exhibits weak activity in initiating lipid peroxidation. However, its potential to produce ROS such as hydroxyl radical through Fenton reaction, is very high causing damage to lipids.[14] Effective scavenging of H_2O_2 can therefore prevent oxidative damage. The decomposition of H_2O_2 into water may depend on the antioxidant compounds present in the extract. As they are good donors, they can accelerate the conversion of H_2O_2 to H_2O. Figure 3.3 depicts the hydrogen peroxide scavenging activity of NBb compared to SJb, PMb, NBr, SJr and PMr. NBb was capable of scavenging hydrogen peroxide in a dose-dependent manner and was tested in the range of 50-1000 µg/ml. Results indicate that it had stronger hydrogen peroxide scavenging activity compared to SJb > PMb > NBr > SJr, where as PMr did not respond in the concentration range mentioned earlier. The order of IC_{50} values are: PMb (799.9 µg/ml) > SJb (464.03 µg/ml) > NBb (73.55 µg/ml) > gallic acid (55 µg/ml) > quercetin (15 µg/ml). At higher concentrations from 200-1000 µg/ml, NBb showed higher scavenging compared to standard quercetin.

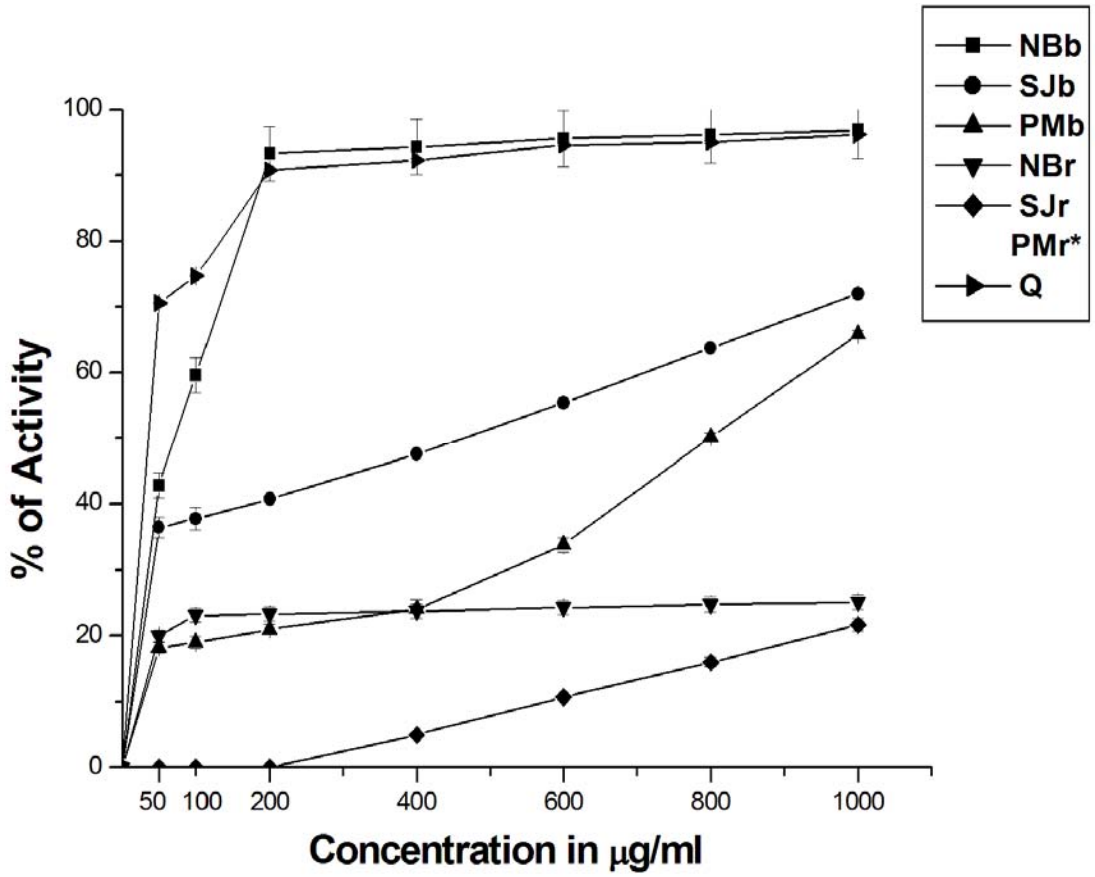

Figure 3.3: Hydrogen peroxide scavenging activity of different concentrations of methanolic extracts of NBb: Njavara black bran; SJb: Sujatha bran; PMb: Palakkadan matta bran; NBr: Njavara black rice; SJr: Sujatha rice; PMr: Palakkadan matta rice; Q: Quercetin.. values are expressed as the mean±SD of 3 x 2 (n = 6) determinations at all concentrations (50-1000 μg/ml). *nd = not detected.

Greater scavenging of hydrogen peroxide by NBb may be attributed to the phenolics in it, which could donate electrons to hydrogen peroxide, thus converting it to water [15] as shown below:

$H_2O_2+2H^+ + 2e- \rightarrow 2H_2O$

There are no reports in the literature on hydrogen peroxide scavenging effect of rice bran/rice varieties. However, hydroxyl radical scavenging effects of pigmented and nonpigmented rice varieties have been reported by Nam *et al.* (2006)[13] by ESR method. Gill *et al.* (2010) have reported activty of *Benincasa hispida* seed used in Ayurveda that showed 63.7% at a concentration of 200 µg/ml, whereas NBb showed 93.3% of activity.This results shows significantly higher activity of NBb compared to ayurvedic seed.[16]

3.5 Reducing power

For the measurement of the reductive ability, we investigated the Fe^{3+}/Fe^{2+} redox system in the presence of the methanolic extracts of samples over the concentration range of 200-1000 µg/ml. The reducing capacity of compounds serves as an indicator of its potential antioxidant activity.[17] Figure 3.4 shows the reductive ability of the NBb, which is significantly higher compared to NBr > PMb > SJb > SJr > PMr. The reducing potential of NBb is also higher compared to the standard quercetin. As with the free radical scavenging assays, the reducing potential of extracts increased in a dose-dependent manner with significant reducing potential for NBb. Based on the results, it can be concluded that NBb is an efficient electron donor capable of neutralizing free radicals compared with the staple varieties. This property would enable the antioxidant extract to convert free radicals to more stable products, terminating radical- initiated chain reactions. Chotimarkorn *et al.* (2008)[3] studied the antioxidant properties of bran extracts of five long-grained rice cultivars available in Thailand. According to them, at 100 µg/ml, the rice bran extracts showed reducing power in the range (0.12-0.55). NBb (Figure 3.4) showed reducing power of about 0.5 absorbance at this concentration. In another study, Laokuldilok *et al.* (2011)[5] reported that pigmented rice varieties had higher reducing power. Choi *et al.* (2007) [9] have also reported that the highly pigmented red sorghum and black rice have relatively higher reducing power

than white rice, millets and barley. These reports indirectly support our results that Njavara has higher reducing power.

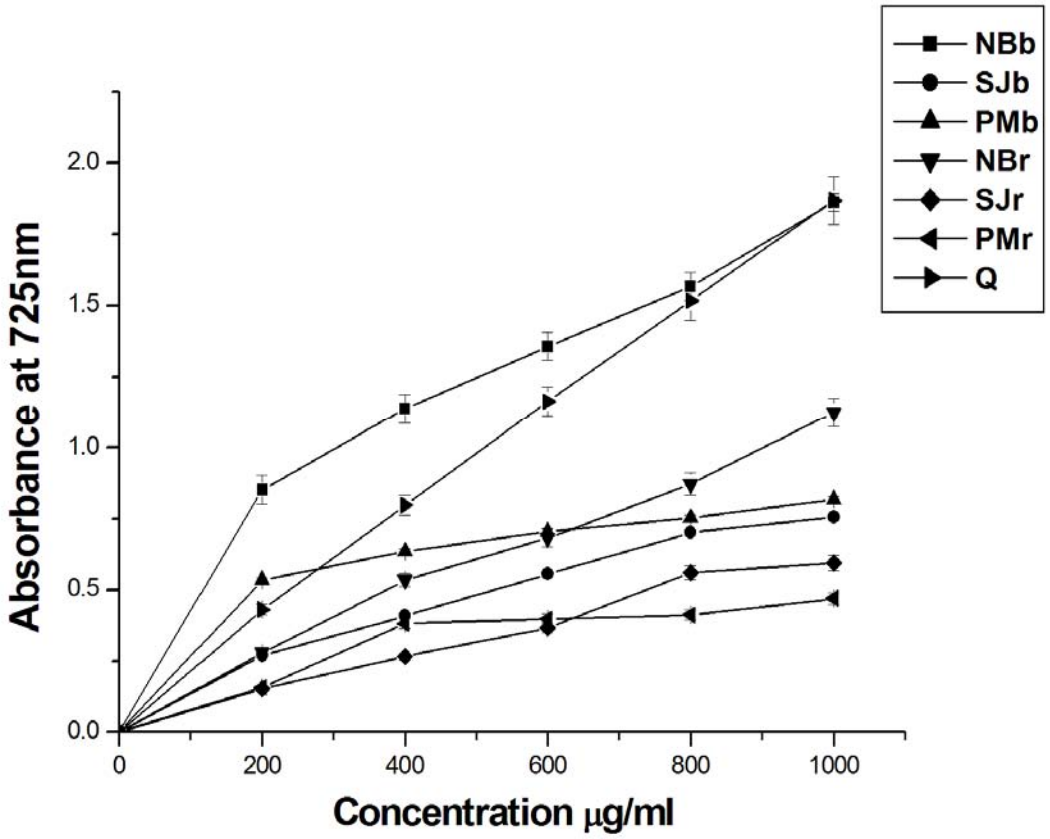

Figure 3.4: Reducing powers of different concentrations of methanolic extracts of NBb: Njavara black bran; SJb: Sujatha bran; PMb: Palakkadan matta bran; NBr: Njavara black rice; SJr: Sujatha rice; PMr: Palakkadan matta rice; Q: Quercetin. Values are expressed as the mean±SD of 3 x 2 (n = 6) determinations at all concentrations (200-1000 μg/ml).

3.6 *In vivo* Anti-inflammatory activity

Since Njavara is used as medicinal rice especially, in the treatment for rheumatoid arthritis, the extract was checked for its anti-inflammatory activity in comparison with staple varieties. The anti-inflammatory activity of extracts of NBb, SJb, PMb, NBr, SJr and PMr against acute edema (induced by carrageenan) on Sprague Dawley rats (Figure 3.5) is shown in Table 3.2 at a dose of 5 mg/kg body weight and the results are comparable to that of the standard drug diclofenac (Voveran). At 3rd h, NBb showed 66.6% edema inhibition where as NBr, PMb, PMr, SJb, SJr showed low inhibition. At 5th h, NBb inhibited edema formation maximum, to an extent of 83.3% (Figure 3.6 and 3.7) whereas NBr, PMb, PMr, SJb and SJr showed in the range of 16-66 % respectively. Compared with standard drug diclofenac as well as NBr, PMb, PMr, SJb and SJr, NBb showed significant inhibition at lower dose (5 mg/kg).

Carrageenan-induced local inflammation (paw edema or pleurisy) is a commonly used method to evaluate the efficacy of nonsteroidal anti-inflammatory drugs (NSAID) and also in determining the role of mediators involved in vascular changes associated with acute inflammation.[18,19] In the present study, the edema inhibition by NBb reached maximum and it was more pronounced in the second phase, suggesting its inhibitory effect on prostaglandin production as a major mechanism by which the extract exerts anti-inflammatory effect. There are no previous reports on comparison of *in vivo* anti-inflammatory effect of NBb and NBr extracts, with staple varieties.

Gorzalczany *et al*. (2011) have reported the *in vivo* anti-inflammatory activity of *Lithrea molleoides* which is traditionally used as a folk medicine for the treatment of various diseases related to inflammation.[20] The methanolic extract of leaves of this plant has shown only 46% and 37% inhibition at 3rd h and 5th h respectively at a dose of 100 mg/kg. The inhibition by NBb is highly significant compared to above results as it showed above 50% inhibition at 3rd and 5th h at a lower dose of 5 mg/kg. Similarly, Viji and Helen (2008) have reported the anti-inflammatory activity of *Bacopa monniera* used for the treatment of inflammation as 75% and 82% at 3rd and 5th h inhibition, at a higher dose of 100 mg/kg.[21]

Figure 3.5: Sprague Dawley rats

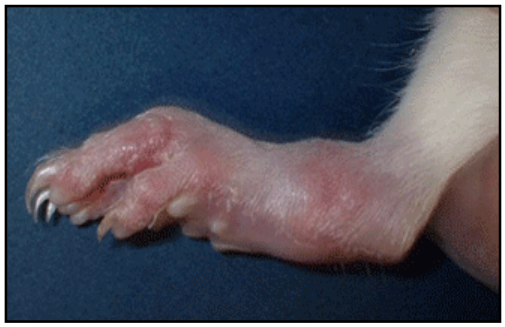

Figure 3.6: Photograph showing rat paw edema at 0 h after treating with Njavara rice bran extract (NBb)

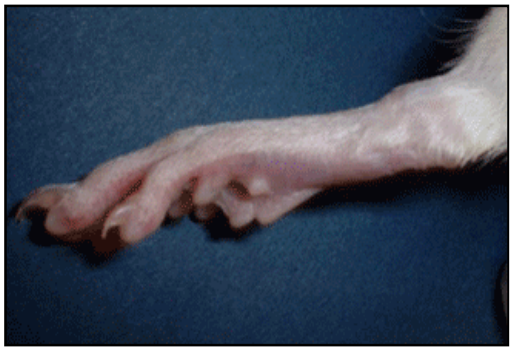

Figure 3.7: Photograph showing rat paw edema at 5^{th} h after treating with Njavara rice bran extract (NBb)

Table 3.2: Anti-inflammatory effects (*in vivo*) of the Njavara Black bran (NBb), Sujatha bran (SJb), Palakkadan Matta bran (PMb), Njavara Black rice (NBr), Sujatha rice (SJr), Palakkadan Matta rice (PMr) and diclofenac.

Treatment group	Dose	Inhibition (%)	
		3^{rd} h	5^{th} h
NBb	5 mg/kg	66.60 ± 1.22^{b}	83.30 ± 1.52^{a}
SJb	5 mg/kg	33.30 ± 0.61^{f}	33.30 ± 1.22^{d}
PMb	5 mg/kg	45.50 ± 0.83^{d}	53.80 ± 0.98^{c}
NBr	5 mg/kg	55.50 ± 1.00^{c}	66.69 ± 0.44^{b}
SJr	5 mg/kg	11.10 ± 0.20^{g}	16.60 ± 0.30^{e}
PMr	5 mg/kg	36.40 ± 0.66^{e}	53.80 ± 0.39^{c}
Diclofenac	20 mg/kg	72.00 ± 2.08^{a}	86.00 ± 2.48^{a}

[a-g]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 $P < 0.05$.

Very recently Yodmanee *et al.* (2011) [22] have published their results on colour, phenolic content and antioxidant properties of pigmented rice varieties grown in southern Thailand. The increase in number of research papers on rice recently, shows the importance of antioxidant, sources through regular diet. The present study reports on the antioxidant effects of Njavara rice bran and rice as compared with staple, pigmented (Palakkadan Matta) and white (Sujatha) varieties. Reports on antioxidant activity of rice extracts are very limited in the literature especially, on Njavara rice and as such, our study is the first report. In conclusion, we can say that NBb and NBr showed higher

chemical indices, antioxidant activity and anti-inflammatory effect compared to corresponding rice and bran of the staple varieties.

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CHAPTER 4

Phytochemical Investigations and Quantification of Bioactive Compounds of 'Njavara' as Compared to Staple Varieties

The studies based on the activity of methanolic extract as discussed in Chapter 3 confirmed that the active part of Njavara is its rice bran. Hence we undertook the phytochemical investigation (activity guided) of the methanolic extract and also the quantification of the bioactive compounds in comparison with staple varieties. Even though the petroleum extracts showed weak radical scavenging activity, there are reports of bioactive compounds like oryzanols, being present in the bran of rice varieties. Oryzanols are reported to have physiological effects of lowering plasma cholesterol, reducing platelet aggregation, decreasing hepatic cholesterol biosynthesis, increasing faecal bile acid excretion, decreasing cholesterol absorption, nerve imbalances, menopausal disorders and aortic fatty streak formation.[1] Hence the petroleum ether extracts of Njavara rice bran and rice were identified and analysed for oryzanols in comparison with staple varieties by analytical HPLC and quantification was also carried out.

4.1 Phytochemical investigation and quantification of bioactive compounds from petroleum ether extracts

4.1.1 Plant material and extraction

100 g lots of 'Njavara black' (NBb), 'Sujatha' (SJb) and 'Palakkadan Matta' (PMb) and rice of 'Njavara black' (NBr), 'Sujatha' (SJr) and 'Palakkadan Matta'(PMr) were collected from Njavara Eco farm . The rice bran was stabilized as explained in Chapter 2 section 2.1. The rice bran NBb, SJb and PMb and rice of NBr, SJr and PMr were extracted using petroleum ether and concentrated as explained in chapter 2 section 2.2. The petroleum ether extract residue of NBb (8.61 g), SJb (6.79 g) and PMb (5.52 g) rice bran and NBr (1.80 g), SJr (1.57 g) and PMr (1.98 g) rice were made up to a definite volume in chloroform solvent and stored in refrigerator until further analysis for oryzanol content.

4.1.2 Identification of oryzanols

For identification of oryzanols, reverse phase analytical HPLC with solvent system of acetonitrile, dichloromethane, and acetic acid (42:3:5, v/v/v): methanol, n-butyl alcohol, water (45:1:4, v/v/v) in the ratio of 75:25 (v/v), at a flow rate of 1ml/min was selected.[2] Samples of rice bran and rice were prepared in mobile phase with concemtration of 0.5 mg/ml. Figure 4.1 shows the HPLC profile of oryzanol in NBb. Similar profile were obtained for NBr, SJb, PMb, SJr and PMr. The identification of compound peaks were based on the congruence of retention times with standard oryzanol,[2] mass spectral (MS) analysis[3] and comparing with literature. Thus, the (M+1) peak and the peaks of major fragmentation of the compounds are:

Cycloartenyl ferulate (Compound **I**): (calculated for $C_{40}H_{59}O_4$ (M+1), 603.43) 603.61 (M+1), 409.37, 195.32, 177.26.

24-methylene cylcoartanyl ferulate (Compound **II**): (calculated for $C_{41}H_{61}O_4$ (M+1), 617.45) 617.64 (M+1), 439.12, 195.28, 177.26.

Campesteryl ferulate (Compound **III**): (calculated for $C_{38}H_{57}O_4$ (M+1), 577.42) 577.63 (M+1), 383.63, 195.28, 177.26.

β-sitosteryl ferulate (Compound **IV**): (calculated for $C_{39}H_{59}O_4$ (M+1), 591.43) 591.85 (M+1), 397.52,195.35, 177.30.

Cycloartanyl ferulate (Compound **V**): (calculated for $C_{40}H_{61}O_4$ (M+1), 605.45) 605.56 (M+1), 411.17, 195.28, 177.26.

The peaks marked as **I**, **II**, **III**, **IV** and **V** were identified as cycloartenyl ferulate, 24-methylene cylcoartanyl ferulate, campesteryl ferulate, β-sitosteryl ferulate and cycloartanyl ferulate respectively (Figure 4.1). The chemical structures of the compounds **I**, **II**, **III**, **IV** and **V** identified in NBb petroleum ether extract are shown in Figure 4.2.

4.1.3 Quatification of oryzanols in petroleum ether extract

The peaks corresponding to the compounds **I**, **II**, **III**, **IV** and **V** were quantified using standard 24-methylene cylcoartanyl ferulate. Standard solutions of 24-methylene cylcoartanyl ferulate were prepared with concentrations of 0.12, 0.14, 0.16, 0.18 and 0.2 µg /20 µl (injection volume) and run by HPLC. The standard solutions exhibited linear relationship between area response (Y) and concentration of sample injected (X), with the correlation coefficient (R^2) of 0.9982 and equation of calibration curve was Y =

23408500 X - 2469950. The quantification values of individual oryzanols and total oryzanol content are shown in Table 4.1. Among the oryzanols, the content of 24-methylene cylcoartanyl ferulate is 3 and 9 fold higher in NBb than in SJb and PMb. It is seen that the total oryzanol content is 2.7 and 7 times higher in NBb than in SJb and PMb, whereas in rice extracts of NBr, SJr and PMr, the values were less. Hence, the content of each of the oryzanols as well as the total oryzanols, are significantly higher in NBb (rice bran) compared to staple varieties and corresponding rice samples. There are only very few reports in the literature on oryzanol content of rice extracts. Our study is the first attempt to determine oryzanols in Njavara rice samples. Iqbal *et al.* (2005) have reported the oryzanol content to be 0.802 mg/g in rice bran 'Super kernel', whereas in NBb, it is 1.84 mg/g, the methodology being comparable in both cases.[4]

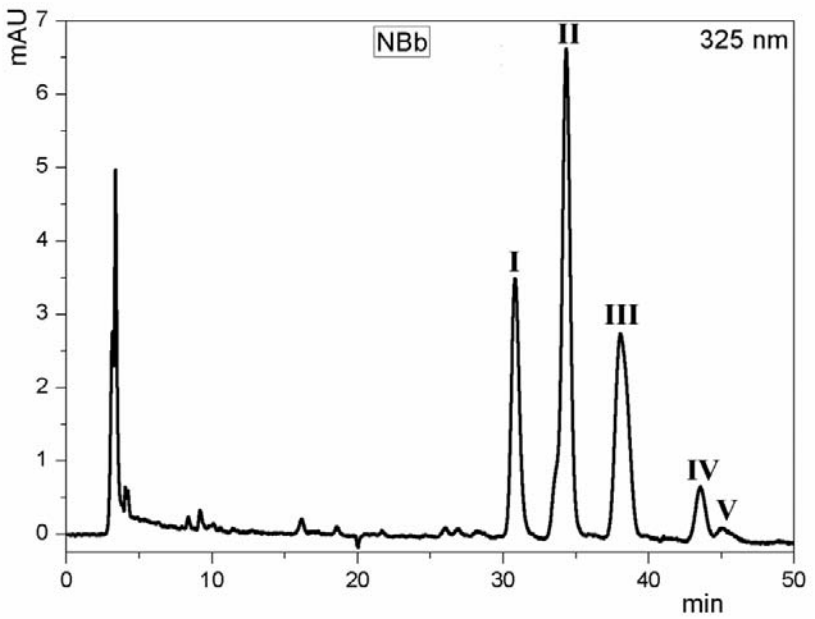


Figure 4.1 Reverse phase HPLC/PDA chromatographic profiles of oryzanols in NBb (Njavara 'black' rice bran) of petroleum ether extract. Peak identification: **I.** cycloartenyl ferulate; **II.** 24-methylene cycloartanyl ferulate; **III.** campesteryl ferulate; **IV.** β-sitosteryl ferulate; **V.** cycloartanyl ferulate.

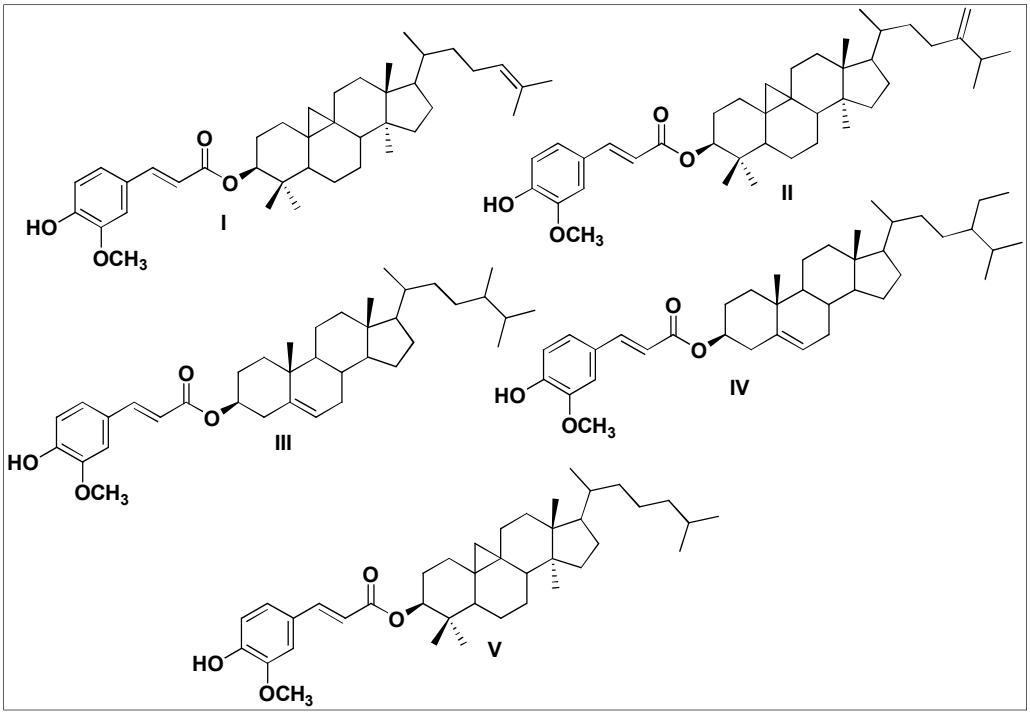


Figure 4.2: Chemical structures of the compounds **I.** cycloartenyl ferulate; **II.** 24-methylene cycloartanyl ferulate; **III.** campesteryl ferulate; **IV.** *β*-sitosteryl ferulate; **V.** cycloartanyl ferulate.

Kong and Lee (2010) have reported the oryzanol content of two black rice cultivars (Heugjinjubyeo and Heugkwangbyeo) as 0.07 mg/g and 0.03 mg/g which are lesser than NBr (0.15 mg/g).[5] Hence, the literature reports as well as the present study comparing Njavara with staple varieties, show that the total oryzanol content of Njavara is significantly higher.

TABLE 4.1: Oryzanols in NBb, SJb, PMb, NBr, SJr and PMr * of petroleum ether extract

Compounds	NBb#	SJb#	PMb#	NBr$	SJr$	PMr$
I	0.38 ± 0.02[a]	0.13 ± 0.01[b]	0.03 ± 0.00[c]	0.03 ± 0.00[c]	0.02± 0.00[d]	0.02±0.00[d]
II	0.91 ± 0.04[a]	0.30 ± 0.01[b]	0.10 ± 0.01[c]	0.05 ±0.00[d]	0.04±0.00[e]	0.05 ±0.00[d]
III	0.42 ± 0.01[a]	0.19 ± 0.01[b]	0.09 ± 0.00[c]	0.05 ±0.00[b]	0.02± 0.00[e]	0.05± 0.00[d]
IV	0.07 ± 0.00[a]	0.03 ± 0.00[c]	0.02 ± 0.00[d]	0.01± 0.00[f]	0.01± 0.00[e]	0.01± 0.00[e]
V	0.06 ± 0.00[a]	0.02 ± 0.00[b]	0.01 ± 0.00[c]	0.01 ± 0.00[c]	0.00 ± 0.00	0.01±0.00[c]
Total	1.84 ± 0.07[a]	0.67 ± 0.03[b]	0.25 ± 0.01[c]	0.15± 0.00[d]	0.09± 0.00[f]	0.14± 0.00[e]

*NBb: Njavara Black bran; SJb: Sujatha bran; PMb: Palakkadan Matta bran; NBr: Njavara Black rice; SJr: Sujatha rice; PMr: Palakkadan Matta rice.

mg/g dry weight of bran

$ mg/g (dry weight of rice)

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

4.2 Phytochemical investigation and quantification of bioactive compounds in methanolic extract of NBb

4.2.1 Plant material and extraction

The medicinal rice, 'Njavara black' (NBb) was collected from the 'ECO FARM' Karukamanikalam at Chittoor, Palakkad, Kerala as explained in Chapter 2, section 2.1. 'Activity-guided' fractionation and isolation of compounds was followed. Methanolic extract residue of NBb (16.77 g) from 250 g of bran obtained after Soxhlet extraction as explained in Chapter 2 was suspended in 500 ml water and partitioned with (5 x 250 ml) of diethyl ether. The diethyl ether extracts were combined and the removal of solvent in the rotary evaporator yielded 2.30 g of crude extract. The diethyl ether extract gave 91% DPPH radical scavenging activity at 1000 µg/ml. The extract showed 81% inhibition towards rat paw edema, in the *in vivo* anti-inflammatory study at 5 mg/kg dose. It also gave a positive shinoda test and green colour with $FeCl_3$ that indicated the presence of flavonoids. The higher antioxidant and anti-inflammatory effect of the diethyl ether extract made us to investigate further for the isolation of bioactive flavonoid compounds.

4.2.2 Isolation and characterization of major compounds from diethyl ether residue

The diethyl ether extract (2 g) was then subjected to careful column chromatography using silica gel (40 g, 100-200 mesh) starting with 100% hexane as eluent and thereafter gradually raising the polarity (hexane:ethyl acetate; 100:0 to 0:100) based on the separation observed, after examining the fractions, by TLC. A total of 118 fractions (A_1-A_{118}) of 40-50 ml each were collected. They were further pooled together according to similarities in TLC into twelve major fraction (B_1-B_{12}) pools. These 12 major fractions were tested for DPPH radical scavenging actity. Fraction B_3 (500 mg) which was DPPH inactive, UV inactive and iodine active appeared to be single spot in TLC analysis, was subjected to spectroscopic characterisation. The proton ^{1}H and ^{13}C analysis showed the fraction to be mixture of fatty acids. This fraction was further esterified for GCMS analysis. Upon GCMS analysis three major peaks were obtained (Figure 4.3). Identification of the components was achieved by comparing the retention time and the mass spectra of the samples with standard library available with

instrument.[6-9] Three major peaks at RT values 27.6, 31.208 and 31.52 were identified as esters of a) hexadecanoic acid, b) 10- octadecenoic acid and c) octadecanoic acid respectively at m/z 270, 296 and 298 (Figure 4.3). These compounds may be occurring as derived products from glycerides in rice bran.

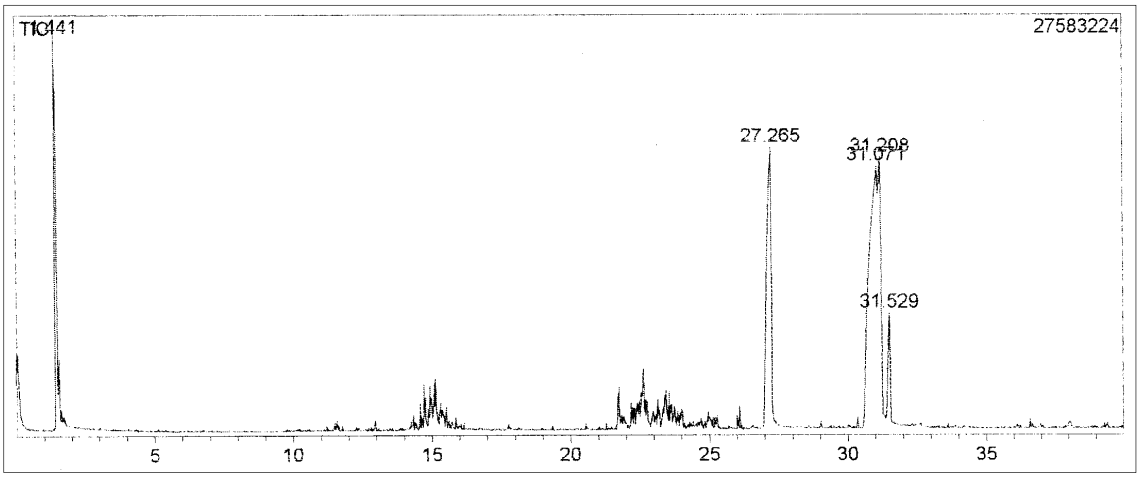


Figure 4.3: GCMS profile of the esters

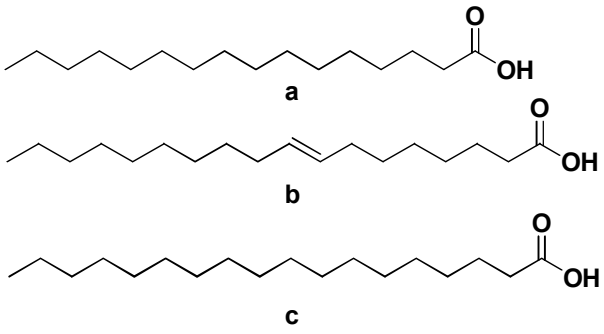


Figure 4.4: Structures of the fatty acids a) hexadecanoic acid, b) 10- octadecenoic acid and c) octadecanoic acid

Fraction B_{11} (300 mg), active towards DPPH, UV and iodine active was a polar mixtures based on TLC analysis, could not be further fractionated by silica column chromataograhy. This mixture was further chromatographed on a Sephadex LH-20 column by elution with methanol (MeOH) to give twenty fractions (C_1-C_{20}) fractions.

