STUDIES ON THE BIOACTIVE NATURAL ANTIOXIDANTS FROM OILSEEDS

Thesis submitted to
Cochin University of Science and Technology
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
In
CHEMISTRY

BY
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APRIL 2005

DECLARATION

I hereby declare that the thesis entitled **"STUDIES ON THE BIOACTIVE NATURAL ANTIOXIDANTS FROM OILSEEDS"** embodies the results of investigations carried out by me at the Agroprocessing and Natural Products Division of the Regional Research Laboratory (CSIR), Thiruvananthapuram, as a full-time Research Scholar under the supervision of Dr.C.Arumughan and Dr. A. Jayalekshmy and that no part of this thesis has been presented before for any other degree.

Renu-R

Thiruvananthapuram
April 12, 2005

Renuka Devi.R

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This is to certify that the thesis entitled **"STUDIES ON THE BIOACTIVE NATURAL ANTIOXIDANTS FROM OILSEEDS"** is an authentic record of the research work carried out by Mrs. Renuka Devi. R under my supervision in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Chemistry of the Cochin University of Science and Technology, and further that no part of this thesis has been submitted elsewhere for any other degree.

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Renukadevi.R

C O N T E N T S

Dedicated to my teachers,
friends and family

ABSTRACT

Oxidation and consequent generation of reactive oxygen species (ROS) is the major cause for deterioration of food lipids. ROS have also been implicated in a variety of degenerative diseases. Epidemiological and experimental evidences suggest close links between ROS and diseases such as cancer, CVD, diabetes, cataract etc. In the human body, ROS is balanced by an array of endogenous and exogenous antioxidants. A disturbance in the pro-oxidant-antioxidant balance in favour of the former, is termed as oxidative stress and can cause potential damage including lipid peroxidation and consequent cytotoxicity.

Antioxidants are substances that when present at low concentrations compared to that of an oxidisable substrate significantly delays or inhibits oxidation of that substrate in food products or in living systems. Antioxidants are either endogenous to the body or derived from the diet. Several types of synthetic antioxidants like BHT, BHA, TBHQ etc. are also used in the food industry. However, findings and subsequent publicity has fostered significant consumer resistance to the use of synthetic food additives as antioxidants, colourants etc. and therefore food industry is in search of potential natural antioxidants from edible sources.

The major dietary sources of antioxidant phytochemicals are cereals, legumes, fruits, vegetables, oilseeds, beverages, spices and herbs. In the present study, we have focused on rice bran and its byproducts. Rice is one of the oldest of food crops and has been a staple food in India from very ancient times. It is also the staple food for about 60% of the world's population. Rice bran is a byproduct of the rice milling industry and is a potential commercial source of a healthy edible oil viz. rice bran oil and a variety of bio-active phytochemicals.

Defatted rice bran (DRB), a byproduct of rice bran oil extraction, is also a good source of insoluble dietary fiber, protein, phytic acid, inositol, vitamin B and a variety of other phytochemicals. Though the antioxidant potential of DRB has been demonstrated, it still remained a relatively unexplored source material, which demanded further investigation especially with regard to its detailed phytochemical profile leading to practical application. The focus of the present investigation therefore has been on DRB primarily to establish its phytochemical status and feasibility of using it as a source of bio-active phytochemicals and natural antioxidants leading to value addition of DRB otherwise used as cattle feed. To gain a better understanding of the value of rice bran as a source of phytochemicals, five popular rice varieties of the region viz. PTB 50, PTB 39, PTB 38, JAYA, and MO 10 and a wild variety (*oryza nivara*) that is mainly used for medicinal applications in traditional ayurvedic system were characterized along with commercial samples of rice bran. The present study also explains the feasibility of a process for the extraction, enrichment, and isolation of antioxidant compounds from DRB. The antioxidant potential of the extracts were evaluated both in bulk oils and in food relevant model emulsions, using standard *in vitro* models. Radical scavenging effects, indicative of possible biological effects, were also evaluated. The thesis consists of four chapters.

Chapter 1 reviews the literature on oxidation and formation of reactive oxygen species (ROS), oxidative stress and its major consequences, and the role of antioxidants in preventing lipid peroxidation. It also gives comprehensive data on the natural distribution of dietary phytochemicals with special emphasis on oleaginous plant products. This chapter also updates the literature on major rice phytochemicals, their antioxidant and biological effects and highlights the relevance of the present investigation.

Chapter 2 describes in detail about the materials and methods employed for the present investigation. Six rice varieties namely PTB 50, PTB 39, PTB

38, JAYA, MO 10 and *oryza nivara* obtained from Rice Research Station, Kayamkulam, (Kerala) as well as commercial samples of rice bran and rice bran oil were used for the study. The rice bran and rice bran oil samples were defined in terms of their chemical composition and chemical characteristics respectively. The phytochemical profile of full fat rice bran (FFB), defatted rice bran (DRB), and rice bran oil (RBO) in terms of oryzanols, ferulic acid, and tocols were determined with modified HPLC protocols. The feasibility of a process for the extraction, enrichment, and isolation of antioxidant compounds from DRB is also explained. For enrichment of TPC, oryzanol and ferulic acid content of the crude extracts, sequential extraction technique was employed. The isolation of components present in the extract was carried out by column chromatography. The isolated compounds were identified using IR, UV, NMR and MS data. The antioxidant potentials of the crude extract, enriched fractions, and the isolated compounds were then evaluated using standard *in-vitro* models. For assessing the antioxidant efficacies of the DRB extracts in bulk oils, Schall Oven Test method (60^{o}C) and differential scanning calorimetry (150^{o}C) were used. To evaluate the activity of the extracts in food relevant emulsions, linoleic acid emulsion method and the β-carotene bleaching test were used. Radical scavenging effects (indicative of possible biological effects) of DRB extracts were studied using the stable DPPH radical and the superoxide radicals generated *in-situ* by the xanthine-xanthine oxidase system. Synthetic antioxidants viz. BHT and TBHQ were used as reference compounds.

Chapter 3 consists of results and discussions. Rice bran, from major cultivars of the region were analysed for their chemical profile. Significant varietal variations were observed in the levels of different nutrients in rice bran. The mean values for major constituents were dry matter (89.1%), fat (16.8%), protein (10.1%), crude fiber (11.3%), ash (11.4%), and available carbohydrates (50.5%). The mean energy content was 393.5 Kcal/100g. The mean values

(ppm) of various minerals followed the order P (13608) > K (9520) > Mg (3844) > Ca (362) > Fe (216) ~ Na (190) > Mn (99) > Zn (39) > Cu (4), with P and Cu being the most and least abundant minerals respectively.

Rice bran oil obtained from the major cultivars were analysed for their chemical characteristics as well as fatty acid composition. The values obtained for FFA, saponification value, iodine value, and unsaponifiable matter were 12.3%, 182.9, 97.3 and 4.8% respectively. RBO had exceptionally high unsaponifiables as compared to that of other edible oils. Major fatty acids of RBO were 16:0, 18:0, 18:1, and 18:2 with mean values of 21.6%, 2.0%, 41.8%, and 32.5% respectively, with saturated to unsaturated ratio of approximately 1:3. The fatty acid profile of RBO is thus close to the ideal ratio of saturated: mono unsaturated: poly unsaturated of 1:1.5:1.

The oryzanol, tocopherol and tocotrienol composition of RBO from the major cultivars were analysed by standardized HPLC protocols. On an average, RBO contained about 2% oryzanols. The various oryzanol components identified in RBO include stigmasteryl ferulate, cycloartanyl ferulate, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, campesteryl ferulate and β-sitosteryl ferulate with the latter four compounds accounting for more than 95% of the total oryzanols in RBO. RBO was rich in chromanols (tocols) with concentrations ranging from 1042 to 1648 ppm. Seven tocols except β-T_3 were separated, identified, and quantitated in all the rice varieties. The major tocopherol isomer in all varieties was α-T, whereas γ-T_3 was the major tocotrienol. However, γ-T_3 was the predominant vitamin E homolog accounting for about 42-70% of the total vitamin E compounds in the selected varieties.

The defatted meal which remained after the extraction of oil from rice bran was also analysed for oryzanols and tocols. On HPLC analysis, extracts of DRB prepared with various solvents was found to contain significant

amounts of oryzanols, tocols as well as ferulic acid. Kinetic studies were designed to select appropriate solvent and to optimise other process parameters like material-solvent ratio, time, temperature etc. for extraction of antioxidants from defatted rice bran. Methanol was found to be the most efficient solvent, with respect to the yield of TPC, oryzanols and ferulic acid from DRB. Other optimized conditions included a material-solvent ratio of 1:15 and a time of extraction of 10 hours using a Soxhlet extractor. The yield of methanol extracts of DRB from the major cultivars ranged from 3.2 to 5.0%. The sugar content of the extracts ranged from 18.8 to 33.8%, protein from 17.9 to 25.0%, TPC from 5.3 to 8.4% and ash from 3.9 to 5.1%. The oryzanol content of the extracts ranged from 2358 to 6602 ppm, ferulic acid from 2541 to 4376 ppm and tocols from 110 to 284 ppm. 24-methylene cycloartanyl ferulate (~45%), and cycloartenyl ferulate (~25%) represented the major oryzanols of the DRB extracts. γ-tocotrienol (~70%), and α-tocopherol (~10%) were the major chromanols.

Enrichment of antioxidants in crude methanol extract was achieved by sequential extraction and fractionation. For this, the CME was re-extracted with less polar organic solvents like ethyl acetate, acetone, ether etc. From this, acetone was found to be the best solvent for ferulic acid and tocols. For further purification of the acetone extract (AE), sequential extraction technique was employed. For this, the dry AE was re-extracted with hexane to give a soluble fraction enriched in lipophilic compounds (AE-LP) and a residue enriched in polar compounds (AE-PP). Considering the bioactive phytochemicals of interest, AE-LP was enriched in oryzanols, and tocols and AE-PP in ferulic acid. Column chromatography was employed to isolate components present in the crude extract. The two pure compounds obtained were identified to be β-sitosterol and tricin based on UV, IR, NMR and MS data. Of these, the flavone tricin is of special phytochemical interest because of its rare occurrence.

The crude extract (CME), the enriched fractions (AE, AE-LP, and AE-PP) and the pure phytochemicals (oryzanols, tocols, ferulic acid, tricin and sterol) were then subjected to a number of antioxidant and antiradical activity assays using standard *in-vitro* models. To evaluate the antioxidant potential of the extracts and its phytochemical constituents in bulk oils, Schall Oven Test method and differential scanning calorimetry (DSC) were used. The results demonstrated that in bulk oils, some of the DRB extracts (AE-PP) were either equally efficient or better than BHT and that at identical concentrations AE-PP, AE-LP, and AE performed better than the phytochemical constituents ozyzanols, ferulic acid and tocols with respect to PV, DV and DSC data. The increase in activity with fractionation might be due to the enhanced levels of antioxidants in the resultant fractions compared to CME. To evaluate the antioxidant potential in food relevant systems, linoleic acid emulsion method and the β-carotene bleaching test were used. The DRB extracts and its phytochemical constituents proved to have significant activity in these emulsion models as well. Contrary to the bulk oil system (SOT & DSC), where the DRB extracts (AE-PP) were either equally efficient or better than BHT, the latter was more effective in emulsions. None of the pure phytochemicals tested performed better than BHT or TBHQ, both in bulk oils and emulsions indicating that the synergistic effects of phytochemicals in the extracts including that of proteins, sugars and unidentified polyphenols could be contributing to the observed efficacy of DRB extracts. The results further suggest that the DRB extracts could be used both in bulk oils and in food emulsions as natural antioxidants.

The antiradical efficacies of DRB extracts and their phytochemical constituents were studied using the stable DPPH radical and the superoxide radicals generated *in-situ* by the xanthine-xanthine oxidase system. The DPPH radical scavenging activity of ferulic acid, and Tmix was greater than that of BHT and the activity of AE-PP was equal to that of BHT. It was also

found that the DPPH scavenging activity of the fractions AE, AE-LP and AE-PP could be largely attributed to the levels of TPC and ferulic acid in the fractions. H-donating capacity (as evaluated by the DPPH method here) is an important biologically significant property of antioxidants to convert potentially damaging ROS (oxyl and peroxyl radicals) into non-toxic species, and in this context DRB could be a good source of such antioxidants. For evaluating the superoxide scavenging activity of the extracts, the cytochrome C and NBT methods were used. The superoxide scavenging activities of the extracts also followed the order of their TPC and ferulic acid contents. Moreover, the various phytochemical constituents of DRB extracts viz. ferulic acid, tricin and T_{mix} also exhibited excellent superoxide radical scavenging activity thus directly supporting the superior antiradical efficacies of DRB extracts.

The conclusions arrived from the present investigation is summarized in chapter 4. The study establishes the feasibility of utilization of rice bran, an abundantly available renewable resource, as a commercial source for natural antioxidants. DRB, as the byproduct of rice bran oil extraction, could contain substantial amounts of antioxidant phytochemicals like oryzanols, tocols and ferulic acid that could be harnessed as a source for natural antioxidants. It is also shown that the DRB retained the entire ferulic acid in rice bran as ferulic acid is not amenable to hexane extract (RBO) as practiced in the industry. Detailed information regarding the antioxidant activity of DRB extracts and their phytochemical constituents were provided. The extracts could be used both in bulk oils and in food emulsions as natural antioxidants. They possessed substantial hydrogen donating capacity too and were found to be effective against superoxide radicals. More over, the residue after antioxidant extraction could still be used as cattle feed.

ABBREVIATIONS

24-M CFE	-	24-methylene cycloartanyl ferulate
AAPH	-	2,2'-azobis (2-amidino propane) dihydrochloride
AAS	-	Atomic absorption spectrometer
ABTS	-	Azino bis (3-ethyl benz thiazoline-6-sulfonic salt)
AE	-	Acetone extract
AE-LP	-	Lipophilic phase of acetone extract
AE-PP	-	Polar phase of acetone extract
AOAC	-	Association of Official Analytical Chemists
AOM	-	Active oxygen method
ARDS	-	Adult respiratory distress syndrome
BHA	-	3-tertiary-butyl-4-hydroxy anisole
BHT	-	3,5-di-tertiary-butyl-4-hydroxy toluene
BPH	-	Brown plant hopper
BSA	-	Bovine serum albumin
CAD	-	Cinnamic acid derivatives
CAT	-	Catalase
CFE	-	Cycloartenyl ferulate
CL	-	Chemiluminescence
CME	-	Crude methanolic extract
CVD	-	Cardiovascular diseases
DMRT	-	Duncons Multiple Range Test
DNA	-	Deoxy ribonucleic acid
DOC	-	Deoiled cake
DPPH	-	1,1-diphenyl-2-picryl hydrazyl
$DPPH_{REM}$	-	DPPH remaining
DRB	-	Defatted rice bran
DSC	-	Differential Scanning Calorimetry
DV	-	Diene value
EDA	-	Electron donating ability
ESR	-	Electron spin resonance
FA	-	Ferulic acid

FFA	-	Free fatty acids
FFB	-	Full fat rice bran
FIR	-	Far-infrared irradiated
GABA	-	γ-amino butyric acid
GC	-	Gas chromatography
GSH	-	Glutathione
GSH-Px	-	Oxidised glutathione
GSSG-Red	-	Reduced glutathione
HDL	-	High density lipoprotein
HIV-1	-	Human immuno deficiency virus type 1
HPLC	-	High pressure liquid chromatography
IPA	-	Isopropyl alcohol
IT	-	Induction time
IUPAC	-	International Union of Pure and Applied Chemistry
IV	-	Iodine value
LDL	-	Low density lipoprotein
MMT	-	Million metric tons
MPO	-	Myeloperoxidase
NBT	-	Nitroblue tetrazolium
NER	-	Non-enzymic reactions
NFE	-	Nitrogen free extractives
OSI	-	Oxidative stability index
OYL	-	Oryzanol
p-AV	-	para-anisidine value
PCA	-	Principal Component Analysis
PER	-	Protein efficiency ratio
POD	-	Peroxidase
PV	-	Peroxide value
RBD	-	Refined, bleached, and deodorized
RBO	-	Rice bran oil
RNA	-	Ribonucleic acid
ROS	-	Reactive oxygen species
RT	-	Retention time

SE	-	Stabilisation effectiveness
SOD	-	Superoxide dismutase
SOT	-	Schaal Oven Test
SV	-	Saponification value
TBARS	-	Thiobarbituric acid reactive substances
TBHQ	-	Tertiary butyl hydroquinone
T_{mix}	-	1:1 mixture of α-T and γ-T_3
TPA	-	12-*O*-tetradecanoyl-phorbol-13-acetate
TPC	-	Total phenolic content
UV	-	Ultraviolet
XOD	-	Xanthine oxidase

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CHAPTER 1

INTRODUCTION

1.1.Preamble

In recent years, phytochemicals in human health have assumed an unprecedentedly important status in the light of new evidences for their preventive and therapeutic properties against degenerative diseases. It is a paradox that oxygen that is essential for life, is also shown to be toxic. The toxic effects of normal oxygen metabolism is due to the release of free oxygen radicals and other reactive oxygen species (ROS), which can attack major inter- and extra cellular components such as proteins, unsaturated lipids, DNA and RNA. In the living cells, atmospheric $^3O_2$ undergoes a four-electron reduction process to yield water, thus producing superoxide radical, hydroxy radical and hydrogen peroxide as reactive intermediates.

Sometimes the body's immune system produce ROS in response to viral and bacterial infection. In addition to this, environmental factors such as pollution, radiation, cigarette smoke and herbicides can also generate free radicals. But the toxic effects of ROS are combated regularly by a number of endogenous defense and protective mechanisms which include various enzymes and non-enzymatic antioxidants in the living cells. The capacity of such protective systems, however, gradually decreases with age leading to imbalance in redox-status with oxidative stress gradually overwhelming. The excess free radicals produced have been implicated in a number of disorders including cardio vascular diseases (CVD), cataract, cancer, rheumatism and ageing.

Phytochemicals are secondary metabolites in plants and many of them are incorporated into foods or used as food supplements or nutraceuticals or as pharmaceuticals, that can function *in vivo* to complement or boost the endogenous defense systems. Thus functional foods, nutraceuticals or phytoceuticals capable of providing additional physiological benefits such as preventing or delaying onset of chronic diseases are now considered alternative health care. The functional components could be an essential macronutrient or

micronutrient, or a non-nutritive component, that are required in quantities more than the recommended dietary allowances.

In addition to the above mentioned *in vivo* functions, antioxidants have other well defined roles of preventing lipid peroxidation which occurs during the processing and storage of lipid containing foods. Though many synthetic compounds like BHA, BHT, TBHQ etc. are efficient antioxidants, their use is being restricted because of their possible toxic and carcinogenic effects. Thus the health concern of synthetic food additives and evidence in phytonutrients as chemo preventive agents led to resurgence in bio- and chemo prospecting of plants for potential therapeutic properties. Furthermore, mounting cost of modern health care and contra indications of chemical entities based therapues coupled with recent experimental and epidemiological evidences in favour of phytochemicals have encouraged people to accept the concept of alternative health care.

1.2. Oxidation

As oxidising agent, oxygen oxidises another atom or molecule by accepting a pair of electrons from it. Both these electrons must be of antiparallel spin so as to fit into the vacant spaces in the π^*orbitals of ground state oxygen molecule (Fig.1.1). This imposes a restriction on electron transfer which tends to make oxygen accept its electrons one at a time, and contributes to the fact that oxygen reacts sluggishly with many non-radicals. The ground state oxygen molecule with two unpaired electrons has a permanent magnetic moment and would exist in three closely grouped energy states if it was placed in a magnetic field. Hence it is also known as triplet oxygen [1, 2]. However, such a spin restriction is not there in the more reactive singlet oxygen which is produced from ordinary triplet oxygen by an input of energy. Net spin for the two outermost electrons is zero, there is no magnetic moment and hence there would not be any splitting of energy in an applied magnetic field. Hence the name singlet oxygen (Fig.1.1). There are two singlet excited states of oxygen, the $^1\Delta gO_2$ state having an energy 22.4 Kcal above the ground state and $^1\sum gO_2$,

with an energy 37.5 Kcal above the ground state. The term singlet oxygen generally refers to the less energetic $^1\Delta gO_2$ and is not a radical [1,3]. Most of the damaging effects of oxygen could be attributed to the formation of free oxygen radicals [1].

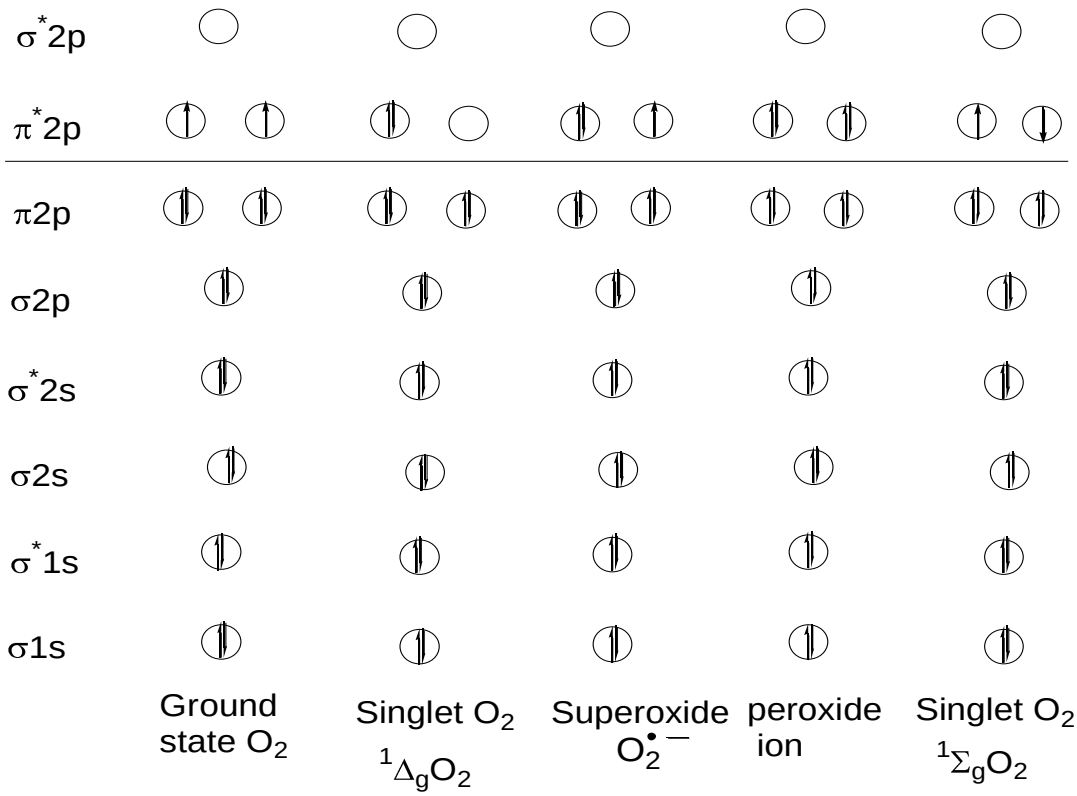


Figure.1.1. Bonding in diatomic oxygen molecule

1.3. Free oxygen radicals and reactive oxygen species (ROS)

A free radical is any species capable of independent existence that contains one or more unpaired electrons. Free radicals of importance in living organisms include hydroxyl ($OH^{\cdot}$), superoxide ($O_2^{\cdot -}$), nitric oxide ($NO^{\cdot}$) and peroxyl ($RO_2^{\cdot}$) radicals. Peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and ozone (O_3) are not free radicals but can easily lead to free-radical reactions in living organisms. The term " reactive oxygen species" (ROS) is often used to include all the above radical and non radical species [4]. Their chemical and biological sources are given in Tables 1.1 and 1.2 respectively.

Table.1.1. Chemical sources of reactive oxygen species [1- 4]

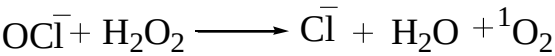
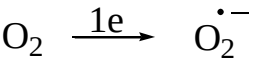
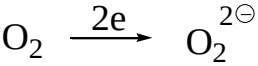
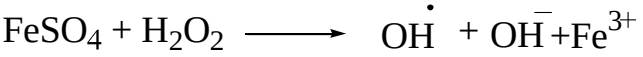
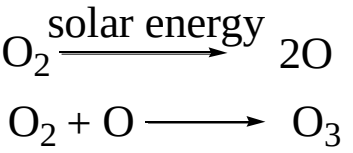
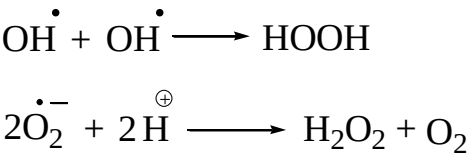
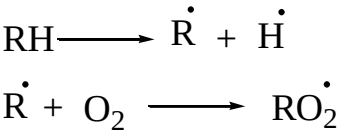
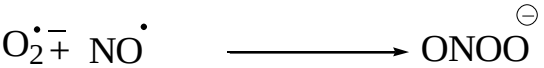
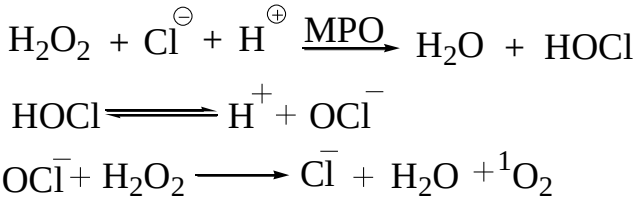
ROS	Chemical formation/exogenous sources
Singlet oxygen ($^1O_2$)	Photosensitized oxidation , and by the reaction 
Superoxide anion ($O_2^{\cdot -}$)	Univalent reduction of O_2 by hydrated electrons 
Peroxide ion (O_2^{2-})	Two electron reduction product of O_2 
Hydroxyl radical ($OH^{\cdot}$)	Radiolysis of H_2O, photolysis of H_2O_2, Fenton reaction 
Ozone (O_3)	Polluted air, reaction of O_2 with oxygen atoms produced by its photodissociation 
Hydrogen peroxide (H_2O_2)	Radiolysis or photolysis of H_2O, combination of two $OH^{\cdot}$, dismutation of $O_2^{\cdot -}$ 
Hypochlorous acid (HOCl)	Enzymatic reactions
Peroxyl radicals ($RO_2^{\cdot}$)	Lipid peroxidation 
Nitric oxide ($NO^{\cdot}$)	Smoke
Nitrogen dioxide ($NO_2^{\cdot}$)	Smoke
Peroxynitrite ($ONOO^-$)	By the reaction 

Table.1.2. Biological sources of reactive oxygen species [1- 4]

ROS	Endogenous sources
Singlet oxygen ($^1O_2$)	Photosensitized oxidation & during phagocytosis by the enzyme *myeloperoxidase* (MPO) 
Superoxide anion ($O_2^{\cdot -}$)	Enzyme systems (eg. *xanthine oxidase*), autoxidation reaction of oxyhaemoglobin, electron transport chains of mitochondria & endoplasmic reticulum, phagocytosis
Hydroxyl radical ($OH^{\cdot}$)	Radiolysis of H_2O , Fenton reaction
Hydrogen peroxide (H_2O_2)	Enzyme systems (eg. *glycollate oxidase*), phagocytosis, dismutation of $O_2^{\cdot -}$
Hypochlorous acid (HOCl)	During phagocytosis by *myeloperoxidase* (MPO) 
Peroxyl radicals ($RO_2^{\cdot}$)	Lipid peroxidation
Nitric oxide ($NO^{\cdot}$)	From vascular endothelial cells catalysed by *nitric oxide synthase*
Nitrogen dioxide ($NO_2^{\cdot}$)	Combination of $NO^{\cdot}$ with O_2 at body temperature
Peroxynitrite ($ONOO^-$)	From vascular endothelial cells

1.4.Oxidative stress

Free radicals and other reactive oxygen species (ROS) are constantly formed in the human body. Some of this production is a chemical accident, such as generation of $OH^{\cdot}$ by our constant exposure to low levels of radiation from the environment and of $O_2^{\cdot -}$ by leakage of electrons from electron transport chain. Other production of these species is deliberate and beneficial, for e.g. the formation of $NO^{\cdot}$ by the vascular endothelial cells and the production of $O_2^{\cdot -}$, H_2O_2 etc. during phagocytosis. It can be harmful also and free radical mechanisms have been implicated in the pathology of several human diseases including cancer, atherosclerosis, rheumatoid arthritis and neurodegenerative diseases [2,5].

There are ofcourse antioxidant defences in the human body to balance the normal rate of production of oxygen derived species. Tilting the balance in favour of the oxygen-derived species can upset cell biochemistry and "oxidative stress" is the term that refers to this imbalance. Most cells can tolerate mild oxidative stress as they have repair systems which recognise and remove oxidatively damaged molecules, which are then replaced. In addition, cells may increase the antioxidant defences in response to the stress [4, 5].

Oxidative stress causes lipid peroxidation and consequent damages to proteins and DNA. Severe oxidative stress results in cell damage and death. It has been implicated in numerous human diseases [1,7]. Thus the role of free radical reactions in human disease/ageing (Table 1.3), toxicology (Table 1.4), biology (Table 1.5), and in the deterioration of food (lipid peroxidation) has become an area of intensive investigation.