The C_{18} fraction gave a yellow powder which was purified by re-crystallization with MeOH to give pure compound **VI** (24 mg). It was analyzed by various spectroscopic techniques. Methanolic solution of compound **VI** responded positively towards Shinoda test and imparted an intense green colour with ferric chloride solution indicating that V**I** 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 4.5).

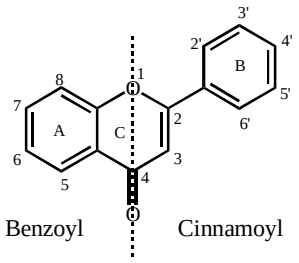


Figure 4.5: Skeleton of flavonoid

The UV spectra of flavonoids are readily distinguished from those of other flavanones in that they exhibit a low intensity band II absorption which often appears as a shoulder to band I peak.[10] The UV spectrum of **VI** (Figure 4.6a) was suggestive of a flavonoid with a flavonone skeleton from the high intensity band I peak (352 nm) and a shoulder band II peak (269 nm). Further, the bathochromic shift of band I with NaOMe without a decrease in intensity showed the presence of a free 4'-OH group while the bathochromic shift of band II with NaOAc, indicated the presence of a free 7-hydroxyl group. The bathochromic shift of band I in the presence of $AlCl_3$/HCl indicated the presence of a free 5-OH group (Figure 4.6b). To confirm the structure of **VI**, other spectroscopic techniques were employed. The IR spectrum showed absorptions arising from –OH and >C=O groups at 3313 cm^{-1} and 1654 cm^{-1} respectively. The ^1H NMR spectrum (Figure 4.7) showed a sharp singlet at 13.04 indicating the presence of the phenolic -OH group at C5 position.[10] This sharp peak is the result of the strong hydrogen bonding between the carbonyl group at the C4 position and the 5-OH group. Protons at the 6^{th} and 8^{th} positions appeared separately as doublets at 6.27 (J = 2.5 Hz) and 6.57 (J = 2.0 Hz) respectively. The H-6 doublet consistently occurred upfield than

the H-8 doublet. These signals are clearly distinguished from each other because of their widely different paramagnetic induced shifts.[10] The two proton singlet at 7.40 indicated the B-ring aromatic protons. The sharp singlet at 3.89 integrating for six protons clearly indicated the presence of two methoxy group in the B ring. The 2' and 6' protons appeared downfield due to the deshielding influence of C-ring functions on them. Thus, it could be concluded that the B-ring of compound V**I** is substituted but symmetrically. Singlet at 6.76 could be attributed to the proton at C3, adjacent to the carbonyl group. Furthermore, the ^{13}C NMR spectrum (Figure 4.8) clearly showed 13 carbon signals (exclusive of 3 additional symmetric carbons), including the characteristic signal of α, β unsaturated >C=O group at 183.1 and the others at 164.8, 163.4, 158.9, 149.1 (2C), 140.9, 122.3, 105.6 (2C), 105.2, 104.7, 99.7, 94.9, 56.9 (-OCH_3 x 2). The mass spectrum of compound **VI** (Figure 4.9) gave the molecular ion peak as the base peak at m/z 331 $[M+H]^+$.The melting point of the compound was found to be 267-268°C.

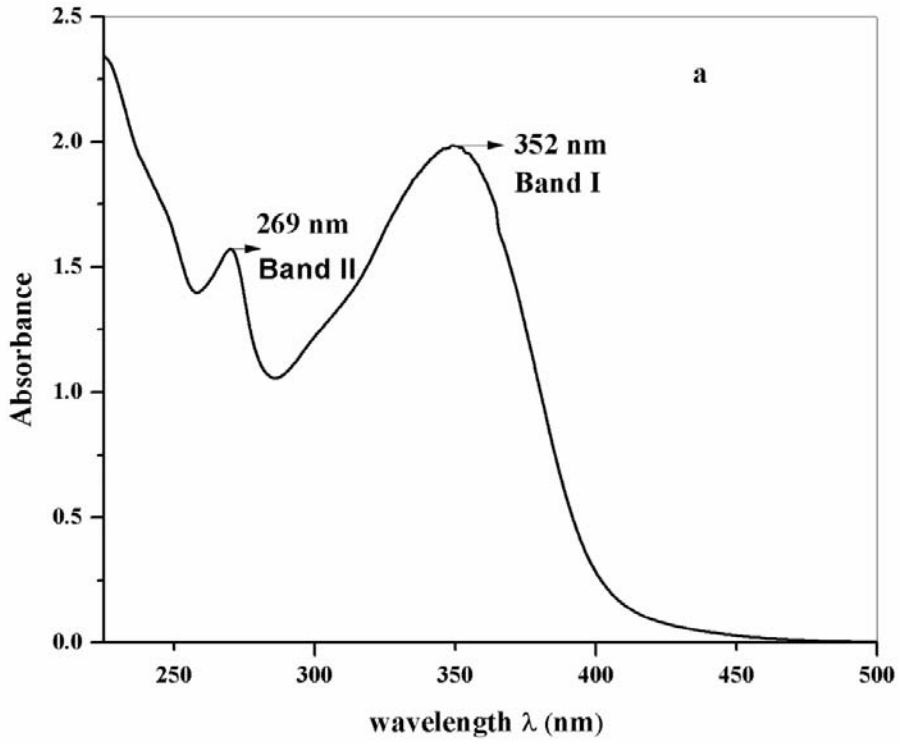


Figure 4.6a: UV spectra of compound **VI** in methanol

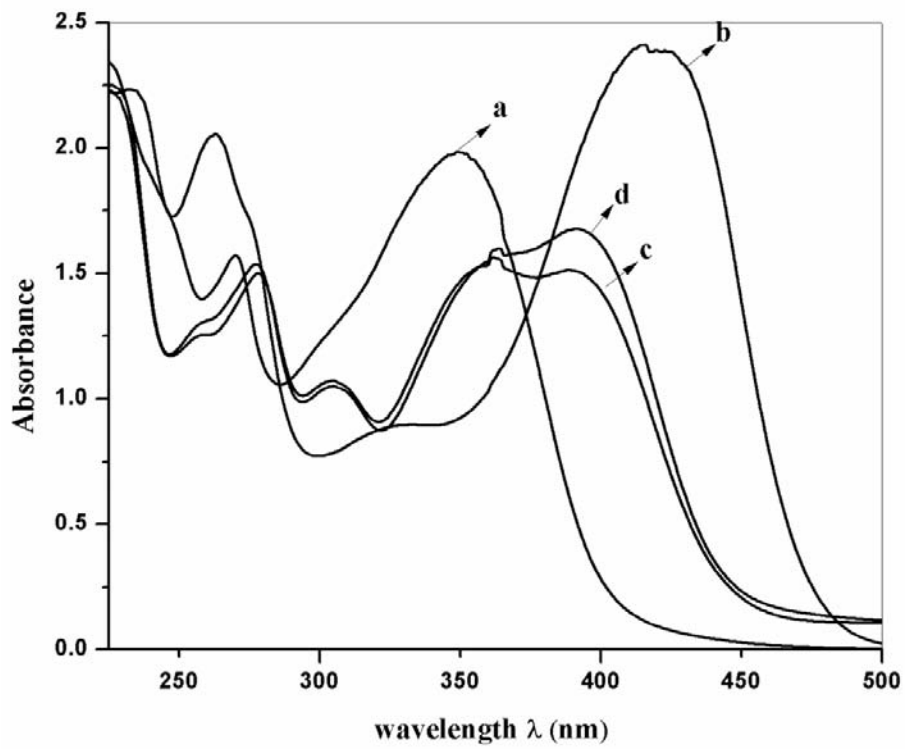


Figure 4.6b: UV spectra of compound **VI** a) Methanol b) Sodium methoxide c) Aluminium chloride/HCl d) Sodium acetate

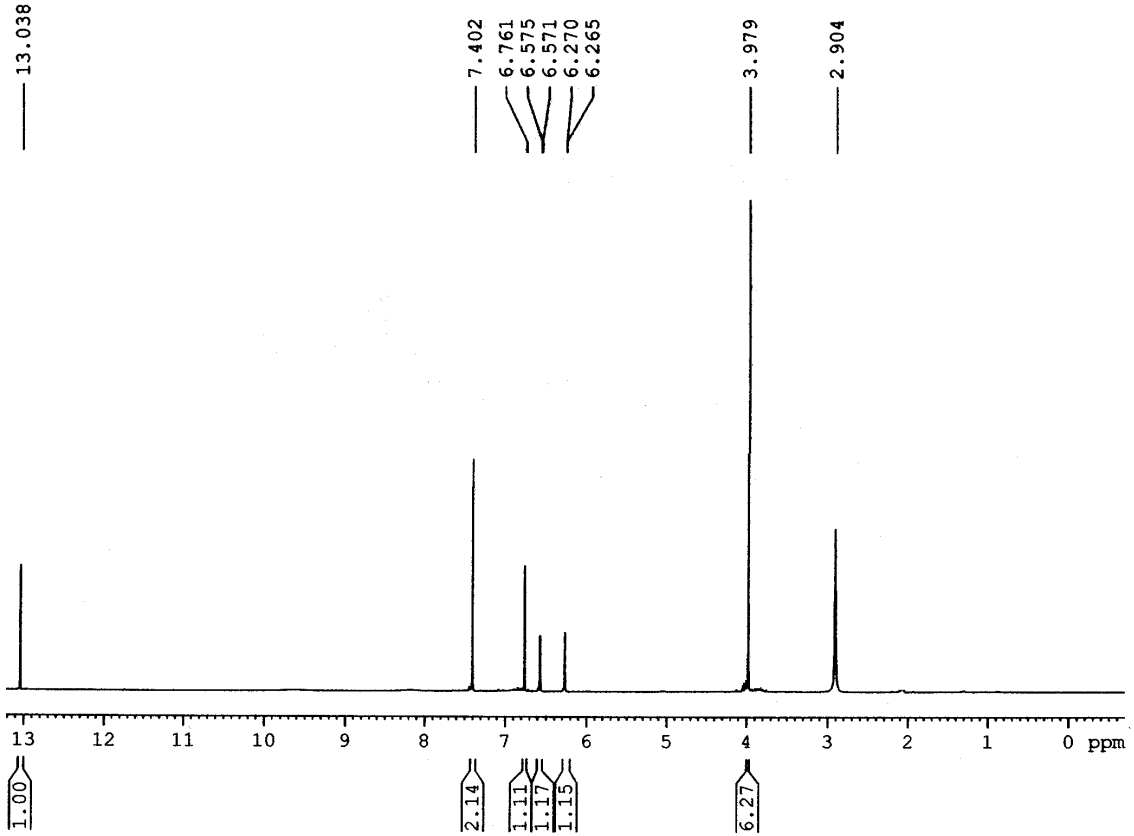


Figure 4.7: ^{1}H NMR spectrum of compound **VI**

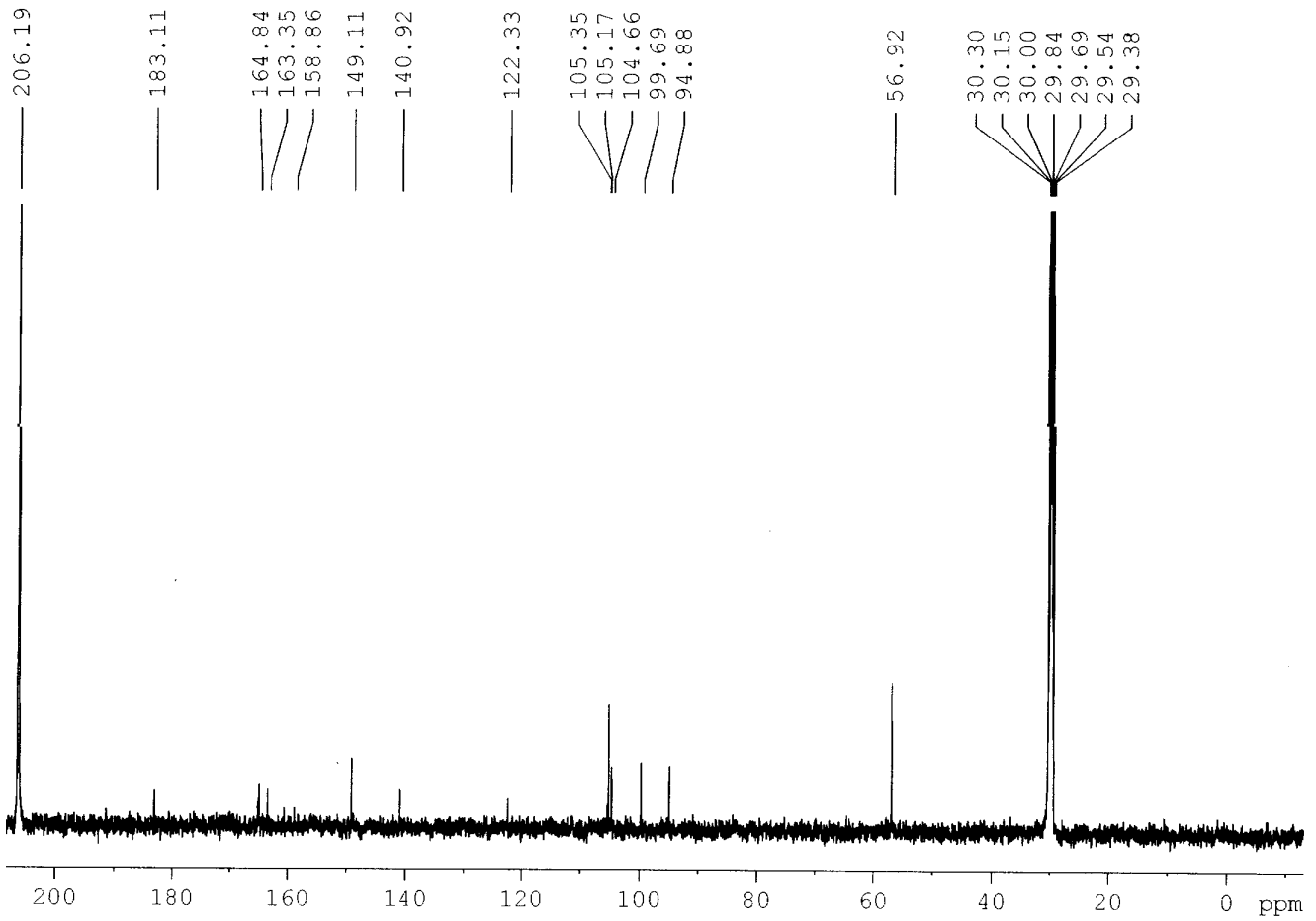


Figure 4.8: ^{13}C NMR spectrum of compound **VI**

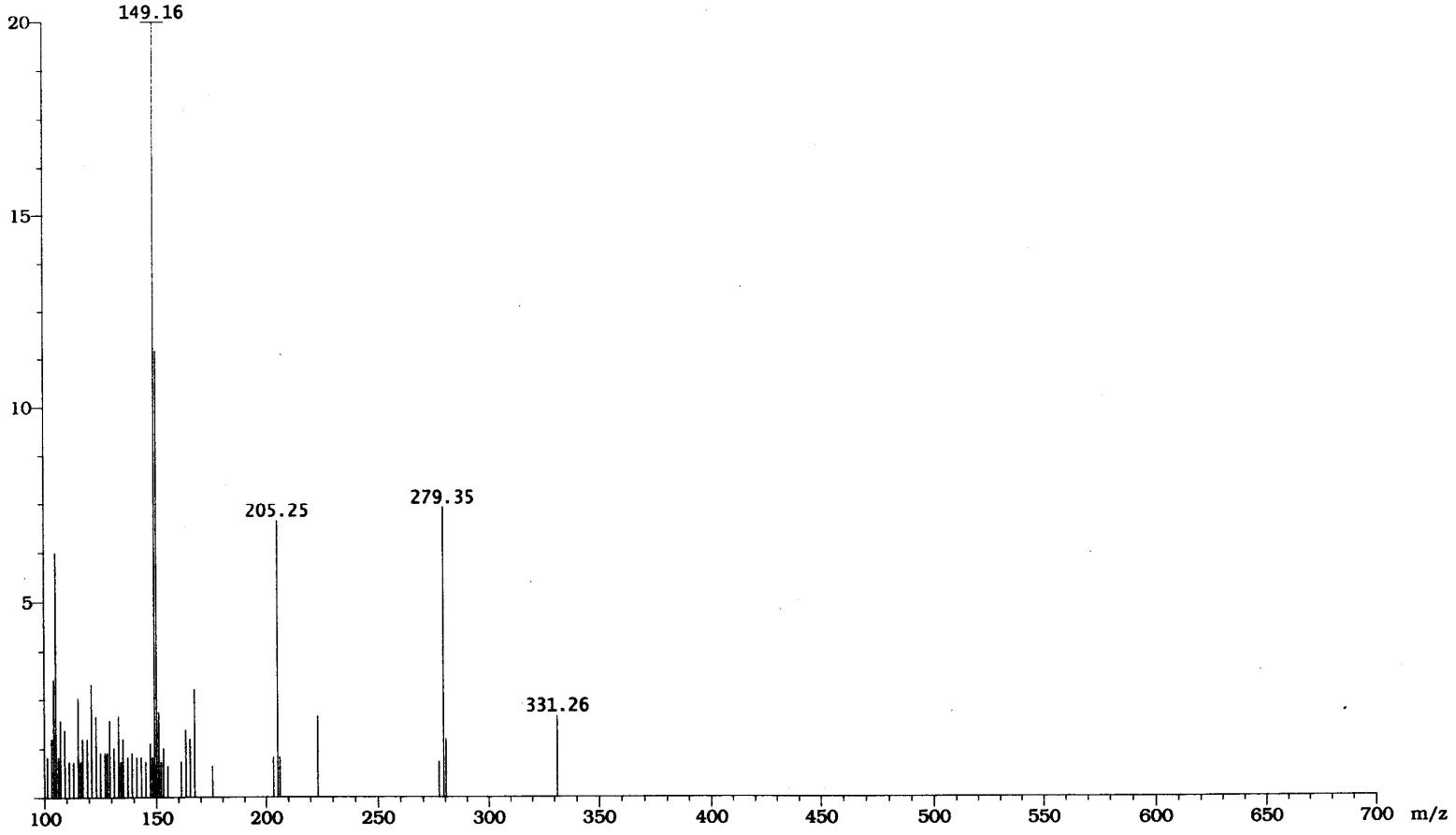


Figure 4.9: Mass spectrum of compound **VI**

From all the above spectral details and by comparing with the reported data,[12] compound **VI** was confirmed as 5, 7, 4'-trihydroxy-3', 5'-dimethoxyflavone known as tricin as shown below (Figure 4.10).

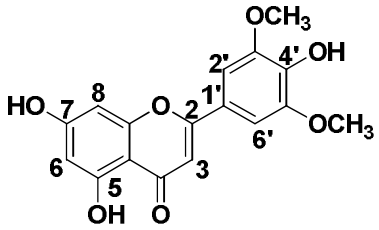


Figure 4.10: Structure of compound **VI**

Tricin (**VI**). Pale yellow crystal. m.p.°C: 267-268°C. UV: λ_{max} (MeOH) 245 (sh), 269, 305 (sh), 352; +NaOMe 262, 275 (sh), 323, 419; +$AlCl_3$/HCl 277, 305, 365, 389; +NaOAc 277, 306, 363 (sh), 394 IR: υ_{max} (KBr) 3313, 2928, 2851, 1654 cm^{-1}. HRFAB-MS: m/z 331.26, calcd for CHO $[M+H]^+$, 331.07). ^1H NMR (CD_3COCD_3): δ 13.04 (1H, s, 5-OH), 7.40 (2H, s, H-2' and 6'), 6.76 (1H, s, H-3), 6.57 (1H, d, *J* = 2 Hz, H-8), 6.27 (1H, d, *J* = 2.5 Hz, H-6), 3.99 (6H, s, -OCH_3 x 2). ^{13}C NMR (CD_3COCD_3): δ 183.1 (C-4), 164.8 (C-2), 163.4 (C-9), 158.9 (C-5), 149.1 (C-3', C-5'), 140.9 (C-4'), 122.3 (C-1'), 105.6 (C-2', C-6'), 105.2 (C-10), 104.7 (C-3), 99.7 (C-6), 94.9 (C-8), 56.9 (-OCH_3 x 2).

Tricin is widely distributed in Gramineae family. Tricin was first isolated in the free form from rust infected wheat (*Triticum dicoccum* L.) leaves.[11] It was later found to be typically distributed in grasses, sedges and palms,[12-14] including many important cereal crop plants, such as wheat, rice, barley, sorghum, oat and maize. In rice tricin is reported in leaves, rice husk and bran.[12,15]

The early steps of flavonoid biosynthesis[16] involve the stepwise addition of malonyl CoA (polyketide pathway) and p-coumaroyl CoA (phenylpropanoid pathway), mediated by the sequential action of chalcone synthase and chalcone isomerase, to give rise to naringenin chalcone and the flavanone, naringenin, respectively (Figure 4.11). B-

ring hydroxylation is introduced by both the flavanone 3'-hydroxylase (F3'H) and flavonoid 3, 5' hydroxylase (F3', 5'H) to give rise to their respective products, eriodictyol and 5'-hydroxyeriodictyol. Followed by the action of flavone synthase (FNS) that introduces a double bond between C-2 and C-3, and ultimately gives rise to tricetin. The later step in tricin biosynthesis involves the sequential O-methylation of tricetin to its 3'-monomethyl-(seigin) and 3', 5'-dimethyl-(tricin), with small amounts of 3', 4', 5'-trimethyl ether derivatives (Figure 4.11). The stepwise methylation of tricetin is catalyzed by flavone O-methyltransferase (TaOMT2).[17]

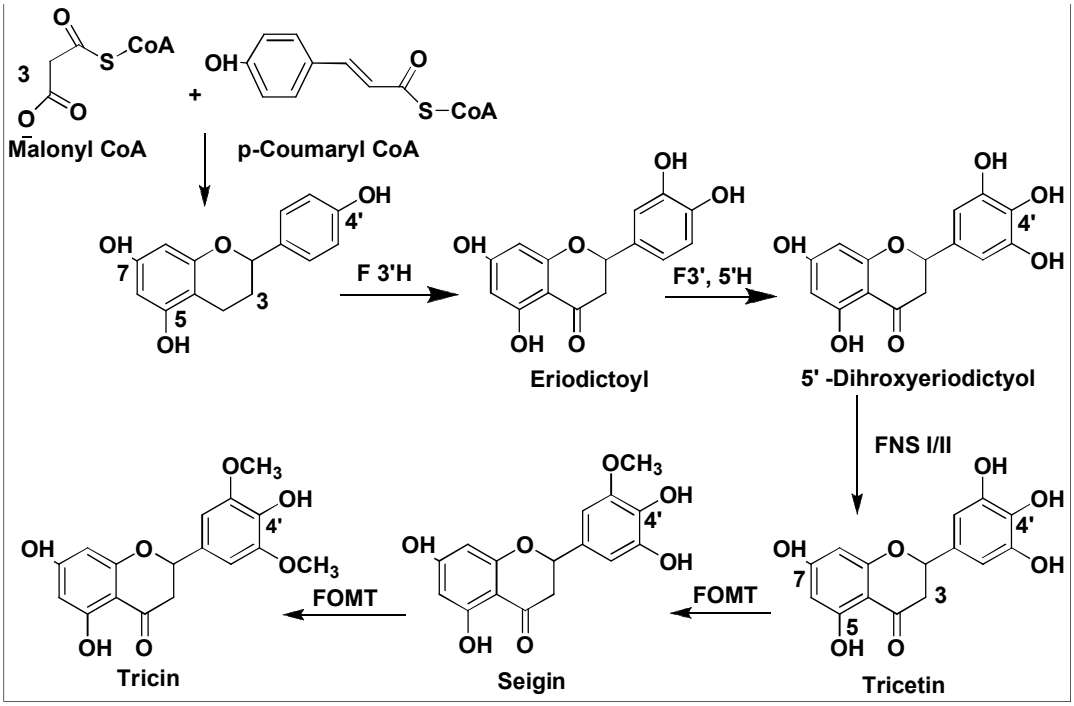


Figure 4.11: Proposed biosynthetic pathway of tricin, F 3'H- flavonoid 3'-hydroylase, F 3', 5' H- flavonoid 3', 5'-hydroxylases, FNS- flavone synthase, FOMT- flavone O-methyltransferase

The C_5-C_6 fraction was subjected to preparative HPLC on a 250 mm x 20 mm i.d., 5µm, YMS-Pack R&D ODS column (YMC Co., Ltd. Japan) with (28:72) CH_3CN:H_2O at a flow rate of 20 ml/min at 330 nm (λ_{max}) gave two fractions D_1 and D_2 (Figure 4.27 **a**). D_1 upon crystallisation in HPLC grade methanol gave compound **VII** (3.1 mg). Methanolic solution of compound **VII** also responded positively towards Shinoda test and imparted an intense green colour with ferric chloride solution

indicating that V**II** is a flavonoid derivative. The UV spectrum of V**II** (Figure 4.12) was suggestive of a flavonoid with a flavonone skeleton from the high intensity band I peak (335 nm) and a shoulder band II peak (272 nm). The bathochromic shift of band II with NaOAc and band I in the presence of $AlCl_3$/HCl indicated the presence of a free 7-OH and 5-OH group, as observed in tricin. Absorptions in IR spectrum at 3367 cm^{-1} and 1646 cm^{-1} confirmed –OH and >C=O groups respectively. The bathochromic shift with a decrease in intensity to band I on addition of NaOH indicated the absence of a free 4'-OH group and indicated a substituent at 4'-OH (Figure 4.12). The ^{1}H NMR spectrum (Figure 4.13) showed a sharp singlet at 12.78 confirmed the presence of the phenolic free 5-OH group. Protons at the 6th and 8th positions appeared separately as doublets at 6.15 (J = 2 Hz) and 6.45 (J = 2.0 Hz) respectively. The two proton singlet at 7.29 indicated the B-ring aromatic protons. The sharp singlet at 3.88 integrating for six protons clearly indicated the presence of two methoxy group in the B ring. Singlet at 6.70 could be attributed to the two protons at C3, adjacent to the carbonyl group. This pattern of ^{1}H NMR spectrum indicated the presence of tricin moiety, which was confirmed by a peak at m/z 331 in mass spectra. Furthermore, the signal at 183.1 in ^{13}C NMR spectrum (Figure 4.14) clearly confirmed the characteristic signal of α, β unsaturated >C=O group in the tricin moiety. One proton doublet of doublet (J = 8, 2 Hz) at 6.74, one proton doublet (J = 8 Hz) at 6.66, one proton singlet at 6.94 and three proton singlet at 3.71 indicated the presence of a guaicyl moiety. One proton doublet at 4.90, multiplet at 4.25-4.22 integrating for one proton and two protons doublet of doublet at 3.78 (J = 12, 5.5 Hz) and 3.41 (J = 12, 3.5 Hz) indicated the presence of glyceryl moiety. Furthermore, the ^{13}C NMR spectrum (Figure 4.14) clearly showed 24 carbon signals (exclusive of 3 symmetrical carbon atoms), including the characteristic signal of α, β unsaturated >C=O group at 183.1 and the others at 165.4, 164.6, 163.3, 158.9 , 154.6 (2C), 148.0, 146.6, 140.1, 133.7, 127.6, 120.1, 115.3, 111.0, 106.0, 105.3, 105.1 (2C), 99.9, 95.0, 88.1, 73.6, 61.2, 57.0 (2 –OCH_3), 56.3 (–OCH_3). DEPT-135 spectrum confirmed the presence of only one –CH_2-OH in the glyceryl moiety and the remaining two are >CH-OH connected to tricin and guaicyl moiety (Figure 4.15). The Figure 4.16 shows the ^{1}H -^{13}C correlation by HMQC and is depicted in Table 4.2. The mass spectrum of compound **VII** (Fig. 4.17) gave the molecular ion peak as the base peak, at m/z 527 $[M+H]^+$.