Table.1.3. Role of free radical reactions in human diseases and ageing [1, 2]

Disease/Disorder	Free radical related event
Cardiovascular diseases	Oxidative modification of LDL after an initial damage to vascular endothelium by other means
Cancer	DNA damage
Adult respiratory distress syndrome (ARDS)	Damage to lungs
Brain & spinal cord injury	Tissue damage after the injury
Radiation damage	Damage to proteins, lipids, and DNA
Keshan disease	Mediated by oxidative stress
Cataract	Oxidation of lens proteins
Ageing	May be due to tissue damage

Table.1.4. Some toxins that impose oxidative stress in human diseases [1, 2]

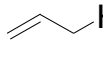
Toxins	Consequences
Cigarette smoke	Destruction of lung elastic fibres leading to lung cancer
Asbestos, herbicides	Lung damage
Alcohol	Depletion of vitamin E and GSH
CCl_4 & benzene	Damage to liver & bone marrow respectively
Acetaminophen, metal ions	Depletion of GSH
Air pollutants	Peroxidation of lung lipids
UV light	DNA damage

Table. 1. 5. Role of free radical reactions in biology [1, 2, 4]

ROS	Deleterious Effect
Singlet oxygen ($^1O_2$)	Oxidation of lipids and proteins especially in the retina of the eye
Superoxide anion ($O_2^{\cdot -}$)	Oxidation of membrane lipids, reduction of cytochrome C
Hydroxyl radical ($OH^{\cdot}$)	Membrane damage, DNA damge & strand breakage
Ozone (O_3)	Irritating to eye, nose, and lungs, cross-linking of proteins, peroxidation of lipids, DNA damage
Hydrogen peroxide (H_2O_2)	Inactivation of enzymes
Hypochlorous acid (HOCl)	Damage to proteins, tissues, & DNA, cell death caused by cholesterol chlorohydrins
Peroxyl radicals ($RO_2^{\cdot}$)	LDL oxidation
Nitric oxide ($NO^{\cdot}$)	Excess $NO^{\cdot}$ causes hypotension & insufficient amounts cause hypertension
Nitrogen dioxide ($NO_2^{\cdot}$)	Peroxidation of membrane lipids
Peroxynitrite ($ONOO^-$)	Damage to proteins, lipids, and DNA

1.5. Lipid peroxidation and consequences

Autoxidation is the spontaneous free radical reaction of organic compounds with oxygen [8]. Autoxidation of lipids is termed as lipid peroxidation [9] and has a profound role in quality deterioration of lipid containing foods as well as in a number of destructive biological processes [10]. It can occur either by enzymic or non-enzymic reactions (NER). Autoxidation and photo-oxygenation are two aspects of the NER between oxygen and unsaturated fatty acids [11].

Mechanism of lipid peroxidation : It is a free radical chain process consisting of chain initiation, propagation and termination steps (Fig.1.2). The key event in initiation is the formation of a lipid radical, $R^{\cdot}$. This can occur by thermal or photochemical homolytic cleavage of an RH bond or by hydrogen atom abstraction from RH by an initiator free radical, where RH represents an unsaturated fatty ester or ester with H attached to an allylic carbon atom (). The propagation step normally begins with the addition of molecular oxygen to $R^{\cdot}$ and the second step of propagation, the rate-limiting step, is abstraction of a hydrogen atom from RH by peroxyl radical $ROO^{\cdot}$ to generate ROOH (lipid hydroperoxide) and another radical $R^{\cdot}$. In the termination step, radicals combine to give non-initiating and non-propagating species [12].

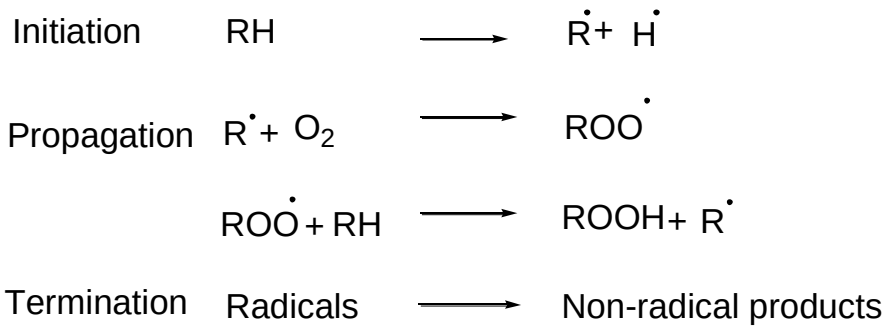
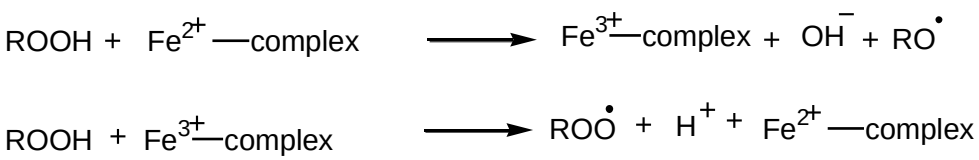


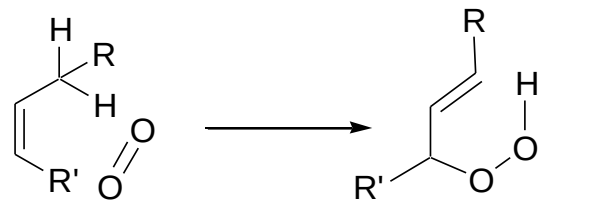
Figure.1.2.Chain sequence for free radical autoxidation

Autoxidation is promoted by heat and light, by metals and by radical producing species which act mainly on the initiation step. The detailed nature of the initiating step is not fully understood, but one important process is the metal catalysed decomposition of hydroperoxides to produce initiating radicals as shown below [1] and thermal initiation is possible in heated samples [11].



There is some evidence that photo-oxygenation may be responsible for the first formed hydroperoxides. It involves interaction between a double bond and singlet oxygen produced from ordinary triplet oxygen by light in the presence of a sensitizer such as chlorophyll. The reaction is not a chain reaction and there is no induction period. Instead oxidation occurs by the –ene mechanism in which oxygen becomes attached to either of the olefinic carbon atoms with attendant migration and stereomutation of the double bond as shown below.

$$S + h\nu \longrightarrow {}^1S \xrightarrow{{}^3O_2} {}^3S + {}^1O_2$$



Hydroperoxide

Photo-oxygenation is a quicker reaction than autoxidation and is unaffected by antioxidants, though singlet oxygen quenchers like carotene can inhibit it. But autoxidation is affected by antioxidants [11].

Hydroperoxides decompose readily and spontaneously at 160^{o}C and the peroxyl radical concentration can become relatively high under such conditions, thus leading to the formation of polymers as observed in frying oils. Hydroperoxides may also decompose to produce alcohols, aldehydes, alkyl formates, ketones, hydrocarbons etc. [13]. A generalized scheme for autoxidation is given in Fig.1.3.

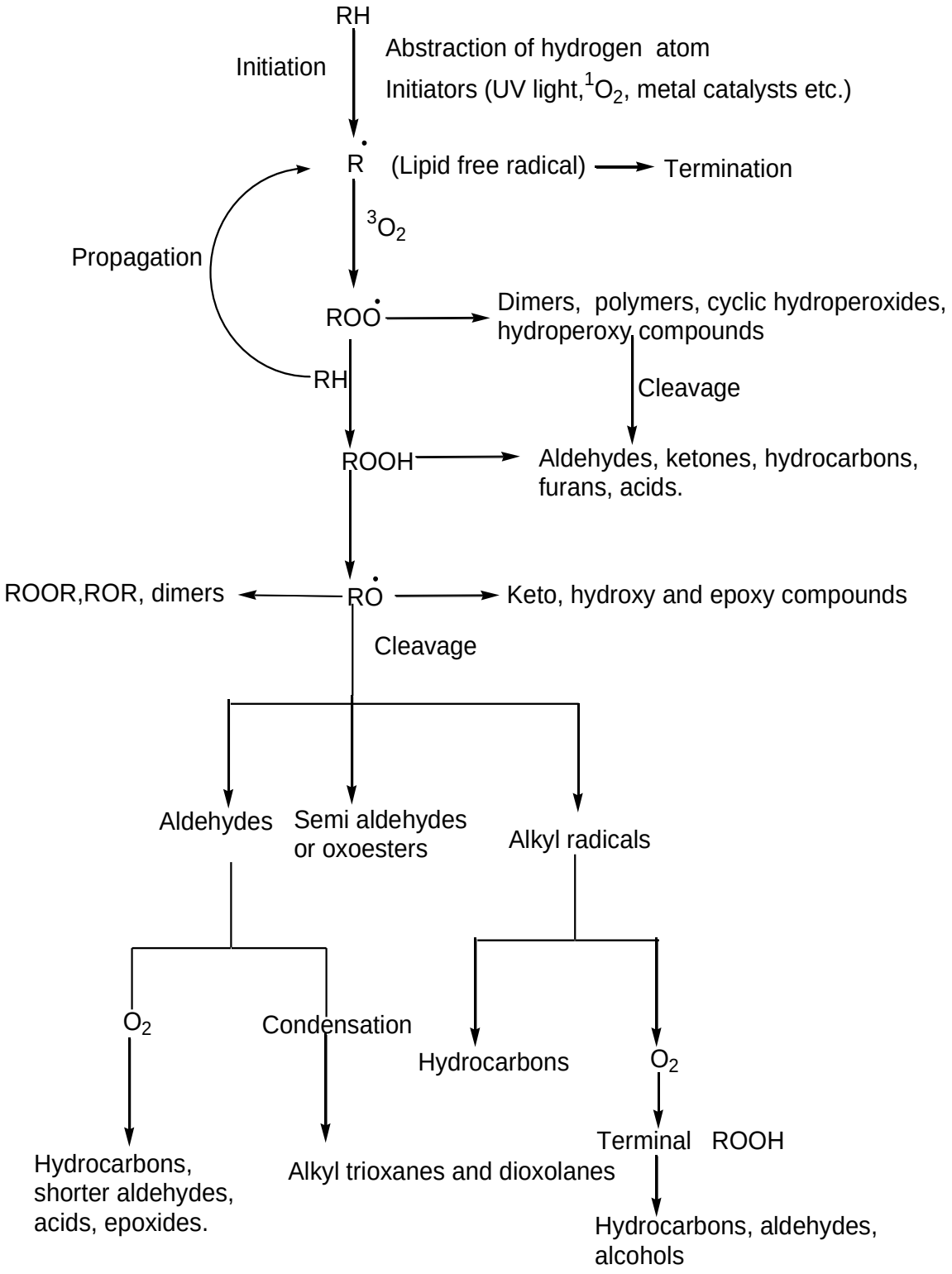


Figure.1.3. Generalised scheme for autoxidation of unsaturated lipid fatty acids.

Consequences of lipid peroxidation: Oxidation of unsaturated lipids is a major cause of food quality deteioration. The breakdown products of hydroperoxides such as alcohols, aldehydes, ketones, and hydrocarbons generally possess offensive off-flavours [13]. These compounds may also interact with other food components like proteins and bring about changes in their functional and nutritional properties. Lipid oxidation can also lead to the destruction of essential fatty acids and lipid soluble vitamins [14]. Oxidation of the poly unsaturated fatty acids of the biomembranes decreases its fluidity, increases the 'leakiness' of the membrane to substances that do not normally cross it (such as Ca^{2+} ions) and inactivate membrane bound systems [1]. It has also been implicated in several pathological conditions including ageing, hepatotoxicity, hemolysis, heart disease, cancer, tumour promotion, inflammation and iron toxicity [14].

1.6. Antioxidants

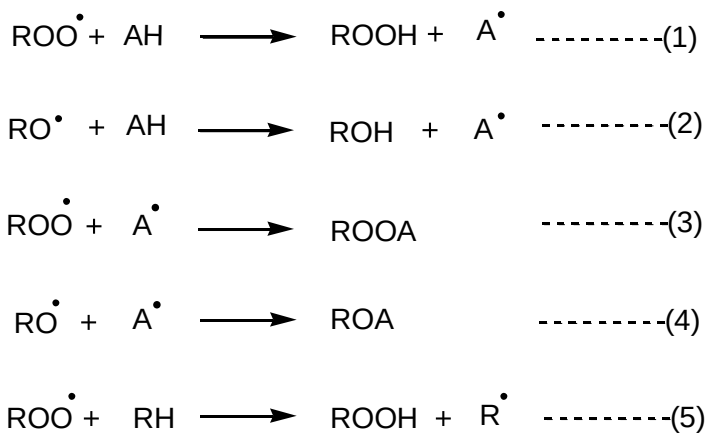
The term antioxidant is defined by Halliwell and Gutteridge as any substance that when present at low concentration compared to that of an oxidisable substrate significantly delays or inhibits oxidation of that substrate [15]. Antioxidants are either endogenous to the body or derived from the diet. Diet derived antioxidants may be of plant or animal origin (eg. musle peptides, carnosine and anserine) and some compounds with antioxidant activity could also be formed during processing of food related products (eg. Maillard reaction products). Several types of synthetic antioxidants are also used in the food industry [14]. Based on their major modes of action, antioxidants could be classified as free radical terminators, oxygen scavengers (reducing agents), metal chelators (sequestrating agents), singlet oxygen quenchers, and enzyme inhibitors [1,5, 11,14].

Free radical terminators may prevent lipid peroxidation either in the initiation stage itself by scavenging initiating $OH^{\cdot}$ radicals (eg. flavonoids) or

in the propagation step by scavenging peroxy radicals- known as the chain breaking antioxidant action (eg. BHA, BHT, TBHQ, propyl gallate, and vitamin E) [11,14]. Ascorbic acid is a good reducing agent [5]. Amino acids and phospholipids act by chelating catalytic metals. β-carotene, ascorbic acid and tocopherol are efficient singlet oxygen quenchers [14]. Some enzymes, such as various cytochrome P-450 isoforms, lipoxygenases, cyclo-oxygenases and xanthine oxidase, are potentially pro-oxidant and can generate radicals. Certain flavonoids and phenyl propanoids are effective inhibitors of these enzymes [16].

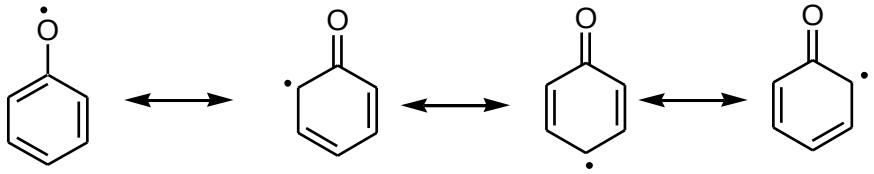
1. 6. 1. Mechanism of action of phenolic antioxidants

Antioxidants (AH) interfere with lipid oxidation by rapid donation of a hydrogen atom to lipid radicals (Reactions 1 and 2). The latter reactions compete with chain propagation reaction (5).



The above reactions are exothermic in nature. The activation energy increases with increasing A-H and R-H bond dissociation energy. Therefore, the efficiency of the A-H increases with decreasing A-H bond strength. The resulting phenoxyl radical itself must not initiate a new free radical or be subject to rapid oxidation by a chain reaction. In this respect, phenolic antioxidants are excellent hydrogen or electron donors and in addition, their radical intermediates are relatively stable due to resonance delocalisation and

lack of suitable sites for attack by molecular hydrogen. The phenoxy radical formed by reaction of a phenol with a lipid radical is stabilized by delocalisation of unpaired electrons around the aromatic ring, as indicated by the valence bond isomers [13].



1. 6. 2. Endogenous antioxidants

The defensive mechanisms in the human body include both enzymatic & non-enzymatic antioxidants, as well as repair & replacement systems. Both the acqueous and lipid parts of cells are protected by antioxidants. *Catalase* (CAT), oxidized glutathione (GSH–Px), reduced glutathione (GSSG-Red), *superoxide dismutase* (SOD) and glutathione (GSH) appear to reside in the aqueous part of the cell and can thus protect it. Protection of cell membranes (consisting of phospho-lipid bilayer) and other lipids is achieved by three mechanisms: radical scavenging, lipid repair, and lipid replacement.

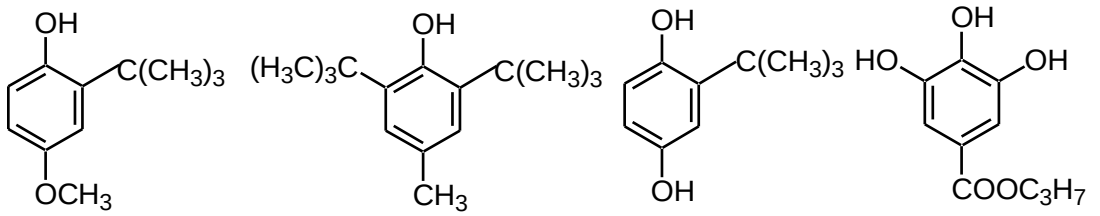
The co-operation of α-T and vitamin C is probably the most important antioxidant mechanism protecting LDLs in human plasma against peroxidation. Aqueous phase of plasma contains ascorbic acid as the major antioxidant. Sequestration of metal ions in forms unreactive in radical reactions provides an important part of extracellular antioxidant defence [17].

The self-defence systems against the toxic effects of oxygen free radicals may be supported by antioxidative compounds taken as foods, cosmetics, and medicine particularly in the elderly. Several types of antioxidants both synthetic and natural are added to foods to prevent lipid oxidation mediated quality deterioration. However, research published over the last few decades, and subsequent publicity has fostered significant consumer

resistance to the use of synthetic antioxidants and therefore food industry is in search of potential natural antioxidants [14].

1. 6. 3. Synthetic antioxidants

In general synthetic antioxidants are compounds with phenolic structures of various degrees of alkyl substituition. Substitution of hydrogen atoms in the *ortho*- and *para*- positions with alkyl groups increases the electron density of the OH moiety by an inductive effect and thus enhances its reactivity toward lipid radicals. Synthetic antioxidants currently permitted for use in foods are BHA (3-tertiary-butyl-4-hydroxy anisole), BHT (3,5-di-tertiary-butyl-4-hydroxy toluene), PG (propyl gallate), and TBHQ (tertiary butyl hydroquinone).



BHA BHT TBHQ Propyl gallate

BHA and BHT are extremely fat soluble, monohydric phenolic antioxidants. Both assert a good carry-through effect, although BHT is slightly better than BHA in this respect. Due to their volatile nature, both BHA and BHT are important additives used in packaging materials because they are able to migrate into foods. A synergestic effect has been shown to exist when BHT and BHA were used in combination.

TBHQ is diphenolic and is regarded as the best antioxidant for protecting frying oils against oxidation. It provides good carry-through protection similar to that of BHA and BHT. It is adequately fat soluble. Its maximum allowed concentration for use (either alone or in combination with BHA and BHT) is 0.02% or 200 ppm, based on the fat content of foods,

including essential oils. Propyl gallate is a a triphenolic antioxidant and is sparingly H_2O soluble. It functions particularly well in stabilizing animal fats and vegetable oils. PG chelates iron ions and forms an unappealing blue-black complex.

TBHQ is not permitted in Europe and Canada due to the lack of adequate toxicological information. The use of synthetic antioxidants is becoming increasingly restricted as many of them are reported to be carcinogenic in experimental animals and this has resulted in an increased interest in the investigation of newer sources of natural antioxidants [13,18].

1. 6. 4. Natural antioxidants (Phyto chemicals)

Phytochemicals are specific plant constituents that have the potential of being incorporated into foods or food supplements as nutraceuticals (any non-toxic food extract supplement that has scientifically proven health benefits for both disease treatment and prevention) or into pharmaceuticals, that can function *in vivo* as complements to the endogenous defense systems [19].

Harborne identified the three major classes of phytochemicals as terpenoids, phenolic metabolites and alkaloids and other nitrogen-containing plant constituents [20]. The most important phenolic metabolites are flavonoids, phenolic acids, tannins, lignans and stilbenes. They are formed from simple phenyl propanoids produced *via* the shikimic acid pathway (Fig.1.4). Tocopherols, tocotrienols, carotenoids, steroids, limonoids and phenolic diterpenes form the major terpene antioxidants. Alkaloids, glucosinolates, amines, amino acids, peptides etc. are among the major nitrogen–containing phytochemicals. Ascorbic acid, phytic acid and phospholipids are some other well known phytochemicals with antioxidant properties [5, 19].

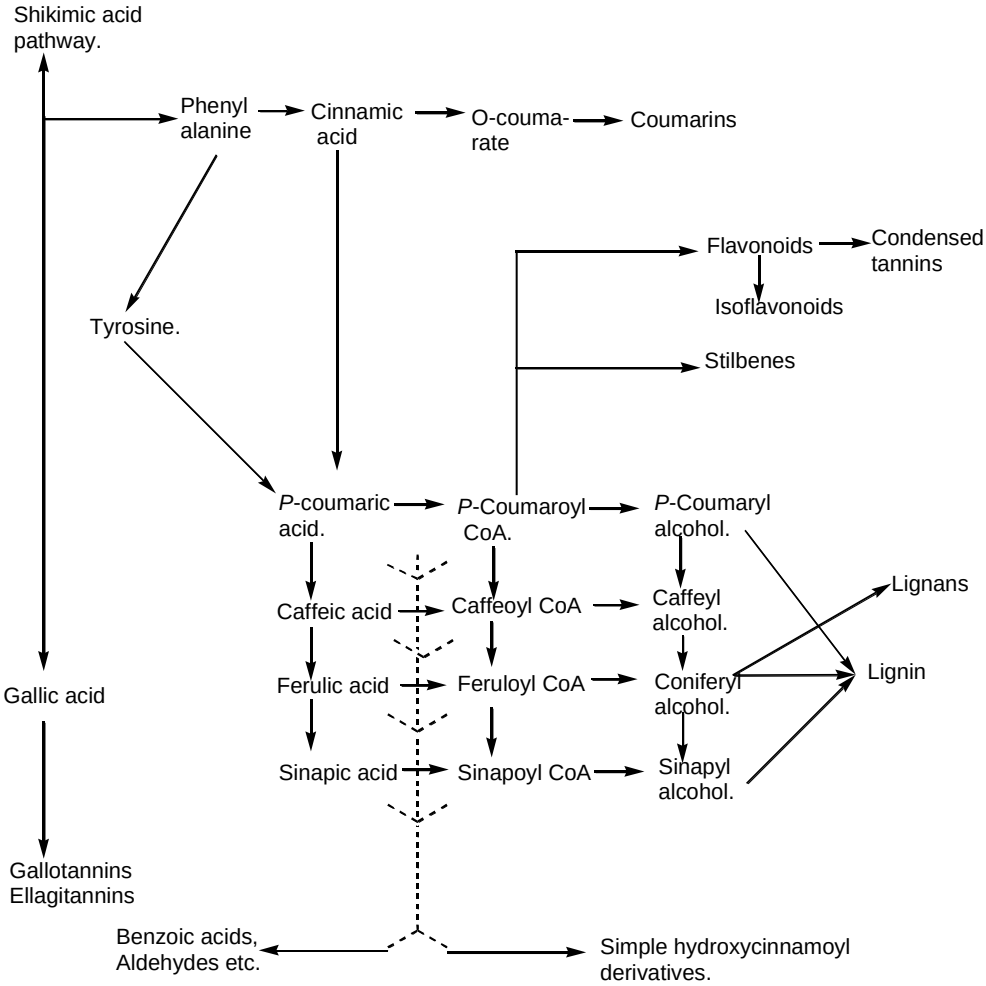


Figure.1.4. Schematic illustration of the origins of varied phenolics from the simple phenyl propanoids

Phenolic acids are formed *via* the shikimic acid pathway [21] and constitute both benzoic acids (C_6-C_1) [22, 23] and cinnamic acids (C_6-C_3) [22, 24, 25]. The phenolic acids are present both in the free form as well as conjugates (eg.chlorogenic acid) [24,25]. In plants, flavonoids exist in a variety of structural forms (Fig.1.5) like chalcones, dihydrochalcones, retrochalcones, flavanones [26], flavones, flavonols, flavanols (catechins) [27], isoflavones [28], anthocyanins [29], and as flavonoid glycosides, all having the basic (C_6-C_3-C_6) configuration [30].

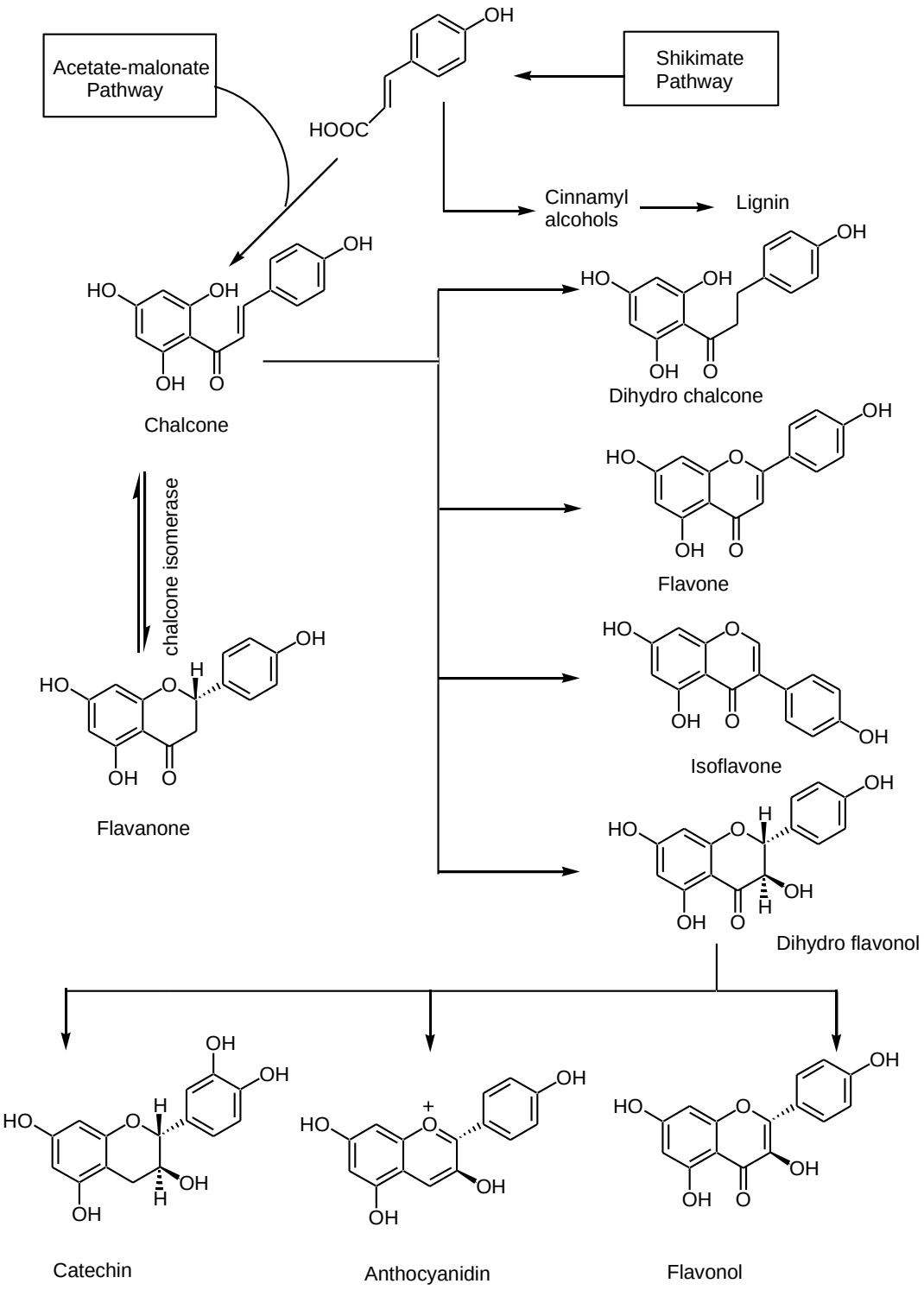


Figure.1.5. Currently proposed interrelationships among flavonoid monomer types

Tannins can be classified as condensed or hydrolysable, based on their structure and reactivity towards hydrolytic agents/acids [31]. Hydrolysable tannins are esters of phenolic acids and a polyol, usually glucose and yield gallic acid (gallo tannins) [31] and ellagic acid (ellagi tannins) [32] on hydrolysis. Proanthocyanidins (syn condensed tannins) are complex flavonoid polymers made of elementary flavan-3-ol units [31]. Lignans are dimers of phenyl propanoid ($C_6 - C_3$) units linked by the central carbons of their side chains [28]. Stilbenes are 1,2-diaryl ethenes [28].

The most important terpene antioxidants are tocopherols, trienols, carotenoids, steroids, limonoids and phenolic diterpenes [5,19]. The eight naturally occurring vitamin E compounds are α-, β-, γ- and δ- tocopherols and the corresponding trienols [33,34]. Carotenoids represent a class of structurally related colorants having a basic symmetrical tetraterpene skeleton formed by the conjunction of two C20 Units. Those carotenes containing oxgen in addition to carbon and hydrogen are referred to as xanthophylls [35].

Plant sterols are steroid alcohols. They resemble cholesterol, the predominant sterol found in animals, both in their chemical structure and biological function. They are made up of a tetracyclic cyclopenta [a] phenanthrene ring and a long flexible side chain at the C-17 carbon atom. The 3- hydroxyl group of free sterols may be esterified by a fatty acid or a phenolic acid to give steryl esters, or it may be β- linked to the 1'-position of a carbohydrate to form either steryl glycosides [36]. Phenolic diterpenes are formed by the aromatisation of terpenes [37]. Limonoids are terpenes present in citrus fruits [19].

Comprehensive data on dietary sources, major modes of antioxidant action and health effets of phenolic compounds (Table.1.6), terpenoids (Table.1.7), and nitrogen-containing compounds and others (Table.1.8) are given below. Chemical structures of some representative compounds belonging to the major classes of phytochemicals are given in Figure 1.6.

Table.1.6. Comprehensive data on phenolic phytochemicals [21-32]

Phytochemicals/Dietary sources	**Major mode of antioxidant action/ Health effects**
Phenolic acids (red wines, cereals, potato, tea, coffee, berries, beetroot etc.)	Free radical acceptors and chain breakers (anticancer & hepatoprotective effects)
Flavonoids (fruits & vegetables)	Metal chelation, chain breaking, $^1O_2$ quenching (vasodilatory, anticarcinogenic, anti-inflammatory, antibacterial, immune-stimulating, anti-allergic, antiviral, and estrogenic effects, inhibiton of various enzymes (eg. *phospholipase A2, cyclo-oxygenase, lipoxygenase, glutathione reductase, and xanthine oxidase*) & peroxidation of LDL, vitamin C sparing, protection against liver diseases, vascular disorders and heart diseases)
Tannins	
1.Hydrolysable tannins (oak galls & berries)	(decrease plasma triglycerides, antitumour effects)
2.Condensed tannins (legumes, grapes, cereals)	Free radical scavenging, $^1O_2$ quenching. (vascular protecting, anti-cancer, anti-histaminic, anti-viral, anti-caries, and anti-inflammatory activities)
Lignans (flax seed & sesame seed)	Free radical scavenging (anti-estrogenic estrogenic, & anti-cancer properties)
Stilbenes (grapes, pea nut)	(anti-inflammatory, anti-mutagenic and anti-cancer activities)

Table.1.7. Comprehensive data on terpenoid phytochemicals [19, 33-37]

Phytochemicals/Dietary sources	Major mode of antioxidant action / Health effects
Tocochromanols (cereals, nuts, legumes, seeds, vegetable oils)	Free radical scavenging , $^1O_2$ quenching. (anticancer, lipid lowering, & membrane stabilising effects, effective against certain diseases (CVD, arthritis & cataract), inhibit nitrosation of amines, also a dietary nutrient)
Carotenoids (fruits, vegetables, palm oil)	Most effective natural quenchers of $^1O_2$. (provitamin A activity, regulation of vision, growth & reproduction, essential for the normal cellular differentiation of most epithelia and immuno modulatory effects)
Sterols (vegetable oils, cereal grains, nuts)	Anti- polymerisation activity (anti-neoplastic, anti-inflammatory, hypocholestreolemic, anti-pyretic, and immune modulating activity)
Phenolic diterpenes (rosemary, sage)	Antioxidant activity (anti-carcinogenic)
Limonoids (citrus fruits)	(chemotherapeutic, protect lung tissues).

Table.1.8. Comprehensive data on nitrogen-containing & other important phytochemicals [5, 14, 19]

Phytochemicals/Dietary sources	**Major mode of antioxidant action/ Health effects**
Nitrogen- containing metabolites	
Amino acids, peptides	Metal chelation (effective against lipid peroxidation)
Alkaloids	Radical scavenging, $^1O_2$ quenching
Glucosinolates (cabbage, cauliflower)	(Induce detoxifying enzymes)
Others	
Phospholipids (legumes, cereals)	Metal chelation, act as synergists with tocopherols & flavonoids
Phyticacid (legumes, cereals, oilseeds)	(anti- carcinogenic activity)
Ascorbic acid (vegetables, citrus fruits)	Reducing agent, $^1O_2$ quencher (effective against lipid peroxidation in plasma)
Dietary fiber (vegetables, fruits, nuts)	(Soluble fibres reduce lipid & glucose, insoluble ones prevent colon cancer)

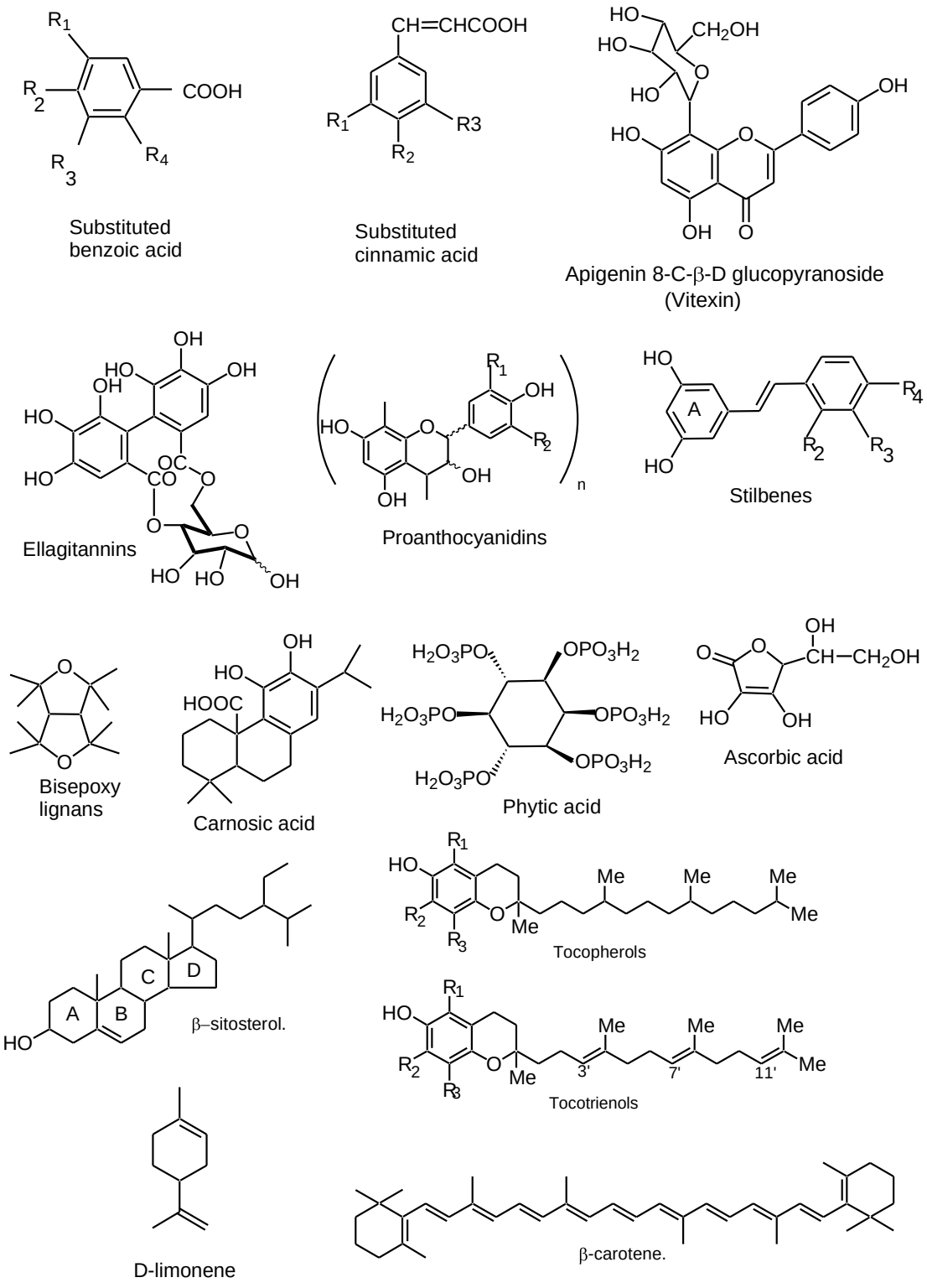


Figure.1.6. Chemical structures of some important phytochemicals

1.6. 4.1. Natural distribution of dietary phytochemicals

The major dietary sources of antioxidant phytochemicals are cereals, legumes, fruits, vegetables, oilseeds, beverages, spices and herbs [14]. Fruits and vegetables contain various antioxidants like flavonoids, phenolic acids, nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids and amines), ascorbic acid (vitamin C), β-carotene, vitamin E, fiber, isothiocyanates, phytic acid (IP_6) etc.[5]. Antioxidants from cereals and legumes include phenolic acids and their derivatives, flavonoids, tocols, phospholipids, amino acids, peptides, phytic acid, ascorbic acid, chlorophyll derivatives and sterols. Rosmanol, carnosol, carnosic acid (labiatae), gingerol, curcumin (Zingiberaceae) are some of the important antioxidant compounds obtained from spices and herbs [14]. Beverages like tea (catechins) and wine (proanthocyanidins) are also good sources of antioxidants [31].

1.6. 4. 2 . Antioxidants from oilseeds

Oleaginous plant products are excellent sources of antioxidants. Different types of oil-bearing seeds have been used since antiquity for human consumption and other essential needs. Antioxidants present in these oilseeds are given in Tables 1.9 [38,39].

Table.1. 9. Antioxidant profiles of various oil seeds [38-39]

Oleaginous plant products	Antioxidants identified
Chia	Flavonols (Kaemferol, quercetin, myricetin) and their glycosides, chlorogenic and caffeic acids
Corn	Ferulic acid, γ-T, phospholipids (PC, PI, PE), carotenoids (xeaxanthine & lutein), sterols (β-SS, SS, CS)
Cotton seed	Rutin, phenolic acids (*t*-FA, *t-p*-CA, *P*-HBA, *t*-CA)
Flaxseed	Phenolic acids [*t*-FA (major), *t*-SA, *t-P*-CA, *t*-CA, *P*-HBA], lignans, (seico-isolariciresinol diglucosides), condensed tannins, sterols [β-SS, CS, Δ^5-AS (major), SS, Δ^7–SS, BS), tocopherols [γ- (major), β- and δ- forms), phytic acid
Mustard	Tocols (α-T, γ-T & δ-T), phenolic acids [*t*-SA & *P*-HBA (major), *c*-SA & *t*- FA]
Olive	Phenolic acids (*p*-CA, SYA, VA, FA, CA, *P*-HBA, PCA), tyrosol, hydroxy tyrosol
Palm fruit	Tocopherols and trienols (α-, β-, γ-, and δ- forms of both), carotenoids (α-, β-, and γ- carotenes, lycopenes, xanthophylls), sterols (β-SS, SS, CS)
Peanut	Taxifolin, tocopherols (α-, β-, γ-, δ-), gums (phospholipids & glycolipids), phytic acid, condensed tannins, luteolin, phenolic acids (t-*p*-CA, SYA, *t*-FA, *t*-CA, *P*-HBA)
Rapeseed	Flavonoid glycoside (1-*O*-β-D-glucopyranosyl sinapate), phytic acid, phenolic acids [SA (major), *P*- HBA, VA, GA, PCA, SYA, *P*-CA, FA and CA], condensed tannins
Rice bran	γ-oryzanols, sterols (β-SS, CS, SS), phospho lipids (PC, PE, PI, PA), tocopherols (α-, β-, γ-, and δ- forms), tocotrienols (α-, γ-, and δ- forms), isovitexin, *t*-ferulic acid
Sesame	Lignophenols (sesamin, sesamolin, sesamolinol, sesaminol), pinoresinol diglucosides, phenolic acids (*t*-FA, *t*-CA, *t-p*- CA),γ-T
Soybean	Isoflavones as glucosides of daidzein, genistein, and glycitein, phytic acid, tocopherols (α-,γ-,δ-), sterols (β-SS, CS, SS), phospholipids (PC, PE, PI, PA, PS, PG), phenolic acids (CGA, iso CGA, CA, FA, *P*-CA, SYA, VA, SA, *P*-HBA, and salicylic acid)

Abbreviations used *c*: *cis*, *t:trans*, T: tocopherol, T_3 : tocotrienol
Sterols : β-SS: β–sitosterol, CS: Campesterol, Δ^5– AS : Δ^5-avenasterol, SS: Stigmasterol, Δ^7–SS : Δ^7–stigmasterol, BS :Brassicasterol
Phospholipids : PE: phosphatidyl ethanol amine, PI : Phosphatidyl inositol, PA: phosphatidic acid, PS : phosphatidyl serine, PG: phosphatidyl glycerol, PC : phosphatidyl choline
Phenolic acids : SA: Sinapic acid, *P*-HBA: *Para*-hydroxy benzoic acid, VA: Vanillic acid, GA: Gentisic acid, PCA: Protocatechuic acid, SYA: Syringic acid, *P*-CA: *Para*-coumaric acid, FA : Ferulic acid, CA: Caffeic acid, CMA : Cinnamic acid, CGA : Chlorogenic acid , iso CGA : Iso chlorogenic acid

1.7. Rice

Rice (*Oryza sativa* L.) is the staple dietary cereal of about 3 billion Orientals [40]. Rice accounts for 21, 14 and 2% of global energy, protein and fat supply respectively [41]. In developing countries, rice accounts for 71% Kcal/capita/day, 27% of dietary energy supply, 20% dietary protein and 3% dietary fat [42]. Rice bran, the major byproduct of the rice milling industry is also the source of a high quality vegetable oil viz. rice bran oil (RBO). RBO has attracted much medical attention due to its strong hypocholesterolemic properties primarily attributable to its fatty acid composition and high levels of antioxidant phytochemicals like oryzanols, tocopherols and tocotrienols. Defatted rice bran, the predominant byproduct of RBO extraction is a good source of insoluble dietary fiber, protein, phytic acid, inositol and vitamin B [43]. With an annual production of 550 to 600 million metric tons (MMT), rice is the second largest cereal next to wheat and is the main staple food for about 60% of the world's population. About 90% of the world's rice is produced and consumed in Asia with China and India accounting for about 85%. Rice (paddy) production in India has been 120 to 130 MMT during the past few years [44].

1.7.1. History and origin

Rice is one of the oldest of food crops and has been in cultivation in India, China, Java and East Africa from very ancient times. There are records of rice in ancient Hindu scriptures and literature of India, some of them dating back to 1,300 B.C. Carbonized paddy grains and husks have been found in the excavations of Hastinapur (Uttar Pradesh) dated 1,000–800 B.C. and impressions of paddy on clay lumps and remnants of husk have been found in Lothal in Gujrat, which is considered to be a south-ward extension of the Harappa and Mohenjodero culture of Indus Valley Civilization assigned to Ca 2,300 B.C. Available information supports the inference that *O. sativa* evolved in South-East Asia (India or Indo-China) [45].

1.7.2. Genetic diversity

Rice is from the genus Oryza and is comprised of 21 species, only two of which are cultivated: *Oryza sativa* and *Oryza glaberrima*. *Oryza sativa* is believed to have originated in South East Asia, while Oryza glaberrima originated in West Africa. Three sub-species of *O. sativa* have evolved. Indica is the tropical one, Japonica is the temperate, and Javanica, believed to have evolved in Indonesia, is intermediate between Indica and Japonica. Indica types generally have long slender grains and cook dry and flaky, whereas Japonica types have short round grains and cook moist and sticky. Glutinous ("sticky") and nonglutinous varieties exist for all sub-species. Japonica varieties tend to have lower amylose content and hence more sticky than the Indica varieties [46].

1.7. 3. Botany

Rice is a semiaquatic annual grass which can be grown under a broad range of climatic conditions. It does not have a rhizome, leaves are long and narrow, 30-50cm x 1.2-2.5cm, slightly pubescent with spiny hairs on the margin. Inflorescence is a terminal panicle varying from close and compact in some to loose and spreading in others. Spikelets are generally single, but in some in clustures of 2-7, number of spikelets varying from 50-60 to 200-300, large numbers being usually associated with smaller size and a densely packed arrangement. Lemma and palea surrounds the kernel, variously coloured, golden yellow, red, purple, brown or smoky black and becomes straw or light yellow when the grain ripens. The grain varies in size from 5 to 14.5 mm long and 1.9 to 3.7 mm broad and the length/breadth ratio defines the size and shape of the grain. Kernel is most commonly white, occasionally red, purple or brown [45].

1.7. 4. Structure and composition of rice grain (paddy)

The structure of the mature rice grain (caryopsis) is shown in Fig.1.7. The principal parts of the grain are the hull, pericarp, seed coat, nucellus, embryo, aleurone layer and endosperm. The hull formed from lemma and palea is the outer covering for the caryopsis and comprises 18-20% by weight of the rough rice. Hulls are low in protein, fat and starch but high in crude fiber, crude ash (mostly silica), and dietary fiber (Table 1.10). Removal of the hull from rough rice by dehulling exposes the rice caryopsis. The outer four morphologically distinct layers of the caryopsis are the pericarp, seed coat (tegmen), nucellus, and aleurone (Fig. 1.7). Along with much of the embryo, these layers comprise the bran portion of the rice grain which accounts for 5-8% of the brown rice weight.

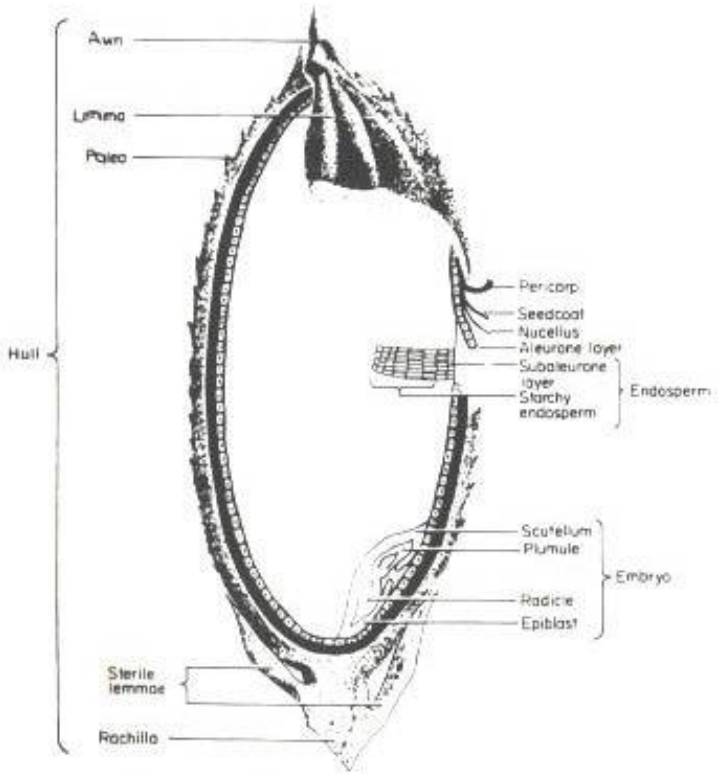


Figure.1.7. Structure of the mature rice grain

The bran is the most nutritious part of the caryopsis (Table 1.10). Rice bran is rich in lipids, protein, minerals, vitamins, phytin, trypsin inhibitor, lipase and lectin. Compared with other cereal brans, rice bran, with germ, is a little higher in fat content but is comparable in protein, fiber and ash. Further milling of the rice caryopsis to get milled (white) rice results in the removal of subaleurone layer and a small part of the starchy endosperm. This fraction referred to as "polish" comprises 3-4% by weight of brown rice (Table 1.10). Polish contains only slightly less protein and lipid but considerably more starch than the bran. When brown rice is commercially milled to white rice, the bran and polish fractions are not separated. This entire milling fraction is called rice bran and is the source for rice bran oil [44, 48].

Table.1.10. Proximate composition of rice and its byproducts [47]

Constituent (%dry basis)	Rough rice	Brown rice	Milled rice	Hulls	Bran	Embryo	Polish
Protein (Nx5.95)	6.7-8.3	8.3-9.6	7.3-8.3	2.3-3.2	13.2-17.3	17.7-23.9	13.0-14.4
Crude fat	2.1-2.7	2.1-2.3	0.4-0.6	0.4-0.7	17.0-22.9	19.3-23.8	11.7-14.4
Crude fiber	8.4-12.1	0.7-1.2	0.3-0.6	40.1-53.4	9.5-13.2	2.8-4.1	2.7-3.7
Crude ash	3.4-6.0	1.2-1.8	0.4-0.9	15.3-24.4	9.2-11.5	6.8-10.1	6.1-8.5
Starch	62.1	77.2	90.2	1.8	16.1	2.4	48.3-55.4
Dietary fiber	19.1	4.5	2.7	77.3	27.6-33.3	-	-

Full fat rice bran for human food should be suitably treated after milling to deactivate the naturally occurring lipase enzymes, which if untreated, would rapidly deteriorate the oil in the bran. Methods proposed to stabilize bran are based on altering the moisture content, temperature, or pH to destroy the activity of lipase. These include processes involving heat treatments, low-temperature storage, chemical treatment, control of relative humidity during storage and oil extraction immediately after milling [49].

The milling product of greatest commercial importance is white rice and rice bran is the major byproduct. The old method of dehulling yields bran mixed with hulls with significantly lower oil content (7-15%) and is not suitable for oil extraction. The modern method of separate dehulling and polishing using shellers yield high quality bran with 18-22% oil content. Modern rice mills today are employing this method.

Until recently, rice bran was predominantly used as an ingredient in livestock feeds, which provides a good source of protein, fiber, minerals, vitamins and fat. Due to overall composition, nutritional profile, functional characteristics and apparent hypoallergenicity, rice bran has many applications in a healthy diet that is high in dietary fiber and low in saturated fat. It is also beneficial in reducing the risk of cardiovascular diseases and colon cancer. Stabilised RB is used as ingredient in ready- to- eat cereals, in baked products like multigrain or high fiber breads, muffins, cookies, crackers, pastries, pan cakes and waffles, in pasta products, in healthy snacks and desserts [43].

Rice bran protein is of relatively high nutritional value. Reported PER values range from 1.6 to 1.9 and digestibility is around 73%. Rice bran contains atleast 78% of the rice kernel thiamine, 47% of the riboflavin and 67% of the niacin. The major carbohydrates present in rice bran are cellulose, hemicelluloses and starch due to contamination from endosperm during milling. Sugars in rice bran range from 3-8%, sucrose being the predominant one. Antinutritional compounds reported in rice bran include trypsin inhibitor,

pepsin inhibitor, hemaglutinin, phytates, and an antithiamine factor. Fortunately, activity of these compounds is relatively low and can be inactivated by heat treatment [43,48]. Minerals and vitamins found in rice bran are listed in Table 1.11.

Table.1.11. Minerals and vitamins in rice bran [50]

Minerals		**Vitamins**	
Component	Content (ppm)	Component	Content (ppm)
Aluminium	53-369	Vitamin A	4
Calcium	140-1310	Thiamine	10-28
Chlorine	510-970	Riboflavin	2-3
Iron	190-530	Niacin	236-590
Magnesium	8650-12300	Pyridoxine	10-32
Manganese	110-877	Panthothenic acid	28-71
Phosphorous	14800-28700	Biotin	0.2-0.6
Potassium	13650-23900	Myoinositol	4600-9300
Silicon	1700-16300	Choline	1300-1700
Sodium	0-290	*p*-aminobenzoic acid	0.7
Zinc	80	Folic acid	0.5-1.5
		Vitamin B_{12}	0.005
		Vitamin E	150

Rice bran oil (RBO), the most significant product from rice bran is considered to be one of the highest quality vegetable oils in terms of its cooking qualities, shelf life, and fatty acid composition [51]. RBO is generally obtained from the bran by solvent extraction with hexane [48,52]. The major component of crude RBO is triglycerides (80%) (Table.1.12) with three major fatty acids, (palmitic, oleic and linoleic) accounting for more than 90% of the total fatty acids (Table 1.13).

RBO is noted for its unusually high content of unsaponifiable matter unlike most other vegetable oils, with phytosterols being its major constituent. Oryzanols, present at 0.96-2.9% of oil, are ferulic acid esters of triterpenoid alcohols and plant sterols. Tocols are present in relatively high quantities at 0.1%. α-T, α-T_3 and γ-T_3 are the major ones. The hypocholesterolemic properties of RBO is attributed to its unsap matter constituents and are associated with alterations in liver cholesterol content and excretion of fecal sterols and bile acids [53, 54]. Rice oil fed to hamsters has shown cholesterol lowering properties [55].

Table.1.12. Composition of Rice Bran Oil [48]

Component	Content (%)
Saponifiable lipids	90-96
Neutral lipids	88-89
Triglycerides	83-86
Diglycerides	3-4
Monoglycerides	6-7
Free fatty acids	2-4
Waxes	3-4
Glycolipids	6-7
Phospholipids	4-5
Unsaponifiable lipids	4.2
Phytosterols	43
Sterol esters	10
Triterpene alcohols	28
Hydrocarbons	18
Tocopherols	1

Table.1.13. Chemical characteristics and fatty acid composition of Rice Bran Oil [48]

Characteristics	RBO
Chemical parameters	
Acid value	1.2
Iodine value	104.0
Saponifiable value	188.0
Unsaponifiable matter	4.2
Fatty acid composition %	
C14:0	0.3
C16:0	15.0
C18:0	1.7
C18:1	43.0
C18:2	37.4
C18:3	1.5
C20:0	0.6
C22:0	-

The distillates, soaps, and waxes produced in the oil refining process are sources for many potentially valuable and useful products like fatty acids, oryzanols, tocopherols, trienols, gums, waxes and inositol which are used in pharmaceutical and other applications [43]. An important byproduct of RBO extraction is defatted rice bran (DRB), a good source of insoluble dietary fiber and protein. It is more bland and easier to incorporate into many food products. DRB is also a source of such compounds as dibasic calcium phosphate, phytic acid, inositol, vitamin B, and protein concentrates [43,49].

1.7. 5. Current status of Rice Phytochemicals

1.7. 5. 1. Chemistry

Rice seeds are known to contain important antioxidative compounds such as tocochromanols [56], oryzanols [57], phenolic acids (ferulic, sinapic, syringic, *p*-coumaric, and vanillic) [58], and phytic acid [59]. Much work has been done on the oryzanols and tocols of rice bran. Chemical structures of some important rice phytochemicals are given in Figure.1.8.

Oryzanols, the ferulate esters of triterpenoid alcohols and plant sterols are well known for their strong hypocholesterolemic properties [60]. The many pharmacological uses of oryzanols include growth accelerating action in animals, regulation of estrous cycle as demonstrated in rats and ability to promote skin capillary circulation. Oryzanol is also reported to have anti-itching and anti-dandruff action and has been used in cosmetics and dentrifice preparations. Oryzanols has also been shown to be a good antioxidant for fats and oils [references in 61].

The individual oryzanol compounds identified so far include *trans*-ferulates of [cycloartanol, 24-methylene cycloartanol, cycloartenol, campestanol, campesterol, Δ-7 isomer of campesterol, sitosterol, Δ-7 isomer of sitosterol, stigmastanol, stigmasterol, cycloeucalenol, and 24-methylene cholesterol], *cis*-ferulates of cycloartenol, 24-methylene cycloartanol and sitosterol], two stereo isomers of 24-hydroxy-24-methyl-cycloartanol ferulates, 25-hydroxy-24-methyl-cycloartanol ferulates, and *trans*-caffeates of cycloartenol and campesterol [62]. Nothing is known to date about the biosynthesis of oryzanols [63].

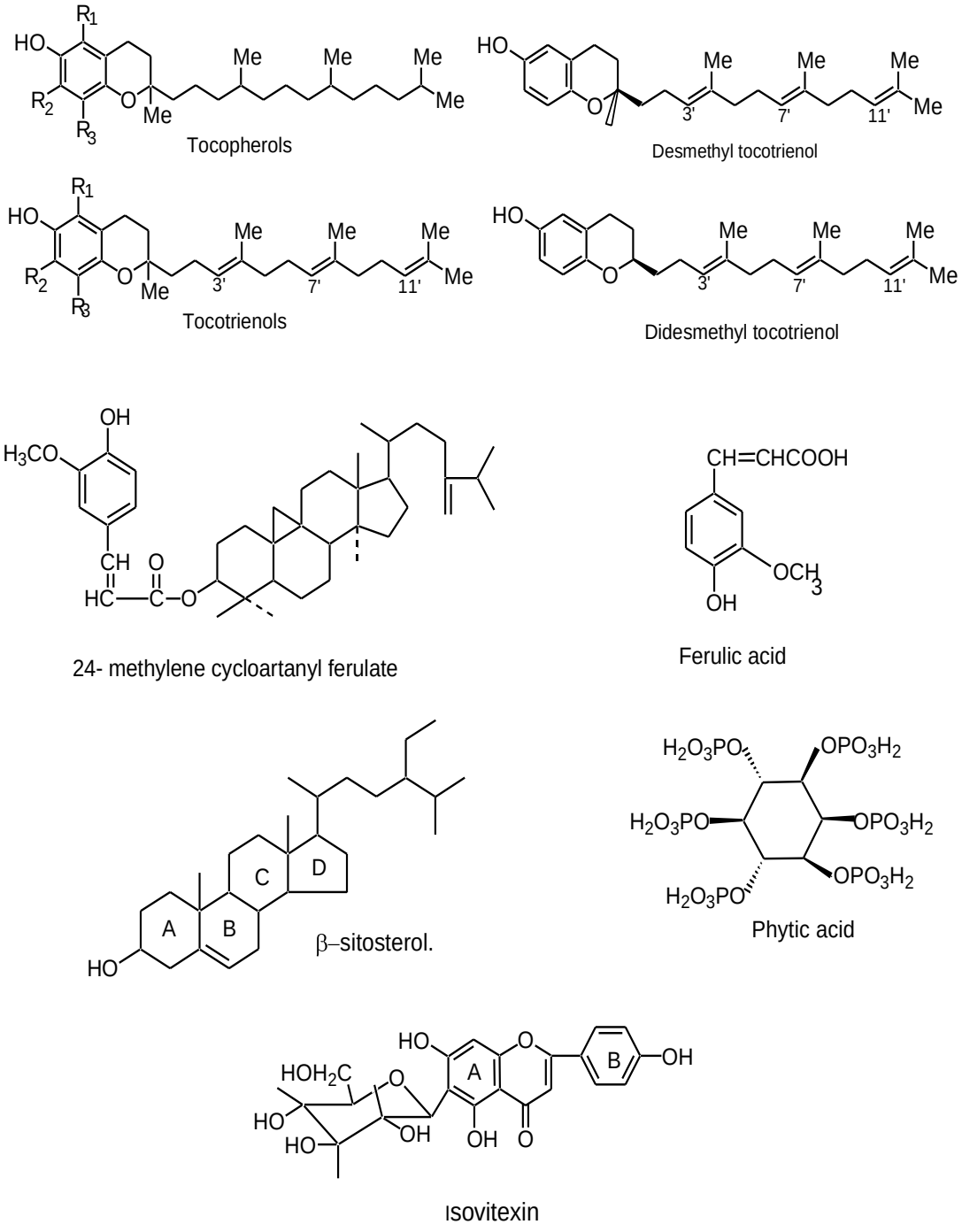


Figure.1.8. Bioactive phytochemicals in rice

Tocopherols and tocotrienols (Vitamin E compounds) are derivatives of 2-methyl-6-chromanol onto which is attached a 16-carbon isoprenoid side chain at C-2 which is saturated in the former and is unsaturated at C-3', C-7', and C-11' in the latter (Fig.1.8). The eight naturally occurring compounds are α-, β-, γ- and δ- tocopherols and α-, β-, γ-, and δ- tocotrienols. The homologues α- (5, 7, 8), β- (5, 8), γ- (7, 8), and δ-(8) differ in the number and positions of the methyl groups (given in brackets) on the aromatic ring [33]. These compounds are synthesized by higher plants and cyanobacteria from precursors of two pathways : the isoprenoid pathway and that of homogentisic acid formation. Vitamin E is thus a meroterpenoid (terpenoid incorporation in to molecules of mixed biosynthetic origin) [34].