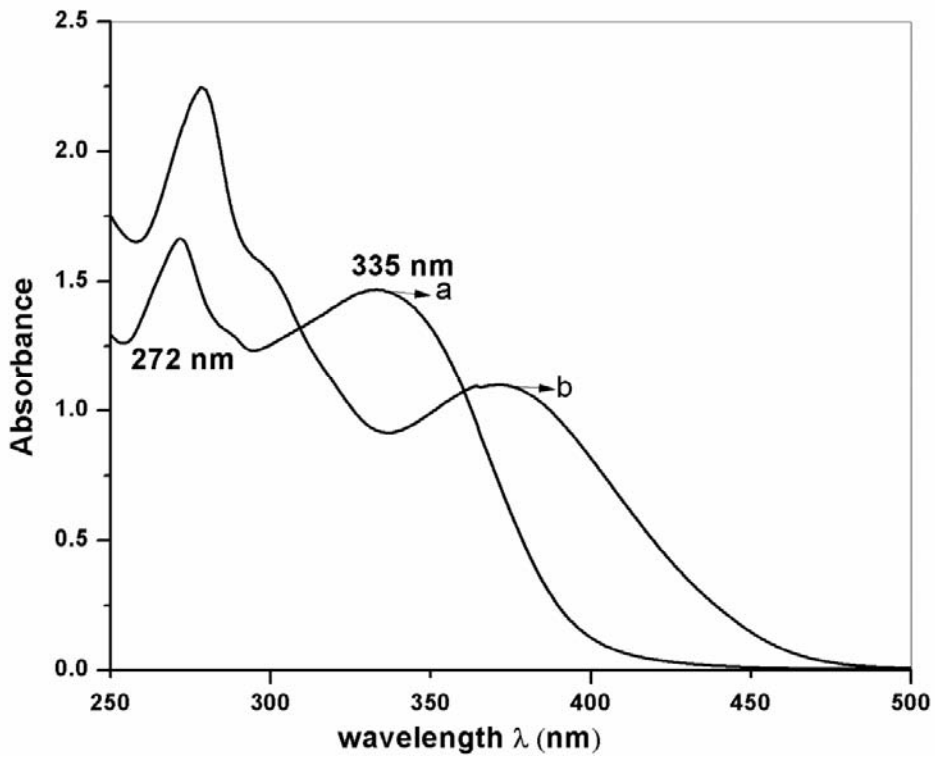


Figure 4.12: UV spectra of compound **VII** a) methanol b) NaOH

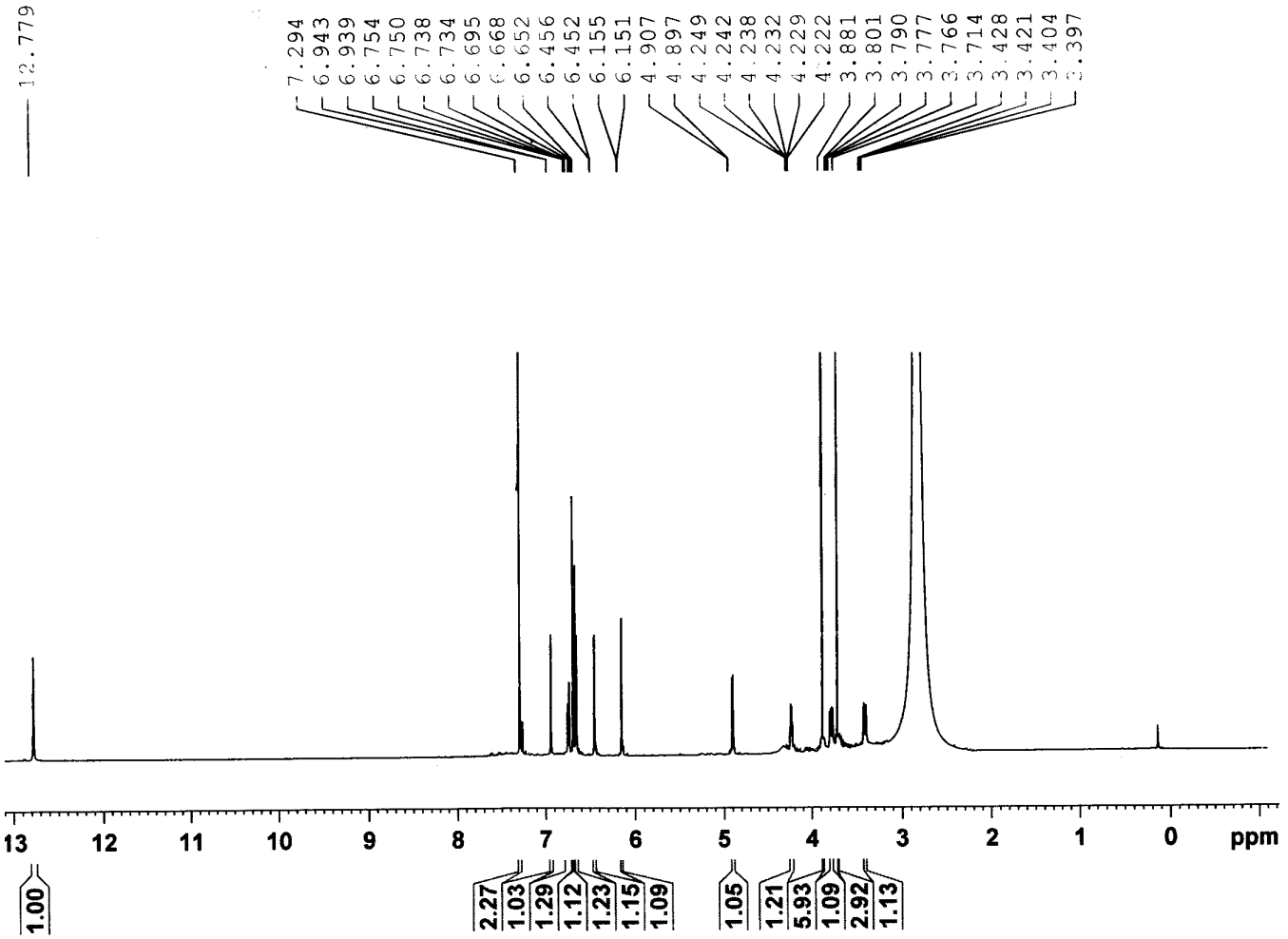


Figure 4.13: ^{1}H NMR spectrum of compound **VII**

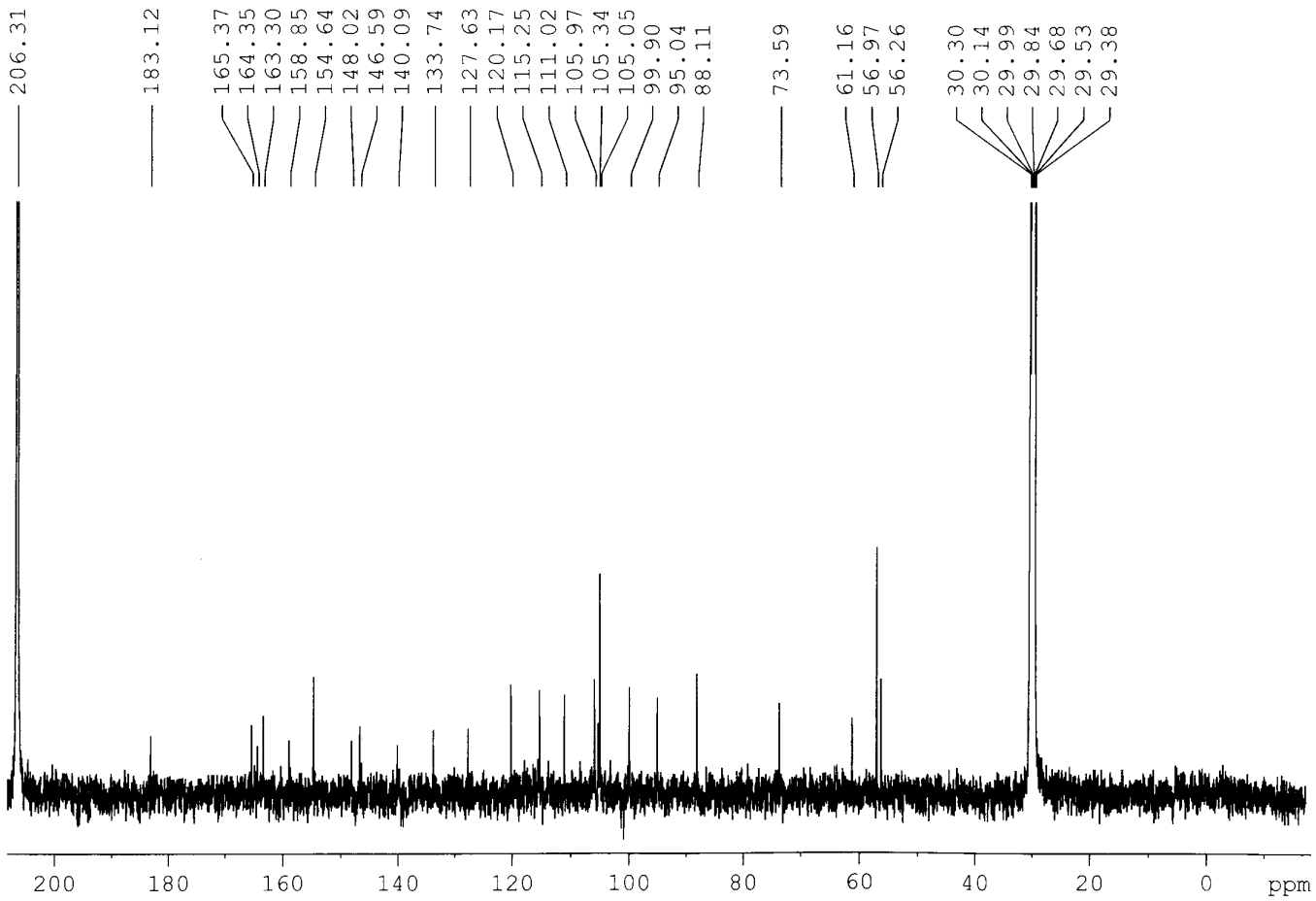


Figure 4.14: ^{13}C NMR spectrum of compound **VII**

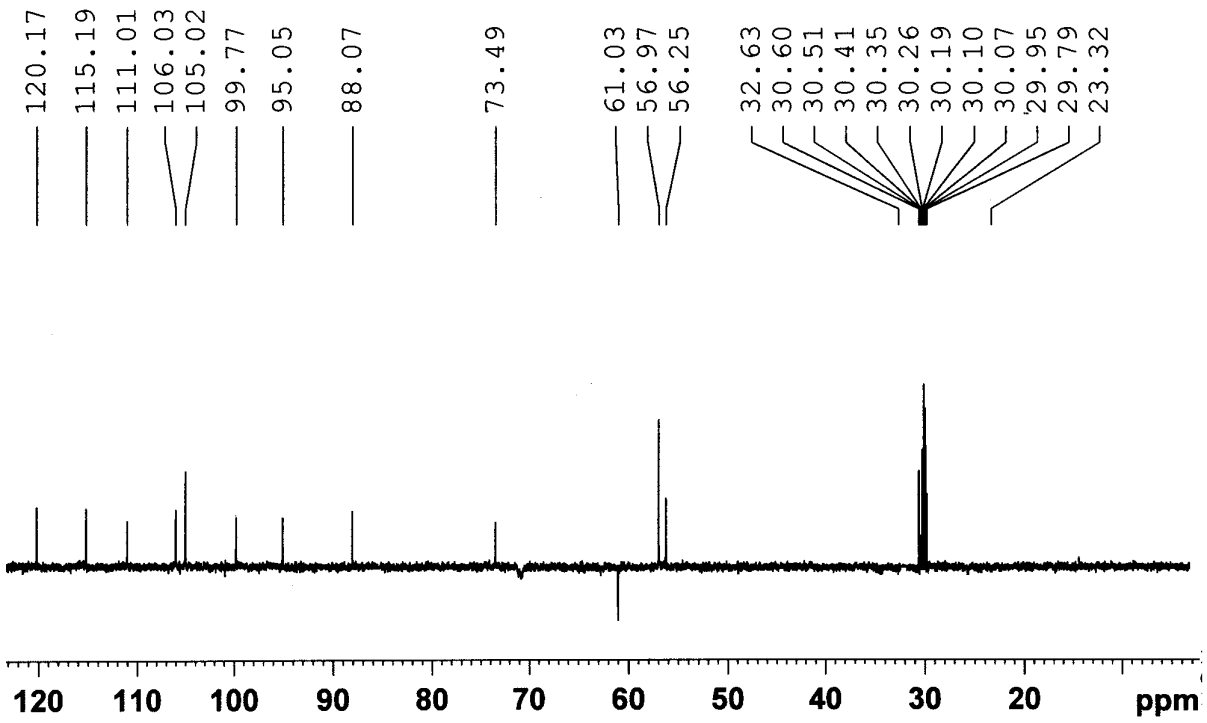


Figure 4.15: DEPT-135 spectrum of compound **VII**

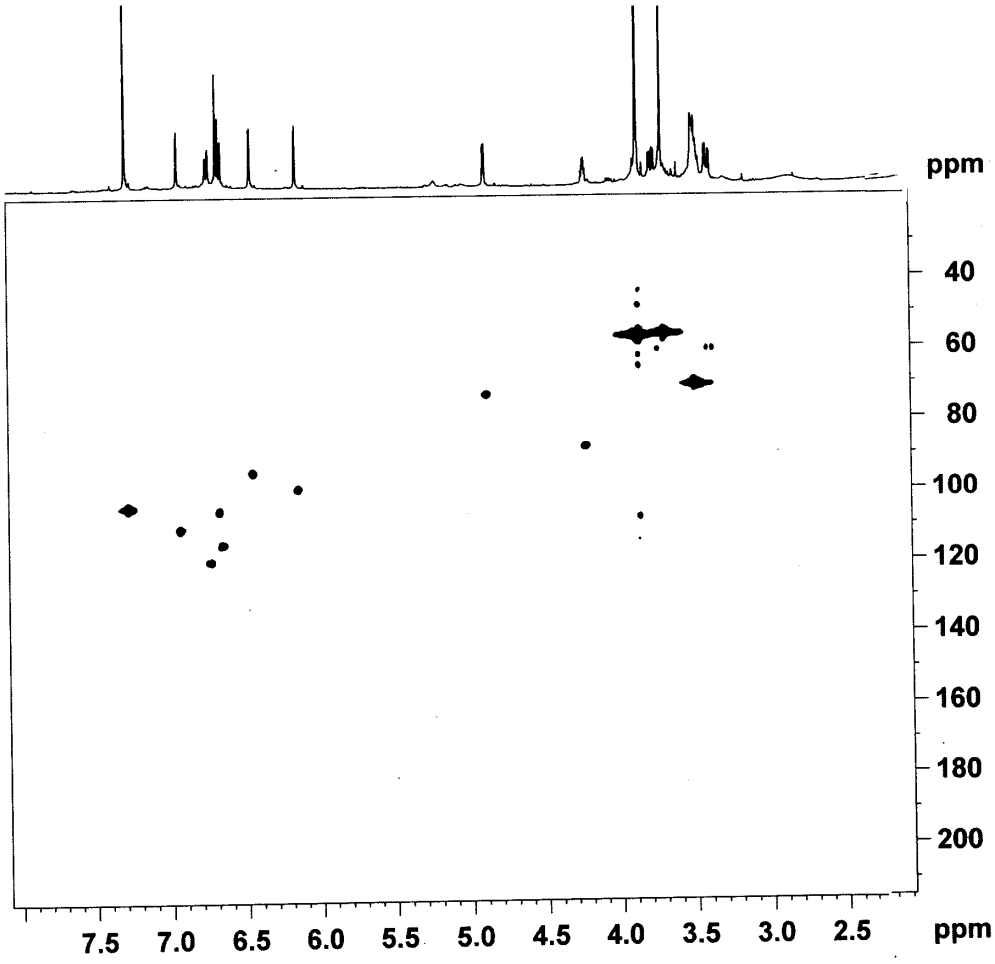


Figure 4.16: HMQC spectrum of compound **VII**

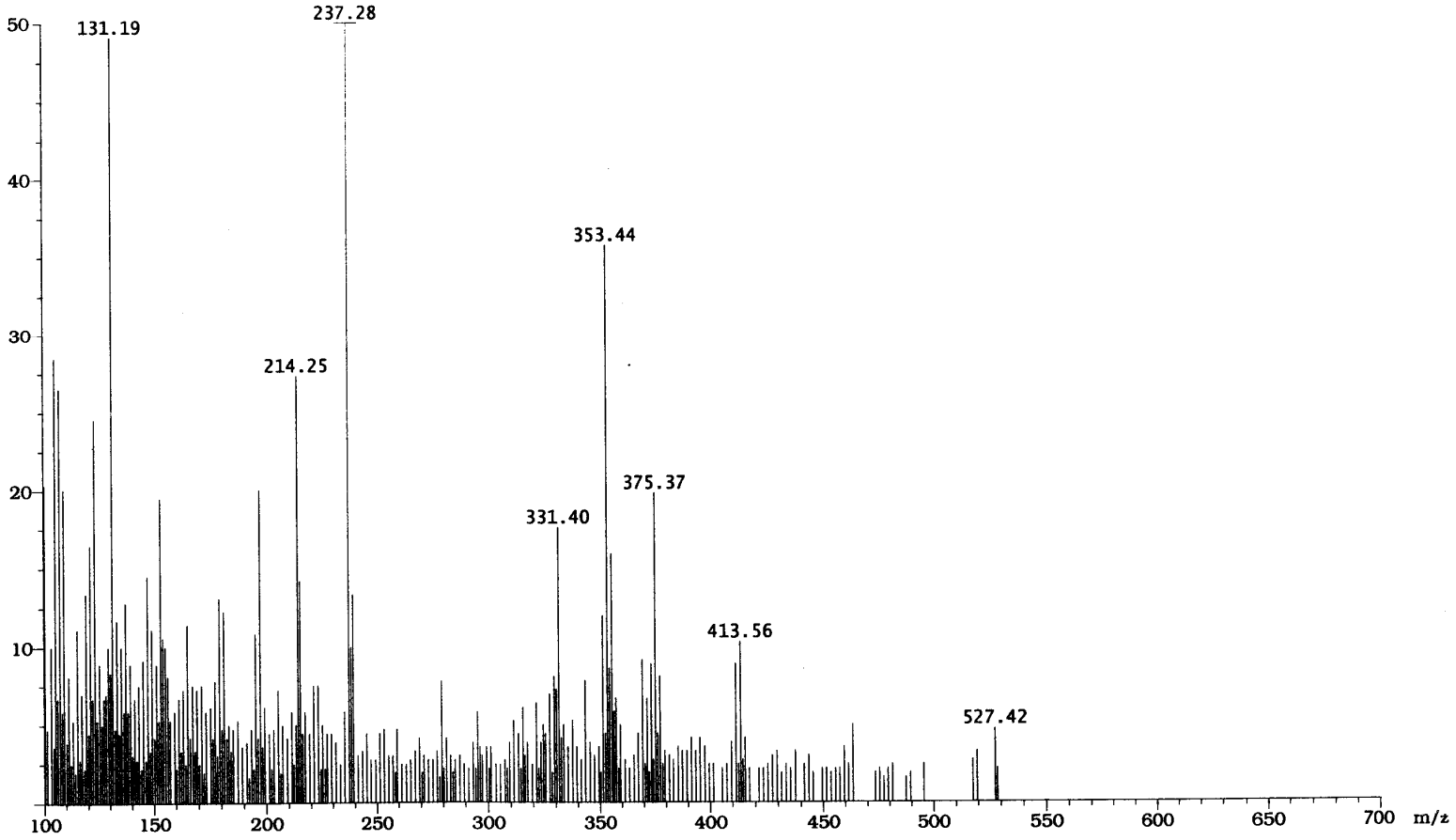


Figure 4.17: Mass spectrum of compound **VII**

Table 4.2: ^{1}H and ^{13}C correlation assignment of chemical shift by HMQC

Atom position	^{1}H (*J*)	^{13}C
3	6.70 *s*	106.0
6	6.15 *d* (2)	99.9
8	6.45 *d* (2)	95.0
2', 6'	7.29 *s*	105.1
3', 5' –OCH_3	3.88 *s*	57.0
15	6.94 *d* (2)	111.0
18	6.66 *d* (8)	115.3
19	6.74 *dd* (8, 2)	120.1
13	4.90 *d* (5)	73.6
12	4.23 *m*	88.1
11	3.78 *dd* (12, 5.5)	61.2
	3.41*dd* (12, 3.5)	
16-OCH_3	3.71 *s*	56.3

Stereoisomerism arises due to two chiral centres at positions 12 and 13. The proton at 13th position give rise to the coupling constant *J* = 5 Hz (Table 4.2) and comparing with literature, the above data confirms the compound **VII** as *erythro* form.[18] From all the above spectral details and by comparing with the reported data[18-20] compound **VII** was confirmed as tricin-4'-O-(*erythro-β*-guaiacylglyceryl)ether as shown below (Figure 4.18).

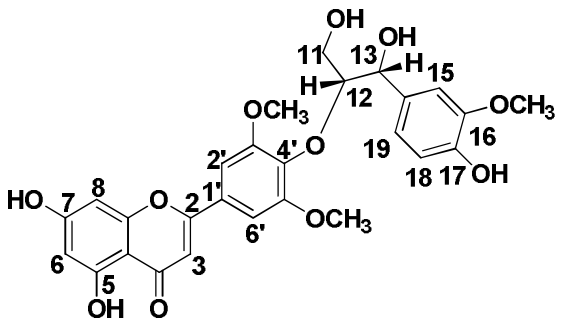


Figure 4.18: Structure of compound **VII**

Tricin-4'-O-(*erythro-β*-guaiacylglyceryl) ether (Compound **VII**). Yellow amorphous powder. UV: λ_{max} (MeOH) 272, 288 sh, 305 sh, 335; +NaOH 278, 298 sh, 371 (decreased intensity); +$AlCl_3$ and HCl 280, 303, 345, 394 sh; +NaOAc 278, 312 sh, 367. [ ]$_D^{26}$ +14.8° (c about 0.05, MeOH). IR: υ_{max} (KBr) 3367, 2921, 2851, 1646, 1610, 1591 cm^{-1}. HR FAB-MS: m/z 527.42 calcd for CHO $[M+H]^+$, 527.15. ^{1}H NMR (CD_3COCD_3): δ12.78 (1H, s, 5-OH), 7.29 (2H, s, H-2', 6'), 6.94 (1H, d, *J* = 2 Hz, H-15), 6.74 (1H, dd, *J* = 8, 2 Hz, H-19), 6.70 (1H, s, H-3), 6.66 (1H, d, *J* = 8 Hz, H-18), 6.45 (1H, d, *J* = 2 Hz, H-8), 6.15 (1H, d, *J* = 2 Hz, H-6), 4.90 (1H, d, *J* = 5 Hz, H-13), 4.23 (1H, m, H-12), 3.88 (6H, s, 3', 5'-OCH_3), 3.78 (1H, dd, *J* = 12, 5.5 Hz, 11-CH_2), 3.71 (3H, s, H-16 -OCH_3), 3.41(1H, dd, *J* = 12, 3.5 Hz, 11-CH_2). ^{13}C NMR (CD_3COCD_3): δ183.1 (C-4), 165.4 (C-7), 164.6 (C-2), 163.3 (C-5), 158.9 (C-9), 154.6 (C-3', 5'), 148.0 (C-16), 146.6 (C-17), 140.1 (C-4'), 133.7(C-14), 127.6 (C-1'), 120.1 (C-19), 115.3 (C-18), 111.0 (C-15), 106.0 (C-3), 105.3 (C-10), 105.1 (C-2', 6'), 99.9 (C-6), 95.0 (C-8), 88.1 (C-12), 73.6 (C-13), 61.2 (C-11), 57.0 (C-3', 5'–OCH_3), 56.3 (C-16 –OCH_3).

D_2 fraction upon crystallisation in HPLC grade methanol gave compound **VIII** (3 mg). Methanolic solution of compound **VIII** also responded positively towards Shinoda test and imparted an intense green colour with ferric chloride solution indicating that V**III** is a flavonoid derivative. The UV spectrum of compound V**III** (Figure 4.19) in methanol and shift reagents gave almost similar UV pattern and λ_{max} as that compound **VII** with high intensity band I peak (334 nm) and a shoulder band II peak (272 nm). The IR spectrum showed absorptions arising from –OH and >C=O groups at 3390 cm^{-1} and 1651 cm^{-1} respectively. The ^{1}H NMR spectrum (Figure 4.20) showed a sharp singlet at 12.78 indicating the presence of the phenolic 5-OH group. Protons at the 6th and 8th positions appeared doublet at 6.15 (*J* = 1 Hz) and singlet at 6.46 respectively. The two proton singlet at 7.29 indicated the B-ring aromatic protons. The sharp singlet at 3.90 integrating for six protons clearly indicated the presence of two methoxy group in the B ring. Singlet at 6.68 could be attributed to one proton at C3, adjacent to the carbonyl group. This pattern of ^{1}H NMR spectrum indicated the presence of tricin moiety, which was confirmed by a peak at m/z 331 in mass spectra. Furthermore, the signal at 183.2 in ^{13}C NMR spectrum (Fig 4.21) clearly confirmed the characteristic signal of *α*, β unsaturated >C=O group in the tricin moiety.

One proton doublet of doublet (J = 8.3, 1.8 Hz) at 6.80, one proton doublet (J = 8.5 Hz) at 6.65, one proton doublet at 6.95 and three proton singlet at 3.70 indicated the presence of guaicyl moiety. One proton doublet at 4.92, multiplet at 4.04 integrating for one proton and two protons doublet of doublet at 3.62 (J = 12.3, 3.3 Hz) and 3.27 (J = 12, 2.3 Hz) indicated the presence of glyceryl moiety. Furthermore, the ^{13}C NMR spectrum (Fig 4.21) clearly showed 24 carbon signals (excluding 3 symmetric carbon atoms), including the characteristic signal of α, β unsaturated >C=O group at 183.2 and the others at 165.3,164.2, 163.3, 158.8, 154.3 (2C), 148.0, 146.8, 140.5, 133.6, 127.7, 120.6, 115.2, 111.4, 106.0, 105.1, 105.0 (2C), 99.9, 95.1, 89.8, 73.9, 61.6, 57.0 (2 $-OCH_3$), 56.2 (16 $-OCH_3$). DEPT-135 spectrum confirmed the presence of only one $-CH_2$-OH in the glyceryl moiety and the remaining two are >CH-OH connected to tricin and guaicyl moiety (Figure 4.22). The Figure 4.23 shows the ^1H -^{13}C correlation by HMQC and is depicted in Table 4.3.The mass spectrum of compound **VIII** (Fig. 4.24) gave the molecular ion peak as the base peak at m/z 527 $[M+H]^+$.