The antioxidant activity of the tocopherols and tocotrienols (grouped as chromanols) is mainly due to their ability to donate their phenolic hydrogens to lipid free radicals. The chromanols seem to be the most efficient lipid antioxidants in nature [33]. Qureshi *et al.* have identified two new tocotrienols [56] in addition to the α-, β-, γ-, and δ- homologues of both tocopherols and trienols [64]. The new compounds are desmethyl tocotrienol, and didesmethyl tocotrienol **(**Fig.1.8). The beneficial effects of vitamin E compounds include anti-cancer activity, lipid lowering effect, nutrient function, prevention of diseases like CVD, cataract, and arthritis, prevention of nitrosoation of amines and immune system maintenance [33,34].

1.7. 5. 2. Antioxidant activity

Yagi *et al.* have demonstrated that campesteryl ferulate and ferulic acid strongly inhibited peroxidation of UV-irradiated linoleic acid as evidenced by diene value measurements, whereas campesterol was prooxidative [65]. Ramarathnam *et al*. have observed strong activity both in the hull and bran-germ fractions of Indica and Japonica rice varieties as assayed by the thiocyanate method. Grain fractions were weaker in activity [66]. Isovitexin, a

C-glycosyl flavonoid was identified as the antioxidant constituent of the methanol extracts of rice hull from long life rice seeds having better activity than the short life seeds in the thiocyanate assay [67]. Ethanolic and methanolic extracts of wild rice and wild rice hull showed antioxidant activity in ground beef and lard as measured by thiobarbituric acid reactive substances (TBARS) and peroxide value respectively. The compound responsible was found to be phytic acid [68].

Dehusked rice and rice husk were reported to possess $DPPH^{\cdot}$ and $OH^{\cdot}$ scavenging activity as assessed by ESR studies [69]. By activity guided fractionation, Asamarai *et a*l. had identified 2,3,6-trimethyl anisole, *m*-hydroxy benzaldehyde, vanillin and syringaldehyde as the antioxidant constituents of wild rice hull [70]. Cyanidin 3-*O*-*β*-D-glucopyranoside and peonidin 3-*O*-*β*-D-glucopyranoside were identified to be the anthocyanin pigments responsible for the activity of ethanol extracted bran fractions in pigmented rice, evaluated by linoleic acid autoxidation and rabbit erthyrocyte membrane systems [71].

The superior radical scavenging activity (DPPH & TBARS) of colored rices to white rices was attributed to the pigments present in the former [72]. Antioxidant activities of some rice milling fractions viz. embryo, bran and milled rice fractions were evaluated by electron donating ability (EDA), PV and TBA values as reported by Chun *et al.* [73]. The antioxidative pigments responsible for the superior ability of black rice seeds to maintain the viability even after long-term storage compared to white rice seeds were found to be cyanidin-3-O-*β*-D-glucoside (C_3G) together with delphinidin 3-*O*-*β*-D-glucoside and pelargonidin 3-*O*-*β*-D-glucoside [74].

Chung *et al.* have studied the superoxide dismutase (SOD) and peroxidase (POD) activities of the seeds and leaves extracts of Korean native and foreign rice varieties [75]. The four chemiluminescence (CL) constituents

isolated from rice bran were found out to be cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, campesteryl ferulate and β-sitosteryl ferulate and their DPPH radical scavenging activities were almost the same as that of ferulic acid [76]. Proteins were identified as the components responsible for the SOD-like activity of fermented rice bran extracts [77]. In another report, Ryu *et al.* has correlated the DPPH scavenging activity of coloured rice extracts with their total anthocyanin contents [78]. Chung *et al*. have examined the free radical scavenging activities of Korean and foreign rice varieties by the flow injection chemiluminescence (FL-CL) and ESR methods [79].

In experiments on the electron donating abilities of rice extracts, Bae *et al.* have observed that hull extracts were better than bran extracts and the activity was not affected much by microwave heating [80]. Nonsaponifiable fraction from rice bran oil was found to inhibit cholesterol autoxidation in an aqueous model system at 80^{o}C with a progressive loss of vitamin E. Oryzanols were found to be more heat stable [81]. According to Xu and Godber, free ferulic acid exhibited greater activity than cycloartenyl ferulate, 24-methylene cycloartanyl ferulate and campesteryl ferulate in inhibiting the formation of linoleic acid hydroperoxides [82]. Cycloartenyl ferulate, campesteryl ferulate and 24-methylene cycloartanyl ferulate were reported to be inhibitors of cholesterol oxidation than any of the four vitamin E compounds viz α-T, α-T_3, γ-T and γ-T_3, with 24-methylene cycloartanyl ferulate being the most effective [83].

Tokuyama *et al.* have patented an active oxygen scavenger contained in a water or organic solvent extract of rice that could be used as a medicament, beauty treatment, preservative or antibiotic [84]. The superior radical scavenging activities of red-hulled rice compared to white or black rice were attributed to the polymeric procyanidins in the former [85]. The major compounds responsible for the radical scavenging activity of Kurosu (Japanese

unpolished rice vinegar) were identified as dihydro ferulic acid and dihydro sinapic acids [86].

In another report, Kikuzaki *et al*. have shown that though the DPPH scavenging activity of ferulic acid was better than that of oryzanols, both were equally effective in inhibiting lipid oxidation as measured under OSI conditions and that the oryzanols were better inhibitors of oxidation in an ethanol-buffer system [87]. Wang *et al*. have compared the antioxidant and antipolymerisation activities of rice bran oil, ferulic acid, oryzanol, sitostanol and sitostanol ferulate in fatty acid methyl esters and soybean oil [88]. The free and bound phenolic contents in rice grains were reported to be to be 2.10 and 3.46 μmol gallic acid equivalents/g grains respectively with the bound phytochemicals contributing about 71% to the total antioxidant activity (expressed as μmoles of vitamin C equivalent/g grain). Also, the free, soluble conjugated and bound ferulic acid contents were 0.7, 9.9, and 142.8 μmol ferulic acid / 100 g grains respectively. [89].

Lee *et al.* have demonstrated that far-infrared irradiated (FIR) rice hulls have superior antioxidant activities compared to intact ones. *O*-methoxy cinnamic acid, *p*-coumaric acid and N-indolyl acetate were detected in intact rice hulls whereas irradiated ones contained more phenolic compounds like 3-vinyl-1-oxybenzene, p-hydroxy benzaldehyde, vanillin, *p*-hydroxy benzoic acid, 4,7-dihydrovanillic acid, and isoferulic acid in addition to those present in the intact hulls [90]. FIR rice hull extracts added in irradiated turkey meat at 0.1% showed activity similar to sesamol at 0.01% [91]. Antioxidant activities of methanolic extracts of rice seeds and milled-rice co-products followed the order rice > rice bran > brown rice when applied to beef mince and was correlated with the total phenolic content [92]. Xu *et al.* have studied the effect of foliar application of selenium on the antioxidant activity of aqueous and ethanolic extracts of selenium-enriched rice [93].

1.7. 5. 3. Biological activity

Dietary incorporation of rice bran oil markedly lowered serum and liver lipids in Wistar albino rats, indicating its hypocholesterolemic effect [94]. The antioxidant function of vitamin E compounds and oryzanols of rice bran against cholesterol oxidation might contribute to its hypocholesterolemic properties [60]. The cholesterol lowering action of oryzanol was reported to be associated with significant reductions in aortic fatty streak formation [95]. Two novel tocotrienols isolated from heat stabilized rice bran viz. desmethyl tocotrienol and didesmethyl tocotrienol exhibited superior efficacy with regard to cholesterol lowering, antioxidant, and antitumor properties in comparison to other known tocotrienols and α-tocopherol [56]. Dose dependent suppression of serum cholesterol by tocotrienol-rich fraction (TRF_{25}, a mixture of tocols isolated from stabilized and heated rice bran containing desmethyl and didesmethyl tocotrienols) in hypercholesterolemic humans is demonstrated by Qureshi *et al.* [96]. A possible mechanism for cholesterol lowering by rice bran is by increasing neutral sterol excretion and reducing lipid digestibility [97].

Defatted rice bran too significantly lowered plasma total cholesterol and very low- and low-density lipoprotein cholesterol levels compared to wheat bran with no reductions in high-density lipoprotein cholesterol [98]. Tsutsumi *et al.* have shown that a water extract of defatted rice bran reduced plasma triglycerides and suppressed visceral fat accumulation in rats that might be induced by pancreatic lipase inhibition [99].

Ling *et al.* have suggested that the enhanced serum HDL cholesterol and apo A-I concentration and the increased antioxidant status might be the mechanisms underlying the antiatherogenic activity of red or black rice [100]. Methanolic extracts of black rice pigmented fraction containing anthocyanins, prevented supercoiled DNA strand scission induced by ROS and suppressed the oxidative modification of human low density lipoprotein. In addition, it reduced the formation of nitric oxide by suppressing inducible *nitric oxide*

synthase expression in murine macrophage RAW 264.7 cells, without introducing cell cytotoxicity [101].

Rice bran water solubles significantly reduced hyperglycerima and rice bran fiber concentrates, reduced hyperlipidemia in Type I and II diabetes mellitus in humans [102]. Moreover, α-lipoic acid, a constituent of rice bran is gaining attention as a potential therapeutic agent for diabetes-induced complications [102]. Anthocyanins and phenolic acids (ferulic, caffeic and protocatechuic) in red and black coloured rices could be useful preventive substances of diabetic cataract formation [103].

Rice extracts also showed strong antimutagenic properties [104]. A-quinolone alkaloid (4-carbomethoxy-6-methoxy-2-quinolone) isolated from the aleurone layer of rice cv. Mihyangbyo (a Korean aromatic rice variety) was able to inhibit growth of cultured human leukemia cells [105]. Shoji *et al.* have isolated a fibronectin-binding protein from rice bran with cell adhesion activity for animal tumor cells [106]. Hyun and Chung have isolated cyanidin and malvidin from the aleurone layer of rice cv. Heugjinjubyeo (an enriched Korean rice variety) that could mediate cytotoxicity against human monocytic leukemia cells by arresting G2/M phase and induction of apoptosis [107].

Extracts of germinated brown rice with enhanced levels of γ-amino butyric acid (GABA) were found to be effective for the improvement of immunoregulatory action [108]. Modified arabinoxylans from rice bran enhanced the production of tumor necrosis factor-α and interferon-γ from human peripheral blood lympocytes [109] and showed immunorestoration effect on cancer patients [110]. Hayashi *et al*. have isolated two hydroxy fatty acids with antitumor activity from a water extract of rice bran [111]. Water soluble polysaccharide fractions of hotwater extract of rice bran and endosperm was shown to possess potent anticomplementary activity [112].

Hexane fractions of methanol extract of rice bran and rice bran oil showed significant and strong positive responses to the intestinal microflora, *bifido bacterium* and *lactobacillus* and this effect may be due to the various

fatty acids of RBO [113]. Antimicrobial activity of 4-hydroxy benzoic acid and *trans*-4-hydroxy-cinnamic acid isolated from rice hulls was reported by Cho *et al.* [114]. Cycloartenyl ferulate, 24-methylene cycloartanyl ferulate and stigmastanol from rice bran showed inhibitory effects on human immuno deficiency virus type I (HIV-1) *reverse transcriptase* [115].

Miyazawa *et al.* have isolated protocatechuic acid methyl ester as the *tyrosinase* inhibitor from black rice bran [116]. Hot water extracts of fermented rice bran showed anti-stress and anti-fatigue effects on rats and mice [117]. At 100 ppm levels, the ester fraction from rice bran oil containing steryl cinnamic acid derivatives was found to inhibit the fungal pathogen, *Sclerotinia sclerotiorum* by 50% [118].

Phytic acid or inositol hexaphosphate (IP_6) present in rice bran was found to possess many beneficial features like anti-cancer activity [119], anti-HIV-1 activity [120], prevention of calcification in biological fluids [121], immune cell stimulation [122], lipid lowering effect [123], and anti-platelet activity [124]. Moreover, various oryzanol components have been shown to have anti-inflammatory activity against 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-induced ear inflammation in mice[125].

1.8. Relevance and objectives of the present investigation

Antioxidant compounds are gaining importance due to their dual role in the food industry as lipid stabilizers and as chemo-preventive agents which suppress excessive oxidation that causes degenerative disorders like CVD, cataract, cancer, ageing etc. Tocopherols and a few natural extracts are widely used as safe antioxidants, but they are not as effective as synthetic antioxidants and their manufacturing cost is high.

Agriproducts processing yields many byproducts having significant potential for value addition. Oilseed meals, the major byproduct of any oil extraction practice could be exploited as sources of safe natural antioxidants. For instance, the phenolic antioxidants in the meals of sesame, soybean, canola,

peanut, cotton seeds, mustard and rapeseed have been characterized and were shown to be effective natural antioxidants.

Rice bran, a potential commercial source of edible oil in rice producing countries, is a byproduct of rice milling industry. India is the second largest producer of rice and rice bran in the world after china, with an annual production of 118.6 MMT paddy corresponding to 80 MMT rice and 10 MMT rice bran. The bran layers of brown rice contain protein rich in eight of the essential amino acids, in addition to calcium, phosphorus, potassium, niacin, fiber, vitamins B & E and an edible oil *viz.* rice bran oil (RBO). RBO has attracted much medical attention due to its strong hypocholesterolemic properties primarily attributable to its fatty acid composition and high levels of antioxidant phytochemicals like oryzanols, tocopherols, tocotrienols and phytosterols.

Defatted rice bran (DRB), a byproduct of RBO extraction is also a good source of insoluble dietary fiber, protein, phytic acid, inositol and vitamin B. Though the antioxidant potential of DRB was known [126], it still remained a relatively unexplored source material, which demanded further investigation especially with regard to its phytochemical composition related to possible health benefits as antioxidants. The focus of the present investigation therefore has been on DRB primarily to establish its phytochemical status and feasibility of using it as a source of bio-active phytochemicals and natural antioxidants leading to value addition of DRB otherwise used as cattle feed. To gain a better understanding of the value of rice bran as a source of phytochemicals, five popular rice varieties of the region viz. PTB 50, PTB 39, PTB 38, JAYA, and MO 10 and a wild variety (*oryza nivara*) that is mainly used for medicinal applications in traditional ayurvedic system were characterized along with commercial samples of rice bran. The present study also explains the feasibility of a process for the extraction, enrichment, and isolation of

antioxidant compounds from DRB. The antioxidant potential of the extracts were evaluated both in bulk oils and in food relevant model emulsions, using standard *in vitro* model systems. Radical scavenging effects, indicative of possible biological effects, were also evaluated.

1.9. Structure of the present investigation

Chapter 1: Introduction-objectives and literature review

Chapter 2: Materials and Methods

Chapter 3: Results and Discussion (divided into 4 sections)

- 3.1. Phytochemical profile of rice bran and rice bran oil
- 3.2. Extraction and enrichment of bioactive pytochemicals from DRB
- 3.3. Antioxidant efficacy of phytochemical extracts from DRB in the bulk oil system
- 3.4. Antioxidant efficacy of pytochemicals from DRB in *in-vitro* model systems

Chapter 4: Summary and Conclusion

References

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Six authenticated rice varieties namely PTB 50, PTB 39, PTB 38, JAYA, MO 10, and *oryza nivara* (a wild variety) were collected from Rice Research Station, Kayamkulam, Alappuzha (Kerala). The agronomical features of the varieties provided in the data bank of the Kerala Agricultural University, Vellayani is given in Table 2.1. Commercial samples of rice bran (full fat and defatted), and rice bran oil were obtained from 'Chakkiyathumooda Solvent Extractions', Ankamali, Ernakulam. Refined, bleached and deodorized (RBD) soybean oil without added antioxidants was supplied by M/s. Sakti Soya's, Pollachi, Tamil Nadu.

2.2. Chemicals and solvents

Authentic standards of tocopherols (α-, β-, γ-, and δ-), tocotrienols (α-, β-, γ-, and δ-), β-carotene, and linoleic acid were purchased from E.Merck (Germany) and fatty acid methyl esters from Sigma Chemical Company, USA. Standard compounds of oryzanols, cycloartenyl ferulate and 24-methylene cycloartanyl ferulate were a generous gift from Ms. Fumi Tsuno, Tsuno Rice Fine Chemicals, Japan. DPPH, NBT, Cytochrome C, Xanthine, Xanthine oxidase, and Ferrous chloride were obtained from Sigma Chemical Company, USA and Tween-20, BHT, and TBHQ from E.Merck (Germany). Acetone-d_6 and $CDCl_3$ for NMR were from Sigma Chemical Company, USA. HPLC grade methanol, acetonitrile, dichloromethane, *n*-butanol, water, *n*-hexane and isopropanol were procured from Merck (India). Other chemicals and reagents were of analytical grade and were obtained from Ranbaxy Chemicals (India).

Table. 2.1. Agronomical Features of The Rice Varieties Selected for The Study

Vareity/ popular name	Place of release	Duration (days)	Grain type	Parentage	Agronomical features
PTB 50 (Kanchana)	RARS, Pattambi	105-110 (ED)	Red	IR x Pavizham	Suitable for Kole and Kuttanad regions and for all seasons. Resistant to blight, blast diseases, and gall midge.
PTB 38 (Triveni)	RARS, Pattambi	95-105 (ED)	White, long, bold	Annapurna x PTB 15	Tolerant to BPH, susceptible to blast and sheath blight.
PTB 39 (Jyothi)	RARS, Pattambi	110-115 (ED)	Red, long, bold	PTB 10 x IR 8	Highly popular variety. Suitable to sandy and alluvial soils (Kole and Kuttanad area) and for all seasons. Susceptible to sheath blight, moderately tolerant to BPH and blast, excessive shedding of grains at maturity.
JAYA	DRR, Hyderabad	120-125 (MD)	White, long, bold	TN 1 x T 141	Very high yield potential, highly susceptible to BPH, plant hopper and other pests.
MO 10 (Remya)	RRS, Moncompu	115-120 (MD)	Red, long, bold	Jaya x PTB 33	Moderately tolerant to BPH and gall midge, sheath blight, suitable for all seasons.
Oryza nivara (Knjavara)	Wild vareity	120-125 (MD)	Red, bold	Progenitor of Asiatic rice	Low yield. Moderately tolerant to pests.

RARS Regional Agricultural Research Station of Kerala Agricultural University

DRR Directorate of Rice Research

RRS Rice Research Station

ED, MD Early and medium duration respectively.

BPH Brown Plant Hopper

2.3. Sampling methods for rice bran

For the present study, both commercial samples of rice bran as well as bran samples prepared from popular rice varieties of the region were used [2.1]. The commercial brans were composite samples obtained from sheller milling. Rice samples representing the varieties PTB 50, PTB 39, PTB 38, JAYA, MO 10, and *oryza nivara* (wild variety) were dehusked and milled using Satake (friction type) milling system (Satake, USA, Houston, TX) to about 10% degree of milling. All the bran samples were then sieved through a 20-mesh size sieve to remove broken pieces of rice and husks and stabilized by heating in an air-oven at 110^{o}C for 30 minutes. The samples were then stored in the dark at -4^{o}C in moisture proof containers and analysed within a week for proximate composition. Oil was extracted from the stabilized bran samples [2.4] and the oil and the defatted meal thus obtained were preserved in the dark at -4^{o}C in moisture proof containers and analysed within two weeks.

2.4. Compositional analysis of rice bran

The proximate chemical composition of rice bran which include moisture, fat, protein, fiber, and ash was determined by the standard procedures of the Association of Official Analytical Chemists [127].

2. 5. Analysis of minerals in rice bran

Determination of Ca, Mg, Zn, Mn, Fe, and Cu contents of rice bran was by the standard procedures of the Association of Official Analytical Chemists [128]. The analysis was performed with a Perkin Elmer® Model AAnalyst^{TM} 100 atomic absorption spectrometer (Perkin Elmer Instruments, Shelton, CT, USA) with deuterium background correction and a Perkin Elmer Lumina® hollow cathode lamp. A standard air-acetylene nebulizer burner system was operated at an air flow rate of 4.0 L/min and an acetylene flow rate of 1.0 L/min. The following wavelengths (nm) were fixed for AAS [Cu (324.8), Mn (279.5), Zn (213.9), Fe (248.3), Ca (422.7), and Mg (285.2)]. To avoid phosphorus interference, Lanthanum salts were added during the analysis

of Ca and Mg. Concentrations were calculated from calibration graphs obtained using standard solutions of each mineral elements.

The Na and K content was estimated by flame photometry [129], with a Systronics model 128 flame photometer. Standard solutions of NaCl and KCl were used at 10 ppm levels. The phosphorus analysis was carried out on a Shimadzu 160-UV-vis spectrophotometer, using the ammonium vanadate-molybdate colorimetric method indicated by the AOAC [128], at a wavelength of 400 nm.

2.6. Chemical characteristics of rice bran oil

Free fatty acids (FFA), saponification value (SV), and iodine value (IV) of rice bran oil was determined according to the standard procedures of the International Union of Pure and Applied Chemistry [130].

2.7. Fatty acid composition of RBO

Fatty acid composition of rice bran oil (RBO) was estimated by trans esterification with 2% methanolic sulphuric acid followed by GC of the fatty acid methyl esters [130]. The analysis was done on a Hewlett-Packard 5890 Series II gas chromatograph (Avondale, USA); equipped with a flame-ionization detector and a cross-linked free fatty acid phase column (FFAP; 30 m x 0.53 mm i.d., 1.0 μm film; Hewlett Packard, Avondale, USA). The column temperature was programmed from 100 to 180^{o}C at the rate of 5^{o}C/min. The injection temperature was 250^{o}C and the detector temperature was 300^{o}C. The flow rate of the carrier gas (nitrogen) was 20 mL/min. Fatty acid methyl esters were identified by comparison of their relative retention times to authentic standards using methyl heptadecanoate as an internal standard. Fatty acid levels were reported as relative proportions of the total composition.

2. 8. Unsaponifiable matter content of RBO

The unsaponifiable matter content of rice bran oil was determined according to the standard procedure of the International Union of Pure and Applied Chemistry [130]. To 5 g oil is added 1 mg BHT, 10 ml ethanol, and 5

ml 60% aqueous KOH and heated for 30'over a water bath. Then the mixture is transferred to a separating funnel with 50 ml distilled water and 50 ml diethyl ether. Repeated the ether extraction thrice more with 30 ml ether each time. The ether extract is washed with water until alkali free and the ether is evaporated and weighed.

2. 9. Oryzanol composition

The oryzanol composition of rice bran oil was analysed by a reversed-phase HPLC method [131]. The analysis was performed using a Shimadzu make HPLC binary system (Kyoto, Japan) with a LC-10 AD model pump, a 7125 model Rheodyne injector (Cotati, California, USA) fitted with a 20 μL sample loop, a SPD-10A UV-visible detector, with a C-R7 Ae plus integrator for data acquisition and display. A Waters μ-bondapak ^{TM}C18 column (4.6 mm i.d. x 25 cm) (Millford, MA) was used in the reversed- phase with the solvent system of acetonitrile, dichloromethane, and acetic acid (88:6:6, by vol) : methanol, n-butyl alcohol, water (90:2:8, by vol) in the ratio of 75:25 (by vol) at a flow rate of 1 ml/min. The UV detector was set at 325 nm. Oil samples were diluted with the mobile phase and analysed. Peak identification was based on comparison of RT values with authentic standards of cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, and a mixture of oryzanols and also by comparison with reports. The various forms were quantified based upon peak areas relative to standard calibration plots obtained with 24-methylene cycloartanyl ferulate, the major oryzanol component in RBO.

2. 10. Tocopherol and tocotrienol composition

The tocopherol and tocotrienol composition of rice bran oil was analysed by a normal-phase HPLC method [132]. The analysis was done on the same HPLC system with a Shim-pack (LC-NH_2 (M)) column (4.6 mm i.d. x 25 cm) used in the normal- phase with a solvent system of *n*-hexane : isopropanol (96:4; v/v) at a flow rate of 1 mL/min. The UV detector was set at 297 nm. Peak identification was based on comparison of RT values with authentic standards of tocopherols and tocotrienols. The various vitamin E

forms were quantitated based upon peak areas relative to standard calibration plots by external standard method. Oil samples were diluted with *n*-hexane and 20 µl of this was injected into the HPLC column.

2.11. Extraction of antioxidants from defatted rice bran

Defatted rice bran was extracted with hexane, ethyl acetate, ethanol and methanol at a material-sovent ratio (1:7.5 – 1:30; g/ml), extraction time (1-10 hrs), at the boiling temperature of the solvent, in a soxhlet extractor. The extract was filtered through a Whatman No.1 filter paper, evaporated to dryness at 50^{o}C with a rotavapor held under partial vacuum and weighed. It was redissolved in methanol and kept under refrigeration.

2.12. Fractionation of Crude Methanolic Extract (CME)

Fractionation of CME was achieved by re-extracting it with less polar organic solvents like ether, ethyl acetate and acetone. Thorough mixing was ensured by means of a cyclomixer and the process was repeated till no more of the solute was going into solution as observed gravimetrically. The extracts were pooled, filtered, and the solvent was removed as above. The lipophilic and polar compounds of the acetone extract (AE) thus obtained were then separated by sequential solvent extraction. For this, AE was re-extracted with nonpolar solvents like hexane to give a soluble fraction enriched in lipophilic compounds (Lipophilic phase–LP) and a fraction insoluble in the nonpolar solvent, enriched in polar compounds (Polar phase – PP).

2.13. Isolation and characterization of pure compounds from CME

Methanolic extract (40 g) was applied on to a 5 cm x 120 cm silica gel column (800 g). The column was eluted with gradients of hexane : ethyl acetate (100% hexane to 100% ethyl acetate). 800 ml fractions were collected. Fractions similar in TLC profiles were pooled. Pure compounds were obtained in fractions eluted with 20% ethyl acetate and 50% ethyl acetate and identified with the help of UV, IR, NMR and MS spectra.

UVspectra were recorded in a Shimadzu 160-UV-visible spectrophotometer and IR spectra in a Nicolet IR spectrometer. NMR spectra

were recorded in a Bruker AVANCE DPX_{300} series NMR spectrometer and chemical shifts are expressed in ppm units with respect to TMS. Mass spectra were recorded in a GC-MS-QP 5050 Shimadzu Spectrometer with EI mode.

UV-Visible spectroscopy of flavonoids: After measurement of the spectrum of the sample in MeOH (the "MeOH spectrum"), 3 drops of NaOMe were added to the cuvette and after mixing, the 'NaOMe" spectrum was recorded and the sample discarded. Six drops of $AlCl_3$ (5g dry $AlCl_3$/100 ml MeOH) were added to fresh flavonoid solution, the sample mixed and the "$AlCl_3$" spectrum measured. HCl (3 drops) was then added, and after mixing, the "$AlCl_3$/HCl " spectrum was measured and the sample discarded. Powdered NaOAc was added to fresh flavonoid solution and mixed thoroughly to record the NaOAc spectrum. The "NaOAc/H_3BO_3 " spectrum was then measured after addition of H_3BO_3 [30].

2.14. Chemical characterisation of crude extracts and fractions

Standard colorimetric procedures were used for the determination of sugar [133], protein [134], and total phenolic contents [135] of the extracts. Ash was determined as described under 2.4.

2.14.1. Determination of sugars

Reagents

1. Anthrone/thiourea: Stock 66% (v/v) H_2SO_4 was prepared by adding 660 ml H_2SO_4 cautiously with stirring and external cooling to 340 ml water in a large beaker. 10 g thiourea and 0.5 g anthrone (9,10-dihydro-9-oxo-anthracene) were dissolved in 1 litre of this acid by warming the mixture to 80-90^oC and stored at 0-4^oC. The colour of the reagent increased slowly with time and the colour yields tended to decline after two weeks.
2. Standard glucose: A stock standard (0.25 g/100 ml distilled water) was diluted to give standards in the range 25-200 μg/ml.

Procedure: Blank and standard amounts of glucose were carried through with each series of unknowns. 1 ml of the test solution (extract) was pipetted into a

glass stoppered tube and 10 ml of anthrone reagent was added. The tubes were swirled to mix the contents and stoppered firmly. They were then placed in a water-bath at room temperature to bring into equilibrium and then into a boiling water bath for 15 minutes. The tubes were then cooled to room temperature in a tap water bath and left in the dark for 20-30 minutes after which the absorbance was measured at 620 nm.

2.14.2. Determination of proteins

Reagents

(1) 2% Na_2CO_3 in 0.1 N NaOH (Reagent A)

(2) Alkaline Cu reagent (Reagent B) – 1 mL (0.5% $CuSO_4$ in 1% Na tartarate) with 50 ml Reagent A.

(3) Folin-Ciocalteau reagent – 1:1 dilution

(4) Protein stock solution (50 mg BSA dissolved in 0.1 N NaOH and made upto 50 ml in a standard flask).

Procedure: 1ml aliquots containing 100-400 μg proteins were mixed with 5 ml each of reagent A and B in separate tubes. After 10 minutes, 0.5 ml of Folins reagent was added to both the tubes. After 30' absorbance was measured at 660 nm against respective blanks. The difference between two readings was used to calculate proteins from a standard curve prepared with 0-600 μg albumin.

2.14.3. Determination of total phenolic content (TPC)

0 to 10 ml standard tannic acid solution (0.1 mg/ml) was taken in a 100 ml volumetric flask containing 75 ml distilled water. 5ml Folin-Ciocalteau reagent and 10 ml of saturated sodium carbonate solution were added to the flask and diluted to 100 ml with distilled water. Mixed well and allowed to stand at room temperature for 30 minutes. Absorbance of the solution was measured in a UV-visible spectrometer (model UV-160A; Shimadzu) at 760 nm. Absorbance was plotted against mg tannic acid/100 ml to get a standard curve. 1 ml of sample was used instead of standard to obtain concentration from the standard curve.

2.15. Phytochemical composition of extracts

Estimation of oryzanols and tocols was carried out as described under 2.9 and 2.10 respectively. The dried extract was redissolved in methanol and the solution was analysed for ferulic acid under the same HPLC conditions as for oryzanols. The compound was identified and quantitated with the help of standard *trans*- ferulic acid.

2.16.Evaluation of antioxidant potency of DRB extracts in bulk oils

In the present study, Schaal oven test method (60^0C) and DSC method (150^0C) were used to understand the antioxiant potential of DRB extracts and their phytochemical constituents in the bulk oil system in comparison with well known synthetic antioxidants like BHT, TBHQ etc.

2.16.1. Antioxidant efficacy using Schaal oven test

Schall oven method was employed to evaluate the effect of antioxidants against oxidation during the accelerated storage of oils [136]. The antioxidants (extracts and pure compounds) were added to fresh refined, bleached, and deodorized soybean oil without added antioxidants. The extracts were tried at 100, 200 and 500 ppm levels. BHT, TBHQ, oryzanols, ferulic acid, and a 1:1 mixture of α–T and γ-T_3, (herein after referred to as Tmix) were tried at 100 and 200 ppm levels. Duplicate samples of 15 g were placed in glass bottles. The jars were kept at 60^oC. Samples were analysed after 3, 6, 9, 12 and 15 days for peroxide value, diene value and *p*-anisidine value to follow the oxidative changes. The base oil without added antioxidants was used as control.

Peroxide value (PV) : Peroxide value was determined according to the standard titrimetric method of the International Union of Pure and Applied Chemistry [130]. To 5 g oil, 30 ml of CH_3COOH-$CHCl_3$ mixture (3:2; v/v) and 0.5 ml saturated KI were added and kept in the dark for exactly 1 minute. It was then titrated against standard $Na_2S_2O_3$ solution using starch indicator.

$$PV = \frac{V Na_2S_2O_3 \text{ x } N\ Na_2S_2O_3 \text{ x } 1000}{W \text{ sample}}$$

Para-anisidine value (PAV): The colorimetric method of Jirusova was used to determine p-anisidine value of oil samples [137]. 2 ml of the fat solution (2% solution in $CHCl_3$) was mixed with 4 ml trichloroacetic acid (1.5% solution in methanol) and 4 ml *p*-anisidine (0.25% solution in methanol) and heated over a water bath at 60^oC for 60 minutes. The absorbance at 400 nm of the colored solution was denoted as E. The absorbance of the common blank (2 ml $CHCl_3$ + 4 ml TCA + 4 ml para anisidine) was denoted as Eo. The absorbance of sample blank (2 ml fat solution + 4 ml TCA + 4 ml methanol) was denoted as E_1.

$$PAV = \frac{(E - Eo - E_1) \times 10}{n}$$

where n is the weight of fat in grams, in 2 ml of the fat solution.

Diene Value (DV): Diene value was estimated by the spectrophotometric method described by Wettasinghe and Shahidi [138]. Briefly, 0.02 – 0.04 g oil was dissolved in isooctane in a 25 ml volumetric flask and the absorbance at 234 nm was noted.

$$DV = \frac{\text{Abs at 234 nm}}{\text{Conc. of the oil (g/100 ml)} \times \text{path length (cm)}}$$

2.16.2. Antioxidant efficacy using differential scanning calorimetry

Differential scanning calorimetry (DSC) was used for studying various heat-related phenomena in materials by monitoring associated changes in enthalpy [139]. Oxidation is an exothermic process and the heat of reaction evolved makes it possible to employ DSC for the evaluation of oxidative stability of oils. The analysis was carried out in a Mettler Toledo DSC 821 instrument (Schwerzenbach, Switzerland) which was calibrated with indium before the analysis with an empty pan as reference.

A sample of ~10 mg was loaded into an aluminium sample pan. The oil without additives was first studied under dynamic heating regime from 90^{o}C to 200^{o}C and the temperature of onset of oxidative changes was noticed from the DSC curve as the point of inflection. The samples were then analysed isothermally at 150^{o}C at a temperature 10^{o}C below the said onset temperature for 45', under a stream of oxygen at 40 ml/minute. The flow of nitrogen was 200 ml/min. The extracts were tried at 100, 200, 500 and 1000 ppm levels. BHT, TBHQ, oryzanols, ferulic acid, and Tmix were tried at 100 and 200ppm levels. The time at which the onset of oxidation occurred was noted and this induction period was taken as indicative of the oxidative stability of oil.

2.17. Evaluation of antioxidant potency using *in-vitro* model systems

The antioxidant potential of DRB extracts were also evaluated in two food relevant emulsion models viz. the linoleic acid emulsion system and the β-carotene bleaching test. Besides these methods, the ability of antioxidants to donate electrons were also evaluated using stable DPPH radical and superoxide radical generated *in-situ* by the xanthine-xanthine oxidase system.

2.17.1. Thiocyanate method–Linoleic acid emulsion system: Antioxidant activity of the extracts in food-relevant model emulsions was evaluated by the thiocyanate method [140]. The reaction mixture consisted of 0.28 g of linoleic acid, 0.28 g of Tween-20, and 50 ml of phosphate buffer (0.2 M, pH 7.0). To 2.5 ml of the above emulsion, 0.5 ml of test sample and 2.5 ml of phosphate buffer were added and incubated at 37^{o}C for 120 hours. The mixture prepared as above without test sample was the control. The samples were withdrawn after each 24 hours. 0.1 ml of the test sample was then mixed with 5.0 ml 75% ethanol, 0.1 ml 30% ammonium thiocyanate, and 0.1 ml of 20 mM ferrous chloride in 3.5% HCl and allowed to stand at room temperature. Precisely 3 minutes after the addition of ferrous chloride to the reaction mixture, the

absorbance at 500 nm was measured. Samples and standards were tried at 100 and 200 ppm levels.

2.17. 2. β-carotene bleaching method : The antioxidant activity of the extracts was also evaluated by the β-carotene-linoleate model system [141]. 0.2 mg of β-carotene, 20 mg of linoleic acid and 200 mg Tween-20 were mixed in 0.5 ml chloroform and the solvent was evaporated under vacuum. The resulting mixture was diluted with 50 ml oxygenated water. To 4 ml of this emulsion, 0.2 ml of test samples in ethanol was added. A solution with 0.2 ml of ethanol and 4 ml of the above emulsion was used as control. The tubes were placed at 50^{o}C in a water bath. Absorbance was taken at zero time (t = 0') and after every 15 minutes. Measurement of absorbance was continued until the color of β-carotene disappeared in the control reaction (t = 120'). Samples and standards were tried at 100 and 200 ppm levels.

2.17. 3. DPPH radical scavenging method: The method of Brand-Williams *et al.* [142] modified by Sanchez-Moreno [143] was follwed. 0.025 g/L $DPPH^{\cdot}$ solution was prepared in methanol. Various concentrations of samples (extracts and standards) were also prepared in methanol. To 3.9 ml of $DPPH^{\cdot}$ solution, 0.1 ml of appropriately diluted sample solution was added and absorbance at 515 nm was measured at different time intervals in a UV-visible spectrophotometer (UV-160A, Shimadzu) for 30 minutes. The $DPPH^{\cdot}$ concentration in the reaction medium was calculated using the following equation obtained by linear regression.

$$A\ 515\ nm = 2936.68\ [DPPH^{\cdot}]_T - 2.18 \times 10^{-3}$$

The percentage of remaining $DPPH^{\cdot}$ was calculated as

$$\%\ DPPH^{\cdot}_{REM} = [DPPH^{\cdot}]_{T=T} / [DPPH^{\cdot}]_{T=0}$$

The percentage of remaining $DPPH^{\cdot}$ against the standard/sample concentration was plotted to obtain the EC_{50} concentration (the amount of antioxidant required to decrease the initial $DPPH^{\cdot}$ concentration by 50%).

2.17.4. Superoxide radical scavenging method

Superoxide radicals generated *in-situ* by the xanthine-xanthine oxidase system was monitored using two different probes viz.cytochrome C [144] and nitro blue tetrazolium [145].

2.17. 4.1. Xanthine – Xanthine oxidase / Ferricytochrome C method

Reagents

Potassium dihydrogen phosphate

Dipotassium hydrogen phosphate

EDTA

Xanthine

Xanthine oxidase

Ferricytochrome C

Solution A

0.76 mg xanthine in 10 ml of 0.01 N sodium hydroxide and 24.8 mg cytochrome C in 100ml 50mM phosphate buffer (pH 7.8) containing 1.85 mg EDTA were admixed freshly.

Solution B

15 mg xanthine oxidase in 5 ml EDTA solution (3.72 mg EDTA/100 ml distilled water).

Procedure

The assay was performed in a 3.0 ml cuvette. The assay mixture consisted of 2.9 ml solution A and 0.05 ml of test sample. 0.05 ml solution B was added to start the reaction and the rate of reduction of ferricytochrome C was determined by using UV-visible spectrophotometer at 550 nm.

2.17. 4. 2. Xanthine – Xanthine oxidase / NBT method

Reagents

Carbonate buffer 50 mM, pH 10.2 containing EDTA

Xanthine (5 mg/ml 0.1 N NaOH)

Xanthine oxidase (10 mg/ml distilled water)

Nitro blue tetrazolium (NBT) (6 mg/ml distilled water)

Procedure

The assay mixture consisted of 1ml carbonate buffer, 0.05 ml xanthine, 0.01 ml NBT, and 0.02 ml test sample. 0.02 ml *xanthine oxidase* was added to start the reaction and the time course for the reduction of NBT into formazan complex was followed for about 15 minutes at 560 nm. The percentage of NBT reduction was calculated as follows:

% NBT reduction = [Rate of change of absorbance of sample] / [Rate of change of absorbance of control] x 100

Radical scavenging ability = 100 - % NBT reduction.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Phytochemical profile of rice bran and rice bran oil

Rice bran is a major byproduct of the rice milling industry and is a potential commercial source of an edible oil viz. rice bran oil (RBO). RBO has attracted much medical attention due to its strong hypocholesterolemic properties primarily attributable to its balanced fatty acid composition and high levels of bio-active phytochemicals like oryzanols, tocopherols, tocotrienols, and sterols. Rice bran is also rich in other dietary nutrients. Defatted rice bran (DRB), a byproduct of RBO extraction is also a good source of insoluble dietary fiber, protein, phytic acid, inositol and vitamin B. Though the antioxidant potential of DRB was also recognized, it is still a relatively unexplored material especially with regard to its phytochemical profile. The focus of the present investigation therefore has been on DRB primarily to establish its phytochemical status and feasibility of using it as a source of bio-active phytochemicals and natural antioxidants leading to value addition of DRB otherwise used as cattle feed. To gain a better understanding of the value of rice bran as a source of phytochemicals, five important rice varieties of the region viz. PTB 50, PTB 39, PTB 38, JAYA, and MO 10 and a wild variety (*oryza nivara*) that is mainly used for medicinal applications in traditional ayurvedic system were characterized along with commercial samples of rice bran. The results from the above studies are presented and discussed in the ensuing sections.

3.1.1. Proximate composition of rice bran

For the present study, commercial samples of full fat and defatted rice bran were obtained from 'Chakkiyathumooda Solvent Extractions', Ankamali, Cochin. The commercial brans were composite samples obtained from sheller milling. Authentic samples of six rice varieties representing PTB 50, PTB 39, PTB 38, JAYA, MO 10, and *oryza nivara* (wild variety) were collected from Rice Research Station, Kayamkulam, Alappuzha. The rice samples were dehusked and milled using Satake (friction type) milling system (Satake, USA,

Houston, TX) to about 10% degree of milling. The brans were sieved through a 20-mesh size sieve to remove broken pieces of rice and husks and stabilized by heating in an air-oven at 110^{o}C for 30 minutes. The samples were then stored in the dark at -4^{o}C in moisture proof containers and analysed within a week for their proximate chemical composition.

The chemical profiles of commercial rice bran and the bran from the selected varieties are given in Tables 3.1 and 3.2 respectively. The commercial sample of full fat rice bran (FFB) contained 88.9 % dry matter, 21.6% fat, 11.2% protein, 14.2% fiber, 11.4 % ash, 41.7 % available carbohydrates and an energy content of 406.1 Kcal/100g (Table 3.1). After defatting using hexane as practised in the industry, the DRB contained very little fat (0.3%) with proportionate increase in other constituents (Fig.3.1).

Significant varietal variations ($P<0.05$) were observed in all constituents though it was less pronounced in the case of dry matter. On an average, rice bran from the selected varieties contained 89.1 % dry matter, 16.8% fat, 10.1 % protein, 11.3% fiber, 11.4% ash, 50.5 % available carbohydrates and an energy content of 393.5 Kcal/100g (Table 3.2). It could also be seen from Table 3.2 that for the varieties the dry matter ranged from 87.9 to 90.4 %, fat from 15.6 to 18.8 %, protein from 9.1 to 10.8 %, fiber from 9.2 to 13.2 %, total ash from 9.9 to 12.3 %, NFE from 48.7 to 54.3% and energy content from 386.8 to 401.5 Kcal/100g.

Rice bran is the outer brown layer, that is usually removed during the milling of brown rice to produce the familiar white grain. During the milling process, approximately 20% by weight of raw rice (paddy) is milled off as shell (husk), and then another about 10% by weight as rice bran on subsequent polishing to yield milled rice. The outer bran layers which constitute approximately 10% by weight of the whole rice kernel have most of the bio-

active phytochemicals concentrated compared to the remaining 90% of the kernel (endosperm) containing mostly starch [147]. Whole rice bran is shown to be rich in lipids, proteins, fiber, minerals and vitamins particularly B-complex and vitaminE [48]. Due to overall composition, nutritional profile, functional characteristics, and apparent hypoallergenicity, it can find many applications in a healthy diet with dietary fiber, phyto nutrients and low in saturated fat [43]. DRB also is a good source of protein, phytic acid, inositol, vitamin B, and insoluble dietary fiber [43]. Both full fat and defatted rice bran and protein concentrates prepared from it could be incorporated into a variety of food products [49].

Scientific data on the chemical composition of rice byproducts become important in order to maximize their utilization [148]. Composition of rice bran vary widely due to factors such as variety, geographical location, milling techniques, pre-treatment etc. Other than genetic diversity and geographical location, pre-treatments like parboiling, sheller and huller milling etc. have profound influence on the yield and composition of rice bran [48].

The older method of huller milling results in bran mixed with husk with consequent variation in proximate composition. Sheller milling yields high quality bran with higher content of oil, protein and carbohydrates and with lesser fiber and ash. Similarly, parboiling (process of soaking of paddy in hot water at 90-95^{0}C) improves the milling quality with out contamination of endosperm with consequent increase in the oil content of the bran [43,48]. Commercial brans usually represent an admixture of the locally cultivated varieties and may also contain both raw and parboiled bran which too can alter their composition to a great extent. This may explain the higher oil content of commercial bran (21.6%) as compared to the maximum value of 18.8% for raw rice bran from the varieties studied here. Monsoor *et al.* have shown that commercial rice bran contained 11.5 to 17.2 % protein, 12.8 to 22.6% fat, 6.2

to 14.4% total fiber, and 8.0 to 17.7% ash, depending on the processing conditions [149]. The chemical composition of commercial rice bran as found in this study also falls within this range. The proportional increases in protein, fiber, ash and NFE of DRB compared to FFB was obviously due to the removal of fat and are in agreement with other reports [150,151].

The range in chemical composition for the varieties studied here were dry matter 87.9 to 90.4 %, fat 15.6 to 18.8 %, protein 9.1 to 10.8 %, fiber 9.2 to 13.2 %, total ash 9.9 to 12.3 %, NFE 48.7 to 54.3% and energy 386.8 to 401.5 Kcal/100g. Significant varietal influence on the chemical composition of rice bran as observed here is also reported for other varieties. For instance, Palipane and Swrnasiri have shown that the protein content ranged from 10.4 to 15.5 %, fat from 22.2 to 26.2 %, fiber from 9.6 to 12.3% and ash from 10.8 to 12.2% for the six popular Sri Lankan rice varieties studied by them [148]. Amissah *et al.* have determined the chemical composition of bran from 15 Ghanan rice varieties and the ranges observed for the various chemical constituents were moisture 7.1 to 13.0%, fat 13.4 to 19.9%, fiber 7.3 to 13.4%, protein 11.5 to 15.4%, ash 8.4 to 22.2% and energy 245-388 Kcal/100g [152]. Large variations in the nutrient composition of bran was also reported for Mexican [153] as well as Iraqi rice varieties [154].

Table. 3.1. Chemical Profile of Commercial Rice Bran

Constituents [a]	(wt %)	
	Full fat rice bran	Defatted rice bran
Dry Matter	88.9 (±0.4)	83.2 (±0.3)
Fat	21.6 (±0.3)	0.3 (±0.1)
Protein	11.2 (±0.4)	13.8 (±0.3)
Crude Fiber	14.2 (±0.1)	17.9 (±0.1)
Ash	11.4 (±0.1)	13.8 (±0.3)
NFE	41.7 (±0.1)	54.2 (±0.6)
Energy (Kcal/100g)	406.1 (±5.7)	274.5 (±6.8)

[a]Each value in the table represents the mean (± standard deviation) of four analyses from 2 replications.

DM : dry matter

NFE (nitrogen free extractives) = 1000 – (fat + protein + fiber + ash) [146].

Energy : [(Fat x 9) + (Protein x 4) + (NFE x 4)] Kcal/100g [146].

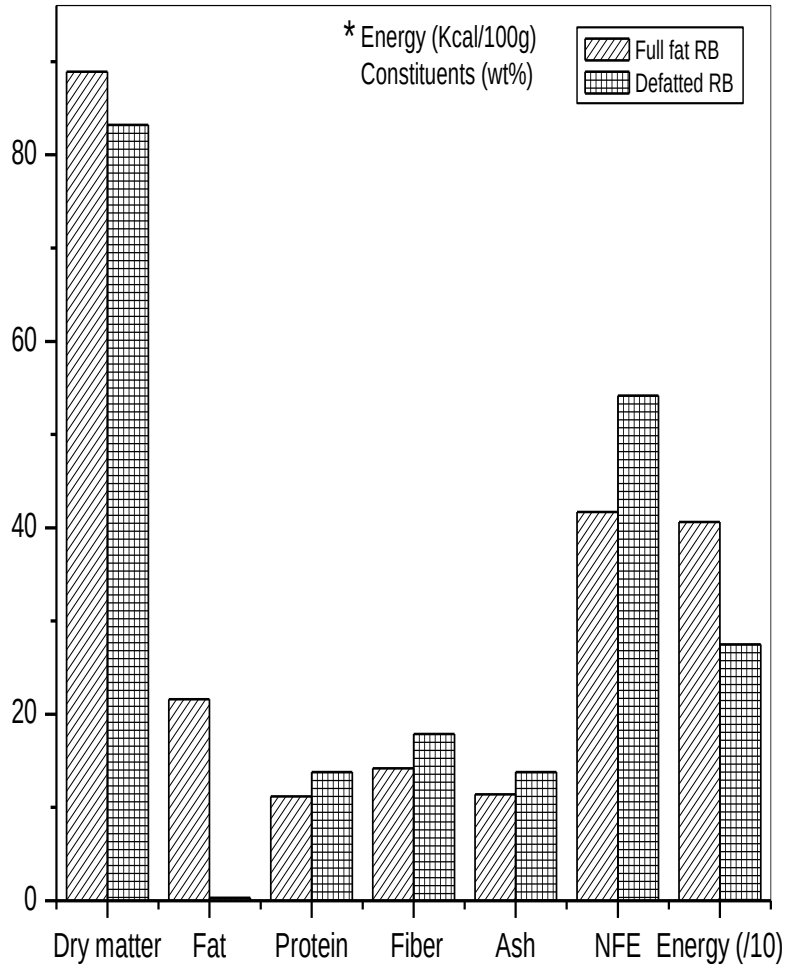


Fig. 3.1.Compositional analysis of full fat and defatted rice bran. *For energy, $1/10^{th}$ of the actual value is given for the sake of convenience of drawing.

Table. 3.2. Proximate Composition of Rice Bran from Popular Varieties of The Region

Variety	chemical constituents (wt%)[a]								
	DM	Fat	Protein	Fiber	Ash			NFE	Energy (Kcal/100g)
					Soluble ash	Insoluble ash	Total ash		
PTB 50	89.1[a] (±0.9)	17. 0[b,c] (±0.5)	9.7[b] (±0.3)	12.1[d] (±0.5)	5.2[b] (±0.3)	7.2[d] (±0.1)	12.4 [c] (±0.3)	48.8[a] (±1.6)	387.2[a] (±4.6)
PTB 39	88.9[a] (±1.1)	17. 2[c] (±0.6)	10.8[d] (±0.4)	10.1[b] (±0.5)	4.9[a] (±0.3)	7.1[d] (±0.3)	11.9 [c] (±0.5)	49.9[a,b] (±1.9)	397.4[c] (±3.6)
PTB 38	89.1[a] (±0.8)	15.7[a] (±0.4)	10.2[c] (±0.4)	10.8[c] (±0.4)	4.7[a] (±0.2)	7.5[e] (±0.3)	12.2 [c] (±0.5)	51.1[b] (±1.6)	386.8[a] (±6.8)
JAYA	87.9[a] (±0.8)	15.7[a] (±0.5)	10.1[b,c] (±0.4)	9.2[a] (±0.3)	4.9[a] (±0.2)	5.9[c] (±0.2)	10.7[b] (±0.4)	54.3[c] (±1.6)	398.6[d] (±0.6)
MO 1O	90.4[b] (±1.0)	18.8[d] (±0.5)	9.1[a] (±0.3)	13. 2[e] (±0.3)	5.6[c] (±0.3)	4.4[a] (±0.1)	9.9[a] (±0.4)	48.9[a] (±1.5)	401.5[e] (±0.7)
Oryza Nivara	89.1[a] (±1.1)	16. 5[b] (±0.4)	10.4[c] (±0.4)	12.4[d] (±0.4)	5.8[c] (±0.2)	5.1[b] (±0.3)	10. 9[b] (±0.5)	49.8[a,b] (±1.7)	389.5[b] (±4.6)

[a]Each value in the table represents the mean (±standard deviation) of six analyses from 3 replications.
[a-e] means within a column with different letters are significantly different ($P<0.05$) according to DMRT.
DM : dry matter; NFE (nitrogen free extractives) = 1000 – (fat + protein + fiber + ash) [146].
Energy : [(Fat x 9) + (Protein x 4) + (NFE x 4)] Kcal /100g [146].

3.1.2. Mineral composition of rice bran from different varieties

Data on the mineral element composition of bran from the selected rice varieties is given in Table 3.3. The inorganic matter was analysed for Na & K by flame photometry. Ca, Mg, Fe, Cu, Mn and Zn were analysed by AAS and phosphorus analysis was done by colorimetry. The average concentrations (ppm) of various minerals among the varieties studied here followed the order P (13608) > K (9520) > Mg (3844) > Ca (362) > Fe (216) > Na (190) > Mn (99) > Zn (39) > Cu (4), with P and Cu being the most and least abundant minerals respectively. The ranges (ppm) observed for various minerals were Na (104 – 280), K (8508- 11202), Ca (162 – 487), Mg (3505 – 4282), Fe (130–325), Cu (2–6), Mn (56–218), Zn (34 – 45), and P (9675–17595).

Cereal products are an important source of a variety of nutrients, comprising macro and micro elements [155]. Rice bran is reported to be a rich source of minerals [43,48]. The significant variations observed here in the mineral contents of bran from different rice varieties is well documented for other varieties. For instance, Al-Bayati *et al.* have determined the mineral composition of bran from 7 Iraqi rice varieties and the ranges (ppm) observed for various minerals by them were Na (77 – 224), K (6200- 15800), Ca (240–890), Mg (500 – 6900), Fe (57-138), Mn (80–190), Zn (40-75), and P (6000–13000) [154]. Kokot and Phuong have analysed the P, K, Mg, Ca, Mn, Zn, Fe, Cu, Al, Na, Ni, As, Mo, and Cd content of Vietnamese and Australian rices and subjected the data matrix to a multivariate data analysis by principal component analysis (PCA). Their results showed that geographical location, grain variety, seasons and soil conditions are the most likely significant factors causing changes in the elemental content between the rice samples [156]. Variations in the mineral composition of bran is also reported for Ghanan [152] as well as Mexican rice varieties [153].

Table. 3. 3. Mineral Composition of Rice Bran from Popular Varieties of The Region

Variety	Minerals in ppm[a]								
	Na	K	Ca	Mg	Fe	Cu	Mn	Zn	P
PTB 50	258^{d} (±20)	9722^{c} (±332)	162^{a} (±12)	4064^{b} (±156)	301^{c} (±25)	2^{a} (±1)	123^{c} (±7)	34^{a} (±2)	9675^{a} (±333)
PTB 39	198^{c} (±14)	9445^{b,c} (±273)	432 ^{b} (±38)	3557^{a} (±127)	130^{a} (±7)	6^{b} (±1)	81^{b} (±5)	45^{b} (±3)	15565^{d} (±638)
PTB 38	280^{d} (±23)	9339^{b,c} (±288)	487^{c} (±41)	3505^{a} (±119)	147^{a} (±13)	5 ^{b} (±1)	59^{a} (±3)	39^{a} (±3)	13699^{c} (±518)
JAYA	104^{a} (±7)	8902^{a,b} (±237)	485^{c} (±47)	3580^{a} (±131)	325^{c} (±27)	5^{b} (±1)	218^{d} (±16)	35^{a} (±2)	11409^{b} (±474)
MO 1O	155^{b} (±11)	11202^{d} (±621)	413^{b} (±34)	4077^{b} (±164)	211^{b} (±16)	2^{a} (±1)	56^{a} (±4)	37^{a} (±3)	17595^{e} (±785)
Oryza Nivara	147^{b} (±12)	8508^{a} (±228)	194^{a} (±16)	4282^{b} (±186)	184^{b} (±12)	5^{b} (±1)	61^{a} (±4)	45^{b} (±3)	13704^{c} (±566)

[a]Each value in the table represents the mean (± standard deviation) of four analyses from 2 replications.
[a-e]means within a column with different letters are significantly different (P< 0.05) according to DMRT.

3.1. 3. Chemical characteristics and fatty acid composition of Rice bran oil (RBO) from different varieties of rice

The chemical characteristics of RBO are given in Table 3.4. The results indicated that the chemical characteristics of RBO for different varieties studied varied in narrow range. The values obtained were; FFA (9.9–14.5%), saponification value (182.1 – 185.9), iodine value (96.1 – 98.3) and unsaponifiable matter (4.5 – 5.1%). Relatively larger variations for FFA can not be attributed to variety as FFA is primarily influenced by lipolytic hydrolysis of the rice bran oil and the lipase activity is controlled by milling conditions and storage of bran. The unsaponifiable matter of RBO was unusually high and was several fold higher than those of other vegetable oils [52]. The exceptionally high content of unsaponifiable matter in RBO could be attributed to some phytochemicals unique to RBO that are present in high concentrations; a detailed discussion on them will follow. RBO therefore is unique among vegetable oils due to its unsaponifiable matter content and its composition.

Fatty acid composition of RBO

The hexane extracts of rice bran (RBO) from different varieties were methylated using methanolic H_2SO_4 and fatty acid composition was estimated by GC. The fatty acid profiles are presented in Table 3.5. Major fatty acids of RBO were 16:0, 18:0, 18:1, and 18:2 with mean values of 21.6%, 2.0%, 41.8% and 32.5% respectively. Myristic (14:0), arachidic (20:0) and linolenic (18:3) acids were also detected but in small concentrations. Thus RBO possesses simple fatty acid composition with three fatty acids (16:0, 18:1, and 18:2) comprising about 95% with saturated to unsaturated ratio of approximately 1:3. It appears that the fatty acid profile of RBO is close to the ideal ratio of saturated: mono unsaturated: poly unsaturated; 1: 1.5:1. RBO, therefore possesses nutritionally significant balanced fatty acid composition besides its high bioactive phytochemical contents.

Fatty acid composition of an oil can be an indicator of its stability, physical properties, and nutritional value [157]. The superior frying stability of RBO is attributed to a balanced linoleic/oleic/linolenic acid ratio and high antioxidant (oryzanols, tocols) levels [158]. Also, the relatively high palmitc acid content in RBO seems to be a promising breeding target for the production of margarines, shortening and frying oil [159]. RBO is rich in linoleic acid, one of the most important essential fatty acids required for growth, physiological functions and body maintenance.

Varietal variations for fatty acid composition was not very significant. However, small but significant variations were seen in the contents of individual unsaturated fatty acids, and $C_{20:0}$. The ranges observed in fatty acid composition as well as the varietal differences observed in this study are by and large are comparable with those of other varieties. For example, Goffman *et al.* have shown that for a germplasm collection consisting of 204 genetically diverse rice accessions, the genotype effects are statistically significant both for lipid content and fatty acid profiles [159]. Gaydou *et al.* have analysed the fatty acid composition of six Malagasy rice bran oils and the ranges observed for the various fatty acids are 14:0 (0.2- 0.4%), 16:0 (16.4- 20.4%), 16:1 (0.1%), 18:0, (1.8-2.2%), 18:1(41.6-48.1%), 18:2 (29.0-37.2%), 18:3 (1.0-1.8%), 20:0 (0.3-0.6%). They have also noted that the vareital variations in fatty acid composition is not significant [160]. Bhattacharyya *et al.* have shown that the major fatty acids of commercial Indian rice bran oils are palmitic, oleic, and linoleic acid [161]. According to Suzuki *et al.* the total lipids of a Thai as well as two Japanese rice varieties consisted of 18:1(38-42%), 18:2 (37-38%), 16:0 (15-17%) and small amounts (1-3%) of 18:0 and 18:3, with only minor differences among the the varieties [162].