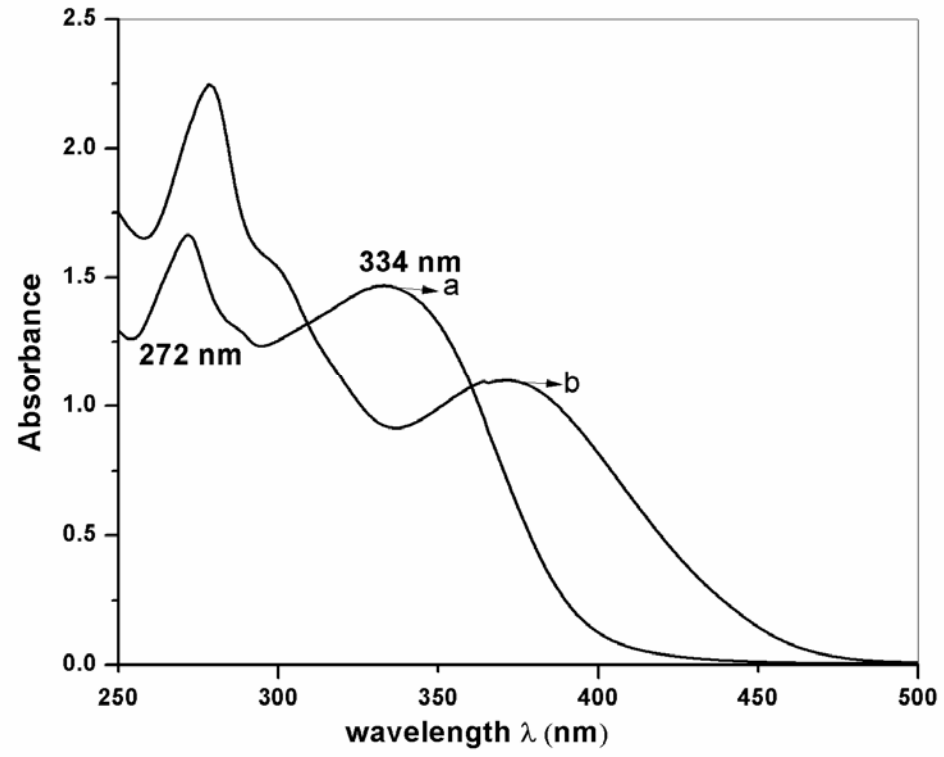


Figure 4.19: UV spectra of compound **VIII in** a) methanol b) sodium hydroxide

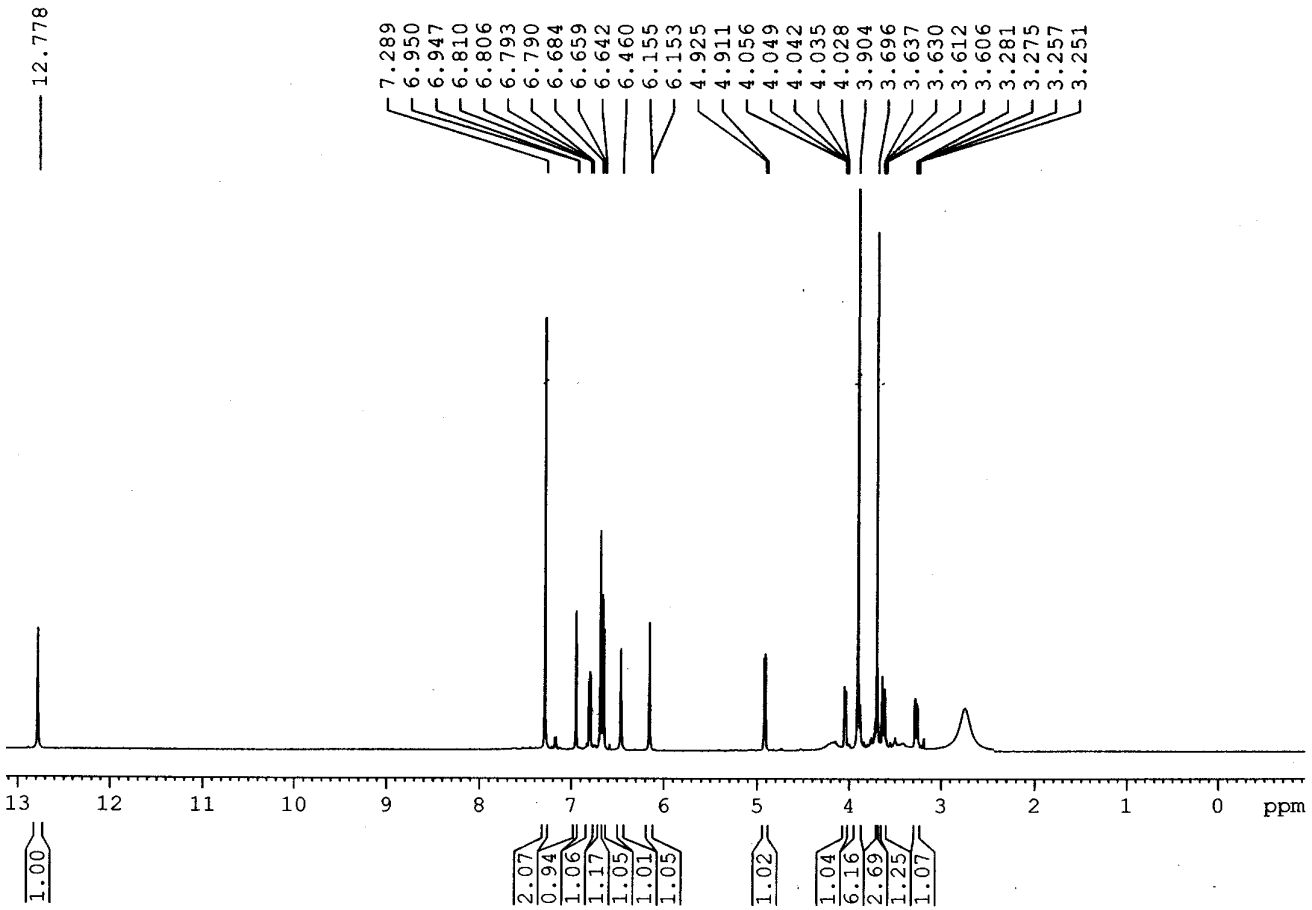


Figure 4.20: ^{1}H NMR spectrum of compound **VIII**

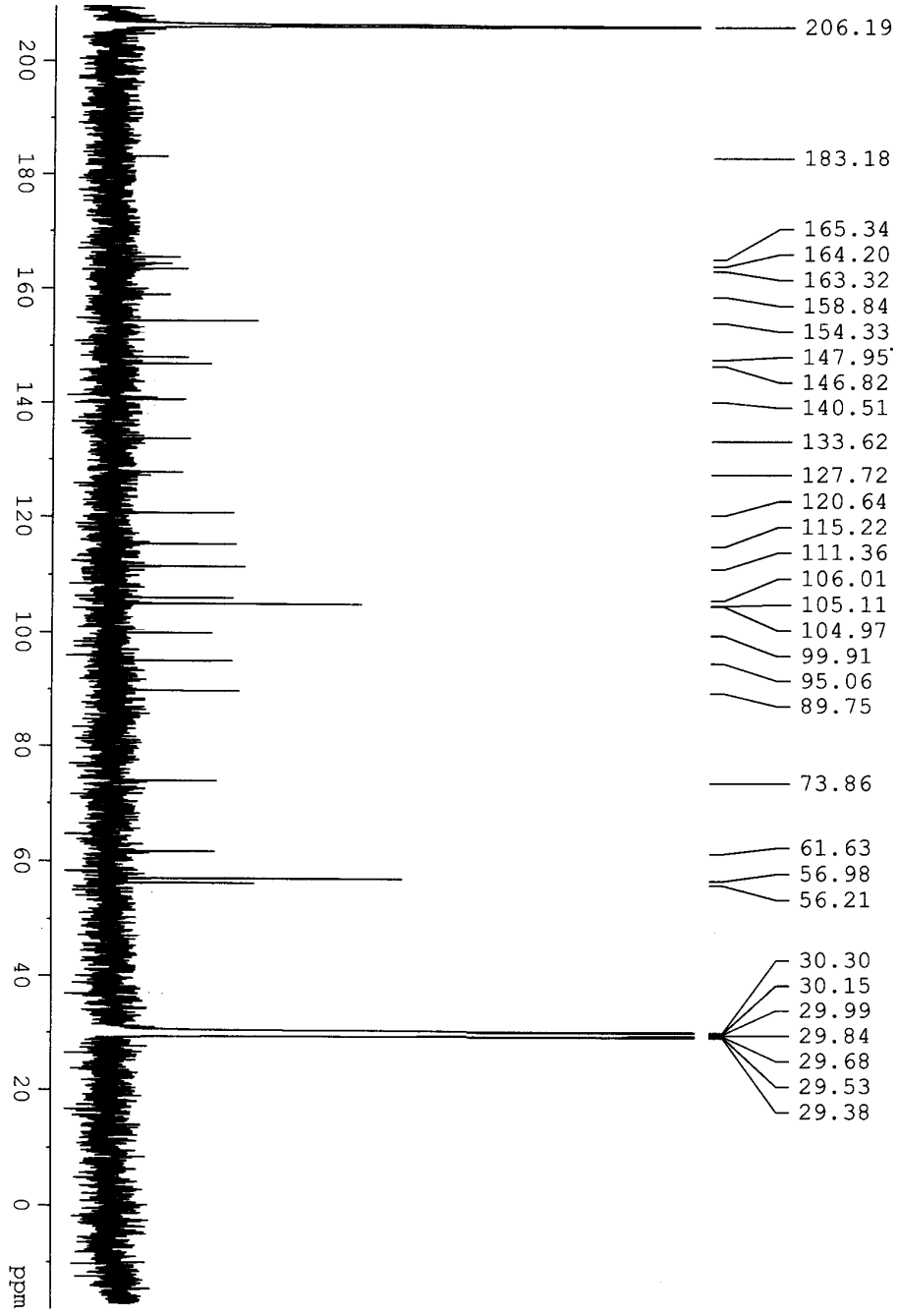


Figure 4.21: ^{13}C NMR spectrum of compound **VIII**

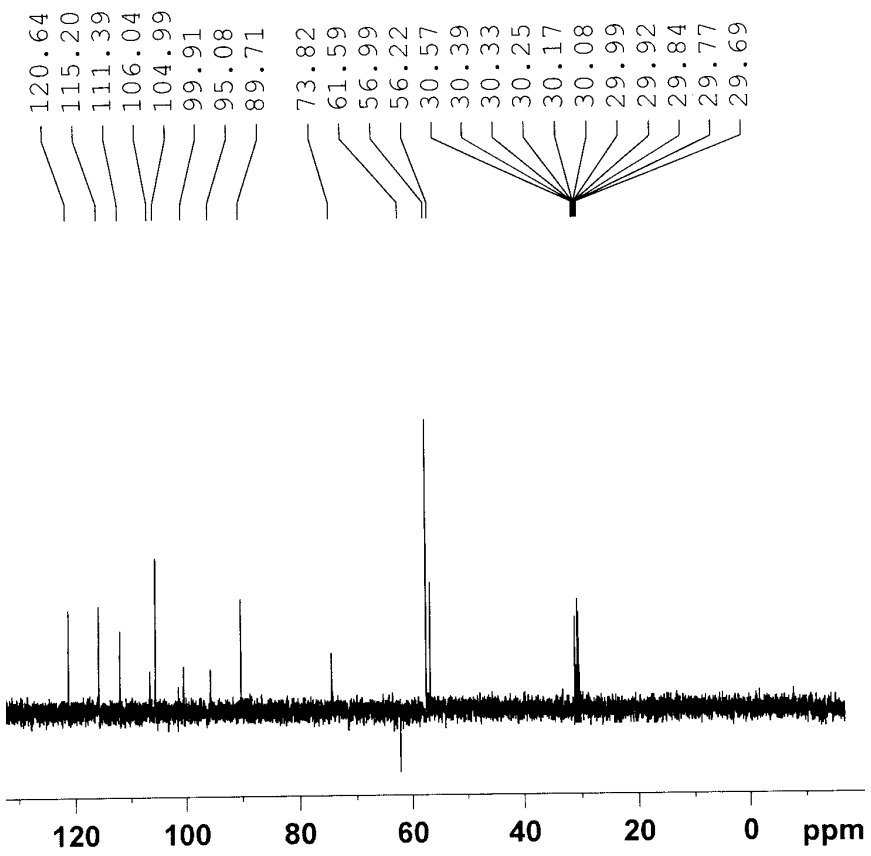


Figure 4.22: DEPT-135 spectrum of compound **VIII**

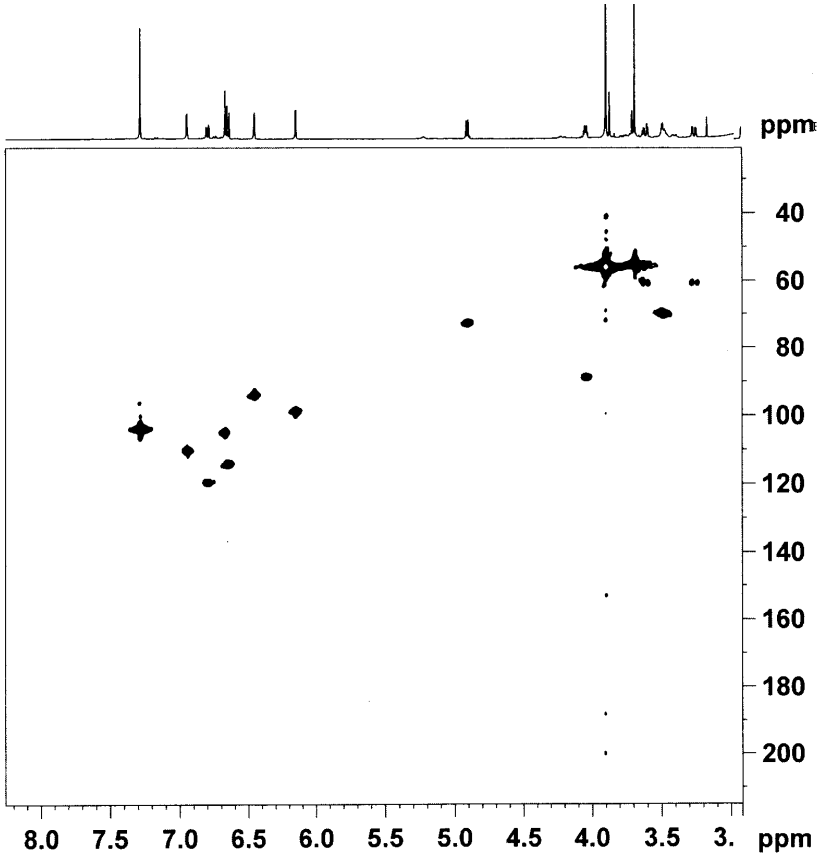


Figure 4.23: HMQC spectrum of compound **VIII**

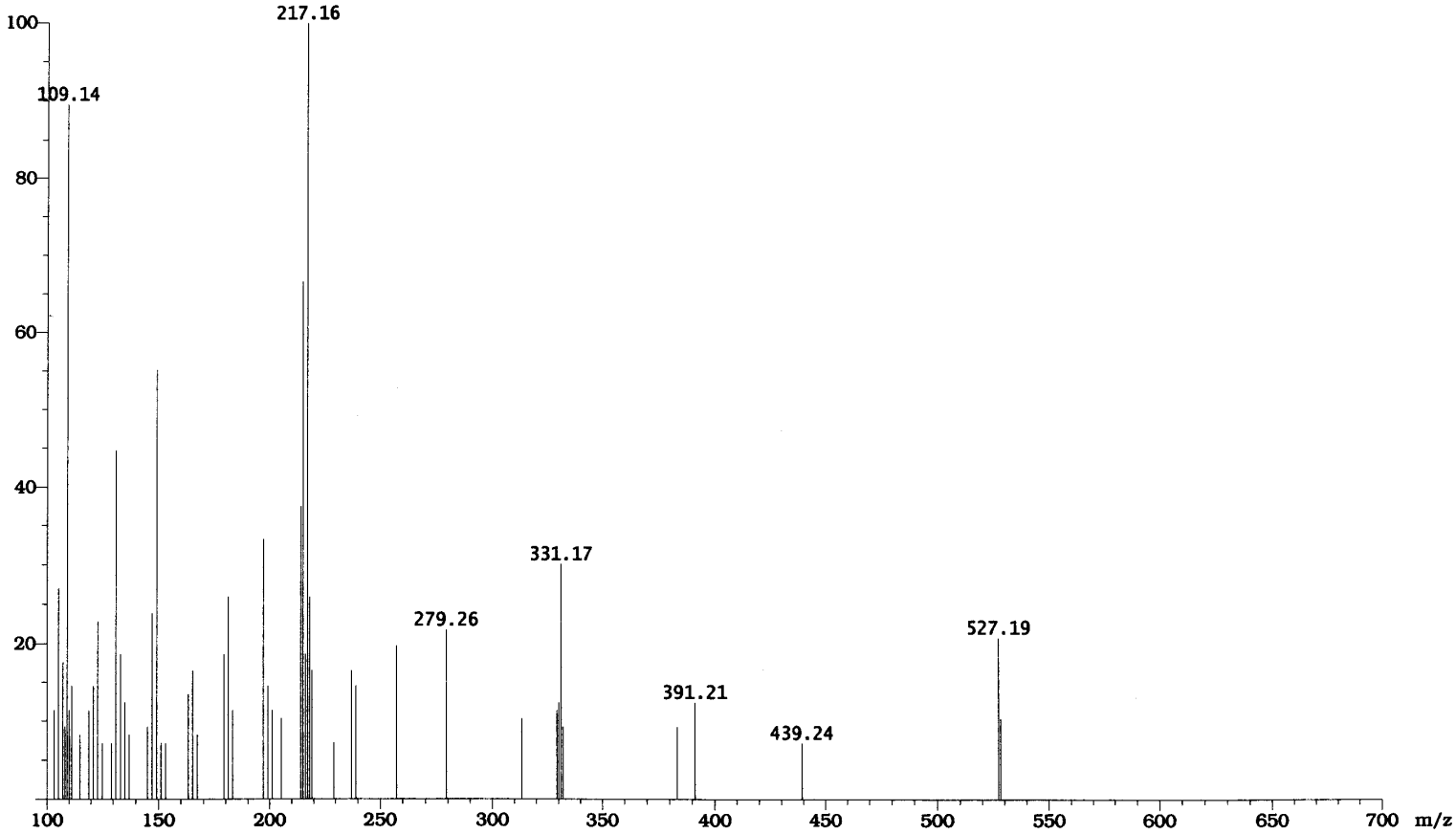


Figure 4.24: Mass spectrum of compound **VIII**

Table 4.3: ^{1}H and ^{13}C correlation assignment of chemical shift by HMQC

Atom position	^{1}H (*J*)	^{13}C
3	6.68 *s*	106.0
6	6.15 *d* (1)	99.9
8	6.46 *s*	95.1
2', 6'	7.29 *s*	105.0
3', 5' –OCH_3	3.90 *s*	57.0
15	6.95 *d* (1.5)	111.4
18	6.65 *d* (8)	115.2
19	6.80 *dd* (8.3,1.8)	120.6
13	4.92 *d* (7)	73.9
12	4.04 *m*	89.8
11	3.62 *dd* (12.3, 3.3)	61.6
	3.27*dd* (12, 2.3)	
16-OCH_3	3.71 *s*	56.2

From all the above spectral details and by comparing with the reported data, compound **VIII** was confirmed as stereoisomer of compound **VII**, the coupling constant of the proton at 13th position gave *J* = 7 Hz (Table 4.3), confirmed compound **VIII** is *threo* form of compound **VII**, thus the compound is tricin-4'-O-(*threo-β*-guaiacylglyceryl) ether as shown below (Figure 4.25).[18]

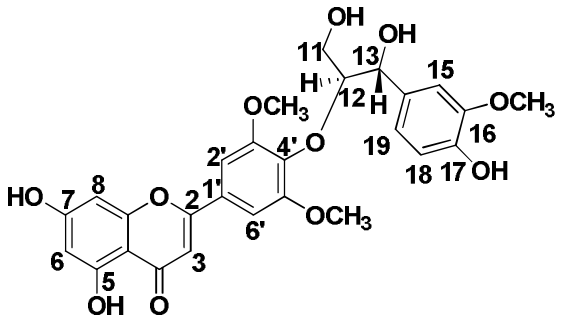


Figure 4.25: Structure of compound **VIII**

Tricin-4'-O-(*threo-β*-guaiacylglyceryl) ether (**VIII)**. Yellow amorphous powder. UV: λ_{max} (MeOH) 272, 287 sh, 300 sh, 334; +NaOH 278, 298 sh, 370 (decreased intensity, stable); +$AlCl_3$ and HCl 280, 303, 347, 392 sh; +NaOAc 278, 312 sh, 367. $[\ ]_D^{26}$ -10° (c about 0.05, MeOH). IR: υ_{max} (KBr) 3390, 2920, 2851, 1651, 1613, 1590 cm^{-1}. HRFAB-MS: m/z 527.19, calcd. for CHO $[M+H]^+$, 527.15. ^1H NMR (CD_3COCD_3): δ12.78 (1H, s, 5-OH), 7.29 (2H, s, H-2', 6'), 6.95 (1H, d, *J* = 1.5 Hz, H-15), 6.80 (1H, dd, *J* = 8.3, 1.8 Hz, H-19), 6.68 (1H, s, H-3), 6.65 (1H, d, *J* = 8.5 Hz, H-18), 6.46 (1H, br s, H-8), 6.15 (1H, d, *J* = 1 Hz, H-6), 4.92 (1H, d, *J* = 7 Hz, H-13), 4.04 (1H, m, H-12), 3.90 (6H, s, 3', 5'-OCH_3), 3.70 (3H, s, H-16-OCH_3), 3.62 (1H, dd, *J* = 12.3, 3.3 Hz, 11-CH_2), 3.27 (1H, *J* = 12, 2.3 Hz, 11-CH_2). ^{13}C NMR (CD_3COCD_3): δ 183.2 (C-4),165.3 (C-7),164.2 (C-2), 163.3 (C-5), 158.8 (C-9), 154.3 (C-3', 5'), 148.0 (C-16), 146.8 (C-17), 140.5 (C-4'), 133.6 (C-14), 127.7 (C-1'), 120.6 (C-19), 115.2 (C-18), 111.4 (C-15), 106.0 (C-3), 105.1 (C-10), 105.0 (C-2', 6'), 99.9 (C-6), 95.1 (C-8), 89.8 (C-12), 73.9 (C-13), 61.6 (C-11), 57.0 (C-3', 5'–OCH_3), 56.2 (16 –OCH_3).

The compounds **VII** and **VIII** belong to non-conventional category of lignans as flavonolignans, the molecular backbone of which is rather unusual with a phenyl propanoid unit linked to the 'A' ring of a flavonoid moiety[21] and have a rare occurrence in the species. These were first isolated from the aerial parts of *Salsola collina* L. (Chenopodiaceae).[22] Later, these compounds were isolated from Poaceae family from *Hyparrhenia hirta* (L.) Stapf, *Sasa veitchii* (Carr.) Rehder and *Avena sativa* L[18,20] Tricin is previously reported in rice bran[15] but the rare flavonolignans (compound **VII** and **VIII**) are first time identified and reported in Njavara and in the *Oryza sativa* species.

4.2.3 DPPH radical scavenging activity of diethyl ether extract of methanolic extract of Njavara and staple varieties

Methanolic extract residue of NBb (6.71 g), SJb (3.91g) and PMb (5.59 g) were obtained from 100 g lots of bran after Soxhlet exraction as explained in Chapter 2 section 2.2. Each residue were suspended in 200 ml water and partitioned with (100 x 5) ml of diethyl ether separately. The diethyl ether residue gave a yield of 934.23 ± 7.47 mg/100g, (390.32 ± 3.12 mg/100g) and (844.07 ± 6.75 mg/100g) from NBb, SJb and PMb bran respectively. The diethyl ether extracts were evaluated for DPPH radical scavenging activity. [DPPH**˙**], a stable free radical with a characteristic absorption at 517 nm, was used to study the radical scavenging effects of extracts. As antioxidants

donate protons to this radical, the absorption decreases. The decrease in absorption is taken as a measure of the extent of radical scavenging. At each concentration of diethyl ether extract of NBb, SJb and PMb, graph was plotted with time versus % $[DPPH^{\cdot}]_{REM}$ for 30 min (Figure 4.26 a, c and e). This graphic representation showed the behaviour of each antioxidant compound. From this the amount of diethyl ether extract required to decrease the $[DPPH^{\cdot}]$ concentration by 50% is obtained (Figure 4.26b, d and f). The EC_{50} values were taken as a measure of antiradical activity. Lower the EC_{50}, higher the antioxidant potential. The EC_{50} values of the three extracts were in the order NBb (259.83 µg/ml) < PMb (340.26 µg/ml) < SJb (457.36 µg/ml). Lower the EC_{50} value of NBb showed that it had significantly higher radical scavenging activity compared to SJb and PMb. Hence diethyl ether extract of NBb was further taken for phytochemical investigation.

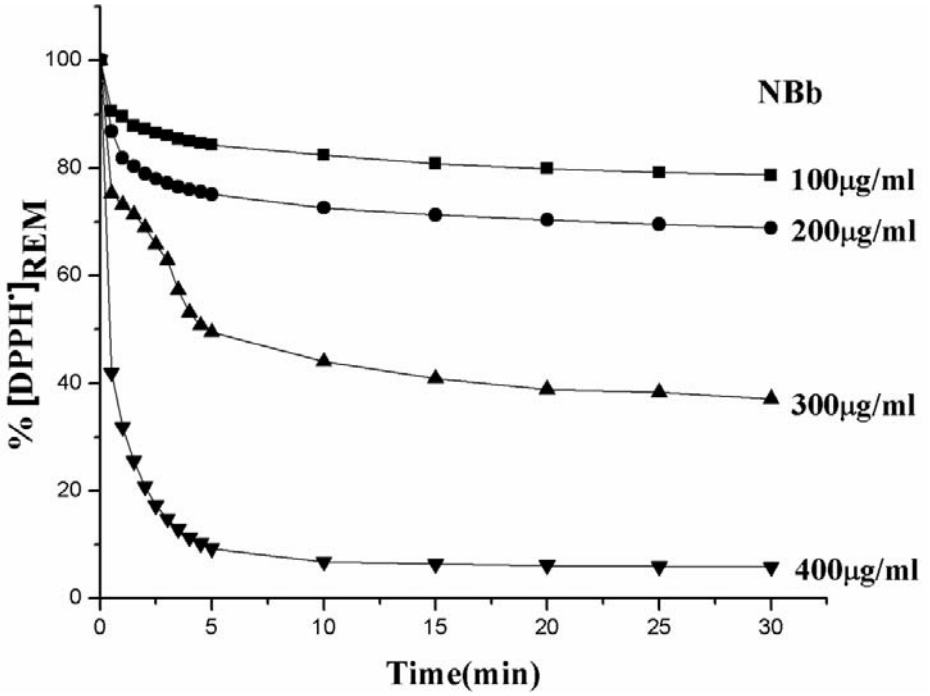


Figure 4.26a: % $[DPPH^{\cdot}]_{REM}$ of diethyl ether extracts of NBb at different concentrations

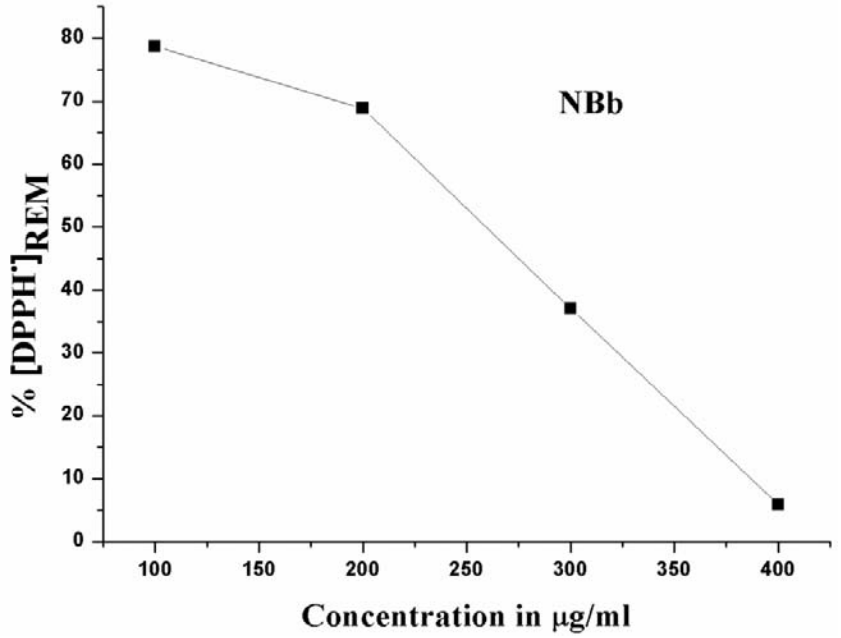


Figure 4.26b: EC_{50} of diethyl ether extracts of NBb

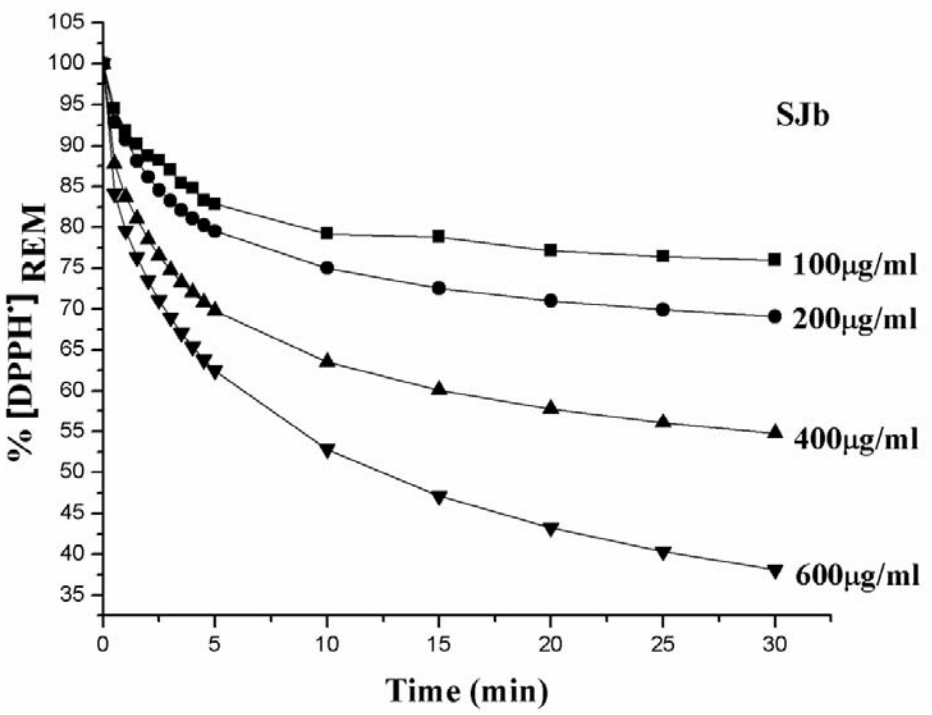


Figure 4.26c: % $[DPPH^{\cdot}]_{REM}$ of diethyl ether extracts of SJb at different concentrations

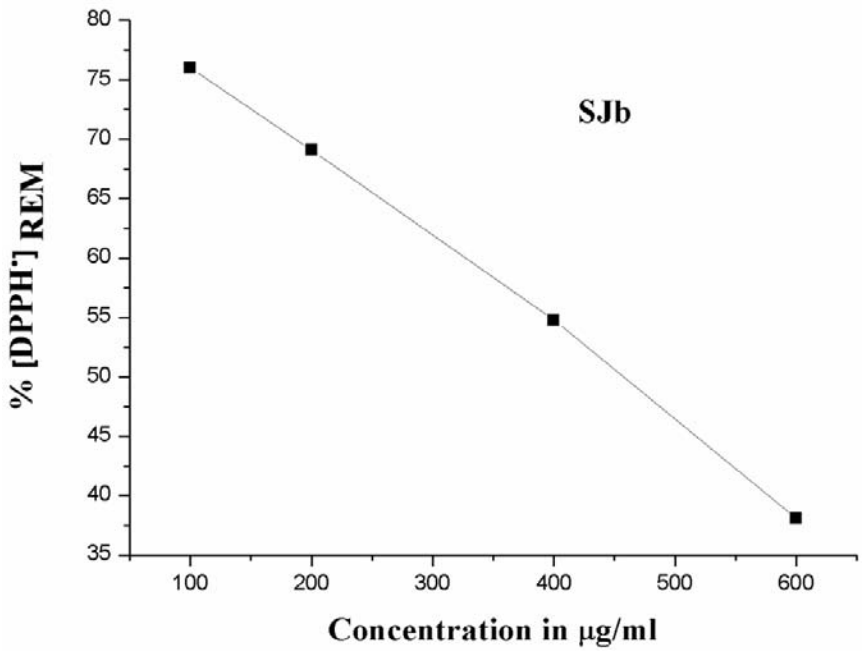


Figure 4.26d: EC_{50} of diethyl ether extracts of SJb

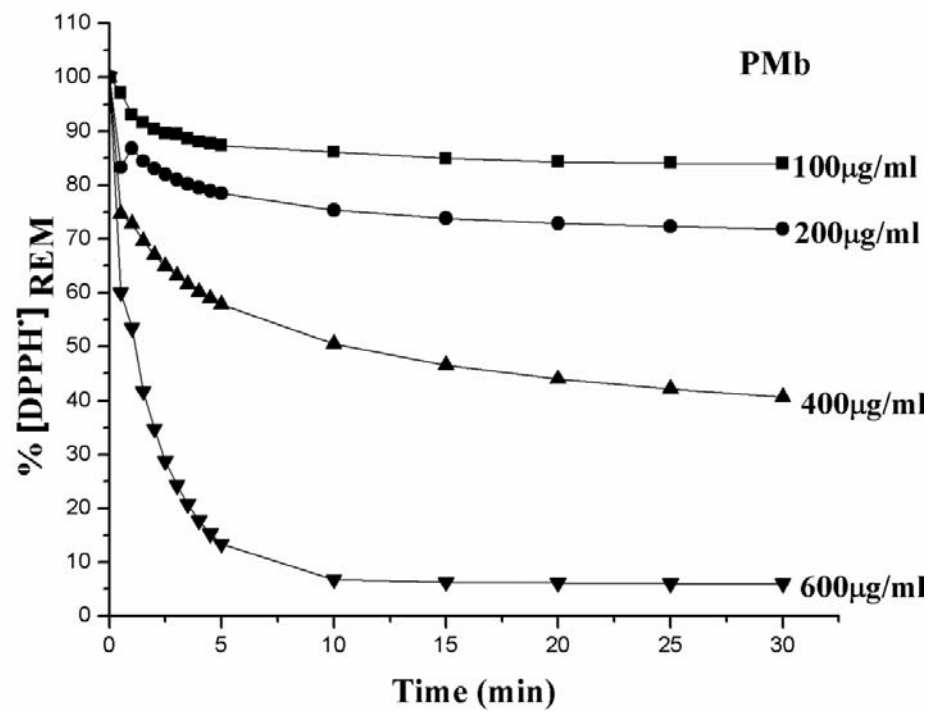


Figure 4.26e: % $[DPPH^{\cdot}]_{REM}$ of diethyl ether extracts of PMb at different concentrations.

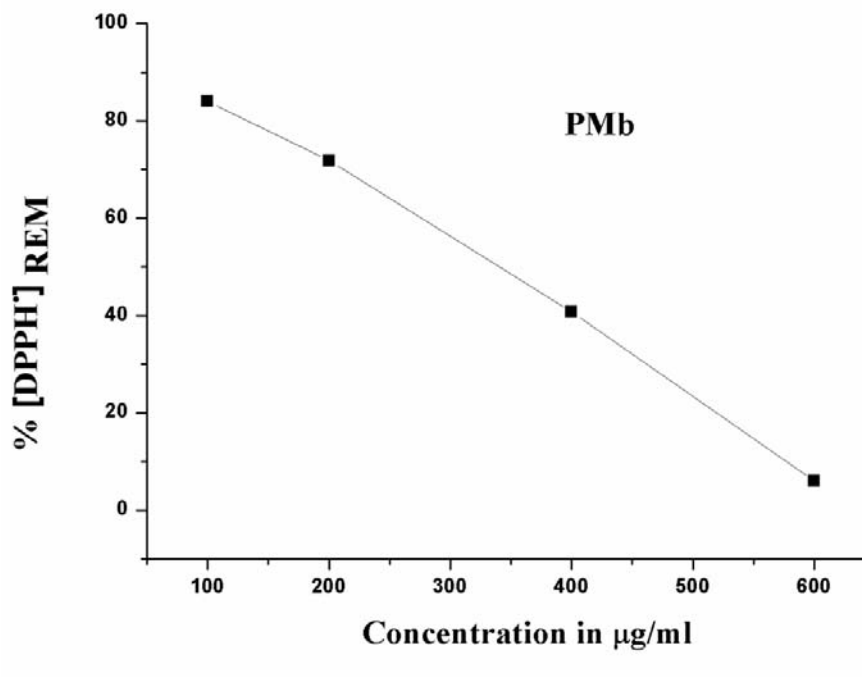
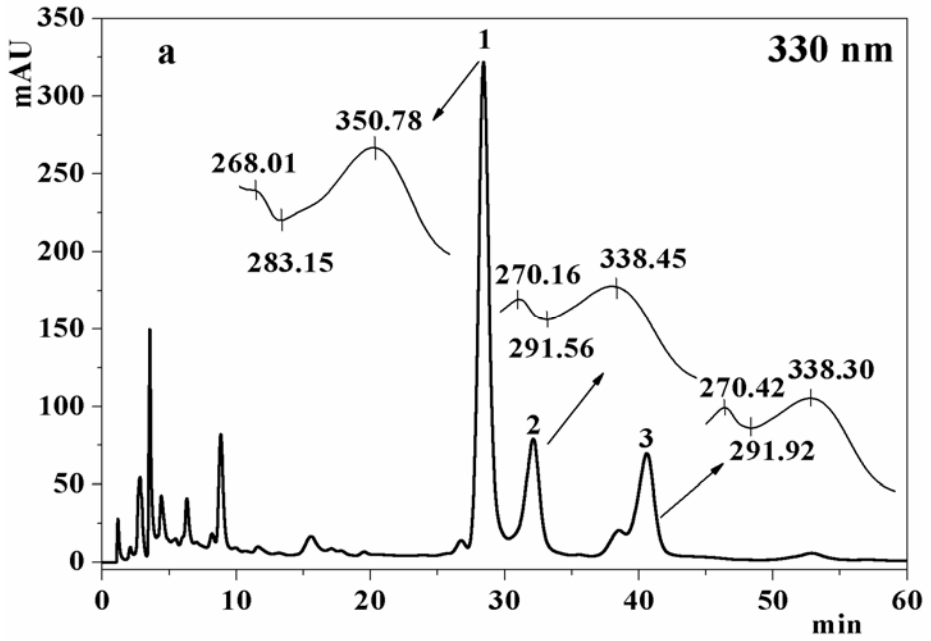


Figure 4.26f: EC_{50} of diethyl ether extracts of PMb.

4.2.4 Quantification of compounds in the diethyl ether extract by HPLC-PDA analysis

After identifying and characterising the compounds present in diethyl ether extract, we focussed our work on the occurrence of these compounds in staple varieties SJb and PMb and their quantification, as NBb showed higher antioxidant activity compared to staple varieties by DPPH radical scavenging activity (section 4.2.3). Reverse phase HPLC was used for the analysis of compounds in NBb, SJb and PMb. Figure 4.27 shows the HPLC-PDA chromatograms of NBb, SJb and PMb with corresponding ultra violet-visible (UV-VIS) spectra. Quantification of each compound in the diethyl ether extract was carried out using pure tricin and tricin 4'-O-(*erythro-β*-guaiacylglyceryl) ether (isolated from Njavara). The peaks corresponding to the compounds **VI**, **VII** and **VIII** were quantified using tricin and tricin 4'-O-(*erythro-β*-guaiacylglyceryl) ether as standards and their solutions were prepared in acetonitrile

with the concentrations 2, 3, 4, 5, 6, 7 and 8 µg/20 µl and run by HPLC. The standard solutions exhibited linear relationship between area response and concentration of sample injected, with the correlation coefficient (R^2) of 0.9976 and 0.9997 respectively. The detection limit for tricin was 0.47 µg/20 µl and % recovery was between 99.05 and 100.37, whereas for tricin 4'-O-(*erythro-β*-guaiacylglyceryl) ether, detection limit was 0.23 µg/20 µl and % recovery was between 99.12 and 100.02. The identification of compound peaks in the experimental samples was based on the congruence of retention times and UV-VIS spectra with those of pure compounds in literature.[18-20] Compounds **VII** and **VIII** were checked for their absorption characteristics and found comparable. Hence, compound **VII** which was available in more quantity was chosen for quantification purpose. Table 4.4 shows the quantification of the compounds **VI**, **VII** and **VIII** in NBb, PMb and SJb rice bran. Tricin is present 39.64 and 16.12 fold higher in NBb, compared to SJb and PMb respectively.