Table. 3. 4. Chemical Characteristics of RBO from Popular Rice varieties of The Region

Variety	Chemical characteristics [a]			
	FFA (%)	S.V.	I.V.	Unsap. (%)
PTB 50	12.5[c] (±0.4)	182.2[a] (±1.4)	96.1[a] (±1.3)	5.0[b] (±0.1)
PTB 39	13.2[c] (±0.5)	182.9[a] (±1.5)	98.3[a] (±1.4)	4.7[a] (±0.1)
PTB 38	14.5[d] (±0.6)	185.9[b] (±1.3)	96.4[a] (±1.2)	5.0[b] (±0.1)
JAYA	11.1[b] (±0.3)	182.1[a] (±1.2)	96.9[a] (±1.2)	5.1[b] (±0.1)
MO 1O	9.9[a] (±0.4)	182.1[a] (±1.6)	97.8[a] (±1.4)	4.5[a] (±0.1)
Oryza Nivara	12.6[c] (±0.3)	182.3[a] (±1.5)	98.2[a] (±1.4)	4.7[a] (±0.1)

[a] Each value in the table represents the mean (± standard deviation) of four analyses from two replications.

[a-f]means within a column with different letters are significantly different ($P < 0.05$) according to DMRT.

Table 3.5. Fatty Acid Composition of Rice Bran Oil from Popular Rice Varieties of The Region

	Fatty acids (%) [a]								
Variety	14:0	16:0	18:0	20:0	T.S.	18:1	18.2	18:3	T.U.
PTB 50	0.4^{a} (±0.1)	21.8^{a} (±0.6)	2.1^{a} (±0.3)	0.9^{a} (±0.1)	25.2^{a} (±1.1)	42.8^{b} (±1.1)	31.1^{a} (±0.7)	$1.4^{a,b}$ (±0.2)	75.3^{a} (±2.0)
PTB 39	0.4^{a} (±0.1)	21.7^{a} (±0.7)	2.0^{a} (±0.3)	0.9^{a} (±0.1)	25.0^{a} (±1.2)	41.8^{b} (±1.0)	32.8^{c} (±0.8)	$1.4^{a,b}$ (±0.1)	76.0^{a} (±2.1)
PTB 38	0.5^{a} (±0.2)	21.5^{a} (±0.6)	2.2^{a} (±0.3)	1.0^{b} (±0.2)	25.2^{a} (±1.1)	38.5^{a} (±0.9)	35.0^{d} (±0.8)	1.6^{b} (±0.2)	75.1^{a} (±2.4)
JAYA	0.4^{a} (±0.1)	21.5^{a} (±0.6)	2.1^{a} (±0.2)	0.8^{a} (±0.1)	24.8^{a} (±1.0)	42.5^{b} (±1.1)	$31.7^{a,b}$ (±0.8)	1.5^{b} (±0.2)	75.7^{a} (±2.2)
MO 1O	0.4^{a} (±0.1)	21.3^{a} (±0.5)	2.0^{a} (±0.3)	1.1^{b} (±0.2)	24.8^{a} (±1.0)	42.3^{b} (±1.0)	$32.2^{b,c}$ (±0.7)	1.2^{a} (±0.1)	75.7^{a} (±2.1)
Oryza Nivara	0.3^{a} (±0.1)	21.7^{a} (±0.7)	1.8^{a} (±0.2)	0.9^{a} (±0.1)	24.7^{a} (±1.2)	42.8^{b} (±1.1)	$32.3^{b,c}$ (±0.6)	1.2^{a} (±0.1)	76.3^{a} (±2.3)

[a]Each value in the table represents the mean (±standard deviation) of six analyses from 3 replications.

[a-c]means within a column with different letters are significantly different ($P<0.05$) according to DMRT.

T.S: total saturated; T.U: total unsaturated

3.1.4. Oryzanol composition of RBO from different varieties of rice

Esters of sterols and cinnamic acid derivatives (CAD) comprise a group of compounds found in seeds of corn, wheat, rye, triticale and rice. They have been shown to lower blood lipid levels [163]. In rice bran, the corresponding compounds include both steryl and terpenyl ferulates commonly known together as oryzanols. The individual compounds so far identified by a combination of chromatographic and spectroscopic techniques include *trans*-ferulates of cycloartanol, 24-methylene cycloartanol, cycloartenol, campestanol, campesterol, Δ^7-campestenol, stigmastanol, stigmasterol, sitosterol, Δ^7-sitostenol, 24-methylene cholesterol [62], Δ^7-stigmastenol, sitostanol [164], 24-methyl cholesterol, and cycloeucalenol [125]. The *cis*-ferulates identified are those of 24-methylene cycloartanol, cycloartenol, sitosterol [62], stigmastanol, 24-methyl cholesterol [125]. A few other compounds reported in rice bran include *trans*-caffeates of cycloartenol and campesterol, 24-hydroxy-24-methyl-cycloartanol ferulate and 25-hydroxy-24-methyl cycloartanol ferulate [62]. Oryzanols are lipophilic and therefore extracted with non polar solvents like hexane commonly used for RBO extraction in the industry. However significant quantity of oryzanols are left in DRB. Oryzanols in RBO and DRB were studied here.

Considering the significance of oryzanols in health and its unique presence in rice bran oil, this part of the study was undertaken to separate and quantify various oryzanol components in RBO from the popular rice varieties of the region. The oryzanol composition of RBO was analysed by a standardized HPLC procedure using a reversed-phase C_{18} column. A number of mobile phases reported were used in succession with a view to obtain maximum resolution among oryzanol components especially between cycloartenyl ferulate (CFE) and 24-methylene cycloartanyl ferulate (24-M CFE) and between 24-methylene cycloartanyl ferulate and campesteryl ferulate. Two of them were especially found good for the purpose. The first one was that proposed by Xu and Godber [164] consisting of methanol,

acetonitrile, dichloromethane, and acetic acid (50:44:3:3; v/v) and was good with respect to the number of components separated using a waters μ-bondapak $^{TM}C_{18}$ column. The second one was that of Norton *et al.* [163] who have found that a mixture of acetonitrile, n-butanol, acetic acid and water (94:3:2:1; v/v) improved the resolution of steryl esters of cinnamic acid derivatives from corn bran. By incorporation of *n*-butanol and water into the mobile phase reported by Xu and Godber, better resolution among various oryzanols could be achieved here, though baseline separation was not obtained between 24-methylene cycloartanyl ferulate and campesteryl ferulate. However, the modified mobile phase yield the best results for separation of oryzanols in RBO as compared to those previously reported. The modified mobile phase consisted of (acetonitrile : dichloromethane : acetic acid) : (methanol : n-butanol : water) (44:3: 3) : (45: 1:4) in the ratio of 75:25. On increasing B concentration, i.e. concentration of (methanol : n-butanol : water) above 25%, 24-M CFE and CFE started merging and below B concentration of 25%, sharpness of the peaks decreased.

Other optimized conditions included a flow rate of 1 ml/minute, a detection wavelength of 325 nm, and a 1:1 mixture of acetonitrile: methanol as the solvent for oryzanol standards. The optimum concentrations for external standards viz. 24-methylene cycloartanyl ferulate, and a standard mixture of oryzanols were 5 and 10 ppm respectively. Similraly for RBO, 1000 ppm (0.1 %) solutions in a 1:1 mixture of acetonitrile and methanol, yielded the best separation among its various oryzanol components.

Peak identification was based on comparison of retention times with authentic standards of cycloartenyl ferulate (CFE), 24-methylene cycloartanyl ferulate (24-M CFE) and a standard mixture of oryzanols and also by comparison with literature reports. The standard mixture of oryzanols used in the study contained ferulates of stigmasterol, cycloartenol, 24-methylene cycloartanol, campesterol, β-sitosterol, and cycloartanol. This mixture on HPLC analysis was resolved into six peaks, even though baseline separation

was not achieved between peaks 3 and 4 (Fig.3.2). The use of CFE and 24-M CFE standards permitted the direct confirmation of peaks 2 (CFE) and 3 (24-M CFE). It could be suggested that the peak retention times of components of γ-oryzanol in the analytical reverse phase HPLC are largely dependent on the number and positions of double bonds as they are related to the polarity of compounds although the relationship is not strong. Components having a double bond on a side chain of the triterpene eluted prior to the components having a double bond in the triterpene [164]. Based on this polarity concept and also by comparison of retention times with literature reports [164, 165, 166], peak 1 was assigned to stigmasteryl ferulate, 4 to campesteryl ferulate, 5 to β-sitosteryl ferulate and 6 to cycloartanyl ferulate. The analysis report provided by the suppliers of the standards (Ms/ Tsuno Rice Fine Chemicals, Japan) supported this. More over, Rogers *et al.* have reported a similar HPLC sequence for a standard mixture of oryzanols provided by the same manufacturer [167]. In addition to the above ferulates, RBO contained small amounts of some unidentified ferulates also (Fig.3.3).

24-methylene cycloartanyl ferulate was used as an external standard in quantitative analysis of oryzanols. Linearity of the method response was assessed with 24-methylene cycloartanyl ferulate (predominant oryzanol in rice bran) at ten concentration levels ranging from 0.1 to 10 ppm in a 1:1 mixture of acetonitrile and methanol. Detector response (area) was found to be linear with a correlation coefficient, r = 0.9999. The accuracy of the method was monitored by determining the recoveries of cycloartenyl ferulate and 24-methylene cycloartanyl ferulate added at levels of 100 μg to a 0.1% solution of RBO and analysed in triplicate. Recoveries were found to be very good at 99.5% for CFE and 99.7% for 24-MCFE. The reproducibility of the method was confirmed by determining oryzanols in the same RBO sample 6 times within the same day under the same analytical conditions. The coefficients of variation were 2.32% (CFE) and 1.65% (24-M CFE). Such detailed analytical approach to oryzanols in RBO as shown here, has not been attemped before.

The oryzanol composition of RBO from the selected varieties as determined using the modified HPLC protocol is presented in Table 3.6. There is significant difference in oryzanol content and composition among the varieties. RBO is rich in oryzanols with concentrations ranging from 1.26% to 1.93%. The concentrations of individual forms in ppm are stigmasteryl ferulate (153 to 625), cycloartenyl ferulate (2234 to 4116), 24-methylene cycloartanyl ferulate (4929 to 9265), campesteryl ferulate (2744 to 3996), β-sitosteryl ferulate (808 to 2300), cycloartanyl ferulate (39 to 268) and 3 unidentified forms, two of which are present only in traces. Concentration of the 3rd unidentified form ranged from 30 to 112 ppm.

24-methylene cycloartanyl ferulate was the predominant oryzanol component in all the varieties accounting for about 37-48% of the total oryzanols. Other major forms were cycloartenyl ferulate (18-25%), campesteryl ferulate (16-30%), and β-sitosteryl ferulate (6-15%), with the respective contributions to the total oryzanol content given in parenthesis. These four oryzanol components together constituted more than 95% of the total oryzanols in all the varieties with 24-methylene cycloartanyl ferulate: cycloartenyl ferulate : campesteryl ferulate: β-sitosteryl ferulate ratio of approximately 4:2:2:1. In the varieties PTB 50, Jaya and *oryza nivara*, the concentration of these four forms followed the order 24-methylene cycloartanyl ferulate> cycloartenyl ferulate >campesteryl ferulate> β-sitosteryl ferulate. Whereas, in the varieties PTB 39, PTB 38, and MO 10, the order was 24-methylene cycloartanyl ferulate> campesteryl ferulate> cycloartenyl ferulate > β-sitosteryl ferulate. Of the varieties studied, PTB 50 possessed the highest total oryzanol content (1.9%), and also the highest contents of cycloartenyl ferulate (4116 ppm), 24-methylene cycloartanyl ferulate (9265 ppm), cycloartanyl ferulate (268 ppm), and β-sitosteryl ferulate (2300 ppm). The varieties PTB 39 and JAYA possessed the highest contents of campesteryl ferulate (3996 ppm) and stigmasteryl ferulate (625 ppm) respectively. Reasons for such wide variations

due to varieties are not explained before. However, genetic factors could be prime determinant.

The total oryzanol content of RBO is reported to range from 1.1-2.6% [61,63], with 24-methylene cycloartanyl ferulate, cycloartenyl ferulate and campesteryl ferulate amounting to about 80% of total oryzanols in rice bran [83]. Also, Bergman and Xu have shown that both genetics and environment influenced the oryzanol contents of Southern United States rice, though the latter had a greater effect [168]. Except for such few reports on overseas rice, detailed analytical data on the oryzanol composition of RBO at the varietal level as presented here is rather rare especially for Indian rice varieties. Though Gopalakrishna *et al.* have reported the oryzanol content of 18 Indian paddy cultivars which ranged from 1.63 to 2.72%, they did not provide any varietal level information on the composition of the oryzanols [169].

The oryzanol present in RBO is reported to have functions similar to vitamin E in promoting growth, facilitating capillary growth in the skin, and improving blood circulation, along with stimulating hormonal secretion [170]. Oryzanols are also known to possess antioxidant activity in various lipid oxidation systems [82, 83, 87] and are shown to be effective against superoxide [171] and DPPH radicals [76]. Campesteryl ferulate component of oryzanol also possessed antioxidant activity [65]. Oryzanol is reported to be associated with significant reductions in aortic fatty streak formation [95]. Cycloartenyl ferulate, 24-methylene cycloartanyl ferulate and stigmastanol from rice bran were shown to have inhibitory effects on human immuno deficiency virus type I(HIV-1) *reverse transcriptase* [115]. Moreover, various oryzanol components have been shown to have anti-inflammatory activity against 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-induced ear inflammation in mice [125]. Furthermore, the cycloartenyl ferulate component of oryzanol was shown to possess anti-tumour activity in mice [172]. Since the antioxidant and biological activities of individual oryzanol components may also differ [83,88],

varietal level information on the oryzanol composition may be useful for breeding purposes.

Oryzanols with such health promoting properties and in such high concentrations are unique to rice bran oil and therefore RBO could be termed as health oil. However, chemical refining employed in the industry removes most of the oryzanols through soap stock, thereby depriving the chemically refined RBO of oryzanols. Alternative methods like physical refining, molecular distillation, super critical fluid extraction are reported to preserve the oryzanols in RBO [169]. Such methods may, in the future could be used to produce far more healthier RBO for human consumption. Recently, a novel physical refining technique was developed at RRL, Trivandrum and commercialized [73]. It is reported that more than 80% of oryzanols is retained in the refined oil by this process.

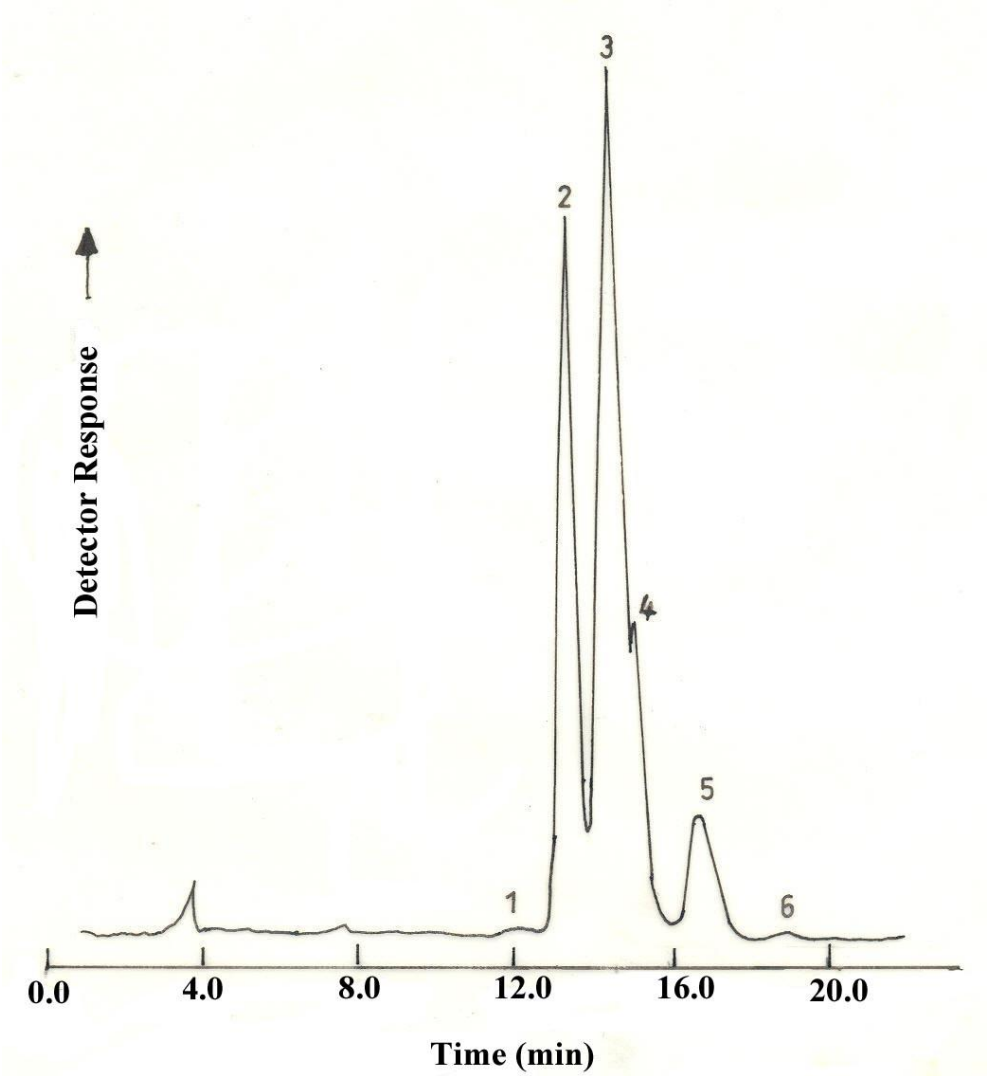


Fig.3.2. Reverse- phase HPLC/UV- Visible detection analysis of a standard mixture of oryzanols (10μg/ml). Peak identification: 1,Stigmasteryl ferulate; 2, Cycloartenyl ferulate; 3,24-methylene cycloartanyl ferulate; 4,Campesteryl ferulate; 5,β-sitosteryl ferulate; 6, Cycloartanyl ferulate.

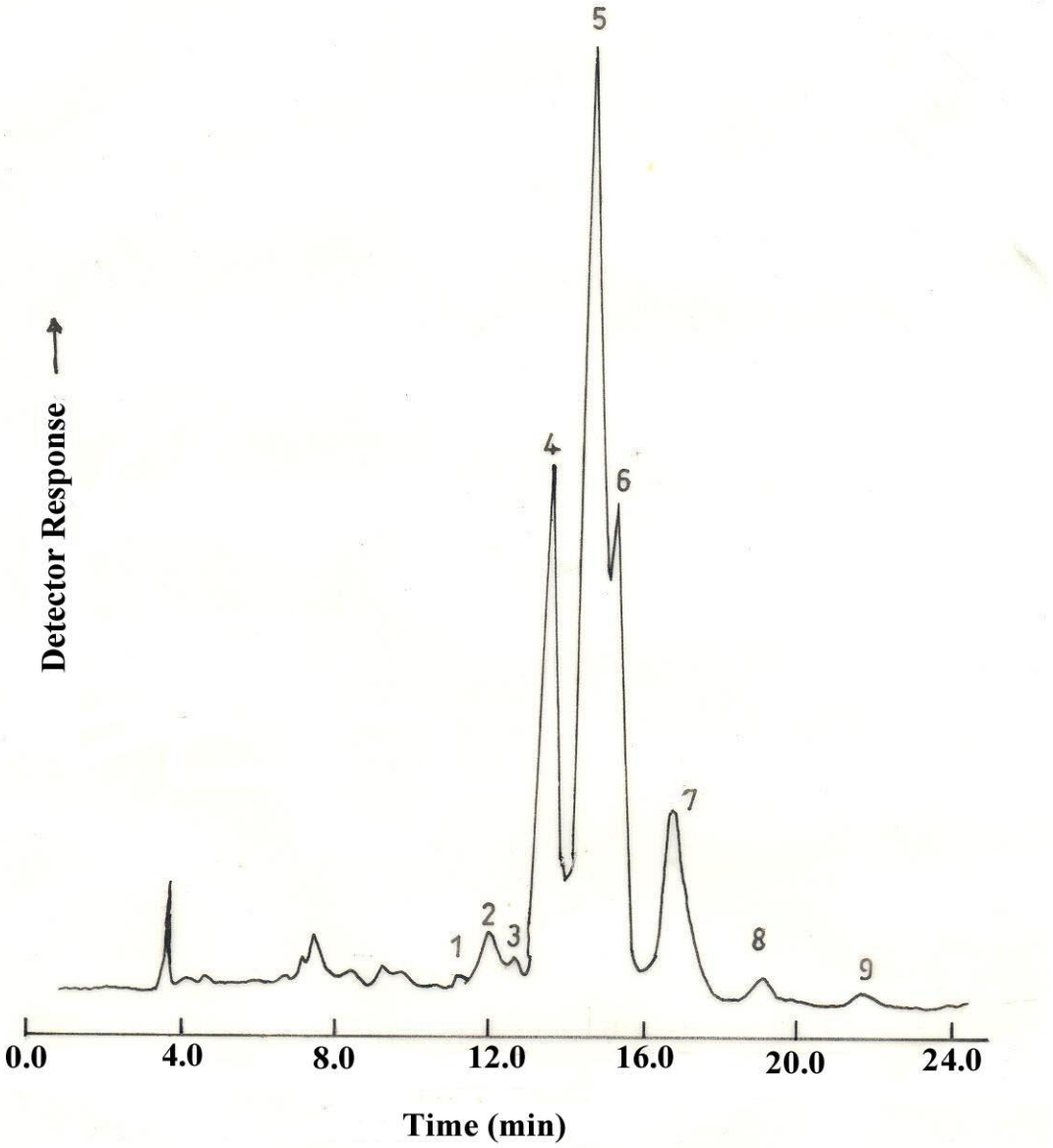


Fig.3.3. Reverse- phase HPLC/UV- Visible detection analysis of oryzanols of RBO. Peak identification: 1,Unidentified; 2,Stigmasteryl ferulate; 3,Unidentified; 4, Cycloartenyl ferulate; 5,24-methylene cycloartanyl ferulate; 6,Campesteryl ferulate; 7,β-sitosteryl ferulate; 8,Cycloartanyl ferulate; 9,Unidentified.

Table. 3. 6. Oryzanol Composition of RBO from Popular Rice Varieties of The Region

Variety	Oryzanols * in ppm [a]									
	1	2	3	4	5	6	7	8	9	Total
PTB 50	Tr	160[b] (± 3)	61[d] (± 2)	4116[f] (± 38)	9265[e] (± 92)	3176[d] (± 30)	2300[f] (± 18)	268[d] (± 4)	Tr	19348[e] (±189)
PTB 39	Tr	291[d] (± 4)	107[e] (± 3)	2624[b] (± 23)	4929[a] (±46)	3996[f] (±38)	1323[b] (± 12)	39[a] (± 1)	Tr	13311[c] (±129)
PTB 38	Tr	363[e] (± 4)	112[f] (± 3)	2705[c] (± 23)	5381[b] (± 46)	2862[c] (± 30)	1529[c] (± 15)	111[b] (± 5)	Tr	13066[b] (±127)
JAYA	Tr	625[f] (± 7)	30[a] (± 1)	4016[e] (± 38)	7943[d] (± 84)	3298[e] (± 30)	2147[e] (± 20)	247[c] (± 4)	Tr	18309[d] (±187)
MO 10	Tr	153[a] (± 3)	40[b] (± 1)	2234[a] (± 20)	5317[b] (± 47)	2744[a] (± 24)	1930[d] (± 20)	251[c] (± 4)	Tr	12672[a] (±121)
Oryza Nivara	Tr	198[c] (± 3)	52[c] (± 2)	3197[d] (± 30)	5558[c] (± 54)	2791[b] (± 30)	808[a] (± 7)	41[a] (± 1)	Tr	12648[a] (±129)

[*]Oryzanols: 1,Unidentified; 2,Stigmasteryl ferulate; 3,Unidentified; 4,Cycloartenyl ferulate; 5, 24-methylene cycloartanyl ferulate; 6, Campesteryl ferulate; 7, β-sitosteryl ferulate; 8, Cycloartanyl ferulate; 9,Unidentified.

[a] Each value in the table represents the mean (± standard deviation) of six analyses from 3 replications.

[a-f] : means within a column with different letters are significantly different ($p<0.05$) according to DMRT.

Tr: trace (<25 ppm)

3.1.5. Tocopherol and tocotrienol composition of RBO from different varieties of rice

Tocopherols and tocotrienols (tocols) seem to be the most efficient lipid antioxidants in nature [33]. The eight vitamers of vitamin E (α–, β–, γ-, and δ–tocopherols and trienols) have different antioxidant and biological activities and have different distribution profiles in foods [174]. Vegetable oils provide the best sources of these bio-active phytochemicals essential in human nutrition. Of these, palm oil and rice bran oil are rich in tocotrienols with γ-T_3 being the predominant form [64]. The antioxidant activity of the tocopherols and tocotrienols (grouped as chromanols) is mainly due to their ability to donate their phenolic hydrogens to lipid free radicals. Qureshi *et al.* have identified two new tocotrienols in rice bran in addition to the α-, β-, γ-, and δ-homologs of both tocopherols and trienols. The new compounds are desmethyl tocotrienol, and didesmethyl tocotrienol [56]. The beneficial effects of vitamin E compounds include anti-cancer activity, lipid lowering effect, nutrient function, prevention of diseases like CVD, cataract, arthritis etc., prevention of nitrosoation of amines, and immune system maintenance [33,34].

Rice bran oil is also a rich source of tocols particularly tocotrienols. The present investigation, therefore attempted to separate, identify and quantify vitamin E homologs in RBO from major varieties of rice grown in the region. The study employed direct injection analysis of oil solutions in *n*-hexane for the tocol analysis of RBO using a normal-phase NH_2 column. It is reported that in direct injection analysis, fouling of the adsorbents may occur due to the co-elution of neutral lipids with hexane [175,176]. However, since hexane is miscible with the mobile phase in normal-phase chromatography, there is the obvious advantage that the tocopherols and trienols will be moving with the mobile phase resulting in longer column life as the risk of more compounds getting trapped on the column is reduced [177]. With regard to the quantitative estimation of tocopherols and trienols also, direct injection analysis was found to be superior to the conventional methods which included saponification of the

oil and TLC separation of the unsaponifiable portion thus obtained with subsequent losses in total recovery [130]. More over, the method is very fast since the oil samples does not require pre treatments.

The HPLC conditions were standardized with respect to flow rate, mobile phase polarity and wavelength of detection using individual standards and their mixtures. Excellent chromatograms were obtained at the optimized conditions of mobile phase composition of *n*-hexane: isopropanol (96:4;v/v) at a wavelength of detection of 297 nm and at a flow rate of 1 ml/minute with the CLC–NH_2 (M) column, though β-T_3 and γ-T were not clearly separated from each other (Fig.3.4).

Peak identification was based on comparison of RT values with authentic standards of tocopherols and tocotrienols. The retention times and relative selectivity for the vitamin E forms in normal- phase HPLC may be explained as follows. The order of elution was α-T > α-T_3 > β-T > β-T_3 > γ-T > γ-T_3 > δ-T > δ-T_3 (Fig.3.4). α-T, the most non polar homolog of the group eluted first and δ-T_3, the most polar homolog of the group eluted last. These observations agreed very well with literature reports that the RT values and relative selectivity for the vitamin E components in normal phase chromatography were presumably attributed to the steric hindrance of the phenolic group by methyl substituents of the chroman ring which led to different degrees of interaction between the molecules and the silanol groups of the silica [175]. The observed resolution of β-T_3 and γ-T was not optimal and hence they were quantitated together. Similar HPLC separations were reported by Dionisi *et al.* in their experiments with a mixture of α–, β–, γ– and δ–tocopherols and the corresponding trienols, the only difference being the elution of γ-T before β–T_3 [178].

In the IUPAC method (1988) for the determination of tocopherols and tocotrienols in vegetable oils and fats, Pocklington *et al.* had quoted an earlier report that the fluorescence intensity of tocotrienols is the same as the

corresponding tocopherols and that the UV absorbance are similar [179]. In accordance with the latter, they have used the calibration factors for tocopherols, for the quantitation of trienols also. But Kramer *et al.* had pointed out that with a fluorescence detector, the integrated area response for equal amounts of α-T and α-T_3 were different [176]. From the analysis of a mixture of all the tocopherols and trienols having equal amounts of the various forms, we had found that the UV responses of the various vitamin E forms were different. Calibration with authentic standards was therefore essential for the accurate estimation of each form.

Thus in the present study, the various vitamin E forms were quantitated based upon peak areas relative to standard calibration plots by external standard method using authentic standards of all tocopherols and trienols. Detector response was found to be linear with a correlation coefficient, γ = 0.9991, for α-T_3 concentrations ranging from 0.02 to 0.2 μg/200 μg oil. Accuracy of the method was evaluated by recovery experiments of α-T_3, γ-T_3, β-T and γ-T added at levels of 10 μg/20 mg oil to sunflower oil. Recoveries were found to be very good, at 99.9% for α-T_3, 99.75% for γ-T_3, 99.13% for β-T, and 100.25% for γ-T. The repeatability of the method was determined with red palm oil. The sample was analysed ten times within the same day under the same analytical conditions. The coefficients of variations were 0.99% (α-T), 1.65% (α-T_3), 8.33% (β-T), 2.37% (β-T_3 + γ-T), 1.52% (γ-T_3) and 3.55% (δ-T_3). The value for β-T was relatively high because of its low concentration (1.91 mg/Kg of oil).