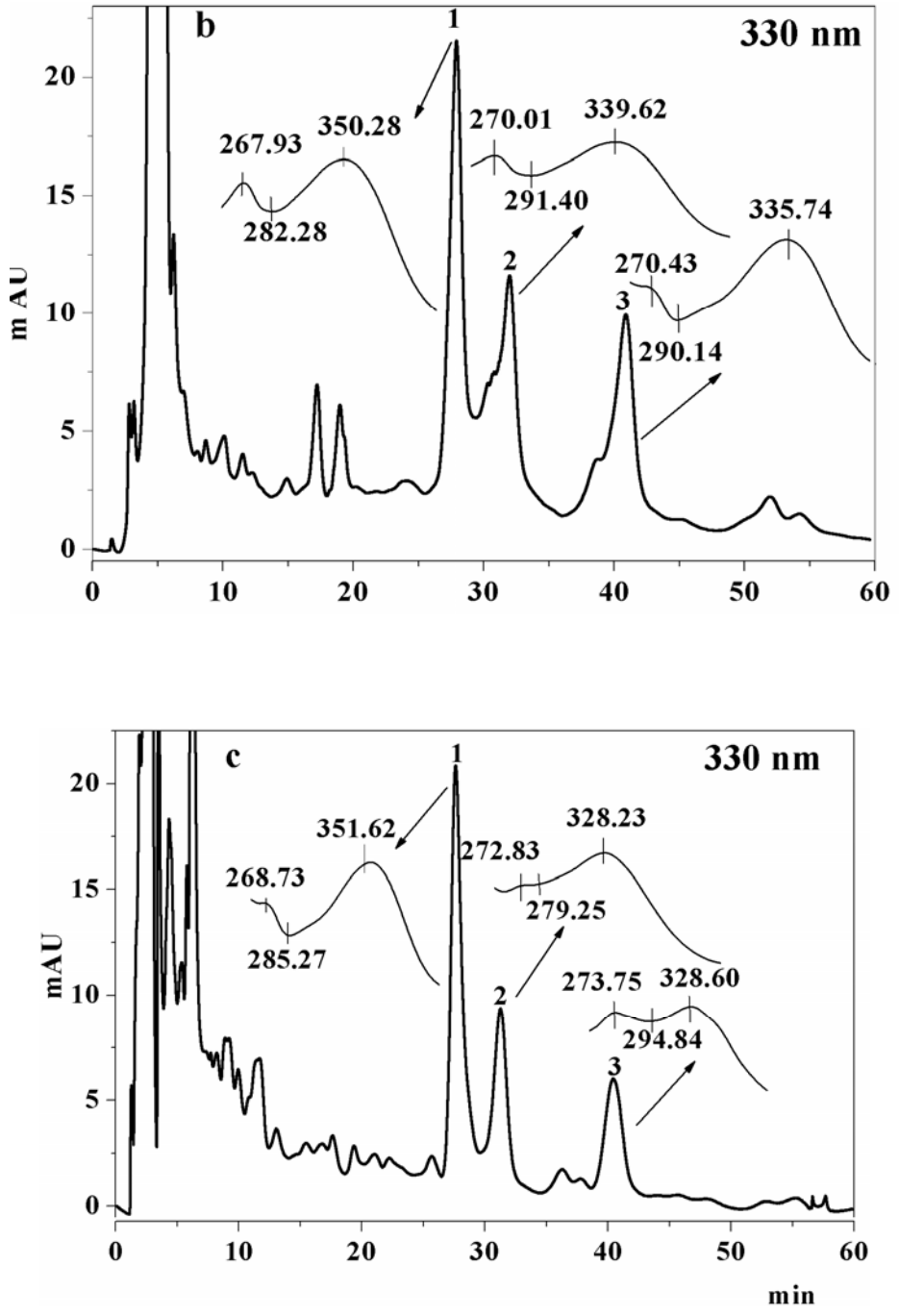


Figure 4.27: HPLC-PDA chromatographic profiles of compounds: tricin (peak **1**), tricin-4'-O-(*erythro*-*β*-guaiacylglyceryl) ether (peak **2**) and tricin-4'-O-(*threo*-*β*-guaiacylglyceryl) ether (peak **3)** with corresponding UV-VIS spectra of diethyl ether extracts of rice bran, at 330 nm in **a**) Njavara black (NBb) **b**) Sujatha (SJb) and **c**) Palakkadan Matta (PMb)

Table 4.4 Content (mg/100g dry weight) of tricin (Compound **V1**), tricin-4'-O-(*erythro-β*-guaiacylglyceryl) ether (TEGE-Compound **VII**) and tricin-4'-O-(*threo-β*-guaiacylglyceryl) ether (TTGE-Compound **VIII)** in Njavara black (NBb), Sujatha (SJb) and Palakkadan Matta (PMb) rice bran

	Content (mg/100g)		
Compound	NBb	SJb	PMb
Tricin	193.05±0.03[a]	4.86±0.01[c]	11.98±0.01[b]
TEGE	57.15±0.09[a]	1.88±0.08[b]	nd[i]
TTGE	64.62±0.03[a]	2.71±0.01[b]	nd[i]

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

[i]not detected

The above studies show that Njavara bran contains tricin, in significantly higher concentration compared to staple rice varieties. A recent review by Zhou and Ibrahim (2010) on tricin highlights the potential of the compound as a multifunctional nutraceutical. In this review, the beneficial health effects of tricin such as antioxidant effect, inhibition of lipid peroxidation, sparing effect on vitamin E in erythrocyte membrane, antiviral, immunomodulatory, antitubercular, antiulcerogenic, antimutagenic, mildly estrogenic, anti-inflammtory and potent anticancer effects are cited.[17] We can see that tricin-4'-O-(*erythro-β*-guaiacylglyceryl) ether (compound **VII)** and tricin-4'-O-(*threo-β*-guaiacylglyceryl) ether (compound **VIII)** are present only in minute quantities in SJb compared to NBb (Table 4.4). In PMb, the peaks 2 and 3 (Figure 4.27 c) showed λ_{max} at 328 nm which is different from the λ_{max} of 338 nm of the peaks 2 and 3 (Figure 4.27a) representing tricin-4'-O-(*erythro-β*-guaiacylglyceryl) ether (compound **VII)** and tricin-4'-O-(*threo-β*-guaiacylglyceryl) ether (compound **VIII)** of NBb. Hence, tricin-4'-O-(*erythro-β*-guaiacylglyceryl) ether (compound **VII)** and tricin-4'-O-(*threo-β*-guaiacylglyceryl) ether are absent in PMb. The peaks with λ_{max} at 328 nm in the HPLC of PMb were not further investigated owing to their very low concentration. The diethyl ether fraction of the methanolic extract of NBb is having

greater radical scavenging activity and the quantification of the compounds tricin, tricin-4'-O-(*erythro-β*-guaiacylglyceryl) ether and tricin-4'-O-(*threo-β*-guaiacylglyceryl) ether in the extracts showed that they are at higher concentration in NBb. The higher activity of diethyl ether extract of NBb can be attributed to the higher concentration of these compounds compared to SJb and PMb. The antioxidant activity and biological effects of these compounds are detailed in Chapter 5.

4.3 References

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Chapter 5

Section A

Antioxidant Activity of the Compounds and their Density Functional Theory (DFT) Studies

Section B

Anti-inflammatory Activity of the Compounds

Section C

Anticancer Activity of Flavonolignans

Chapter 5: Section A

Antioxidant Activity of the Compounds and their Density Functional Theory (DFT) Studies

The diethyl ether extract of Njavara showed higher scavenging activity compared to staple varieties due to the presence of higher concentration of tricin, tricin-4'-O-(*erythro-β*-guaiacylglyceryl) ether (TEGE) and tricin-4'-O-(*threo-β*-guaiacylglyceryl) ether (TTGE), which was discussed in Chapter 4. Hence, the three compounds were subjected to antioxidant studies by scavenging of DPPH radical, superoxide radical anion and hydrogen peroxide. We have discussed the structure activity relationship based on *in vitro* assays in Chapter 1 section 1.9.1.5.2. Theoretical studies based on quantum mechanical models can provide interesting information concerning the relationship between the geometric and electronic structures and the antioxidant properties. A quantum-mechanical investigation using DFT was thus undertaken in order to correlate experimental findings in DPPH radical scavenging with structural features of the compounds and to rationalize the mechanism affecting the scavenging activity.

5.1 Antioxidant activity of compounds

5.1.1 DPPH radical scavenging activity

In the present investigation, the flavonoid and flavonolignans were initially tested for antioxidant capacity using the [$DPPH^{\bullet}$] radical. As explained earlier in Chapter 4 the decrease in absorption is a measure of the extent of radical scavenging activity. Figure 5.1a, c, e and g gives % $[DPPH^{\bullet}]_{REM}$ of tricin, TEGE, TTGE and quercetin at different concentrations. The EC_{50} of the tricin, TEGE, TTGE and quercetin are read from the graph (Figure 5.1b, d, f and h) respectively and are tabulated in Table 5.1. Lower the EC_{50} higher is the antioxidant activity. Hence, the antioxidant activity of the compounds are as follows: quercetin (standard) > tricin > TTGE > TEGE.

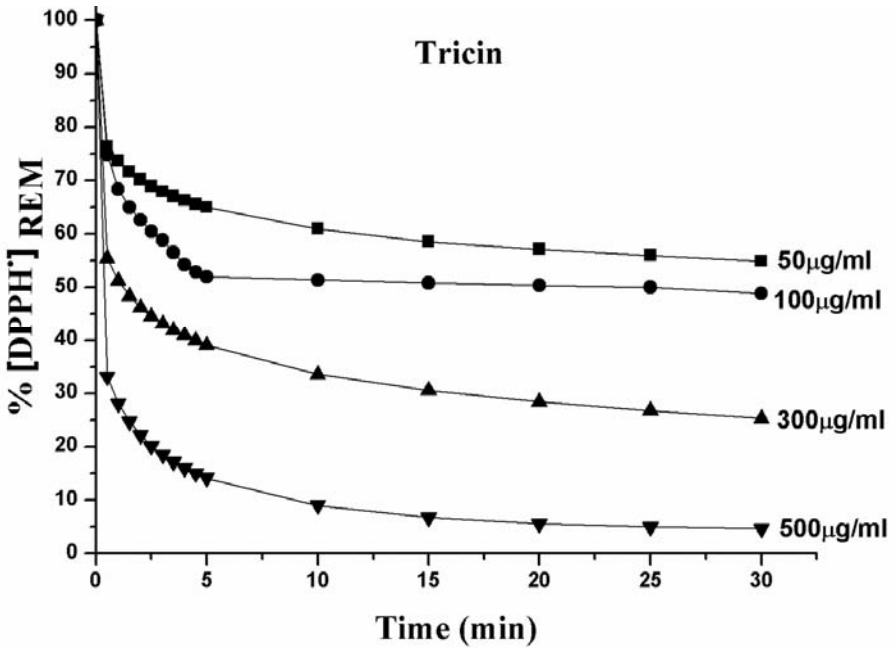


Figure 5.1a: % $[DPPH^{\cdot}]_{REM}$ of tricin at different concentrations

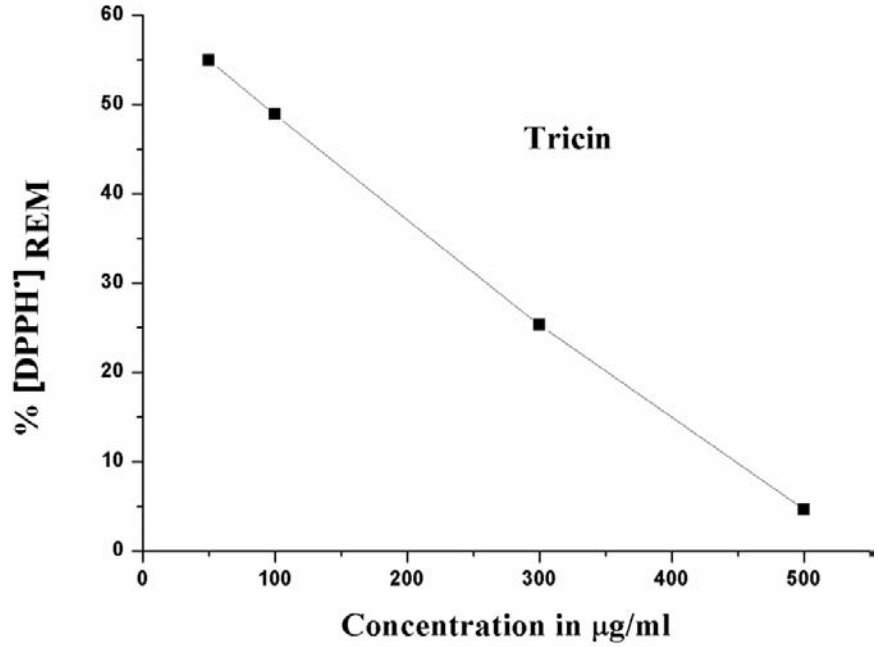


Figure 5.1b: EC_{50} of tricin from graph

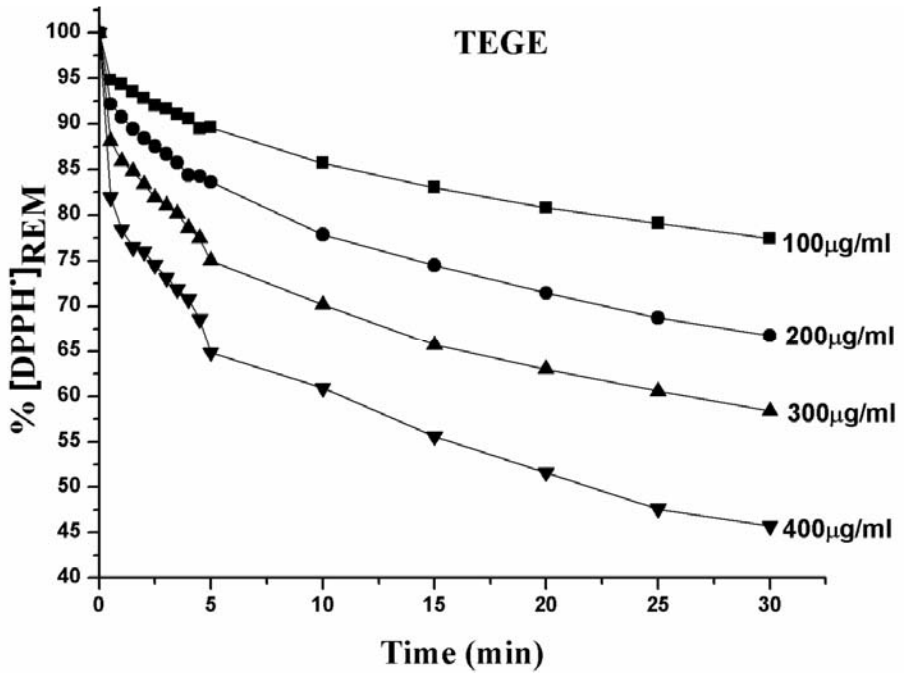


Figure 5.1c: % $[DPPH^{\cdot}]_{REM}$ of TEGE at different concentrations

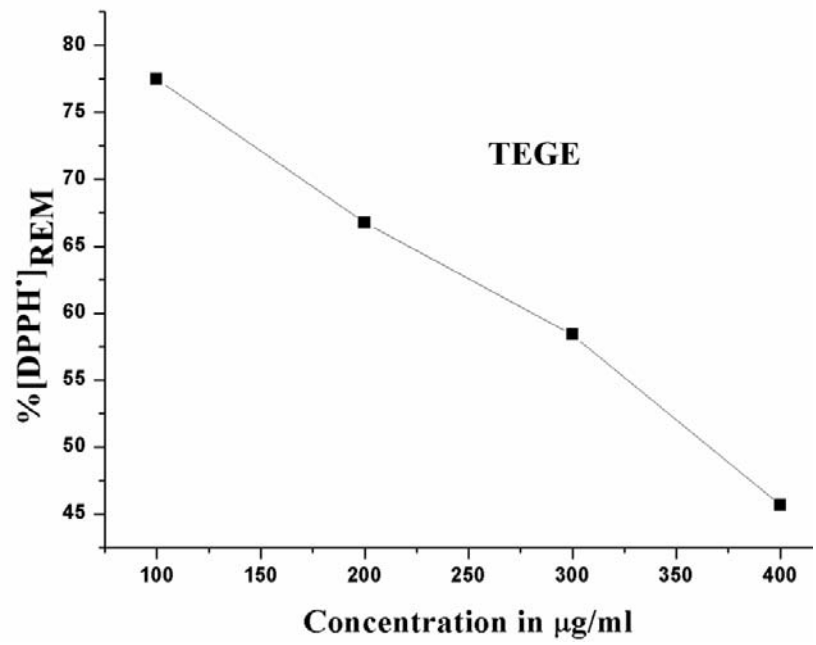


Figure 5.1d: EC_{50} of TEGE from graph

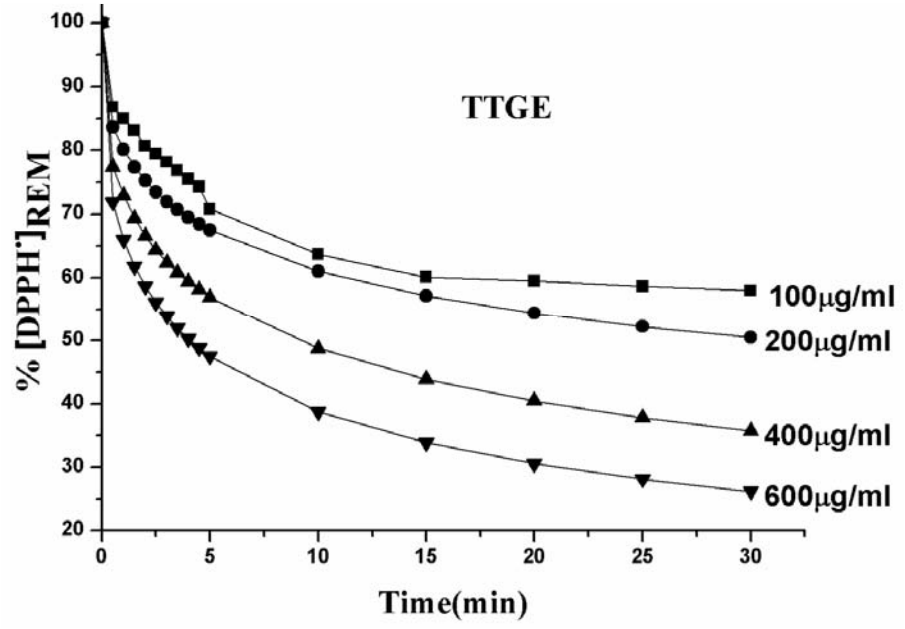


Figure 5.1e: % $[DPPH^{\cdot}]_{REM}$ of TTGE at different concentrations

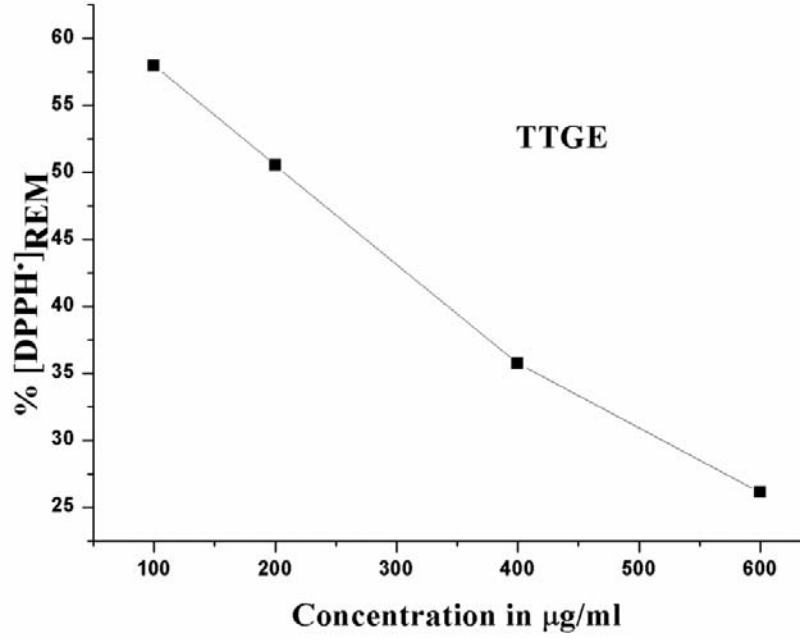


Figure 5.1f: EC_{50} of TTGE from graph

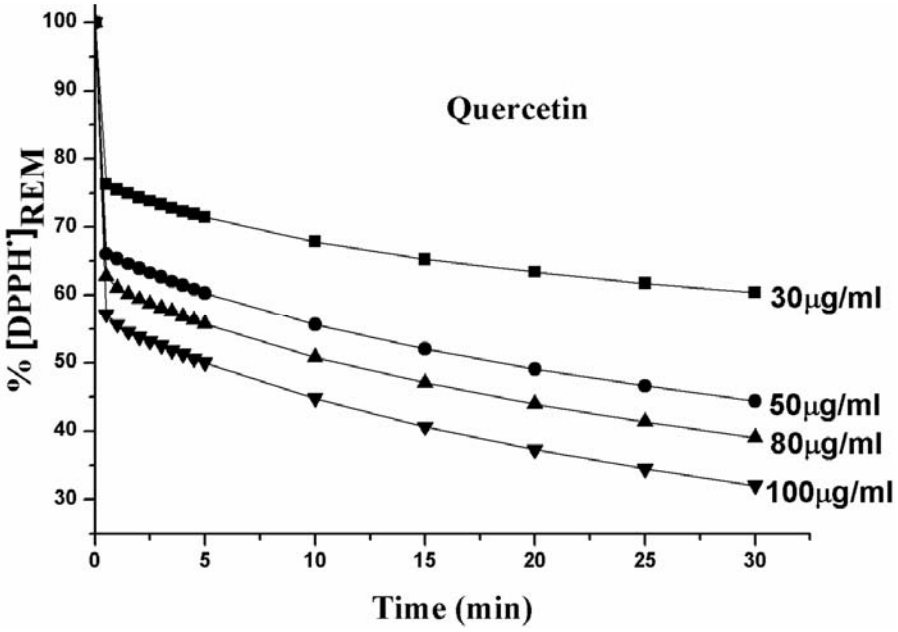


Figure 5.1g: % $[DPPH^{\cdot}]_{REM}$ of quercetin at different concentrations

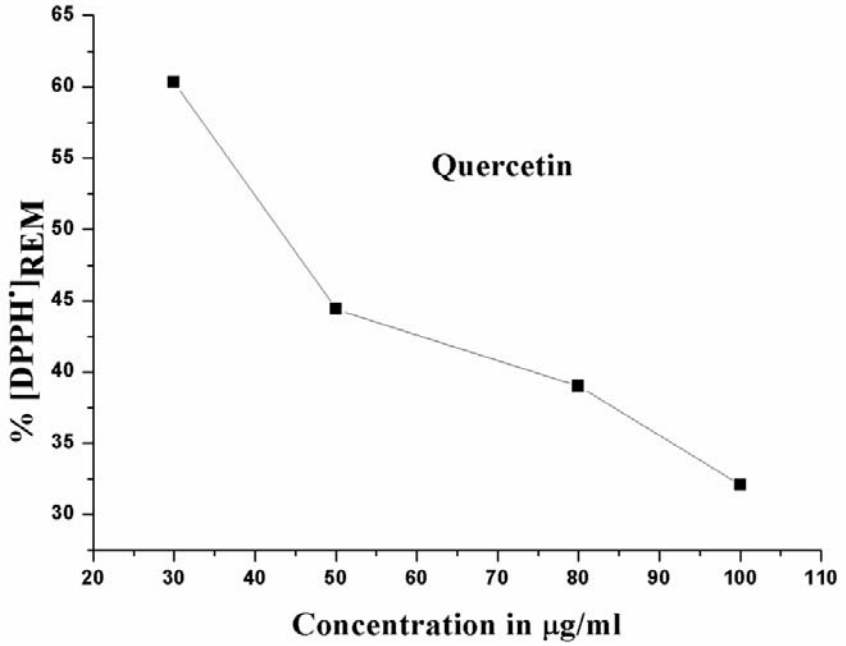


Figure 5.1h: EC_{50} of quercetin from graph

5.1.2 Superoxide anion radical scavenging activity

These compounds were also tested for superoxide anion radical scavenging properties. Figure 5.2 shows dose dependent (%) inhibition of superoxide radical generation, at concentration levels of (50-700 µg/ml) of all the compounds.

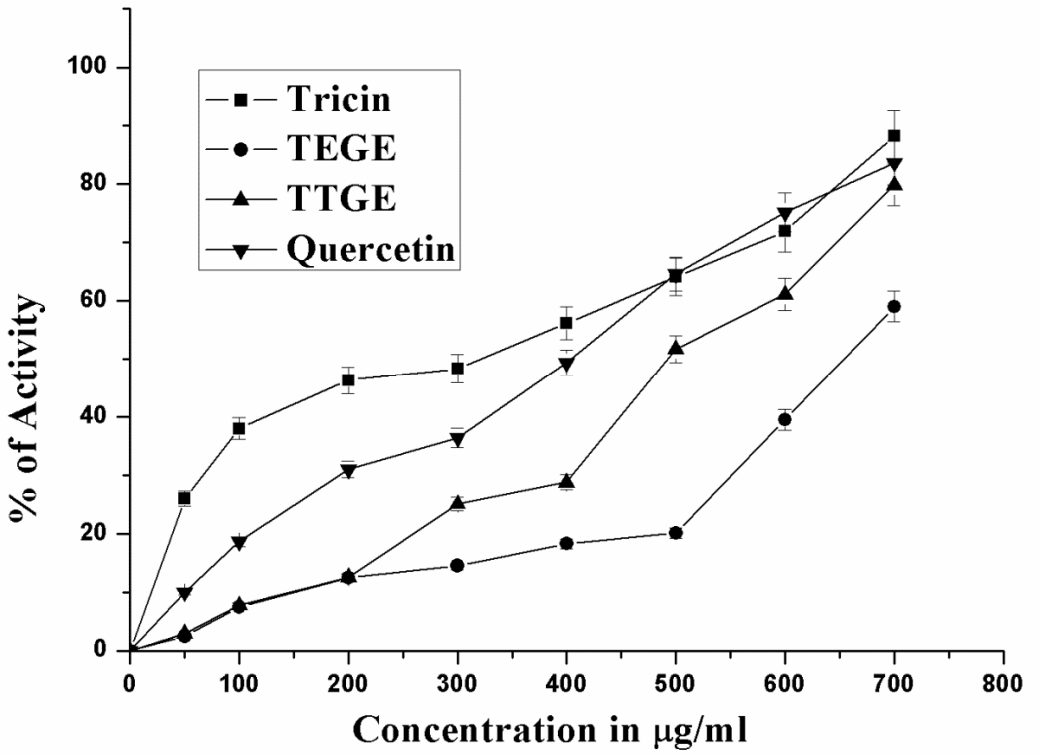
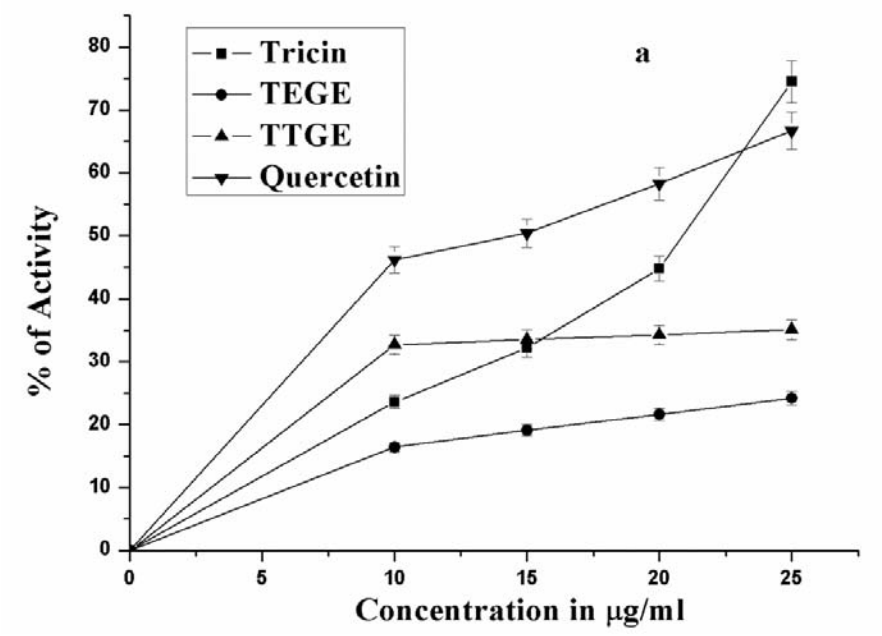


Figure 5.2: Superoxide anion scavenging activity of tricin, TEGE, TTGE and quercetin (standard). Values are expressed as the mean±SD of 3 x 2 (n = 6) determinations at all concentrations (50-700 µg/ml).

Among the compounds, tricin showed a higher scavenging power for the superoxide anion radical, with 88% scavenging capacity, at a concentration of 700 µg/ml. Tricin also showed higher scavenging activity than the standard quercetin, at all concentrations except at 600 µg/ml. But the flavonolignans showed a lower superoxide scavenging power compared to quercetin at all concentrations tested. Between TEGE and TTGE isomer, TTGE showed higher scavenging power, at all concentrations. At lower concentration between 50 to 200 µg/ml, both TEGE and TTGE showed almost similar scavenging activity, with a tendency for higher activity for TTGE > 200 µg/ml. TTGE isomer showed a higher tendency of scavenging activity of 79% at higher concentration (700 µg/ml) almost near to the standard quercetin (83%). The IC_{50} values are depicted in Table 5.1. The activity of the compounds follows the order as shown: tricin > quercetin (standard) > TTGE > TEGE. Among the compounds except standard, the same order of activity is observed as in DPPH radical scavenging activities as shown: tricin > TTGE > TEGE.

5.1.3 Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging ability of the compounds and standard is illustrated in the Figure 5.3a and 5.3b. All the compounds showed higher hydrogen peroxide scavenging activity at lower concentration range 10-200 µg/ml compared to superoxide scavenging activity which showed more effect at higher concentration range of 50-700 µg/ml. At lower concentration range of 10-20 µg/ml, tricin showed only activity in the range of 23–44 % compared to standard (Figure 5.3 a) whereas at higher concentrations (25-200 µg/ml), it showed higher activity in the range of 74-90 % (Figure 5.3b), compared to quercetin (66-89 %). Between TEGE and TTGE isomer, TTGE showed higher scavenging power, at all concentrations. The IC_{50} values are given in Table 5.1. The activity of the compounds follow the order: quercetin (standard) > tricin > TTGE > TEGE. Among the compounds excluding standard the same order of activity is observed as in DPPH radical and superoxide radical anion scavenging activities as shown: tricin > TTGE > TEGE.



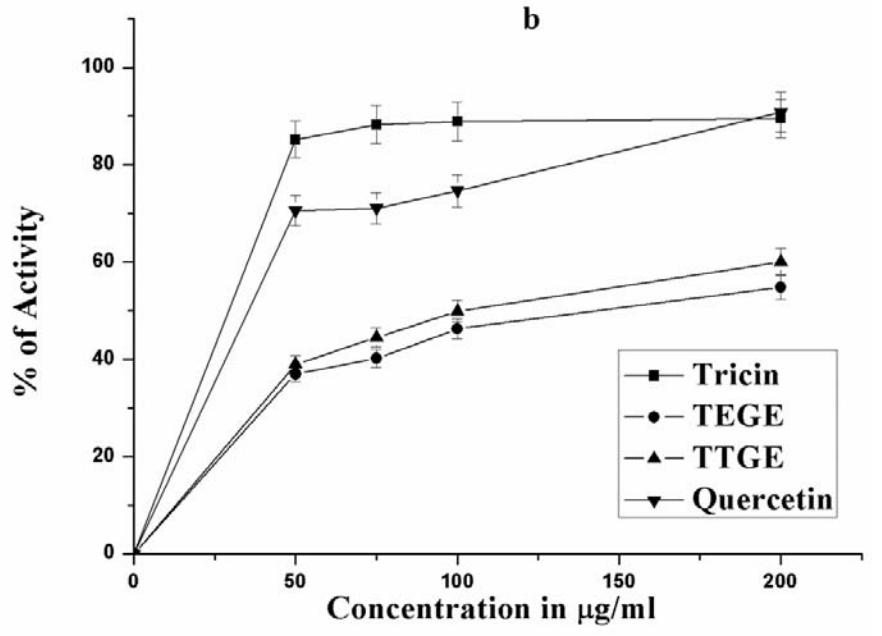


Figure 5.3: Hydrogen peroxide scavenging activity of tricin, TEGE, TTGE and Quercetin at **a**) 10-25 μg/ml **b**) 50-200 μg/ml. Values are expressed as the mean±SD of 3 x 2 (n = 6) determinations at all concentrations (10-200 μg/ml).

Table 5.1: Antioxidant capacity of tricin, TEGE, TTGE and quercetin

Compound	Scavenging capacity		
	($DPPH^{\bullet}$)	($O_2^{-\bullet}$)	H_2O_2
	EC_{50}	IC_{50}	
Tricin	90.39±0.03[c]	325.43±0.16[d]	20.65±0.01[c]
TEGE	352.04±0.16[a]	654.31±0.33[a]	145.61±0.07[a]
TTGE	208.00±0.09[b]	492.62±0.24[b]	103.86±0.01[b]
Quercetin	42.98±0.02[d]	406.27±0.20[c]	14.31±0.01[d]

[a-c]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 $P<0.05$. Values are expressed as the mean±SD of 3 x 2 (n = 6).