The chromanol profile of RBO is given in Fig.3.5. Seven tocols except β-T_3 were detected in all the rice varieties studied here. The tocopherol and trienol composition of RBO from the varieties is given in Table 3.7. RBO was rich in chromanols with concentrations ranging from 1042 to 1648 ppm. The concentration range of various tocols in ppm were α–T (154 to 257), α–T_3

(105 to 169), β–T (6 to 9), β–T_3 (not detected), γ–T (114 to 173), γ–T_3 (486 to 1114), δ–T (30 to 50), and δ–T_3 (45 to 96).

γ–tocotrienol was the predominant vitamin E homolog in all the varieties accounting for about 42-70% of the total tocols. Other major forms were α–T (10 to 21%), α–T_3 (7 to 15%), and γ–T (7 to 13%), with the respective contributions to the total tocol content given in parenthesis. These four compounds together constituted more than 90% of the total tocols in all the varieties. In the varieties PTB 50 and PTB 38, the concentration of these four forms followed the order γ–T_3 >α–T> α–T_3 > γ–T. Whereas, in the varieties PTB 39, MO 10, Jaya and *oryza nivara*, the order was γ–T_3 >α–T> γ–T > α–T_3.

Of the varieties studied, MO 10 and PTB 38 possessed the highest amounts of total tocols (1648 ppm) and total tocopherols (466 ppm) respectively. The highest amount of total tocotrienols (~1265 ppm) was found in the varieties *oryza nivara* and MO 10. The variety PTB 38 contained the highest amounts of α–T (257 ppm) and α–T_3 (169 ppm), and the varieties *oryza nivara* and Jaya contained the highest amounts of γ–T_3 (1114 ppm) and γ–T (173 ppm) respectively. The major tocopherol homolog in all the varieties was α-T whereas the major tocotrienol was γ-T_3. On an average, the ratio of tocopherols to tocotrienols in the varieties was approximately 1:2.5.

The concentrations of various tocols (ppm) in crude RBO were reported to be α-T (10-125), α-T_3 (22-142), β-T (2-9), β-T_3 (2-8), γ-T (58-203), γ-T_3 (272-868), δ-T (6-23), and δ-T_3 (27-51), with a total value that ranged from 457 to 1425 ppm [64]. Xu *et al.* have reported that α-T, α-T_3, γ-T and γ-T_3 amounted to about 90% of the total vitamin E compounds in RBO [83]. Our observations also agreed with this. Generally β-T and β-T_3 in edible oils are in low abundance and in RBO, β-T_3 is reported to be the least abundant species with concentrations ranging from 2 to 8 ppm [64]. However, it was not detected in any of the varieties studied here and similar observations were

reported for RBO by other authors also [180,181]. Significant varietal influence on the tocol composition of rice bran oil as observed here is also reported for other overseas varieties. For instance, Bergman and Xu have shown that both genetics and environment influenced the tocol contents of Southern United States rice though the latter had a greater effect [168]. However, such a varietal level information is not available for Indian rice and hence this study presents the first report of its kind on the quantitative estimation of tocols in RBO for major commercial varieties of rice cultivated in the region.

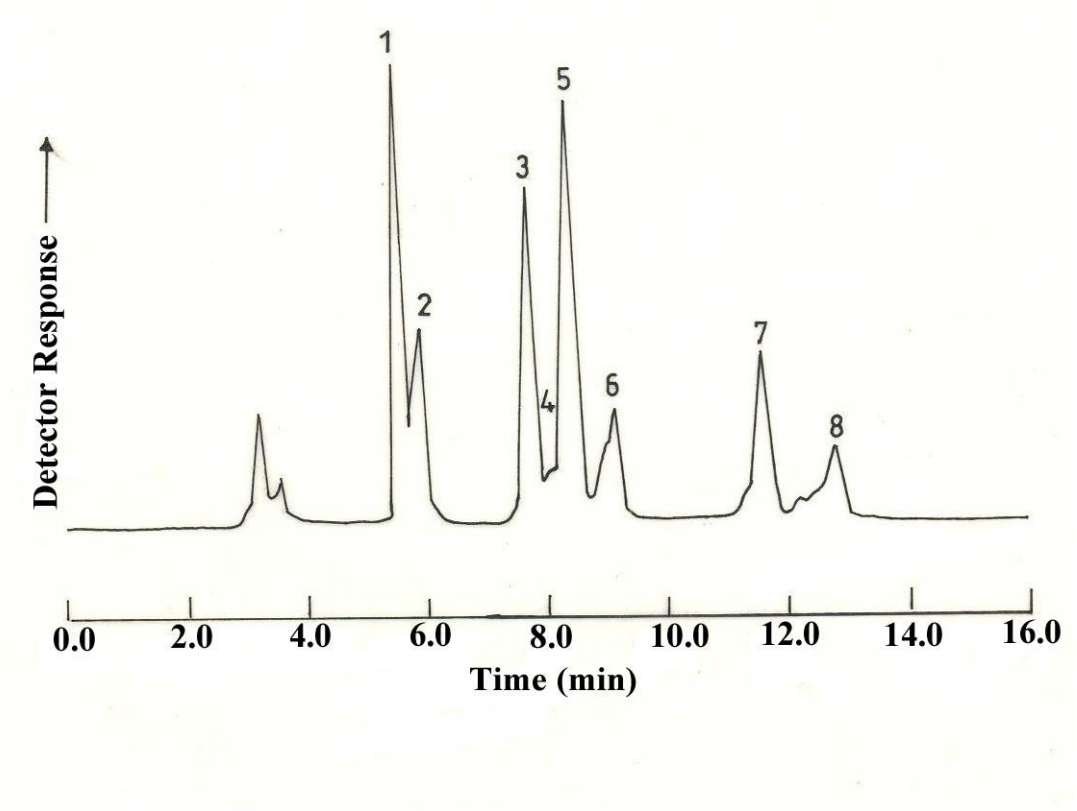


Fig.3.4. Normal- phase HPLC/UV- Visible detection analysis of a mixture of tocopherol and tocotrienol standards (20 μg/ml of each form). Peak identification: 1, α-T; 2, α-T_3; 3, β-T; 4, β-T_3; 5, γ-T; 6, γ-T_3; 7, δ-T; 8, δ-T_3.

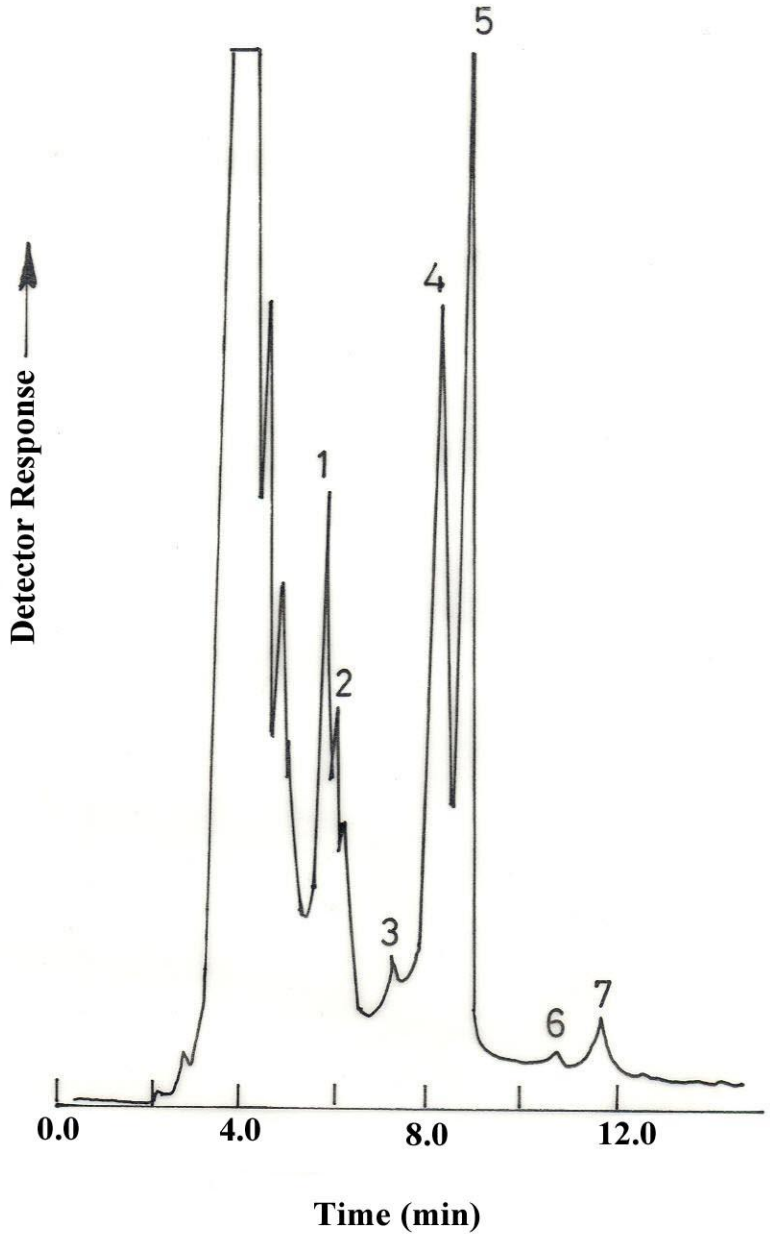


Fig.3.5. Normal- phase HPLC/UV- visible detection analysis of tocopherols and tocotrienols of RBO. Peak identification: 1, α-T; 2, α-T_3; 3, β-T; 4, γ-T; 5, γ-T_3; 6, δ-T; 7, δ-T_3.

Table.3.7. Tocol Composition of RBO from Popular Rice Varieties of The Region

Vareity	Tocopherols and tocotrienols in ppm [a]										
	αT	αT_3	βT	βT_3	γT	γT_3	δT	δT_3	Total T	Total T_3	Total tocols
PTB 50	181[b] (±3)	154[d] (±3)	7[b] (±1)	nd	118[b] (±1)	486[a] (±6)	38[b] (±2)	58[c] (±2)	344 [b] (±7)	698 [a] (±10)	1042[a] (±19)
PTB 39	196[c] (±3)	106[a] (±2)	7[b] (±1)	nd	146[c] (±3)	1001[d] (±10)	49[e] (±1)	96[f] (±2)	398 [c] (±8)	1203 [d] (±14)	1601[d] (±23)
PTB 38	257[e] (±4)	169[e] (±3)	8[c] (±1)	nd	155[d] (±3)	523[b] (±6)	46[d] (±1)	83[d] (±2)	466 [e] (±9)	775 [b] (±11)	1241[b] (±21)
JAYA	204[d] (±3)	120[b] (±2)	6[a] (±1)	nd	173[e] (±3)	757[c] (±7)	50[e] (±1)	90[e] (±2)	433 [d] (±8)	967 [c] (±11)	1400[c] (±20)
MO 1O	194[c] (±3)	137[c] (±3)	9[d] (±1)	nd	157[d] (±3)	1076[e] (±12)	30[a] (±1)	45[a] (±1)	390 [c] (±8)	1258 [e] (±15)	1648[e] (±24)
Oryza Nivara	154[a] (±3)	105[a] (±2)	7[c] (±1)	nd	114[a] (±2)	1114[f] (±10)	43[c] (±1)	53[b] (±1)	318 [a] (±6)	1272 [e] (±13)	1590[d] (±21)

[a]Each value in the table represents the mean (±standard deviation) of six analyses from 3 replications.
[a-f]means within a column with different letters are significantly different ($P < 0.05$) according to DMRT.
Nd – not detected

3.1.6. Distribution of major phytochemicals among full fat rice bran, defatted rice bran and RBO

Oilseeds processing industry produce defatted cake or meal as the by-products in large quantities. Deoiled cake (DOC) is normally obtained after mechanical expression of oil whereas deoiled meal is from solvent extraction. While DOC retains about 5-8% oil and good amount of phytochemicals, the solvent extracted meal normally contains less than 1% oil and less abundance of phytochemicals. Solvent extraction is the method of choice for rice bran. In the emerging context of phytochemicals as preventive and therapeutic agents in human health and disease, the deoiled cake or meal with abundance of phytochemicals could be future commercial source for variety of bioactive phytochemicals primarily as natural antioxidants. A few deoiled cakes from sesame, soybean, canola, peanut, cotton seeds, mustard etc. have been re-examined from this perspective [182]. However, rice bran meal has not been studied in detail. The present study was designed to characterize the major phytochemicals in defatted rice bran and to examine its commercial potential as a source of natural antioxidants.

It is well documented that the major phytochemicals of biological value in rice bran oil are oryzanols, tocopherols, and tocotrienols. It could be presumed that substantial quantities of these compounds might remain in defatted bran also. Though rice bran is reported to contain a significant amount of ferulic acid but the same is not present in RBO. The defatted meal therefore is expected to contain the entire ferulic acid in rice bran. Method for extraction of oryzanols, tocols and ferulic acid from DRB is reported elsewhere.

As for oryzanols and tocols reported elsewhere, an HPLC method was standardised for the separation and quantification of ferulic acid also. For preliminary trials, the mobile phase of *Adom et al.* [89] was followed using isocratic elution with 20% acetonitrile in water adjusted to pH2 with trifluoroacetic acid, and at a wavelength of 280 nm. However, the same HPLC protocol standardized for oryzanols was found to be effective for quantitative

analysis of ferulic acid also. The main advantage of this is the simultaneous detection of both ferulic acid and oryzanols in a single run. The ferulic acid concentration of sample extracts was extrapolated from the pure *trans*-ferulic acid standard curve for which the detector response was linear with r = 0.9998, in the concentration range 0.01– 0.2 μg/20 μl. The recovery of added ferulic acid was 98.23 ± 3.11% (n=6). The HPLC profiles of oryzanols and ferulic acid of DRB extract, tocols of DRB extract, and ferulic acid standard are given in Figs. 3.6, 3.7 and 3.8 respectively.

The contents of total oryzanols, total tocols and total ferulic acid of full fat rice bran as computed from the values obtained for oil (Tables 3.6 and 3.7) and DRB (Table 3.12) is given in Table 3.8. It is based on the assumption that the above compounds in full fat rice bran were distributed between oil and DRB. On an average, defatting of rice bran gave 20% oil and 80% defatted meal. The methanol extractives of defatted bran (optimization of methanol extraction presented in 3.2) is around 4%. It could be deduced from Table.3.8 that about 95% of the total oryzanols and about 98% of the total tocols of full fat rice bran are present in the oil. Small but significant varietal variations were also seen in the distribution of oryzanols and tocols between oil and meal. For the varieties studied, about 92-96% of the total oryzanols and 97-99% of the total tocols of full fat rice bran were present in the oil. It appears that irrespective of the oil content of the bran, these fat soluble antioxidants are getting preferentially distributed in the oil. However, the DRB contained the entire ferulic acid of full fat rice bran as it was not present in the hexane extract of full fat rice bran (RBO). The oryzanol, tocol, and ferulic acid content of full fat rice bran for the varieties studied ranged from 2169-3473 ppm, 183-316 ppm and 66-166 ppm respectively.

Though not from a commercial point of view, oryzanols were identified in the $CHCl_3$-MeOH extracts of defatted bran-germ mixture, hull, and grain fractions of Indica and Japonica rice varieties by Ramarathnam *et al.* [66]. Panfili *et al.* had shown the presence of about 406 ppm of tocols (T & T_3) in

the cake oil of partially defatted wheat germ containing about 2% oil, whereas the wheat germ oil contained about 2287 ppm [151]. Also, Moreau *et al.* have offered a possible explanation for the heat induced increase in the levels of γ-T in corn hulls, suggesting that a significant amount of γ-T is bound to proteins or linked to phosphate or phospholipids and that heat breaks these bonds [183]. As ferulic acid is reported to exist mainly in the cell walls of plants [cited in 86], it could be assumed that ferulic acid is not amenable to hexane extract as practised in the industry. Besides, relatively higher polarity facilitates ferulic acid to remain in the defatted rice bran.

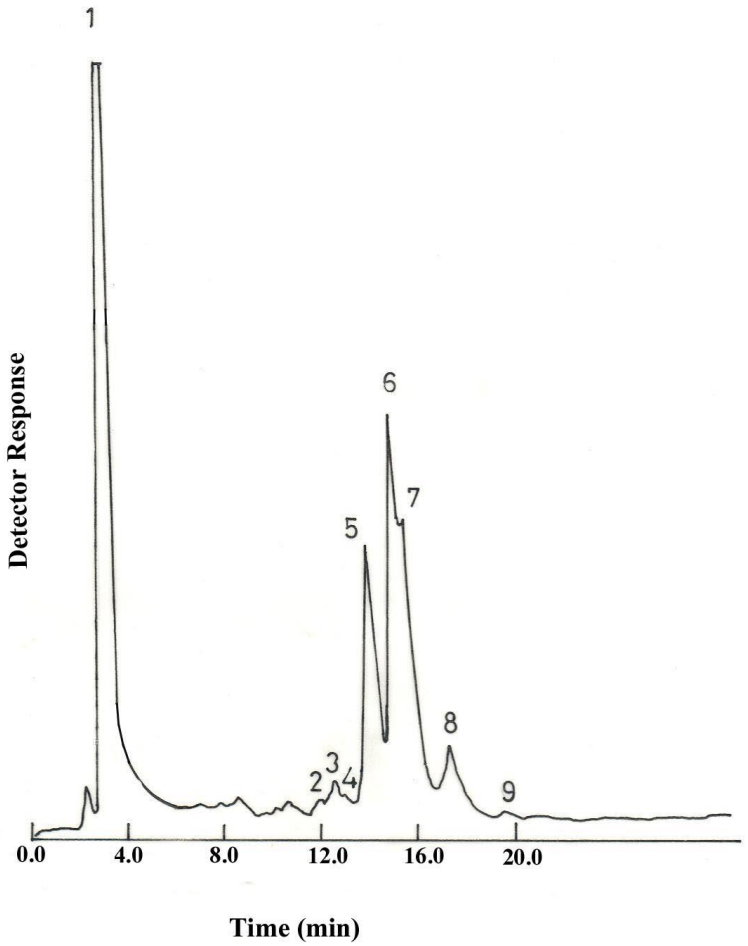


Fig.3.6. Reverse- phase HPLC/UV- Visible detection analysis of oryzanols and ferulic acid of defatted rice bran. Peak identification: 1, Ferulic acid; 2, Unidentified; 3, Stigmasteryl ferulate; 4, Unidentified; 5, Cycloartenyl ferulate; 6, 24-methylene cycloartanyl ferulate; 7, Campesteryl ferulate; 8, β-sitosteryl ferulate; 9, Cycloartanyl ferulate.

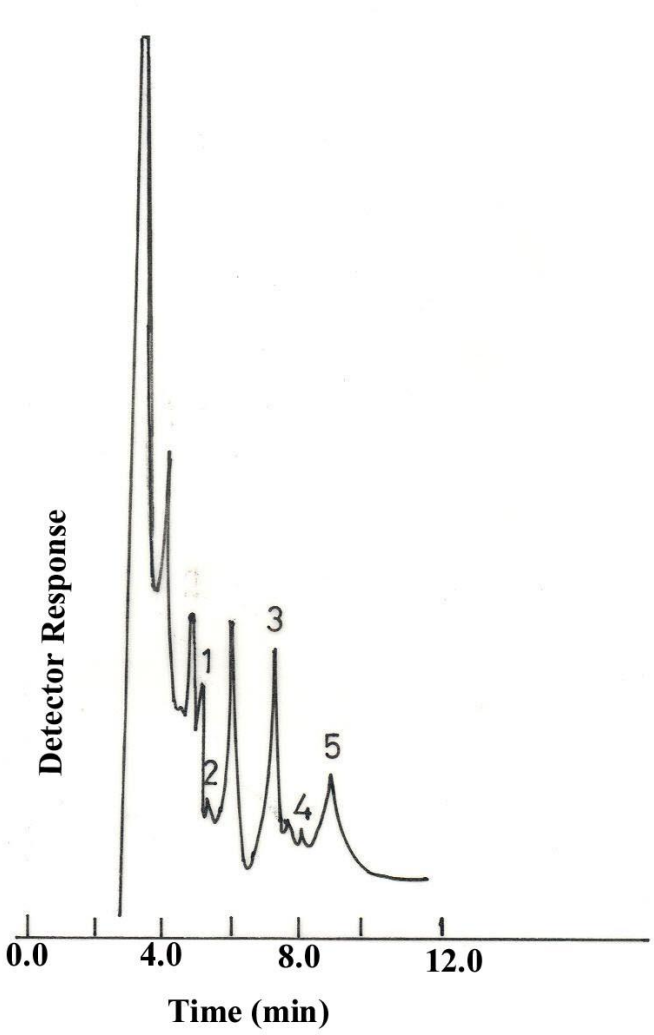


Fig.3.7. Normal- phase HPLC/UV- Visible detection analysis of tocopherols and tocotrienols of defatted rice bran. Peak identification: 1, α-T; 2, α-T_3; 3, γ-T_3; 4, δ-T; 5, δ-T_3.

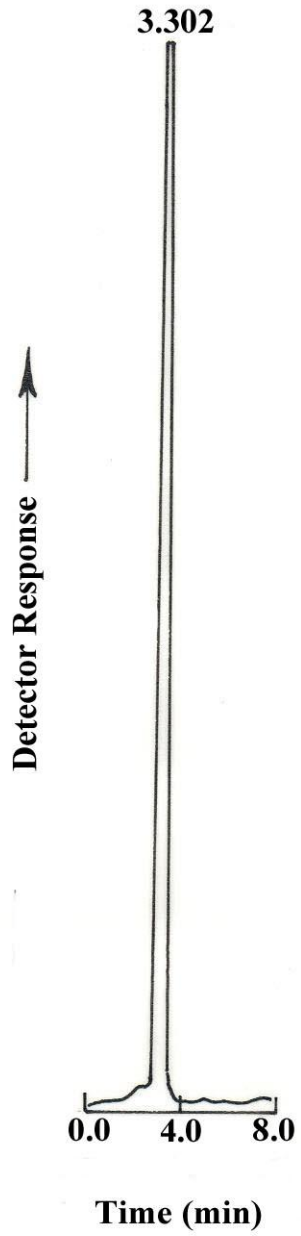


Fig.3.8. Reverse- phase HPLC/UV- Visible detection analysis of ferulic acid standard. Peak identification: 1, Ferulic acid.

Table. 3. 8. Distribution of Phytochemicals among Oil, Defatted Rice Bran (DRB), and Full Fat Rice Bran (FFB) from Different Varieties of Rice

Variety	Phytochemicals in ppm[a]		
	Oryzanols	Ferulic acid	Tocols
PTB 50			
Oil	19348	-	1044
DRB	212	111	7
FFB	3473	92	183
PTB 39			
Oil	13311	-	1603
DRB	146	201	6
FFB	2405	166	280
PTB 38			
Oil	13066	-	1243
DRB	200	146	3
FFB	2224	123	198
JAYA			
Oil	18309	-	1403
DRB	217	82	9
FFB	3052	69	227
MO 10			
Oil	12672	-	1652
DRB	176	81	5
FFB	2530	66	316
Oryza Nivara			
Oil	12648	-	1593
DRB	94	147	5
FFB	2169	122	268

[a] The values given against oil and defatted rice bran (DRB) are expressed on their respective dry weights. The values for full fat rice bran (FFB) are obtained by back calculation and summing of the values for oil and DRB.

3. 2. Extraction and enrichment of bio-active phytochemicals from DRB

The main objective of the present study was preparation of an antioxidant extract from defatted rice bran and to evaluate its efficacy using various *in-vitro* protocols. Kinetic studies were designed to select appropriate solvent and to optimize other process parameters like material-solvent ratio, time, temperature etc. The optimized conditions were then applied to DRB from the six rice varieties. The technique of sequential solvent extraction was used for preparing fractions enriched with phytochemicals from the crude extract. Column chromatographic separation was also employed to isolate and characterize individual compounds other than those discussed before viz. oryzanol, tocols and ferulic acid in the crude extract.

3. 2.1. Extraction kinetics of defatted rice bran

The first attempt was the selection of a suitable solvent for the extraction of phytochemicals from DRB. Once the solvent was fixed with respect to the content and yield of desired compounds in it, other parameters such as time of extraction, solvent-material ratio, temperature etc. could be optimized.

Selection of solvents: Defatted rice bran was extracted with conventional organic solvents used for plant materials. Solvents of different polarities like hexane, ethyl acetate, ethanol and methanol were used for soxhlet extraction as described elsewhere. The extracts were filtered through Whatman No.1 filter paper, evaporated to dryness at 50^{o}C with a rotavapor held under partial vacuum and weighed and were then analysed for total phenols (TPC), tocols, oryzanols and ferulic acid. TPC was measured using the standard colorimetric method. Oryzanols, tocols, and ferulic acid were analysed by HPLC procedures as described before.

Phytochemical profile of crude extracts obtained using different solvents: Solvents with varying polarities were used for direct extraction of DRB. The respective yields of extracts and their phytochemical composition are given in Table 3.9. The various extracts differed significantly in their contents of total extractives in terms of TPC, oryzanols and ferulic acid. Extract yield was maximum (4.9%) with ethanol and least with hexane (1%). The total phenolic content of the extracts ranged from 1% with hexane to 5.5% with methanol. The extracts also contained 3263 (hexane) to 7841ppm (methanol) oryzanols and the ferulic acid content of the extracts ranged from 421 (hexane) to 5782 ppm (methanol). Methanol was thus found to be more efficient with respect to yield of TPC, oryzanols and ferulic acid from DRB. The TPC, oryzanols and ferulic acid contents of DRB were 2204, 316, and 233 ppm respectively when methanol was used as the extractant.

It is reported that in general the amount of extractable substances decreased with decreasing polarity of the solvent [184]. The higher yield of extract with ethanol compared to methanol as observed here might be due to better extractability of soluble sugars with ethanol. Comparitive data on the phenolic content of rice by-products include those of rice husk (1590 ppm) and dehusked rice (25 ppm) by Sripriya *et al.* [69]. Also Ramarathnam *et al.* have reported the total phenolic content of the $CHCl_3$-MeOH extracts of long-life and short-life rice seed hulls to be 295-390 ppm and 190-240 ppm respectively [66].

Polarity of the solvent also affected the extractability of oryzanols and tocols from DRB, with the extractability increasing with increasing polarity of the solvent. According to Chen *et al.* the better extractability of oryzanols and tocols with IPA and methanol compared to hexane could be explained as due to the hydroxyl group of the chroman rings of vitamin E homologs and on

the benzene rings of ferulate esters that might make these compounds more extractable in alcohol than in hexane [166].

Solvent extraction has been established as an efficient method for the removal of free phenolic acids from cereal grains [185]. The phenolic acids of defatted alfalfa, cabbage and spinach extracted with 80% methanol were reported to contain only free or loosely bound acids as methanol extraction would not break ester bonds [186]. Comparative data on the ferulic acid contents of rice and other cereals include 1 ppm in rice grains [89], 2 ppm in defatted rice flour [187], 7640 ppm in defatted durum wheat bran extract [188], 2 to 4 ppm in wheat kernels [189], and 520 ppm in defatted wheat bran [186]. Free ferulic is also reported in rice hulls [190], rice bran [cited in 191and 192], defatted meals of rapeseed and white mustard flours [193], meal of developing barley seeds [194], and in defatted flours of wheat, oats and corn [187]. Thus based on the solubility of the phytochemicals of interest, methanol was selected for subsequent studies to obtain an antioxidant extract from DRB.

Table. 3. 9. Solubility of Bio-active Phytochemicals in Different Solvents*

Solvent	Extract (ppm of DRB)	TPC		Oryzanols		Ferulic acid	
		DRB (ppm)	Extract (ppm)	DRB (ppm)	Extract (ppm)	DRB (ppm)	Extract (ppm)
Hexane	10021[a] (±102)	98[a] (±1)	10012[a] (±108)	31[a] (±1)	3263[a] (±31)	4[a] (±1)	421[a] (±6)
Ethyl acetate	14983[b] (±110)	290[b] (±4)	19035[b] (±116)	59[b] (±1)	3907[b] (±37)	35[b] (±1)	2318[b] (±18)
Ethanol	49026[d] (±223)	1764[c] (±12)	36024[c] (±200)	236[c] (±2)	4777[c] (±46)	148[c] (±1)	2996[c] (±31)
Methanol	40037[c] (±200)	2204[d] (±18)	55027[d] (±220)	316[d] (±4)	7841[d] (±60)	233[d] (±2)	5782[d] (±46)

***10 g DRB was soxhlet extracted with 150 ml solvent for 12 hours. Each value in the table represents the mean (± standard deviation) of four analyses from 2 replications.

[a-d]means within a column with different letters are significantly different ($P< 0.05$) according to DMRT.

Optimisation of material-solvent ratio, temperature and time: In order to optimize parameters that influence the solubility and the yield of phytochemicals, studies were conducted under varying extraction conditions. Thus time course extraction using DRB-methanol ratio of 1:5, 1:10, 1:15, and 1:20 was conducted and the results are presented in Table 3.10 and Fig.3.9. A DRB to methanol ratio of 1:15 was found to be optimum (Table 3.10) and soxhlet extraction (60-70^{o}C) was preferred to ambient temperature (25-30^{o}C) extracion.

Extraction time also influenced the yield of the extract as well as the extractability of phytochemicals. About 75% of the total extract was obtained in the first one hour of extraction and about 97%, by 5th hour. During the next

3 hours, the rate of increase was slow and no further increase was observed after 9 hours (Fig.3.9A). However only about 53% of the total phenolics was obtained in the first one hour and thereafter it increased sharply to 95% by 5th hour and subsequently it was not significant (Fig.3.9B). In the case of ferulic acid, 78% was extracted in the first one hour, reaching 97% by 6th hour (Fig.3.9C). Oryzanols were extracted at faster rate with more than 80% in the first one hour and 97% by 4th hour (Fig.3.9D). Based on these results, the time of extraction was fixed as 10 hours to insure near 100% extraction of phytochemicals for subsequent studies.