5.2 Density Functinal Theory (DFT) studies of the compounds

The free radical scavenging capacity of a flavonoid (ArOH) can be understood *via* the following two mechanisms:

$$ArOH + HO^{\bullet} \rightarrow ArO^{\bullet} + HOH \qquad \text{-----(5.1)}$$

$$ArOH + RO_2^{\bullet} \rightarrow RO_2^{-} + ArOH^{\bullet+} \qquad \text{-----(5.2)}$$

The first one is a hydrogen atom transfer (HAT) mechanism involving a hydroxyl radical while the second one is a single electron transfer (SET) mechanism due to a peroxide radical and both the mechanisms are believed to play important roles in determining the radical scavenging activity of the antioxidants.[1-7] For an effective HAT mechanism, the ArO-H bond dissociation enthalpy (BDE) has to be small to release a reactive phenoxide type radical. This also means that to evaluate the efficacy of the antioxidant activity of a flavonoid, the BDE value can be used as a parameter. For an effective SET mechanism, the ionisation potential (IP) of ArOH has to be small. The SET mechanism will be solvent dependent due to the formation of charged species

($ArOH^+$) whereas the HAT mechanism will be solvent independent as it passes through charge less radicals and neutral species. Radical scavenging activity of the antioxidants is also influenced by structural and environmental features of the compounds in the *in vivo* surroundings.[1-7]

All the compounds tricin, TEGE, TTGE and standard quercetin were optimised using the B3LYP/6-31G* level density functional theory. Figure 5.4 shows the optimized geometries of all the compounds with some important structural parameters. Tricin, TEGE and TTGE showed a twist angle ($\angle C_3C_2C_{1'}C_6$) of 15.5, 19.3 and 19.0 respectively with respect to the central C_2-$C_{1'}$ bond whereas quercetin showed planar geometry (twist angle = 0.0). In all the compounds, the hydroxyl groups interact with nearby oxygen atoms for making intramolecular hydrogen bonds (shown by dotted lines in Figure 5.4) and this will enhance the π-delocalisation in the aromatic ring.[2,3,8,9]

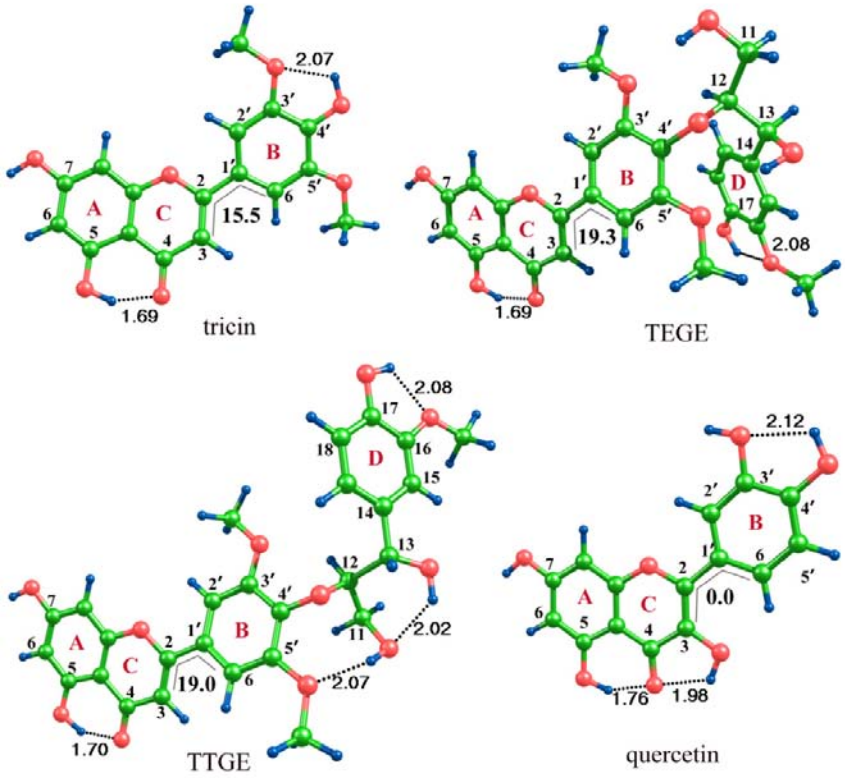


Figure 5.4: The optimized geometries of the flavonoids with their structural features. Bond distances are given in Å and torsion angles are in degrees

The O-H bonds present in these molecules are prone to undergo homolytic cleavage when they interact with a radical such as $DPPH^{\bullet}$. For tricin molecule, three

different phenoxide type radicals are possible by the cleavage of O-H bonds at C_5, C_7 and $C_{4'}$ positions, while the rest of the molecules have five different options. The BDE values of all these possibilities are presented in Table 5.2. For a given system, the most stable radical formed by the O-H bond cleavage is the one which shows the lowest BDE. In the case of tricin, the most stable radical is formed by the cleavage of the $C_{4'}$O-H bond in the C ring as it shows the lowest BDE of 74.3 kcal/mol compared to the BDE for the C_5O-H (97.0 kcal/mol) and the C_7O-H (83.63 kcal/mol) bonds. Similarly, for TEGE and TTGE, the O-H bond cleavage is the easiest at C_{17} position in the D ring and their values are 78.9 kcal/mol and 76.9 kcal/mol, respectively. The O-H bond cleavage of quercetin is favoured at $C_{4'}$ position in the B ring and the corresponding BDE values is 68.03kcal/mol.

Among the system molecules studied here, quercetin shows the lowest BDE for the $C_{4'}$-OH bond, where apart from one hydrogen bond on the $C_{4'}O^{\bullet}$, the planarity of the whole molecule is helpful in delocalizing the unpaired electron. In the case of tricin, the radical formed at $C_{4'}$ position lacks stabilization by hydrogen bond interactions and hence higher BDE is observed compared to quercetin. In general, a radical is more stabilized by the nearby hydrogen bonds than the coplanar nature of the entire molecule. The compounds with increasing order of BDE values are as follows: quercetin < tricin < TTGE < TEGE. In Figure 5.5, BDE values are correlated with EC_{50} values which show that the DPPH radical scavenging activity increases almost exponentially with increase in the BDE values (R^2= 0.9403).

The delocalization of the unpaired electron and its conjugation effects in radical system can be evaluated by spin density distribution. Figure 5.6 shows the relative spin density distribution of the most favourable radicals formed from all the four molecules studied herein. In the case of quercetin and tricin, the spin density is delocalized significantly even up to the B ring whereas for the rest of the molecules, the spin density is confined within only one aromatic ring. In general, lower values of spin density on the oxygen atom is indicative of higher delocalization of the unpaired electron. According to the SET mechanism, the radical scavenging action of the flavonoid antioxidants (ArOH) can be represented as follows:

$$ArOH + RO_2^{\cdot} \rightarrow ArOH^{\cdot +} + RO_2^{-} \qquad \text{---------- (5.3)}$$

$$ArOH^{\cdot+} + H_2O \rightarrow ArO^{\cdot} + H_3O^{+} \quad \text{---------- (5.4)}$$

Table 5.2: The BDE (kcal/mol) and spin density values of the radicals of compounds given in Figure 5.4 (numbering is as given in Figure 5.4)

Compound	Position of cleavage	BDE (kcal/mol) (B3LYP/6-31G*)	Spin density
tricin	C_5-OH	97.0	0.395
	C_7-OH	83.6	0.447
	$C_{4'}$-OH	74.3	0.346
TEGE	C_5-OH	103.1	0.615
	C_7-OH	85.1	0.453
	C_{13}-OH	100.2	0.892
	C_{17}-OH	78.9	0.786
	C_{20}-OH	98.9	0.386
TTGE	C_5-OH	96.8	0.396
	C_7-OH	83.7	0.454
	C_{13}-OH	96.3	0.770
	C_{17}-OH	76.9	0.814
	C_{20}-OH	99.6	0.380
quercetin	C_3-OH	76.7	0.322
	C_5-OH	92.2	0.336
	C_7-OH	83.0	0.403
	$C_{3'}$-OH	70.5	0.357
	$C_{4'}$-OH	68.0	0.295

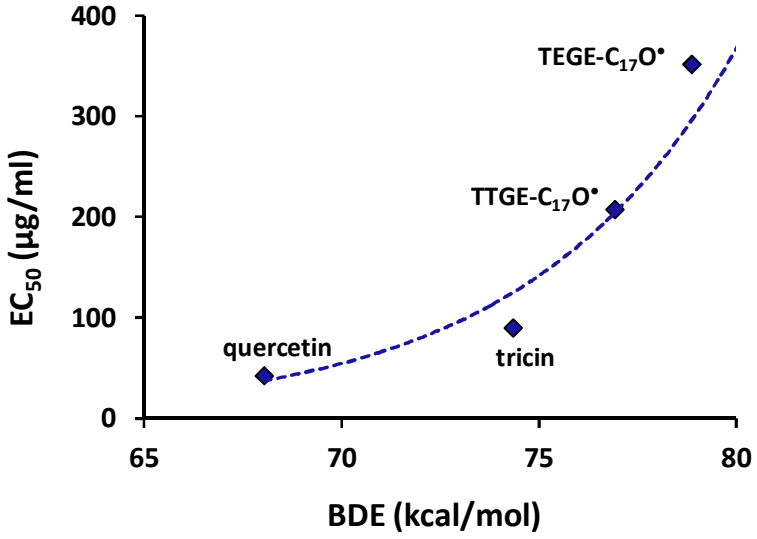


Figure 5.5: Correlation between BDE values and DPPH radical scavenging activity, EC_{50} values

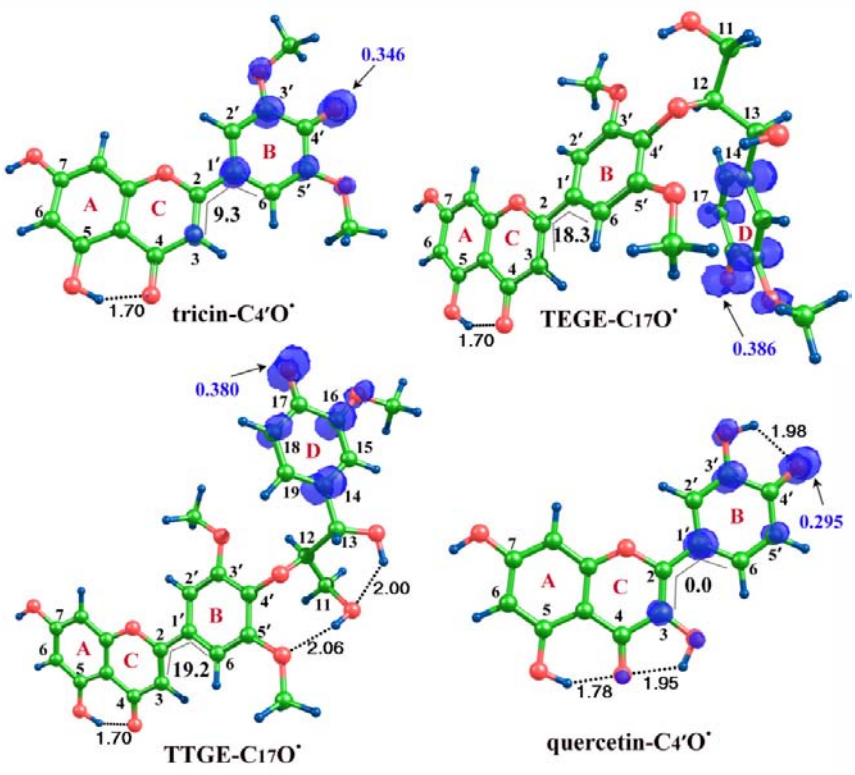


Figure 5.6: The spin density distribution (blue surface) in the most stable radicals of tricin, TEGE, TTGE and quercetin. The bond distances are given in Å and twist angles in degrees.

Table 5.3: Ionization potential (IP) of the compounds given in Figure 5.4

Compound	IP (kcal/mol)
Tricin	125.50
TEGE	121.60
TTGE	121.29
Quercetin	120.08

Further, RO_2^- can interact with H_3O^+ to form ROOH and H_2O. We have calculated the ionization potential (IP) of ArOH to generate $ArOH^{\cdot+}$ and used the IP values (Table 5.3) as a convenient parameter to evaluate this mechanism. The IP values do not show any correlation with the EC_{50} values which means that the HAT mechanism is more important than the SET mechanism for the study of the antioxidant activity of flavonoids. Hence, in this study the flavonoid–tricin, and its flavonolignan conjugates – tricin-4′-O-(*erythro*-*β*-guaiacylglyceryl)ether (TEGE) and tricin-4′-O-(*threo*-*β*-guaiacylglyceryl)ether (TTGE) exhibited antioxidant activity in the order as tricin > tricin-4'-O-(*threo*-*β*-guaiacylglyceryl) ether > tricin-4'-O-(*erythro*-*β*-guaiacylglyceryl)ether. The $DPPH^{\bullet}$ scavenging activity of the compounds were theoretically investigated using B3LYP/6-31G* DFT method. The experimental order of measured free radical scavenging activity of these compounds almost exponentially increased with respect to the increase in the BDE values. Lower the BDE value easier is the O-H bond cleavage and higher will be the radical scavenging activity. Analysis of spin density is useful to evaluate the delocalization of the unpaired electron in the radical species. In general, a radical system with more delocalized spin density is more effective in free radical scavenging. Hydrogen bond interaction around the phenoxide radical centre increases the radical scavenging activity. Moreover, extended conjugation of the -electrons to more than one aromatic ring enhances the stability of the radical systems as well as the radical scavenging activity. In conclusion, the experimental scavenging activity based on DPPH radical activity of the compounds is in same order as that of DFT studies.

5.3 References

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Chapter 5: Section B

Anti-inflammatory Activity of the Compounds

The three compounds tricin, tricin-4′-O-(*erythro-β*-guaiacylglyceryl)ether (TEGE) and tricin-4′-O-(*threo-β*-guaiacylglyceryl)ether (TTGE) showed significant antioxidant activity. As Njavara is used in inflammatory related diseases, we undertook the study of the anti-inflammatory activity of the compounds. Before discussing the results of the anti-inflammatory activity of the compounds, a brief description of the inflammatory process is portrayed here.

5.4 Biochemical markers of inflammation

Inflammation is a complex biological protective response of the body against infectious agents and injury. Although it is a protective response, continous or chronic inflammation is deleterious and paves path to the development of many chronic diseases. Inflammation results in triggering signalling cascades, activation of transcription factors, gene expression, increased levels of inflammatory enzymes, and release of various oxidants and proinflammatory molecules in immune or inflammatory cells. The biochemical markers linked to inflammation have the potential to predict outcomes before, during and after progression of inflammatory diseases. The different biochemical markers of inflammation include lipid derived mediators, redox status, cytokines, chemokines, adhesion molecules and numerous transcription factors that have been linked to inflammation.[1]

5.4.1 Arachidonic acid metabolites: prostaglandins and leukotrienes

Lipid-derived mediators are well positioned to play key roles as signalling molecules in inflammation because they are small molecules, locally acting, rapidly generated and locally inactivated. Arachidonic acid derivatives are important inflammatory mediators in several models of acute and chronic inflammation.[2] Products derived from the metabolism of arachidonic acid, also called eicosanoids, affect a variety of biologic processes, including inflammation and platelet function. Arachidonic acid is derived from cell membrane phospholipids of certain cells in response to some stimulus which activates phospholipase A_2 (PLA_2). Possible stimuli are many and

include antigen-antibody complexes, oxygen free radicals, bradykinin and thrombin. Many enzyme systems are involved in this process including cyclooxygenases (COX) and lipoxygenases (LOX), to name a few. The arachidonic acid cascade generates a family of bioactive lipids, including prostaglandins, thromboxanes and leukotrienes that modulate diverse physiological and pathophysiological responses in the bodyas shown in Figure 5.7.[3]

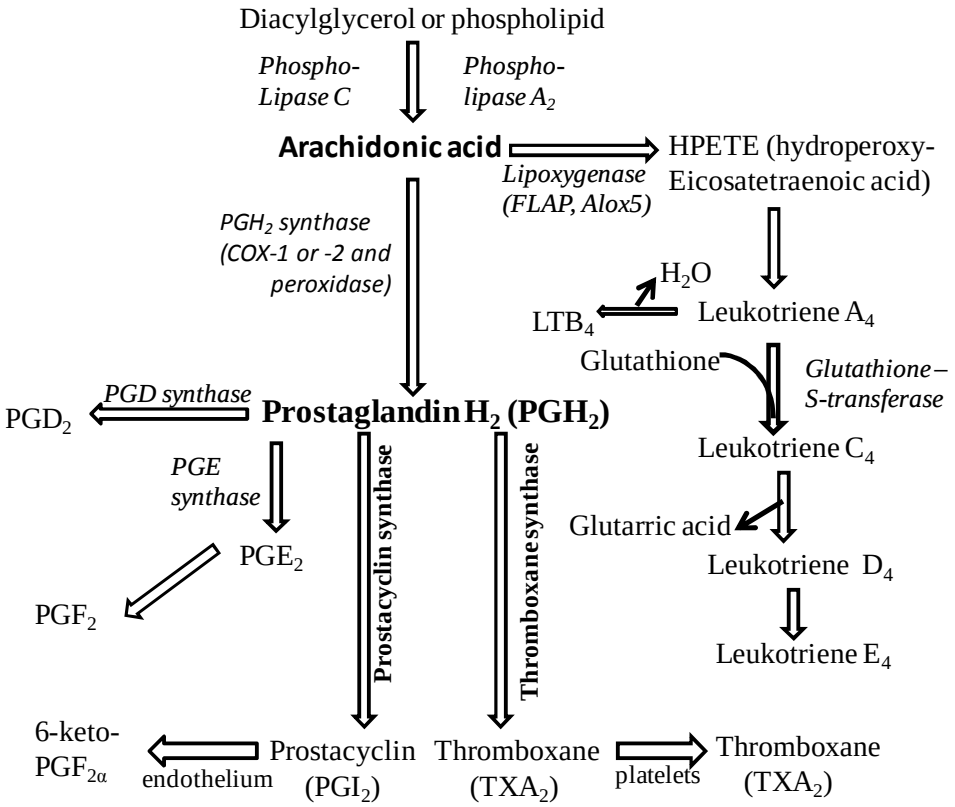

Figure 5.7: COX and LOX pathways of arachidonic acid metabolism

5.4.1.1 Prostaglandins

Prostaglandins are bioactive lipids produced from arachidonic acid by cyclooxygenase (COX) enzymes and specific terminal prostanoid synthase enzymes.[4] Prostanoids are a group of lipid mediators that regulate numerous processes in the body. These processes include regulation of blood pressure, blood clotting, sleep, labour and inflammation. When tissues are exposed to diverse physiological and pathological stimuli, arachidonic acid is liberated from membrane phospholipids by phospholipase

A_2 and is converted to PGH_2 by prostaglandin H synthase (PGHS) or cyclooxygenase (COX).

Three isoforms of COXs have been identified: COX-1, COX-2 and COX-3. COX-1 and -2 are 60% homologous. These isozymes are encoded by two different genes. Different mechanisms stimulate these two different types of cyclooxygenase. COX-1 is constitutively expressed in most tissues and appears to be responsible for maintaining normal physiological functions whereas COX-2 has been shown to be involved in cutaneous inflammation, cell proliferation and skin tumor promotion.[5] The location of the COX-1 enzyme dictates the functions of the prostaglandins it releases. Prostaglandins produced by COX-1 in turn stimulate body functions, such as stomach mucous production and kidney water excretion as well as platelet formation. This isoform was associated with many of the side effects related to NSAIDs therapy such as gastro-intestinal irritation and ulceration.[6] COX-2 is not normally present in all cells but its expression can be increased dramatically by the action of macrophages, the scavenger cells of the immune system. COX-2 is expressed mainly in inflammatory disorders, such as atherosclerosis and converts arachidonic acid to (prostaglandin G_2) PGG_2.[7]

5.4.1.2 Leukotrienes (LTs)

The LTs are a family of lipid mediators derived from arachidonic acid via the 5-lipoxygenase pathway. Leukotrienes together with prostaglandins, thromboxanes and lipoxins, are the major constituents of a group of biologically active oxygenated fatty acids known as eicosanoids.[8] They have been shown to be present at a variety of inflammatory sites and to be generated by cells like polymorphonuclear leukocytes, activated macrophages and mast cells involved in inflammatory response following an inflammatory stimulus.[9,10] The LTs play a major role in the inflammatory response to injury; they have been implicated in the pathogenesis of several inflammatory diseases, most notably asthma, psoriasis, rheumatoid arthritis and inflammatory bowel disease.[11]

Lipoxygenases (LOX) are a family of non heme iron-containing enzymes that insert molecular oxygen into polyunsaturated fatty acids such as arachidonic and linoleic acids.[12] Mammalian lipoxygenases are classified into three types: 5, 12 and 15-lipoxygenase on the basis of the carbon atom of arachidonic acid at which oxygen is inserted. 5-Lipoxygenase and 5- Lipoxygenase activating protein (FLAP), followed by

leukotriene A_4 hydrolase, are the enzymes responsible for the sequential formation of Leukotriene B_4 (LTB_4) from arachidonic acid. 5-LOX is expressed in monocytes and macrophages and contributes to the development and rupture of atherosclerotic plaques.[13] Oxygenation of arachidonic acid at C-12 and C-15 by 12-LOX and 15-LOX generates products which can modulate various neutrophil functions. The 15-LOX is expressed in atherosclerotic lesions of various species.[14,15]

5.4.2 ROS and scavenging enzymes

The human body has several mechanisms to counteract damage by free radicals and other reactive oxygen species. One important line of defence is a system of enzymes, including glutathione peroxidases, superoxide dismutases and catalase; and non-enzymes like glutathione and thioredoxin which decrease concentrations of the most harmful oxidants in the tissues. Catalase is involved in the detoxification of H_2O_2 and glutathione peroxidase catalyses the reaction of hydroxides with reduced glutathione to form glutathione disulphide (GSSG) and the reduction product of H_2O_2. Under the conditions of oxidative stress, superoxide dismutase (SOD) degrades superoxide ($O_2^{-\bullet}$) into oxygen and hydrogen peroxide[16] and protects cells and tissues from superoxide radicals and other peroxides such as lipid peroxides *in vivo*[17] Thus, SOD is a major endogenous cellular defence system in our body, evidenced by its anti-inflammatory activity.[18]

5.4.3 Nitric oxide and nitric oxide synthase

Nitric oxide (NO) is an important signaling molecule regulating the severity of inflammation and has major role in host defence and homeostasis. This free radical is generated in many cells and tissues including neurons, macrophages, neutrophils, endothelial cells, smooth muscle cells, lungs, respiratory tract etc. NO synthesized by activated inflammatory cells regulates the functions of the other cells involved in the inflammatory process. NO is produced in mammalian cells by a group of isoenymes collectively termed NO synthase (NOS). All forms of NOS catalyse the conversion of L-arginine to L-citrulline in nicotinamide adenine dinucleotide phosphate (NADPH) dependent reaction, producing NO from the N-guanidino group of L-arginine.[19]

Lipopolysaccharide (LPS) is a gram-negative bacterial endotoxin and a major factor that contributes to multiple organ failure, including various inflammatory disorders. Progression of inflammation mostly depends on the presence of

proinflammatory cytokines and reactive oxygen/nitrogen species. It is well documented that LPS causes oxidative stress by intensification of proinflammatory cytokines production and by inducing the generation of ROS by different mechanisms.[20,21]

5.5 *In vitro* and *in vivo* anti-inflammatory activity of compounds

For understanding the mechanism of anti-inflammatory action of the compounds isolated, inflammatory response and oxidative stress was induced in the human peripheral blood mononuclear cells (hpBMCs) by LPS. Oxidtion products like MDA, activity of SOD and the activity of inflammatory enzymes like NOS, COX and 5-LOX responsible for inflammation were evaluated using different biochemical assays. The compounds were subjected to anti-inflammatory activity like 5-lipoxygenase, cyclooxygenase activity, superoxide dismutase activity, malondialdehyde level and nitric oxide synthase actity carrageenan induced rat paw edema.There were five groups of cultured hpBMCs for each compound with group 1 treated as normal, group 2 treated as LPS control and rest three groups T_1 (LPS + 2 µg/ml), T_2 (LPS + 5 µg/ml)and T_3 (LPS + 10 µg/ml)as compound treatment groups with three concentration of 2, 5 and 10 µg/ml respectively along with LPS.

5.5.1 5-Lipoxygenase activity

Leukotrienes, the end products of lipoxygenase pathway play a major role in the inflammatory response injury; they have been implicated in the pathogenesis of inflammatory diseases.[22] Pre-treatment of cells with tricin, TEGE and TTGE significantly decreased 5-LOX activity in a dose-dependent manner (Figure 5.8a, b and c). Tricin treated cells were normalised at 5 µg/ml, whereas tricin-4′-O-(*threo*-*β*-guaiacylglyceryl) ether and tricin-4′-O-(*erythro*-*β*-guaiacylglyceryl) ether did not normalise at these three concentrations (2, 5 and 10 µg/ml) but significantly lowered the activity of 5- LOX compared to LPS control.

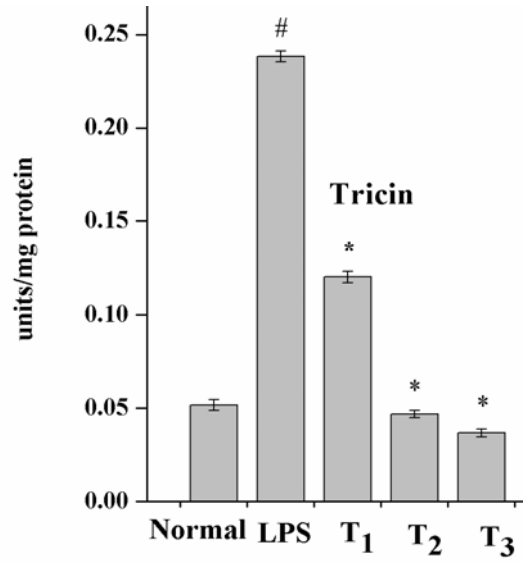

Figure 5.8a: Effect of tricin on level of 5-LOX in LPS induced hPBMCs

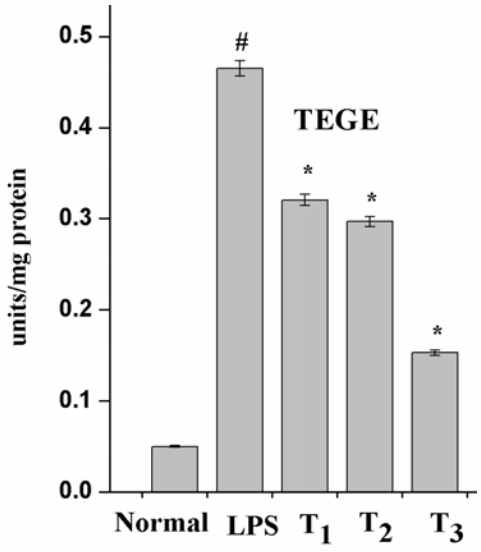

Figure 5.8b: Effect of TEGE on level of 5-LOX in LPS induced hPBMCs

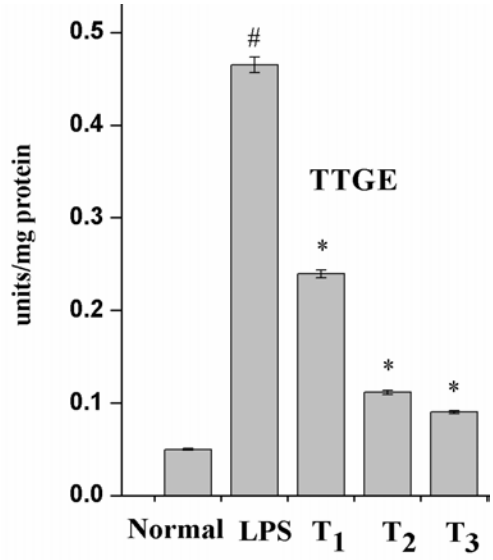

Figure 5.8c: Effect of TTGE on level of 5-LOX in LPS induced hPBMCs (Human peripheral blood monocytes).

Groups treated: 1) Normal 2) LPS control 3) T_1- (LPS+2 µg/ml of compound) 4) T_2 - (LPS+5 µg/ml of compound) 5) T_3- (LPS+10 µg/ml of compound).Values are expressed as average of 6 samples ±SEM in each group

*Significantly different from normal control ($p < 0.05$).

#Significantly different from LPS control ($p < 0.05$).

5.5.2 Cyclooxygenase activity

COX is one of the important enzymes which catalyse the formation of mediators involved in the inflammatory process.Inhibitors of COXs are the main focus of current therapy aimed to modulate pain, inflammation and to control fever.[23] In the present study, tricin, TEGE and TTGE were found to be significantly inhibiting ($p>0.05$) cyclooxygenase enzymes of the arachidonic acid metabolism (Fig 5.9a, b and c). The inhibition was found to be in dose dependent manner in three treatments (2, 5 and 10 µg/ml) of each compound. Tricin treated cells were normalised at 2 µg/ml, whereas TTGE almost normalised at 10 µg/ml and TEGE did not normalise at these three concentrations but significantly lowered the activity of SOD.

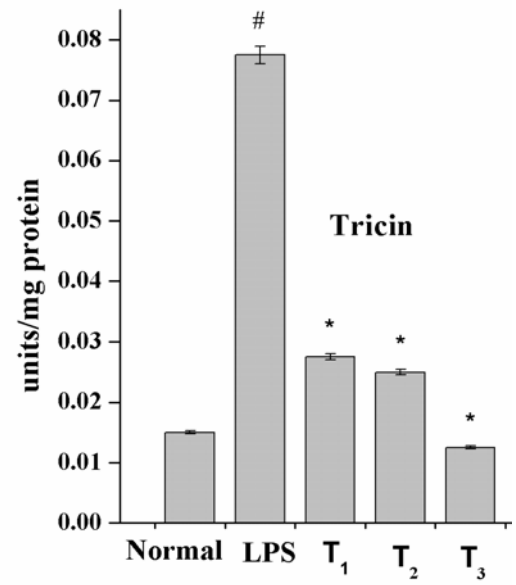

Figure 5.9a: Effect of tricin on level of COX in LPS induced hPBMCs

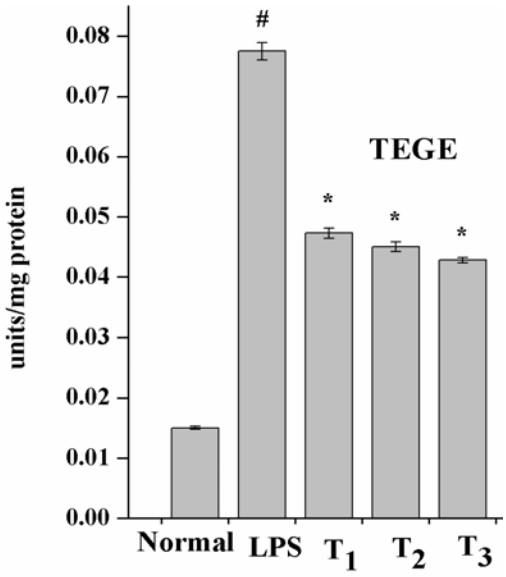

Figure 5.9b: Effect of TEGE on level of COX in LPS induced hPBMCs

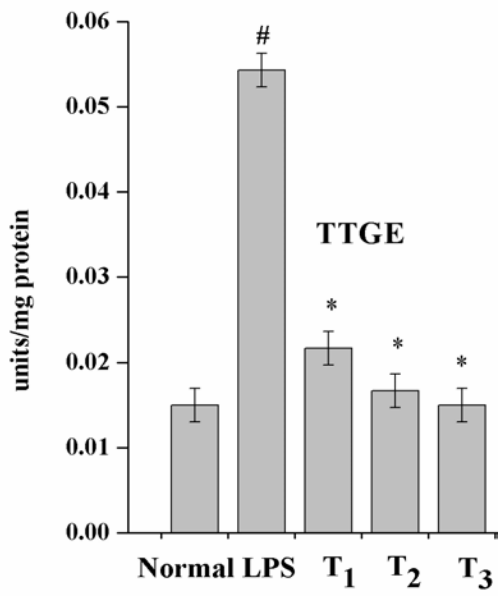

Figure 5.9c: Effect of TTGE on level of COX in LPS induced hPBMCs (human peripheral blood monocytes).

Groups treated: 1) Normal 2) LPS control 3) T_1- (LPS+2 µg/ml of compound) 4) T_2- (LPS+5 µg/ml of compound) 5) T_3- (LPS+10 µg/ml of compound). Values are expressed as average of 6 samples ±SEM in each group

*Significantly different from normal control ($p < 0.05$).

#Significantly different from LPS control ($p < 0.05$).

5.5.3 Superoxide dismutase activity

Cytoplasmic enzyme and superoxide dismutase (SOD) protect the cell contents against oxidizing activity by destroying superoxide and hydrogen peroxide, respectively.[24] SOD is an important protective system that accelerates the dismutation of superoxide anion radicals to hydrogen peroxide and acts as a primary defence, as it prevents further generation of free radicals. SOD, a primary defence, could reduce the oxidative stress and the activation of inflammatory mediators. SOD activity is significantly lowered in the LPS primed group compared to normal group ($p < 0.05$) (Fig. 5.10 a, b and c). Furthermore, when LPS and three treatment groups (T_1, T_2 and T_3) of each compounds tricin, TEGE and TTGE were compared by means of SOD activities, it was found significantly higher in treatment groups in dose dependent manner. Tricin treated cells were normalised at 2 µg/ml, whereas TTGE almost

normalised at 10 µg/ml and TEGE did not normalise at three concentrations (2, 5 and 10 µg/ml) but significantly lowered the activity of SOD.

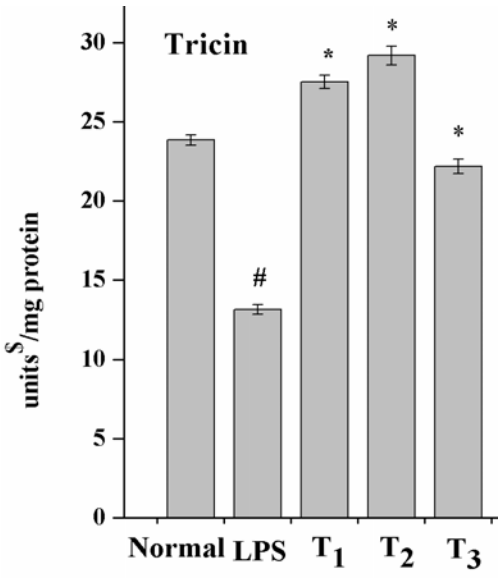

Figure 5.10a: Effect of tricin on level of SOD in LPS induced hPBMCs

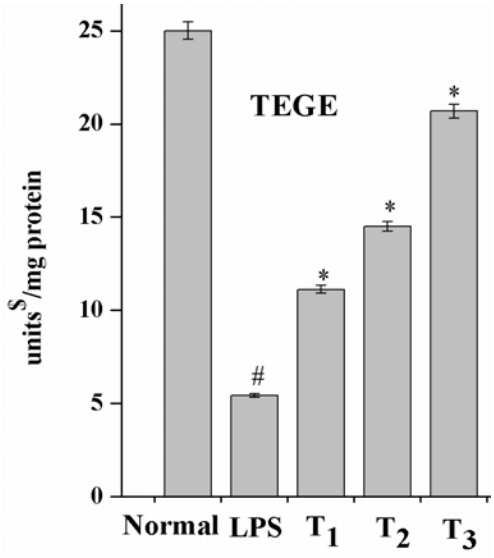

Figure 5.10b: Effect of TEGE on level of SOD in LPS induced hPBMCs

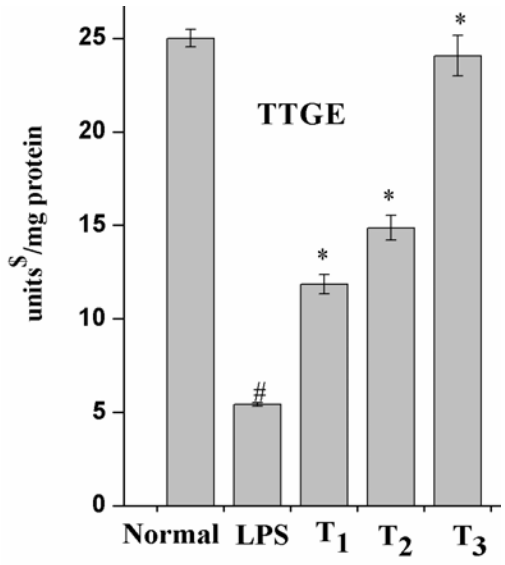

Figure 5.10c: Effect of TTGE on level of SOD in LPS induced hPBMCs (human peripheral blood monocytes). Groups treated: 1) Normal 2) LPS control 3) T_1- (LPS+2 µg/ml of compound) 4) T_2- (LPS+5 µg/ml of compound) 5) T_3- (LPS+10 µg/ml of compound).Values are expressed as average of 6 samples ±SEM in each group

*Significantly different from normal control ($p < 0.05$).

#Significantly different from LPS control ($p < 0.05$).

$SOD-U=enzyme concentration required to inhibit chromogen production by 50 % in 1 min.

5.5.4 Malondialdehyde (MDA) level

The levels of MDA were often used as an indication of oxidative damage and as a marker for free radicals-induced lipid peroxidation. Previous studies have shown that the level of malondialdehyde (MDA) was decreased by antioxidant and anti-inflammatory agents.[25] LPS primed hpBMC cells were pretreated with three different concentrations of 2, 5 and 10 µg/ml (T_1, T_2 and T_3 treatment groups respectively) of tricin, tricin-4′-O-(*erythro-β*-guaiacylglyceryl)ether (TEGE) and tricin-4′-O-(*threo-β*-guaiacylglyceryl)ether (TTGE) (Figure 5.11 a, b and c). In present study, compared with the normal group, the level of MDA contents were increased remarkably in the LPS primed hPBMCs. This stressful condition was reversed by treatment with the compounds. The three compounds provided protective effects in LPS primed oxidation probably by the radical scavenging and antioxidant properties in a dose dependent

manner and were significantly different from LPS control. Tricin treated cells were normalised at 5µg/ml, whereas TTGE normalised at 10 µg/ml and TEGE did not normalise at these three concentrations but significantly lowered the level of MDA.

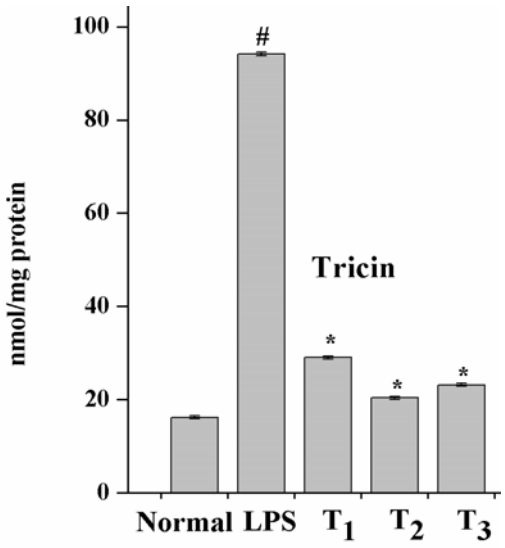

Figure 5.11a: Effect of tricin on level of MDA in LPS induced hPBMCs

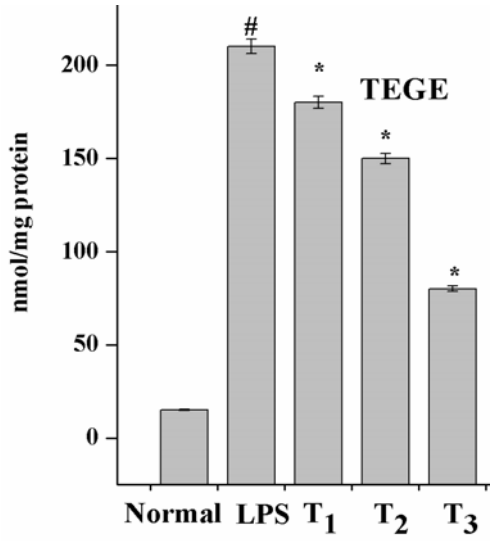

Figure 5.11b: Effect of TEGE on level of MDA in LPS induced hPBMCs

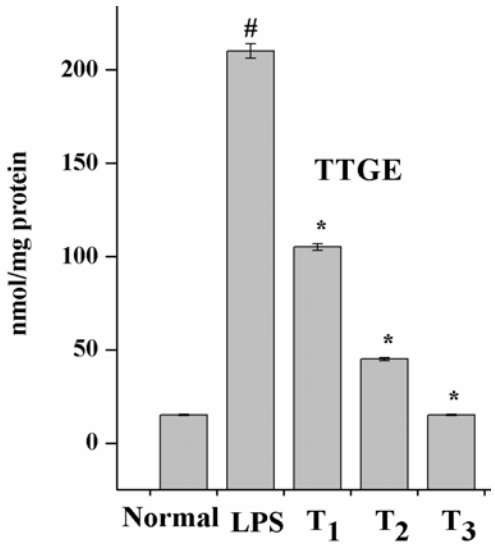

Figure 5.11c: Effect of tricin on level of MDA in LPS induced hPBMCs (human peripheral blood monocytes. Groups treated: 1) Normal 2) LPS control 3) T_1- (LPS+2 µg/ml of compound) 4) T_2- (LPS+5 µg/ml of compound) 5) T_3- (LPS+10 µg/ml of compound).Values are expressed as average of 6 samples ±SEM in each group

*Significantly different from normal control ($p < 0.05$).

[#]Significantly different from LPS control ($p < 0.05$).

5.5.5 Nitric oxide synthase (NOS) activity

Another mediator reported to play an important role in inflammation is NO.[26] Studies have demonstrated that the suppression of biological activities of iNOS by neutralizing antibodies, selective inhibitors or gene targeting have led to a dramatic improvement in the local inflammation and progression of RA and atherosclerosis.[27,28] The three compounds: tricin, TEGE and TTGE significantly inhibited total NOS activity and there by blocked the inflammatory changes exerted by nitric oxide, compared to LPS induced inflammation control (Figure 5.12a, b and c). These results support the ability of the three compounds in blocking the activation of inducible enzymes of inflammation which regulates chronic changes in pathogenesis.

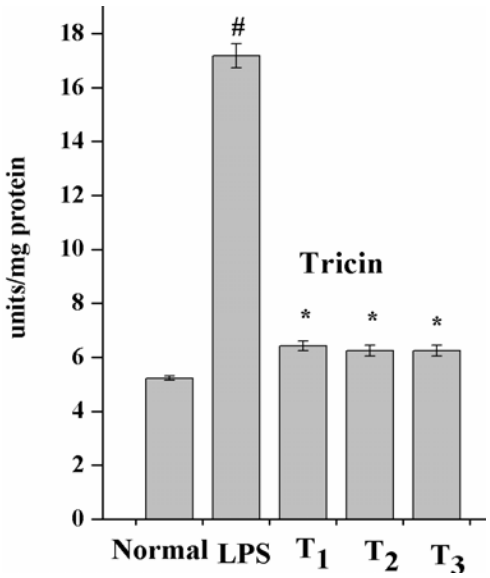

Figure 5.12a: Effect of tricin on level of NOS in LPS induced hPBMCs

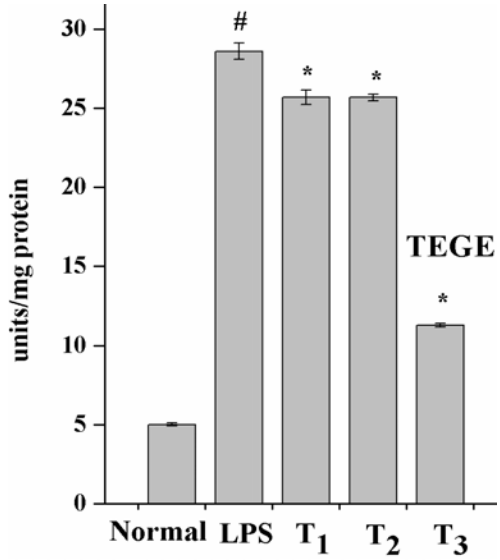

Figure 5.12b: Effect of TEGE on level of NOS in LPS induced hPBMCs (human peripheral blood monocytes.

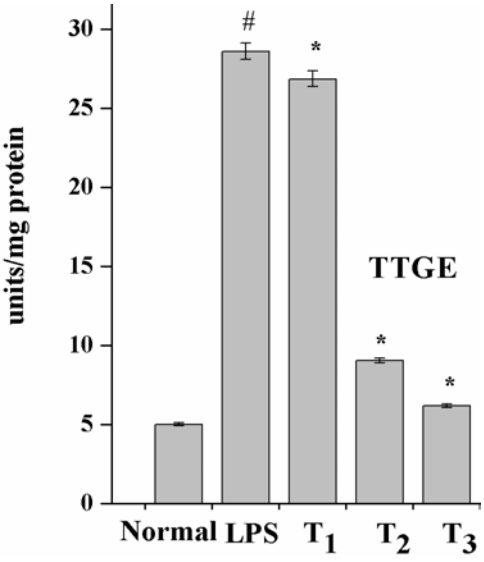

Figure 5.12c: Effect of TTGE on level of NOS in LPS induced hPBMCs (human peripheral blood monocytes. Groups treated: 1) Normal 2) LPS control 3) T_1- (LPS+2 µg/ml of compound) 4) T_2- (LPS+5 µg/ml of compound) 5) T_3- (LPS+10 µg/ml of compound).

Values are expressed as average of 6 samples ±SEM in each group

* Significantly different from normal control ($p < 0.05$).

#Significantly different from LPS control ($p < 0.05$).

5.5.6 Anti-inflammatory activity (*in vivo*)

Studies conducted in hPBMCs showed the dose dependent, anti-inflammatory activity of the three compounds, as evidenced by normalizing the upregulated activity of various inflammatory enzymes like COX, 5-LOX, NOS and alleviating the oxidative stress. Induced paw edema is a working model in the search for new anti-inflammatory drug.[29] The *in vivo* anti-inflammatory activity of tricin, (TEGE), (TTGE) and a standard diclofenac was evaluated by carrageenan induced rat paw edema method. A considerable increase in the percentage of paw edema inhibition was observed in all groups on 3rd and 5th h of carrageenan induction. The edema was significantly ($P<0.05$) reduced or maximum inhibition was observed in the compounds treated group. The anti-inflammatory activity of the compounds against acute edema (induced by carrageenan) is shown in Table 5.4 and the results are comparable to that of the standard drug diclofenac. At 3rd h, compounds tricin and TTGE showed 50% edema inhibition

whereas TEGE showed only 20%. Of the three compounds, tricin inhibited edema formation maximum, to an extent of 70%, followed by TTGE with 66.6% and TEGE with 44.4% at 5th h. Compared to standard drug diclofenac, the three compounds showed better inhibition at lower concentration.

Table 5.4: Anti-inflammatory effect of tricin, TEGE, TTGE and diclofenac on carrageenan induced paw edema in rats

Time	Inhibition (%) of different treatment groups			Drug
	Tricin	TEGE	TTGE	Diclofenac
	(Dose 2mg/Kg)			(Dose 20mg/Kg)
3rd h	50.0±1.44^{b}	20.0±0.57^{c}	50.0±1.44^{b}	72.0±2.08^{a}
5th h	70.0±2.02^{b}	44.4±1.27^{c}	66.6±1.91^{b}	86.0±2.48^{a}

$^{a-c}$Mean±SEM of six determinations followed by different letters in a row are significantly different in the Duncan's test at $P<0.05$.

In the present study, all the compounds showed edema inhibition as compared to diclofenac. The edema inhibition by tricin and TTGE was maximum and it was more pronounced in the second phase, suggesting its inhibitory effect on prostaglandin production as a major mechanism by which compounds exert anti-inflammatory effect. Hence, the pronounced activity of the compounds in the second phase in the *in vivo* experiment supports the *in vitro* activity by downregulating the prostaglandin metabolites, COX and 5-LOX. Moscatelli *et al.* (2006) have studied the *in vitro* anti-inflammatory activity of tricin, based on prostaglandin E_2 levels, cyclooxygenase (COX) and phospholipase activity.[30] Chang *et al.* (2010) mention about the anti-inflammatory effect of salcolin B (identical with tricin 4'-O-(*erythro-β*-guaiacylglyceryl) ether) only, based on inhibition of nitric oxide (NO) in a cell line of mouse macrophages (RAW 264.7).[31] Activated monocytes are a good source of lipid mediators and hence, the study conducted using hpBMCs *in vitro* has given a clear picture that the compounds can inhibit key enzymes involved in inflammation. It was found that the compounds have potency in inhibiting 5-LOX, COX, NOS activities and

oxidative stress. These total effects were confirmed by *in vivo* anti-inflammatory effect. Radical Scavenging, antioxidant efficacy of the compounds are exemplified by the results of different biochemical assays especially, SOD assay, that points towards better utilisation of Njavara (as 'brown rice') as a cardioprotective medicinal food also.

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Chapter 5: Section C

Anticancer Activity of Flavonolignans

Cancer continues to be the second major cause of death worldwide, even after significant progress in pharmaceuticals, which reminds that more research has to be undertaken still further. Many undesirable side effects occur during chemotherapy. Natural remedies, such as the use of plant-derived products in holistic cancer treatment, may reduce such side effects. About 60% of anticancer drugs developed during last 25 years are from natural sources.[1] Apoptosis has always been a target in cancer therapy and plant sources are always negotiated for identifying novel bioactive compounds for induction of apoptosis especially, with minimal side effects.[2] Some of the anticancer agents isolated from plant sources have been discussed early in Chapter 1

Tricin, the flavonoid found in some plant species, Tricin is already reported to be a chemopreventive agent[3] but the tricin conjugates, tricin 4'-O-(*erythro-β*-guaiacylglyceryl) ether (TEGE) and tricin 4'-O-(*threo-β*-guaiacylglyceryl) ether (TTGE), are unexplored in terms of cytotoxic studies. Though, Njavara has been traditionally usedas a health food and in the treatment of rheumatism, inflammatory disorders ,rejuvenation therapy etc. , its application in cancer cure has not been directly verified so far. Recently an anticancer gene, Bowman-Brisk trypsin inhibitor protein, has been identified in Njavara.[4] Rao *et al*. (2010) have shown cell cytotoxic properties for methanolic extracts of Njavara rice bran.[5] Earlier studies show that flavonolignans have cytotoxic effects towards cancer cells. Silibinin, a flavonolignan isolated from 'milk thistle' (*Silybum marianum*), was reported to have anticancer effects against human prostate adenocarcinoma, estrogen-dependent and independent breast carcinoma cells.[6] Hence based on above all reports the flavonolignans, were evaluated for the cytotoxic studies towards three cancer cell lines- colon adenocarcinoma cell line HCT 116, ovarian cancer cell line SKOV3 and breast cancer cell line MCF-7. Before going to the details of results of the anticancer studies, a brief description of apotosis is portrayed below.

5.7 Apoptosis

Cancer is one of the most dreadful diseases of the 20th century and is emerging as a major problem globally; both in more developed and in less developed countries. Cancer mortality in the world, as a whole is more than twice in developing countries. Studies shows that the increase in rate the of cancer is due to the earlier onset of the tobacco epidemic, earlier exposure to occupational carcinogens and the western diet and life style. The recent cancer report, released by WHO, observes that world cancer rates are set to double by 2020.[7]

5.7.1 The biology of cancer

Cancer development is a dynamic and long term process, involves many complex factors with a stepwise progression that ultimately leads to metastasis, an uncontrolled spreading and growth of cancerous cells throughout the body.[8] Cancer cells manifest, to varying degrees, four characteristics that distinguish them from normal cells: (1) uncontrolled proliferation, (2) differentiation and loss of function, (3) invasiveness, and (4) metastasis.[9,10] An abnormal cell that proliferates uncontrollably eventually gives rise to a tumour, or neoplasm, a relentlessly growing mass of abnormal cells. A tumour is counted as a cancer only if it is malignant, that is, only if its cells have the ability to invade surrounding tissue. Invasiveness usually implies an ability to break loose, enter the blood stream or lymphatic vessels, and form secondary tumours, or metastases, at other sites in the body.[11]

5.7.2 Apoptosis

Programmed cell death (PCD) is essential for the development and maintenance of multicellular organisms. PCD plays a central role in embryogenesis and normal adult tissue homeostasis by regulating the balance between cell death and cell proliferation. It is also important for eliminating cells that are potentially harmful to the body, such as cancerous cells and cells harbouring viruses.[12] Many eukaryotic cells that die and are removed in a programmed way undergo an astonishingly stereotypical series of biochemical and morphological changes. The most defining features of this PCD are chromatin condensation, the display of phagocytosis markers on the cell surface, disruption of mitochondria membrane potential and the activation of a family of proteases called caspases that are responsible for the systematic breakdown of the cell, culminating in “cellular suicide”.[13,14] The underlying death process has been called

apoptosis to distinguish it clearly from other cell death programmes.[15] The earliest recognized morphological changes in apoptosis are condensation and segregation of the nuclear chromatin, with the formation of sharply delineated, finely granular masses that become marginalized against the nuclear envelope. Condensation of the cytoplasm (cell shrinkage) translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane is known to be a very early event during the initial stages of apoptosis.[16]

5.8 Cytotoxic studies of flavonolignans on cancer cells

Most anticancer drugs that are in current clinical use kill their target cells by apoptosis.[17-21] Morphology remains the definite criterion for apoptosis and should always be used to confirm any biochemical evidence of apoptosis.[12] Both morphological and biochemical methods were used to investigate the induction of apoptosis in this study. Fluorescence microscopy was used to study chromatin condensation and nuclear fragmentation by fluorescent dyes like Hoechst stain. Biochemical methods of detection included annexin V binding to indicate phosphatidylserine externalization and mitochondrial membrane potential loss. Morpholgical and biochemical methods used in present study are discussed in Chapter 2, section 2.6 and their results are discussed below.

5.8.1 Chromatin condensation

Chromatin undergoes condensation, subsequent to cleavage of structural elements of nucleus by activated caspases, the important enzymes triggered during programmed cell death. In order to understand whether the compounds tricin 4'-O-(*erythro*-*β*-guaiacylglyceryl) ether (TEGE) and tricin 4'-O-(*threo*-*β*-guaiacylglyceryl) ether (TTGE) induced apoptotic cell death, three different cell lines belonging to breast (MCF-7), ovarian (SKOV3) and colon tumours (HCT 116) cancer cells were exposed to different concentrations of compounds for 24 and 48 h. The cells, after indicated period, were stained with the cell-permeable, DNA-specific stain, Hoechst 33342. The cells with intense localized fluorescence in the nucleus were scored as condensed and apoptotic cells. Apoptosis was observed in all the three types of cells by TEGE and TTGE (concentration from 1-100 µg/ml) for 24 and 48 h (Figure 5.13a and b). Treatment with 40 µg/ml of TEGE, after 24 h, increased the percentage of cells with

condensed nuclei to 48 % in HCT 116 cells, 52% in MCF-7 cells and 54% in SKOV3 cells. By 48 h, cell death was increased to 55% in HCT 116, 58% in MCF-7 cells and 59% in SKOV3 cells. Similarly, TTGE at a concentration of 30 µg/ml, induced chromatin condensation in 53% of cells in HCT 116, 48% in MCF-7 cells, 63% in SKOV3 cells at 24 h of TEGE treatment (Figure 5.14a, b and c). More than 50% of apoptosis was shown by TEGE, TTGE and standard drug Camptothecin at concentrations of 40 µg/ml, 30 µg/ml and 1 µg/ml respectively which is shown in Figure 5.15. In all the three cell lines, concentration (1-100 µg/ml of TEGE and TTGE) and time dependent (24 and 48 h) increase in percentage of apoptosis, was observed.

5.8.2 Mitochondrial membrane potential (MMP) loss

Decrease in the mitochondrial membrane potential is an indicator of opening of mitochondrial permeability transition (MPT) pore and is known to be induced by a variety of antitumor agents targeting mitochondrial apoptosis. Most often, mitochondria upon loss of its membrane potential, release its apoptogenic intermembrane protein in to cytosol leading to caspase dependent or caspase independent cell death.[22] To substantiate the role of mitochondria, we have analyzed the status of mitochondrial membrane potential after treatment with the compounds, by staining the cells with potential-specific dye JC-1, followed by analysis using fluorescent microscope. Figure 5.16 shows that the mitochondrial membrane potential loss was induced by both the compounds TEGE (40 µg/ml) and TTGE (30 µg/ml) in all the cells as indicated by the loss of red mitochondrial fluorescence upon membrane potential loss. The study reveals that both the compounds are capable of targeting classical mitochondria mediated apoptotic cell death in cancer cell lines. Since significant mitochondrial potential loss was observed in treated samples, flow cytometry - based quantitation of MMP loss was carried out further using another dye, TMRM.

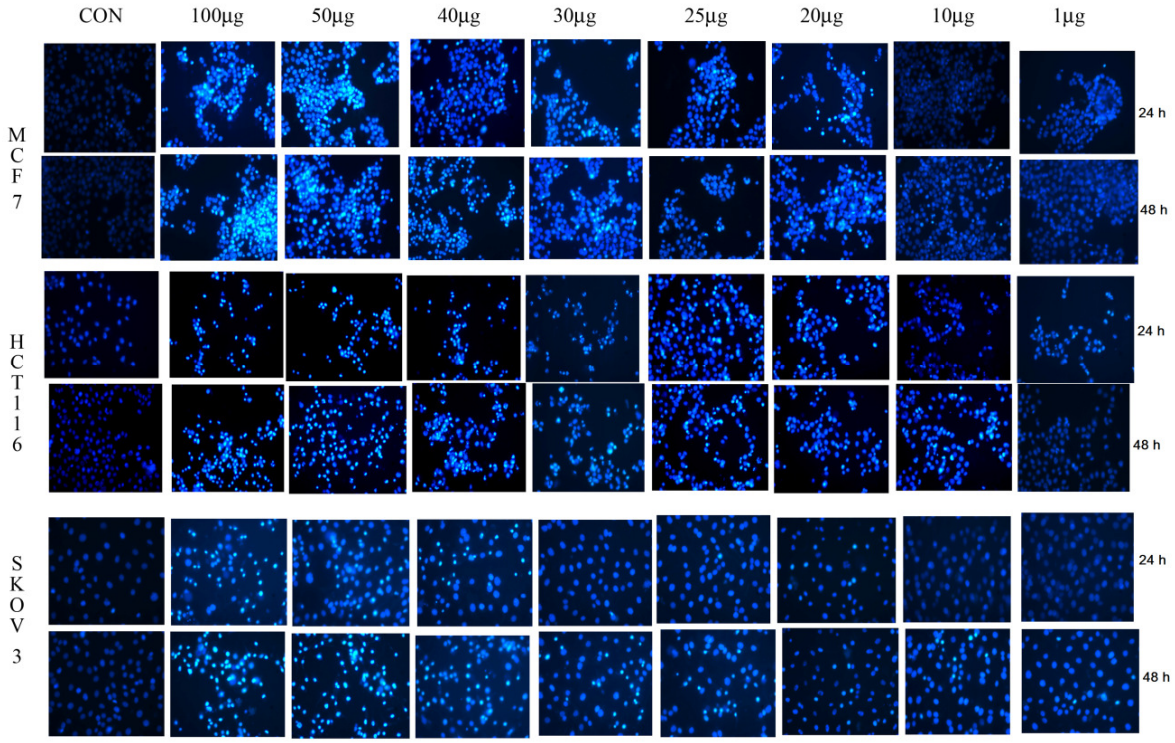

Figure 5.13 a: Images showing the morphology of HCT116, MCF7 and SKOV3 cells as control and the cells treated with TEGE in the chromatin condensation assay by Hoechst staining after 24 and 48 h at concentrations 1, 10, 20, 25, 30, 40 50 and 100 µg/ml. The intensely stained nuclei represent cells with condensed chromatin. The magnification is 20X.

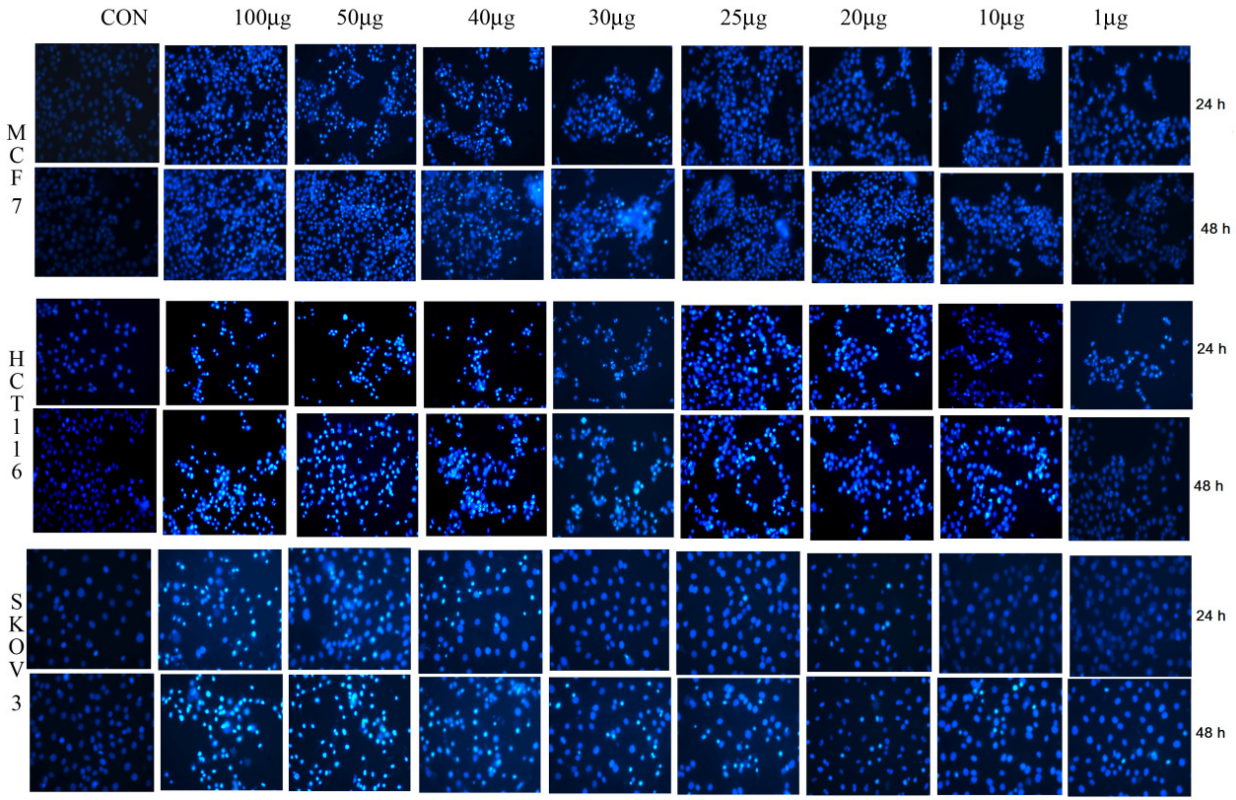

Figure 5.13 b: Images showing the morphology of HCT116, MCF7 and SKOV3 cells as control and the cells treated with TTGE in the chromatin condensation assay by Hoechst staining after 24 and 48 h at concentrations 1, 10, 20, 25, 30, 40 50 and 100 µg/ml. The intensely stained nuclei represent cells with condensed chromatin. The magnification is 20X.

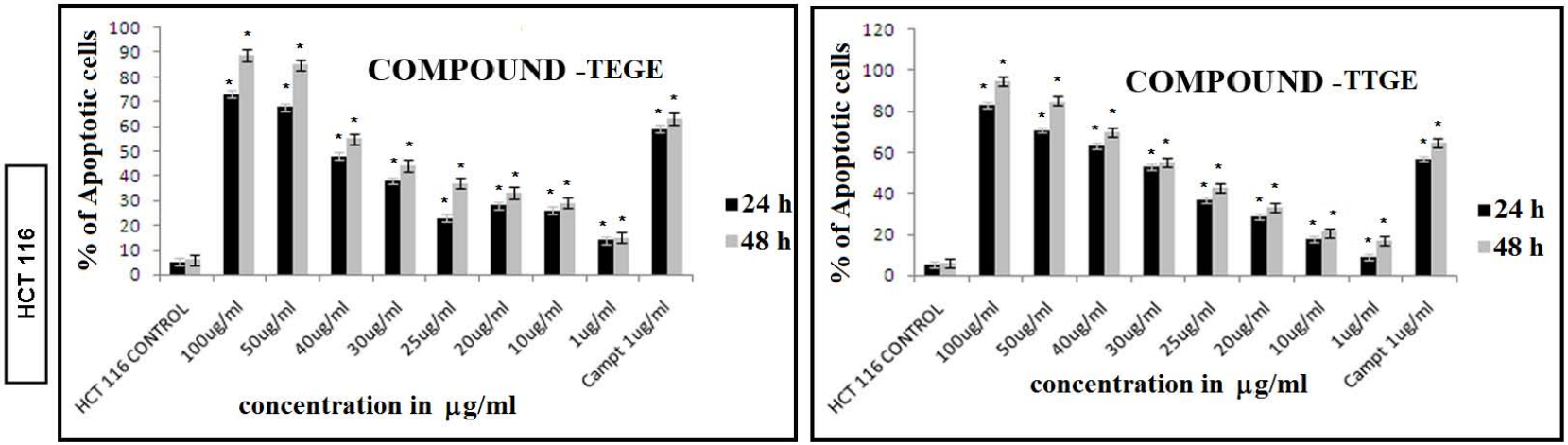

Figure 5.14a: Cells with condensed or fragmented nuclei were taken as the apoptotic population and counted against total number of cells in the field and plotted graphically with percentage of apoptotic cells against different concentrations of compound TEGE and TTGE compared with standard camptothecin (campt-1µg/ml) and a control of cells in a) HCT 116. Results show mean values ± SD of two analyses of three replications (n=6). *Significantly different from control ($P< 0.05$).

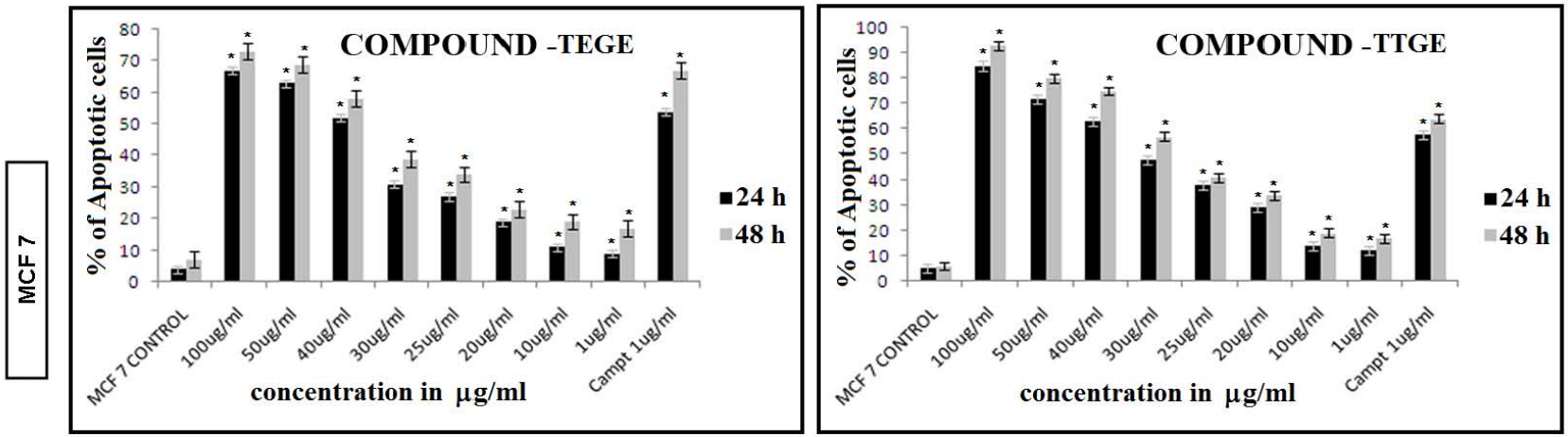

Figure 5.14b: Cells with condensed or fragmented nuclei were taken as the apoptotic population and counted against total number of cells in the field and plotted graphically with percentage of apoptotic cells against different concentrations of compound TEGE and TTGE compared with standard camptothecin (campt-1µg/ml) and a control of cells in MCF-7. Results show mean values ± SD of two analyses of three replications (n=6). *Significantly different from control ($P < 0.05$).

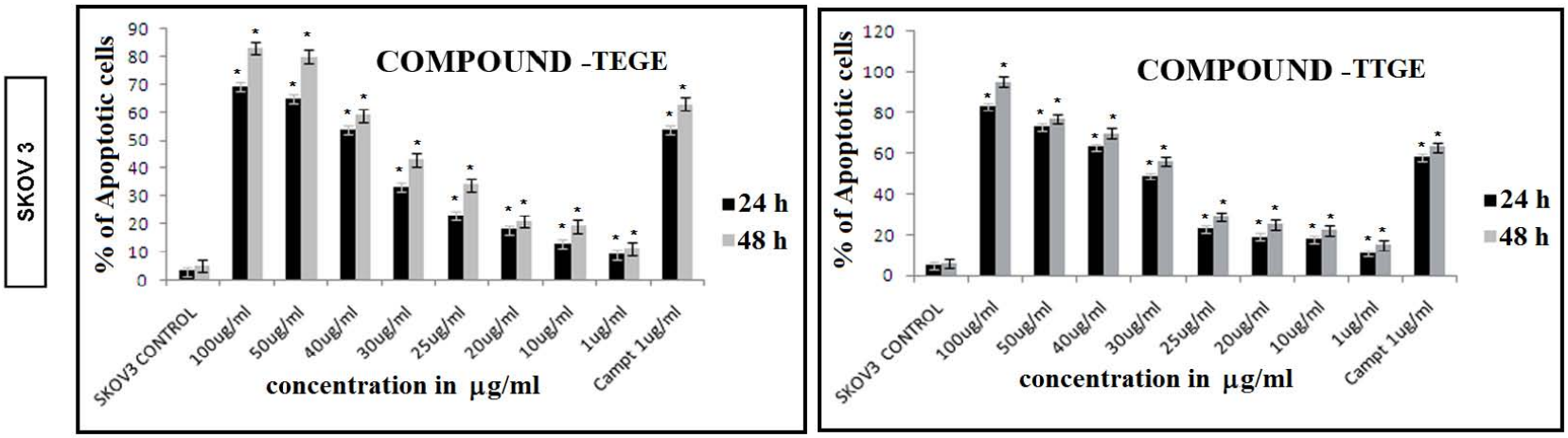

Figure 5.14c: Cells with condensed or fragmented nuclei were taken as the apoptotic population and counted against total number of cells in the field and plotted graphically with percentage of apoptotic cells against different concentrations of compound TEGE and TTGE compared with standard camptothecin (campt-1µg/ml) and a control of cells in SKOV3. Results show mean values ± SD of two analyses of three replications (n=6). *Significantly different from control ($P < 0.05$).

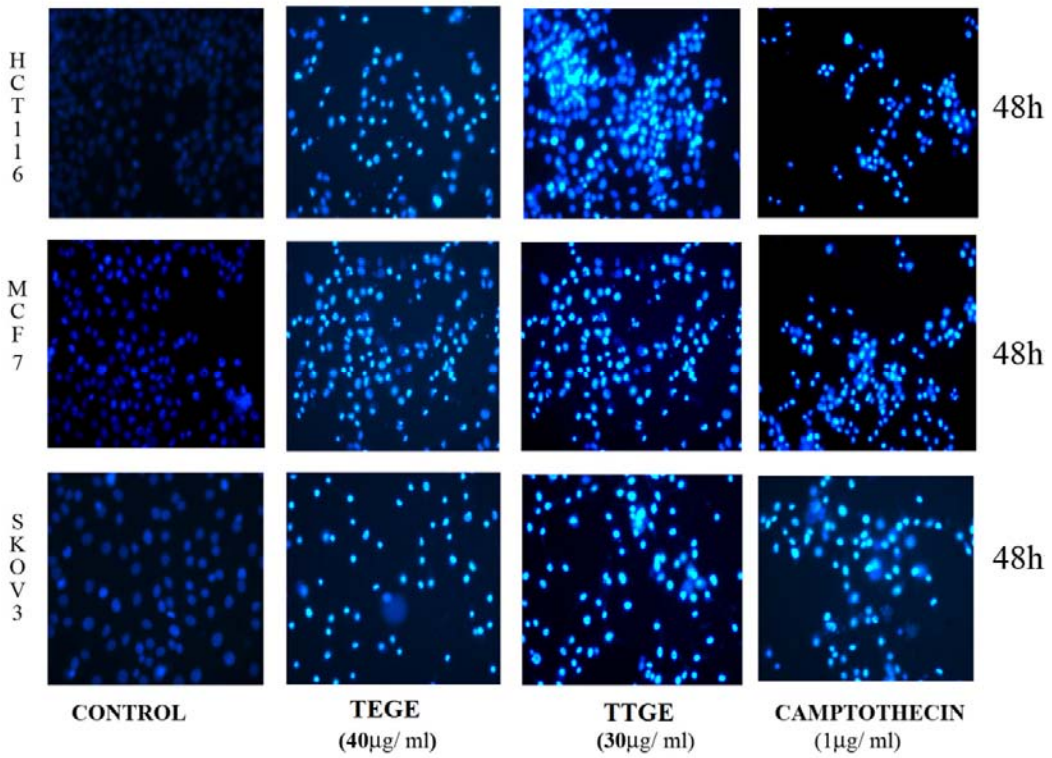

Figure 5.15: Images showing the morphology of HCT116, MCF7 and SKOV3 cells as control and images of the cells treated with TEGE (40 µg/ml) , TTGE (30 µg/ml) and camptothecin (1 µg/ml) in the chromatin condensation assay by Hoechst staining after 48 h. The magnification is 20X.

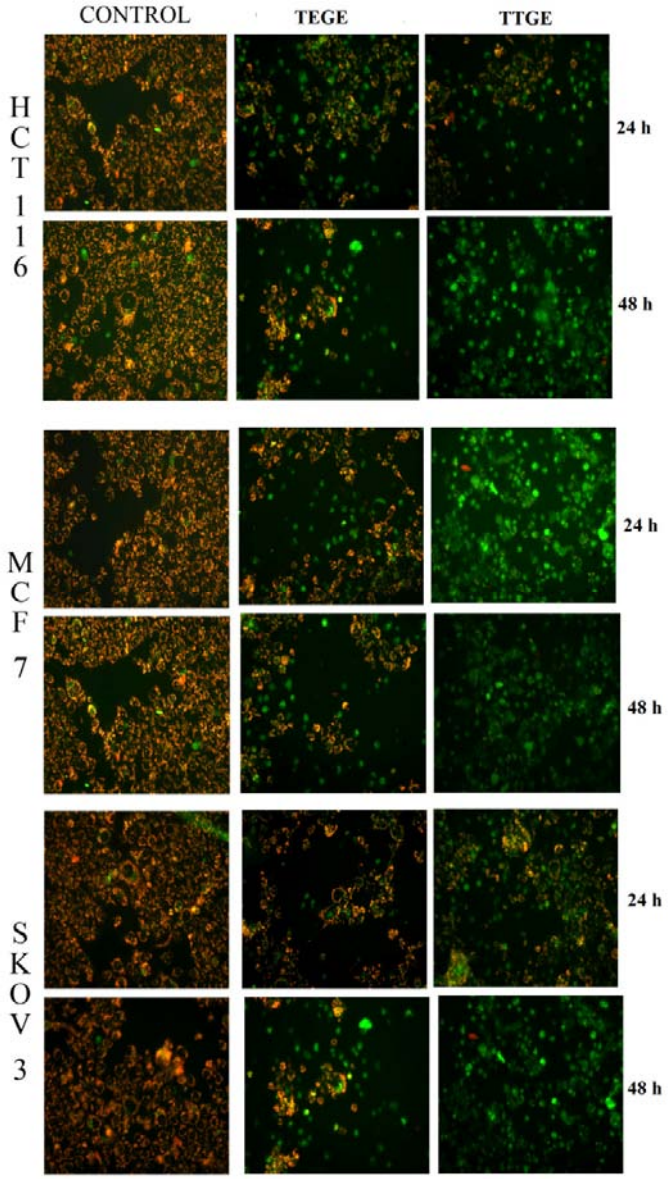

Figure 5.16: HCT 116, MCF-7 and SKOV3 cells pretreated with TEGE and TTGE were stained with lypophilic cation dye- JC-1, which is a sensitive fluorescent probe, to assess mitochondrial membrane potential (MMP) loss. In apoptotic cells the membrane potential is lost and the dye exists as monomer there by showing green fluorescence. Images shown were taken using fluorescent microscopy at 20X magnification.

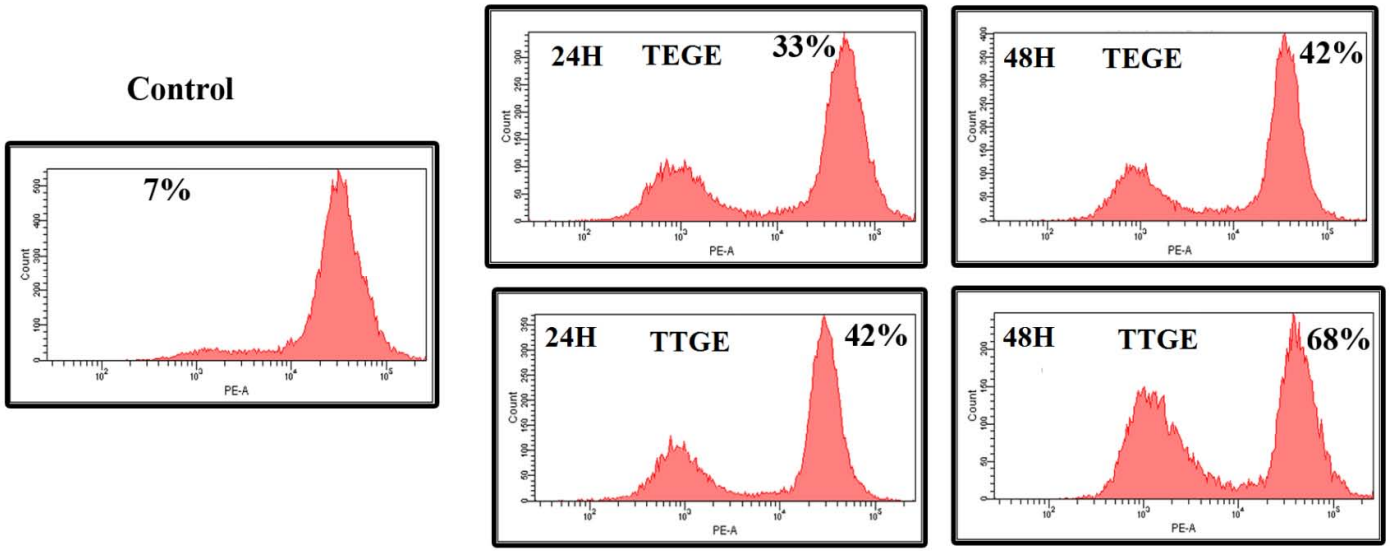

Figure 5.17: HCT 116 cells were treated with the compound TEGE and TTGE for the indicated time point. Then the cells were trypsinized and stained with TMRM as described and analysed by Flow cytometer.

The principle underlying this method was that TMRM cannot stay in mitochondria, upon loss of mitochondrial membrane potential, resulting in the loss of cellular red fluorescence. Thus, TMRM fluorescence was followed after treating HCT 116 cells with the two compounds, for 24h and 48h. Both the compounds significantly induced accumulation of pale red cells in a time-dependent manner (Figure 5.17). In short, our results suggest that both the compounds target the cancer cells and induce apoptosis through mitochondrial-dependent pathways.

5.8.3 Annexin V staining

During the early phase of apoptosis, exposure of phosphatidyl serine (PS) on to the membrane of cells has been observed.[23] One of the simple methods to detect the PS exposure is binding of fluorescent labelled Annexin V to the exposed PS group on to the cell membrane. 'Alexa Fluor 488' labelled Annexin V was employed to study the apoptosis induced by both compounds TEGE and TTGE in HCT 116 cells (that responded maximum among the three cell lines), for 24 h and 48 h. The Annexin V binding was quantified by FACS Aria II. The results are represented in Figure 5.18. As seen from the data, both the compounds significantly increased the Annexin V binding to most of the treated cells compared to untreated control.

This study shows that both the compounds are capable of inducing significant cytotoxicity in all the tumor cells studied. Moreover, preliminary data suggests that the cytotoxicty is associated with apoptotic changes as demonstrated by condensed chromatin and Annexin V exposure on to the surface of the treated cells. These results also show that these compounds target mitochondria and induce its potential loss thereby promoting mitochondrial membrane permeability mediated cell death. Recent reports suggest that mitochondria are the prominent organelle targeted by most antitumor agents currently used in the clinic.[24]

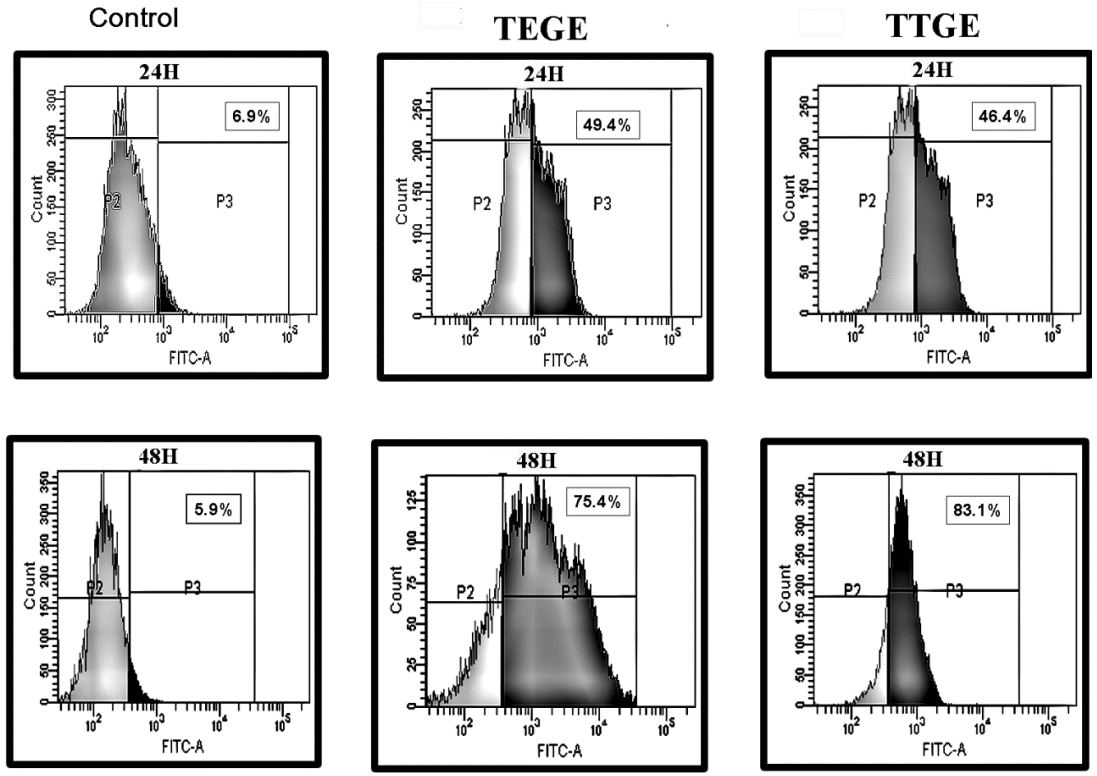

Figure 5.18: Treated cells were stained with fluorescent conjugate Annexin V-Fluoroscein-isothiocynate (Annexin-FITC) conjugate as described in material and methods. The fluorescent signals from 10,000 cells were collected and analysed using FACS ARIA II from Becton, Dickinson and given as histogram.

In conclusion, the study establishes the antitumour effect of the rare flavonolignans namely, tricin 4'-O-(*erythro-β*- guaiacylglyceryl) ether and tricin 4'-O-(*threo-β*-guaiacylglyceryl) ether and their possible role as potential cytotoxic agents against multiple cancer cells, in future. At this juncture, it is to be remembered that tricin, a proven chemotherapeutic agent, occurs at higher concentrations in Njavara. These results also indicate possibility of utilising Njavara in the holistic treatment regimes suggested by many medical experts in diseases like cancer, at least among populations consuming rice.

5.9 References

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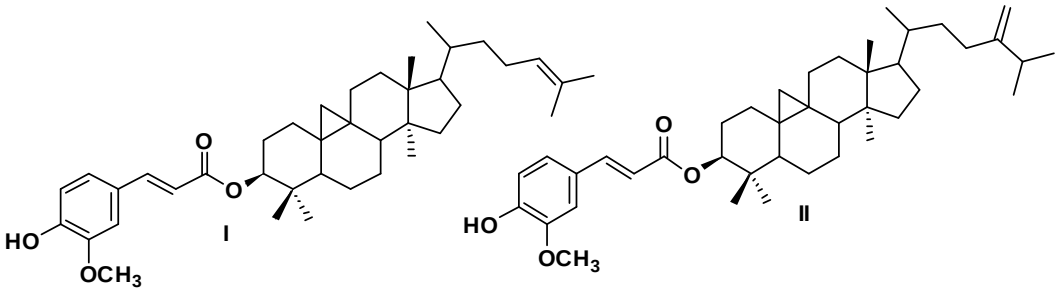
Summary and Conclusions

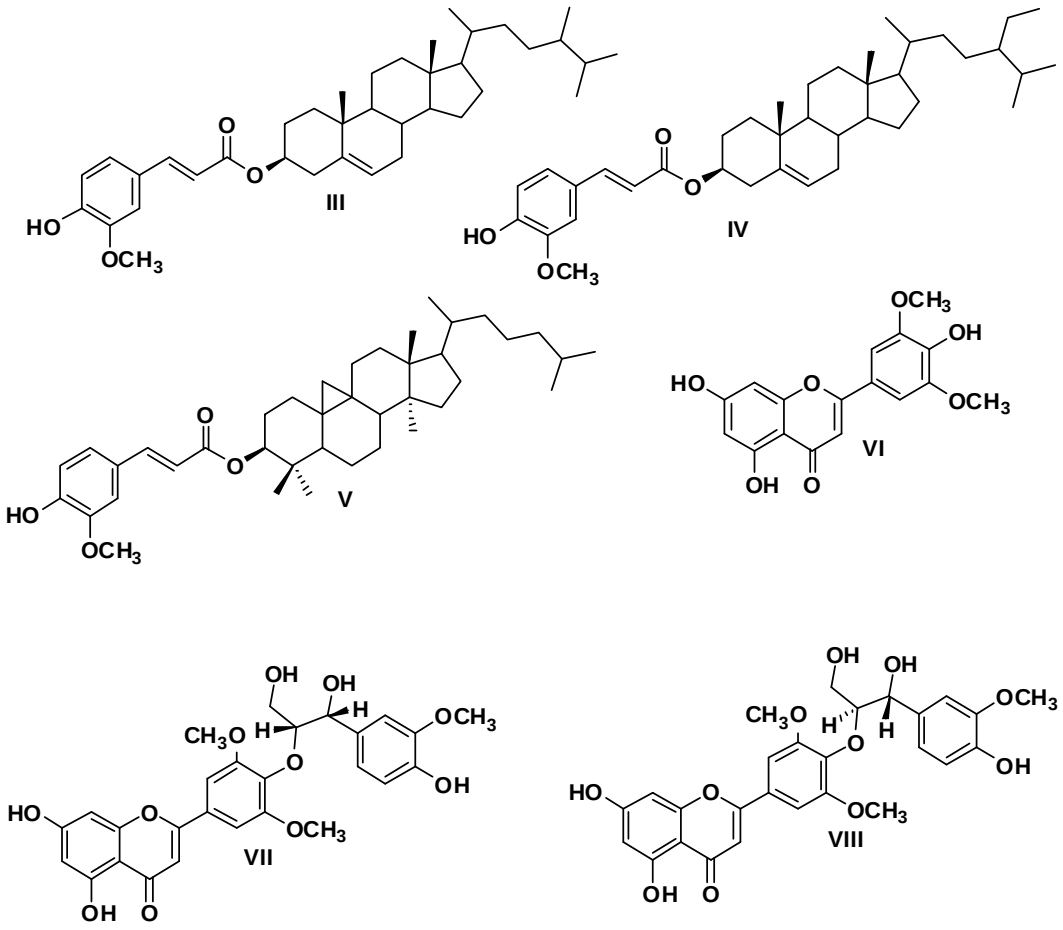
Contemporary importance of free radicals and Reactive Oxygen Species (ROS) as causative agents in degenerative diseases and conditions like atherosclerosis, cancer, inflammation, rheumatism, aging etc. cannot be overlooked. Plants are a potent source of natural antioxidants. India, especially the southern state of Kerala, is well known for its rich biodiversity, medicinal plants and practice of Ayurveda. Recently, there is greater acceptance of 'holistic' treatment regimes to fight degenerative disease conditions using herbal extracts that are rich in antioxidant phytochemicals. Njavara is an important medicinal rice variety endemic to Kerala and traditionally used in Ayurvedic treatment protocols of 'Panchakarma' such as 'Njavarakizhi' and 'Njavara Theppu', for arthritis, paralysis, rejuvenation therapy etc. There are two types of Njavara - 'black glumed' and 'yellow glumed', depending on colour of outer husk. The black type is more preferred by ayurvedic physicians. As there are no previous scientific reports on the bioactive compounds of Njavara and their activity, the present study was undertaken focussing on the antioxidant activity, identification and quantification of bioactive compounds in 'black glumed' Njavara (Njavara Black) as compared to staple varieties Sujatha and Palakkadan Matta, followed by evaluation of antioxidant, anti-inflammatory and anticancer effects of bioactive compounds.

The results of the studies on chemical indices, antioxidant activity and anti-inflammatory effect (*in vivo*) of 'Njavara black' rice bran (NBb) and rice (NBr), in comparison with rice bran and rice of non-medicinal, staple varieties Sujatha(SJ) and Palakkadan Matta(PM). For NBb, the phenolic, flavonoid and proanthocyanidin contents were equivalent to 27.16 mg of gallic acid/g dry weight of bran, 4.50 mg of quercetin/g dry weight of bran and 0.98 mg of catechin/g dry weight of bran, respectively and for Njavara rice and staple varieties (both bran and rice) the corresponding values varied from 0.56 to 5.27 mg of gallic acid/g, 0.03 to 1.04 mg of quercetin/g and 0.05 to 0.37mg of catechin/g dry weight of respective samples. Thus, the total phenolic, flavonoid and proanthocyanidin contents were higher in Njavara rice bran compared to its rice and staple varieties (both bran and rice). Scavenging of 2, 2-diphenyl-1-picryl hydrazyl radical (IC_{50}: 84.66 µg/ml), superoxide anion radical (IC_{50}: 203.16 µg/ml), hydrogen peroxide (IC_{50}: 73.55µg/ml) and reducing power were

significantly higher for NBb compared to Njavara rice extracts as well as bran and rice extracts of staple varieties. The anti-inflammatory effect (*in vivo*) of NBb and NBr were 83.3% and 66.69% respectively whereas, staple varieties showed activity in the range 16.60-53.80%. The anti-inflammatory effect was also significantly higher for Njavara rice bran compared to its rice and other staple varieties.

The phytochemical investigations and quantification of bioactive compounds of petroleum ether extracts of Njavara showed five major oryzanols namely, cycloartenyl ferulate (**1**), 24- methylene cylcoartanyl ferulate (**II**), campesteryl ferulate (**III**), β-sitosteryl ferulate (**IV**) and cycloartanyl ferulate (**V**). These compounds were identified and quantified from petroleum ether extracts of Njavara rice bran and rice, in a comparative study with staple varieties. For NBb, the total oryzanol content was 1.84 mg/g dry weight of bran and for Njavara rice and staple varieties (both bran and rice) the corresponding values varied from 0.67 to 0.09 mg/g dry weight of bran. Quantitatively, the total oryzanol content was 2.7 and 7.3 times higher in NBb than in SJb (Sujatha bran) and PMb (Palakkadan Matta bran) whereas corresponding values were less in rice. Phytochemical investigations of the diethyl ether fraction of methanolic extract of Njavara Black rice bran led to the identification of hexadecanoic acid, 10-octadecenoic acid and octadecanoic acid by GCMS and three very important bioactive compounds namely, tricin (**VI**) and two rare flavonolignans-tricin 4'-O-(*erythro-β*-guaiacylglyceryl) ether (**VII**) and tricin 4'-O-(*threo-β*-guaiacylglyceryl) ether (**VIII**). Tricin and two rare flavonolignans in diethyl ether extract of Njavara rice bran were quantified in comparison with staple varieties by HPLC and showed that tricin (chemopreventive agent) is present 39.64 and 16.12 fold higher in Njavara, compared to Sujatha and Palakkadan Matta respectively. This is the first report on the occurrence of tricin at significantly higher levels in Njavara and occurrence of the two flavonolignans in *Oryza sativa* species.





The bioactive compounds, tricin and the two flavonolignans, were studied for antioxidant and anti-inflammatory effect. The order of activity, based on these assays, is tricin > tricin 4'-O-(*threo-β*-guaiacylglyceryl) ether > tricin 4'-O-(*erythro-β*-guaiacylglyceryl) ether. In order to gain insight into the stability of the radicals formed and the ease with which it is formed from the above three active compounds, Density Functional Theory (DFT) was applied. Free-radical scavenging activity based on DPPH radical scavenging of tricin and the two flavonolignans (tricin conjugates) were explained theoretically in conjunction with isodesmic approach for hydrogen transfer mechanism(HAT) and compared with standard quercetin by the critical evaluation of the factors such as bond dissociation enthalpy (BDE), ionisation potential (IP) and spin

density distribution of its radical. The theoretical studies agree with the results of DPPH radical scavenging activities of the three compounds, in the same order as mentioned above.

Results of *in vitro* anti-inflammatory activity of tricin and two flavonolignans showed that the three compounds substantially attenuated the levels of various inflammatory enzymes like COX, LOX, NOS and oxidative stress (in terms of SOD and MDA values), induced by LPS in cell culture of human peripheral blood mononuclear cells. In the *in vivo* model of the experiment, the three compounds significantly inhibited paw edema (carrageenan- induced) in rats, with tricin and the *threo*- form of flavonolignan showing anti-inflammatory effect of >65% after 5 h, at 2 mg/kg.

Tricin, which is present in higher concentrations in Njavara, has been highlighted as a multifunctional nutraceutical by reviewers, very recently. As it is already established as a known chemopreventive agent, only the flavonolignans were evaluated for anticancer effects, in this study. Chromatin condensation in the three cancer cell lines (colon, ovarian and breast tumours) by Hoechst staining, showed >50% of apoptosis by compounds tricin 4'-O-(*erythro-β*-guaiacylglyceryl) ether and tricin 4'-O-(*threo-β*-guaiacylglyceryl) ether at concentration 40 μg/ml and 30 μg/ml respectively, after 48 h. The study also substantiates that both the compounds targeted cancer cells through mitochondrial membrane potential loss and subsequent chromatin condensation. Both compounds significantly increased the Annexin V binding thus confirming compounds to be potential apoptotic agents. As Hudson *et al*. (2000) has already established the anticancer effects of tricin, only the flavonolignans were evaluated in this thesis work.

To sum up, higher values of chemical indices like phenols, flavonoids and proanthocyanidins and higher antioxidant activity for Njavara confirms that it is a potential source of antioxidants. Our study gave a new insight in to the phytochemical constituents of Njavara and established the presence of three important bioactive compounds- tricin and two rare flavonolignans-tricin 4'-O-(*erythro-β*-guaiacylglyceryl) ether and tricin 4'-O-(*threo-β*-guaiacylglyceryl) ether exhibiting significant antioxidant and anti-inflammatory activity. Higher amount of bioactive oryzanols were also established in the present study. The report of the occurrence of the rare flavonolignans in *Oryza sativa* species, for the first time, forms one of the highlights of our

investigation and throws light on the cytotoxic property of these compounds and Njavara, on cancer cells. Tricin which is already a known chemopreventive and bioactive compound, is present in higher concentrations in Njavara, as evidenced in this work. These results, no doubt, points towards the possibility and better utilisation of Njavara as a health food for cancer patients also. The study also confirmed that the higher antioxidant activity and anti-inflammatory effect of Njavara, compared to staple varieties, were due to the presence of higher levels of tricin and flavonolignans in Njavara compared to staple varieties. The results on the antioxidant and anti-inflammatory activity of Njavara confirm the preferential use of Njavara in indigenous medicine, over staple varieties.

To conclude, the results of this thesis work provides, to the extent possible, a scientific basis to the traditional use of Njavara.

List of publications

1. Isoaltion, Characterisation and Quantification of Tricin and Flavonolignans in the Medicinal Rice Njavara (*Oryza sativa* L.) as Compared to Staple Varieties. **Smitha Mohanlal**, Rathnam Parvathy, Vasantha Shalini, Antony Helen, Ananthasankaran Jayalekshmy, **Plant Foods Hum Nutr** 2011, *66*, 91-96.

2. Chemical Indices, Antioxidant Activity and Anti-inflammatory Effect of Extracts of the Medicinal Rice 'Njavara' and Staple Varieties- A Comparative Study. **Smitha Mohanlal**, Rathnam Parvathy, Vasantha Shalini, Ratheesh Mohanan, Antony Helen, Ananthasankaran Jayalekshmy. Accepted in **Journal of Food Biochemistry** (doi.10.1111/j.1745-4514.2011.00646.x).

3. DPPH Radical Scavenging Activity of Tricin and its Conjugates Isolated from 'Njavara' Rice Bran: Experimental and Theoretical Investigations on Structure-activity Relationships. Manjaly J. Ajitha, **Smitha Mohanlal**, Cherumuttathu H. Suresh and Ananthasankaran Jayalekshmy (Communicated to **Journal of Agricultural Food and Chemistry**).

4. Tricin 4'-O-(*erythro*-β-guaiacylglyceryl) ether and Tricin 4'-O-(*threo*-β-guaiacylglyceryl) ether Isolated from Njavara (*Oryza sativa* L, var. Njavara), Induce Apoptosis in Multiple Tumor Cells by Mitochondrial Pathway. **Smitha Mohanlal**, Sathish Kumar Maney, Thankayyan Retnabai Santhoshkumar, Ananthasankaran Jayalekshmy. (to be communicated shortly to **Journal of Natural Medicine**)

5. Anti-inflammatory and Anti-oxidant Properties of a Flavonoid, Tricin Isolated from Njavara (*Oryza sativa* L.) Rice bran. Vasantha Shalini, Shobha Bhaskar, **Smitha Mohanlal**, Ananthasankaran Jayalekshmy, Antony Helen. (to be communicated shortly to **Phytomedicine**).

Posters Presented

Oral presentation

1. Antioxidant studies and Bioactive compound profiles of Njavara (Oryza sativa L., var. Njavara). **Smitha Mohanlal.** National Seminar on Medicinal Plants. Rajiv Gandhi Centre for Biotechnology, Jagathy, Thiruvananthapuram. 24-25 July, **2008.**

Posters presented

2. Phytochemical Investigation of 'Njavara' (Oryza sativa L, var. 'njavara').**Mohanlal Smitha**, Jayalekshmy Ananthasankaran. In: Proceedings of 5th J-NOST Conference for Research Scholars, Kanpur, IIT. 4-7 Dec, **2009**, p.73.

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