Table. 3.10. Optimisation of Solvent-Material ratio (SMR) for the Extraction of Bioactive Phytochemicals from Defatted Rice Bran*

Weight of DRB (g)	Extract (ppm of DRB)	TPC		Oryzanols		Ferulic acid	
		DRB (ppm)	Extract (ppm)	DRB (ppm)	Extract (ppm)	DRB (ppm)	Extract (ppm)
5	45024^{c} (±218)	2552^{d} (±24)	57011^{d} (±242)	340^{c} (±8)	7640^{c} (±60)	258^{c} (±4)	5798^{c} (±57)
10	44038^{b} (±225)	2376^{c} (±19)	54016^{c} (±216)	335^{c} (±4)	7579^{c} (±66)	254^{c} (±3)	5773^{c} (±60)
15	39061^{a,b} (±136)	1879^{b} (±17)	49021^{b} (±139)	259^{b} (±3)	6692^{b} (±58)	189^{b} (±2)	4884^{b} (±46)
20	37044^{a} (±121)	1366^{a} (±16)	37044^{a} (±111)	210^{a} (±3)	5615^{a} (±46)	146^{a} (±2)	3904^{a} (±37)

***** DRB was soxhlet extracted with 150 ml solvent for 12 hours. Each value in the table represents the mean (± standard deviation) of four analyses from 2 replications. $^{a-d}$means within a column with different letters are significantly different ($P< 0.05$) according to DMRT.

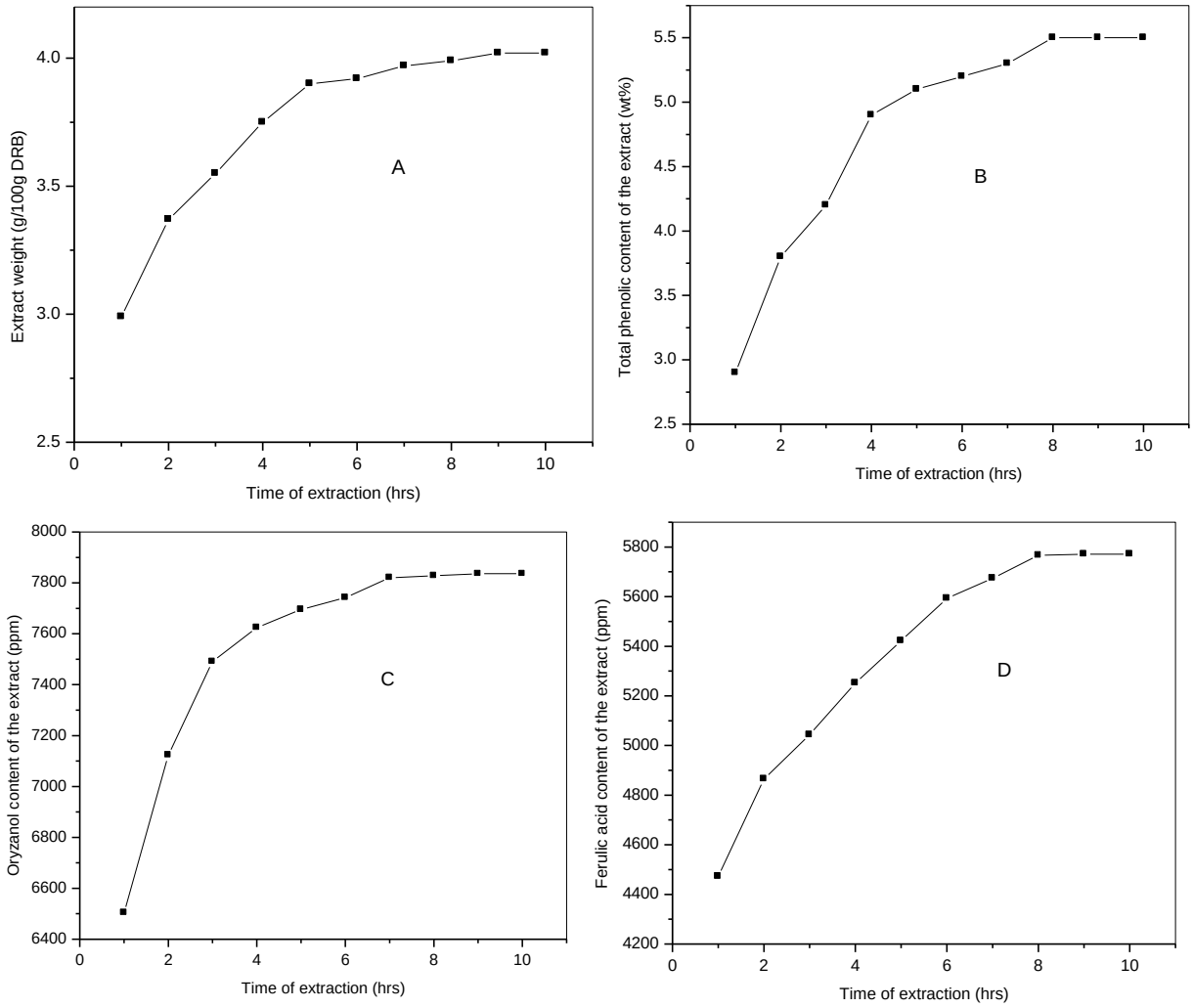


Fig. 3. 9. Effect of time of extraction on the yield of extract from DRB (A) and on the contents of TPC (B), oryzanols (C) and ferulic acid (D) in the methanolic extract.

Phytochemical profile of extracts of DRB from different rice varieties: The optimized extraction conditions for DRB as described before were applied to the six rice varieties. The chemical characteristics and phytochemical compositions of the extracts are given in Tables 3.11 and 3.12 respectively. The yield of the extract ranged from 3.2% (MO 10) to 5.0% (PTB 38). The sugar content of the extracts ranged from 18.8 to 33.8 %, protein 18.0 to 25.0%, TPC 5.3 to 8.4 %, and ash 3.9 to 5.1%.

The oryzanol content of the methanol extracts ranged from 2358 to 6602 ppm, ferulic acid from 2541 to 4376 ppm and tocols from 110 to 284 ppm. Significant varietal variations therefore were observed in the contents of all these phytochemicals, with JAYA having the highest oryzanol and tocol contents and PTB 39, the highest amount of ferulic acid. The detailed oryzanol and tocol profiles of the varieties are given in Tables 3.13 and 3.14 respectively. 24-methylene cycloartanyl ferulate (~45%) and cycloartenyl ferulate (~25%) represented the major oryzanols of the DRB extracts and other identified forms were campesteryl ferulate, β-sitosteryl ferulate, stigmasteryl ferulate and cycloartanyl ferulate. γ-tocotrienol (~70%) and α-tocopherol (~10%) represented the major chromanols and other identified homologs were α-T_3, δ-T_3 and δ-T.

It is assumed that oryzanols, tocols, and ferulic acid in full fat rice bran (FFB) are distributed between oil and DRB. On an average, defatting of rice bran gave 20% oil and 80% defatted meal. The methanol extractives of DRB was around 4%. The oryzanol, tocol, and ferulic acid content of FFB as computed from the values obtained for oil (Table 3.6 and Table 3.7) and DRB (Table 3.12) for the varieties ranged from 2169-3473 ppm, 183-316 ppm, and 66-166 ppm respectively (Table 3.8). The corresponding amounts in DRB were 94-217 ppm, 3-9 ppm and 81-201ppm, respectively. The variety PTB 50 gave the highest oryzanol content in RBO and FFB whereas the variety Jaya gave the highest oryzanol content in DRB and methanolic extract. Similarly, the variety MO 10 had the highest tocol content in RBO and FFB

whereas the variety Jaya gave the highest tocol content in DRB and methanolic extract. Rice bran oil (RBO) thus had 92-96% of total oryzanols and 97-99% of total tocols of the FFB. On an average, FFB had about 16 times oryzanols and 55 times tocols than those in DRB whereas DRB contained the entire ferulic acid in FFB. As compared to DRB, the methanolic extract could be called as phytochemical concentrate as it had about 25 times oryzanols and ferulic acid and about 28 times tocols than those in DRB.

Table.3.11. Chemical Characteristics of Methanolic Extracts of DRB from Different Rice Varieties

Variety	Extract (wt% of bran)	Chemical constituents (wt% of the extract)			
		Sugar	Protein	TPC	Ash
PTB 50	4.3^{c} (±0.1)	33.8^{e} (±0.5)	18.0^{a} (±0.3)	$5.5^{a,b}$ (±0.2)	5.1^{c} (±0.2)
PTB 39	4.6^{d} (±0.1)	18.8^{a} (±0.6)	21.6^{b} (±0.7)	$5.9^{b,c}$ (±0.4)	3.9^{a} (±0.4)
PTB 38	5.0^{e} (±0.1)	19.2^{a} (±0.4)	18.0^{a} (±0.5)	6.1^{c} (±0.4)	5.1^{c} (±0.4)
JAYA	3.3^{a} (±0.2)	28.9^{d} (±0.5)	21.5^{b} (±0.8)	$5.6^{a,b}$ (±0.4)	4.5^{b} (±0.3)
MO 1O	3.2^{a} (±0.1)	25.7^{c} (±0.5)	25.0^{c} (±0.5)	8.4^{d} (±0.3)	4.4^{b} (±0.3)
Oryza Nivara	4.0^{b} (±0.2)	22.3^{b} (±0.4)	21.9^{b} (±0.3)	5.3^{a} (±0.2)	4.7^{b} (±0.2)

[a]Each value in the table represents the mean (±standard deviation) of six analyses from 3 replications.

[a-c] means within a column with different letters are significantly different ($P < 0.05$) according to DMRT.

Table. 3.12. Phytochemical Composition of Defatted Rice Bran and Their Methanolic Extracts from Different Varieties of Rice

Variety	Phytochemicals in ppm[a]					
	Oryzanols		Ferulic acid		Tocols	
	Extract	DRB	Extract	DRB	Extract	DRB
PTB 50	4952[d] (± 53)	212[e] (±2)	2585[b] (± 23)	111[b] (±1)	163[d] (± 5)	7[e] (± 1)
PTB 39	3199[b] (± 37)	146[b] (± 3)	4376[e] (± 38)	201[d] (±1)	146[c] (± 5)	6[d] (±1)
PTB 38	4003[c] (± 56)	200[d] (± 2)	2933[c] (± 34)	146[c] (±1)	110[a] (± 5)	3[a] (± 1)
JAYA	6602[f] (± 71)	217[f] (± 2)	2541[a] (± 23)	82[a] (± 1)	284[e] (± 6)	9[f] (±1)
MO 1O	5500[e] (± 62)	176[c] (± 1)	2546[a] (± 26)	81[a] (± 1)	117[b] (± 2)	5[b] (±1)
Oryza Nivara	2358[a] (± 32)	94[a] (± 1)	3704[d] (± 33)	147[c] (±1)	146[c] (± 6)	5[c] (±1)

[a]Each value in the table represents the mean (± standard deviation) of six analyses from 3 replications.

[a-f]means within a column with different letters are significantly different ($P < 0.05$) according to DMRT.

Table. 3.13. Oryzanol Profile of Methanolic Extracts of Defatted Rice Bran from Different Rice Varieties

	Oryzanols* in ppm[a]								
Variety	1	2	3	4	5	6	7	8	Total
PTB 50	62[d] (±2)	134[e] (±3)	29[b] (±1)	1281[d] (±14)	2699[d] (±22)	300[a] (±4)	444[d] (±4)	Tr	4952[d] (±53)
PTB 39	26[c] (±2)	122[d] (±3)	41[c] (±1)	757[b] (±7)	1058[a] (±12)	833[e] (±7)	359[b] (±4)	Tr	3199[b] (±37)
PTB 38	23[b] (±1)	82[b] (±2)	28[b] (±1)	1133[c] (±12)	1729[c] (±19)	583[d] (±15)	422[c] (±6)	Tr	4003[c] (±56)
JAYA	78[e] (±2)	238[f] (±4)	50[d] (±2)	1912[e] (±20)	3398[f] (±30)	378[c] (±5)	545[e] (±7)	Tr	6602[f] (±71)
MO 1O	63[d] (±2)	106[c] (±3)	10[a] (±1)	1274[d] (±12)	3028[e] (±30)	337[b] (±4)	680[f] (±8)	Tr	5500[e] (±62)
Oryza Nivara	11[a] (±1)	24[a] (±1)	11[a] (±1)	631[a] (±7)	1115[b] (±12)	369[c] (±6)	194[a] (±3)	Tr	2358[a] (±32)

*Oryzanols: 1,Unidentified; 2, Stigmasteryl ferulate; 3, Unidentified; 4, Cycloartenyl ferulate; 5, 24-methylene cycloartanyl ferulate; 6,Campesteryl ferulate; 7, β-sitosteryl ferulate; 8,Cycloartanyl ferulate.

[a] Each value in the table represents the mean (± standard deviation) of six analyses from 3 replications.

[a-f]means within a column with different letters are significantly different ($P < 0.05$) according to DMRT.

Table.3.14. Tocol Profile of Methanolic Extracts of DRB from Different Rice Varieties

Variety	Tocopherols and tocotrienols in ppm[a]								
	αT	αT_3	βT	βT_3	γT	γT_3	δT	δT_3	Total
PTB 50	18^{d} (±1)	15^{b} (±1)	nd	nd	nd	116^{e} (±2)	4^{c} (±1)	9^{c} (±1)	163^{d} (±5)
PTB 39	12^{b} (±1)	13^{a} (±1)	nd	nd	nd	109^{d} (±2)	3^{b} (±1)	$7.^{b}$ (±1)	146^{c} (±5)
PTB 38	$16^{c,d}$ (±2)	16^{b} (±1)	nd	nd	nd	65^{a} (±2)	4^{c} (±1)	7^{b} (±1)	110^{a} (±5)
JAYA	15^{c} (±2)	27^{c} (±1)	nd	nd	nd	236^{f} (±3)	2^{a} (±1)	3^{a} (±1)	284^{e} (±6)
MO 1O	$10^{a,b}$ (±1)	12^{a} (±1)	nd	nd	nd	81^{b} (±2)	4^{c} (±1)	10^{d} (±1)	117^{b} (±2)
Oryza Nivara	$11^{a,b}$ (±1)	16^{b} (±2)	nd	nd	nd	105^{c} (±3)	4^{c} (±1)	9^{c} (±1)	146^{c} (±6)

[a] Each value in the table represents the mean (± standard deviation) of four analyses from 2 replications.
[a-f]means within a column with different letters are significantly different ($P < 0.05$) according to DMRT.
nd: not detected

3. 2. 2. Enrichment of bio-active phytochemicals from defatted rice bran

Fractionation steps as shown in Fig.3.10 were designed to get an extract enriched with beneficial phytochemicals from crude methanolic extract (CME). For this, the CME was re-extracted with less polar organic solvents like ethyl acetate, acetone, ether etc. The extracts were pooled, filtered through Whatman No.1 filter paper and the solvent was removed in rotavapor. The dry residues were weighed, redissolved in methanol and analysed for TPC, oryzanols, ferulic acid and tocols. The results are tabulated in Table 3.15.

There were no significant variations in the recovery percentages (based on CME) among acetone, ethyl acetate and ether (Table 3.15). The selection of these solvents was based on the solubility of targeted compounds in it. Acetone was found to be the best solvent for ferulic acid and tocols and ether for oryzanols. Re-extraction with acetone was able to reduce the amounts of some of the interfering compounds like sugar and protein in the resultant extracts (Table 3.16). Also the resultant acetone extract (AE) was enriched more than 1.5 times in TPC, oryzanols, ferulic acid and tocols as compared to CME (Table 3.15). Based on these results acetone was selected to enrich the phytochemicals at the first stage.

For further purification of the acetone extract, sequential extraction technique was employed. The main objective was to get subfractions more enriched in one or more of the desired bioactive compounds. For this, the dry AE was re-extracted with hexane to give a soluble fraction enriched in lipophilic compounds (AE-LP) and a residue enriched in polar compounds (AE-PP). The dry fractions were weighed, redissolved in methanol and analysed for fat, sugar, protein and minerals (Table 3.16). The TPC, oryzanols, ferulic acid and tocol contents of the fractions are given in Table 3.17.

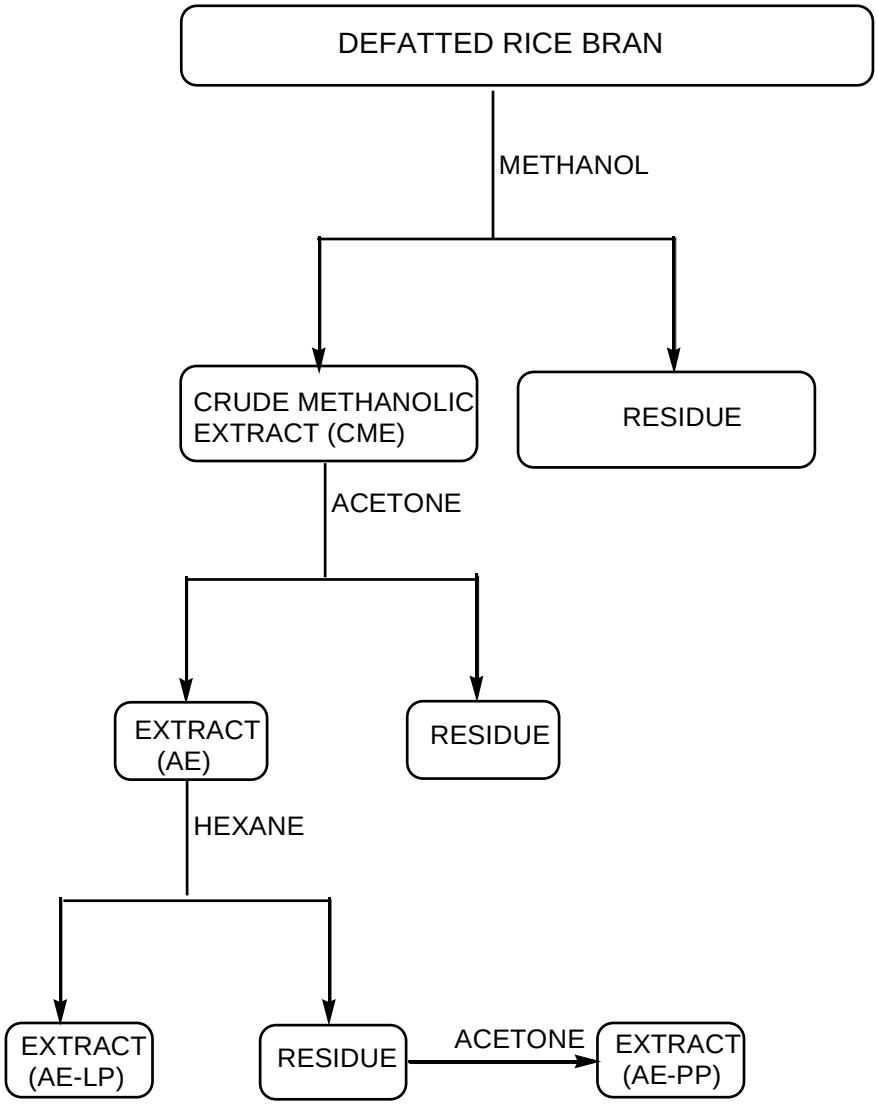


Fig.3.10. Preparation and fractionation of an antioxidant extract from DRB

Table. 3.15. Phytochemical Constituents at the First Stage of Fractionation of Crude Methanolic Extract Using Different Solvents

Solvent	Recovery (%)*	Constituents in the extract[a]			
		TPC (wt %)	Oryzanols (ppm)	Ferulic Acid (ppm)	Tocols (ppm)
Methanol	_	5.5 [a] (±0.2)	7832[a] (±57)	5786 [b] (±33)	146[a] (±2)
Acetone	47.7 [a] (±1.8)	10.7 [d] (±0.3)	14697 [b] (±102)	9204 [d] (±66)	260 [d] (±3)
Ethyl Acetate	46.6 [a] (±1.2)	8.8 [c] (±0.3)	14745 [b] (±108)	7396 [c] (±60)	218 [b] (±3)
Ether	46.1 [a] (±1.4)	7.4 [b] (±0.3)	16270 [c] (±89)	3260[a] (±21)	226 [c] (±3)

[a]Each value in the table represents the mean (± standard deviation) of four analyses from 2 replications.

[a-d]means within a column with different letters are significantly different ($P < 0.05$) according to DMRT.

*Recovery percentages are based on crude methanolic extract.

From Tables 3.16 and 3.17, it could be seen that AE-LP was enriched in fat, oryzanols and tocols more than double as compared to those in CME with lower content of TPC, ferulic acid, sugar, protein and minerals. On the other hand, AE-PP was enriched in TPC and ferulic acid around 3 times compared to those in CME with lower levels of oryzanols, tocols, fat, sugar, protein and minerals.

Table. 3.16. Chemical Constituents at the Second Stage of Sequential Extraction of Crude Methanolic Extract

Extracts*	Recovery (%)**	Chemical constituents in the extract[a]			
		Fat (wt %)	Sugar (wt %)	Protein (wt %)	Minerals (wt %)
CME	_	7.5 [b] (±0.2)	24.4 [c] (±0.9)	22.0 [c] (±0.8)	3.9[d] (±0.1)
AE	47.7 [c] (±1.8)	12.1 [c] (±0.1)	15.1 [b] (±0.9)	13.4 [b] (±0.7)	1.3[c] (±0.1)
AE-LP	26.6 [b] (±0.9)	18.4 [d] (±0.2)	13.6 [a] (±0.7)	12.8 [b] (±0.6)	0.4[a] (±0.1)
AE-PP	19.9 [a] (±0.8)	2.8 [a] (±0.1)	14.9 [b] (±0.4)	8.6 [a] (±0.3)	0.8 [b] (±0.1)

[a]Each value in the table represents the mean (± standard deviation) of four analyses from 2 replications.

[a-d]means within a column with different letters are significantly different ($P < 0.05$) according to DMRT.

*CME: Crude Methanolic Extract; AE: Acetone Extract; AE-LP: Acetone Extract-Lipophilic Phase; AE-PP: Acetone Extract –Polar Phase.

** Recovery percentages are based on CME. For AE-LP & AE-PP, recovery percentages based on AE are 55.8 (±1.9) & 41.8 (±1.8) respectively.

Table. 3.17. Phytochemical Constituents at the Second Stage of Sequential Extraction of Crude Methanolic Extract

Extracts*	Recovery (%)**	Phytochemical constituents in the extract[a] TPC (wt %)	Oryzanols (ppm)	Ferulic acid (ppm)	Tocols (ppm)
CME	_	5.5 [b] (±.0.2)	7832[b] (±57)	5786 [b] (±33)	146 [b] (±2)
AE	47.7 [c] (±1.8)	10.7 [c] (±0.3)	14697 [c] (±102)	9204 [c] (±66)	260 [c] (±3)
AE-LP	26.6 [b] (±0.9)	4.8 [a] (±0.2)	20469 [d] (±129)	944 [a] (±8)	347 [d] (±4)
AE-PP	19.9 [a] (±0.8)	17.7 [d] (±0.4)	978 [a] (±9)	15858 [d] (±113)	51 [a] (±1)

[a]Each value in the table represents the mean (± standard deviation) of four analyses from 2 replications.

[a-d]means within a column with different letters are significantly different ($P < 0.05$) according to DMRT.

*CME: Crude Methanolic Extract; AE: Acetone Extract; AE-LP: Acetone Extract-Lipophilic Phase; AE-PP: Acetone Extract –Polar Phase.

** Recovery percentages are based on CME. For AE-LP & AE-PP, recovery percentages based on AE are 55.8 (±1.9) & 41.8 (±1.8) respectively.

Considering the bioactive compounds of interest, AE-LP was enriched in oryzanols and tocols and AE-LP in ferulic acid (Fig.3.11). Of the various extracts and fractions, AE-PP had significantly higher quantities of TPC and ferulic acid, AE-LP that of fat, oryzanols and tocols, and CME the highest quantities of sugar, protein and minerals. Thus the fractionation procedures employed here was able to reduce the contents of some of the interfering compounds like sugar, protein, minerals etc. and enhanced the amounts of beneficial compounds like oryzanols, tocols, ferulic acid etc. in the resultant extracts.

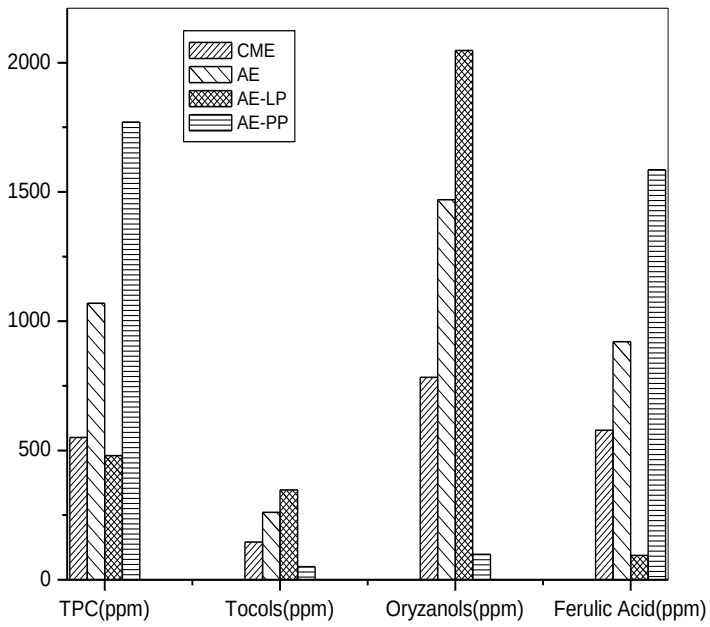


Fig. 3.11. Effect of fractionation on the enrichment of TPC, oryzanols, ferulic acid and tocols in the resultant extracts and fractions. For TPC, 1/100th of the respective value and for oryzanols and ferulic acid, 1/10 th each of the respective values are given for the sake of convenience of drawing.

The crude extract as well as the fractions could therefore be called as phytochemical concentrates (Table 3.17) as compared to their contents in DRB (Table 3.9). CME thus had TPC, oryzanols, ferulic acid and tocols more than 25 times than those in DRB. The acetone extract was far more concentrated by more than 55 times in TPC, 45 times in oryzanols, 40 times in ferulic acid, and 45 times in tocols than those in DRB. While the lipophilic phase of AE (AE-LP) had more than 20 times TPC, 65 times oryzanols, 4 times ferulic acid and 65 times tocols, the polar phase of AE (AE-PP) had more than 90 times TPC, 2.5 times oryzanols, 70 times ferulic acid and 8 times tocols as compared to those in DRB.

3. 2. 3. Isolation and characterisation of other compounds in CME

Experiments were designed to isolate and characterize individual compounds in the crude methanolic extract (CME). For this, CME was subjected to silica gel column chromatography with gradient elution using petroleum ether : ethyl acetate in the order of increasing polarity and fractions of 100 ml were collected and concentrated. The fractions were then grouped according to their TLC pattern. Pure compounds were obtained in fractions termed I and III eluted with petroleum ether : ethyl acetate in the ratios of 80:20 and 50:50 respectively. Compound I (obtained from fraction I) was crystalised from petroleum ether and compound II (obtained from fraction III) was crystallized from ethyl acetate. The compounds were then characterized by UV, IR , NMR and MS techniques.

Structrual Analysis of Compound I : In the IR spectrum of the compound, peaks at 3438.3 cm^{-1}, 2946.6 cm^{-1}, and 1639.5 cm^{-1} were due to –OH strech, –CH strech of CH_3 and CH_2 groups and –CH stretch of –CH=CH respectively. In the ^1H NMR spectrum, characteristic signals were observed at δ 3.5 (1H, m) and at δ 5.3 (1H, d) which were due to the protons on carbon-3 and carbon-6 (olefinic proton) respectively. Normal tertiary methyls showed peak at δ 0.68 (3H,s,18 Me), secondary methyls at δ 0.91 (3H, d, 21 Me) and at δ 1.0 (6H, d, 26 & 27 Me) and primary methyls at δ 0.86 (3H, t, 29Me). Peak at δ 1.9 (2H, t)

showed the presence of allylic proton (on carbon-7), and that on carbon-4 was observed at δ 1.86 (2H, d). The CH_2 protons on carbon-2 were observed at δ 2.0 (2H, m). The ^{13}C NMR spectrum showed the signals for the 29 carbon atoms which were sorted out by DEPT experiments into CH_2 (carbons 1, 2, 4, 7, 11, 12, 15, 16, 22, 23, and 28), CH (3, 6, 8, 9, 14, 17, 20, 24, 25) and into CH_3 (19, 18, 21, 26, 27 and 29) and the remaining signals on ^{13}C were due to the quartenary carbons 5, 10 and 13. In the EI-MS, the molecular ion was observed at m/z 414. Based on the spectral data, the compound was identified as β-sitosterol (Figure 3.12).

Structural Analysis of Compound II : In TLC, the compound showed a single spot which was yellow in colour on spraying with 10% methanolic sulphuric acid followed by charring at 100^oC. It also gave characteristic flavonoid colour reactions, i.e. pink with Mg turnings and con. HCl. The UV spectrum of the compound exhibited absorption maxima typical of flavones at 245 nm and 349 nm. The spectra before and after addition of shift reagents indicated that they were characteristic of flavonoid components showing bathochromic shifts with the reagents due to the presence of phenolic OH groups in their A and B rings (Fig. 3.13). The spectral λmax values in MeOH are 245 (sh), 270, 302(sh), and 349; +NaOMe 264, 277(sh), 329, and 418; +$AlCl_3$ 257, 275, 309, 364 and 396; +$AlCl_3$/HCl 254, 279, 307 and 361; +NaOAc 263, 277(sh), 321(sh), 416; +NaOAc/H_3BO_3 269, 350. The bathochromic shift of band I with NaOMe without a decrease in intensity showed the presence of a free 4'-hydroxyl 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-hydroxyl group.

In the IR spectrum of the compound, the band at 1662 cm^{-1} belonged to the conjugated keto group and the band at 3332 cm^{-1} showed the presence of hydroxyl groups (Fig. 3.14).

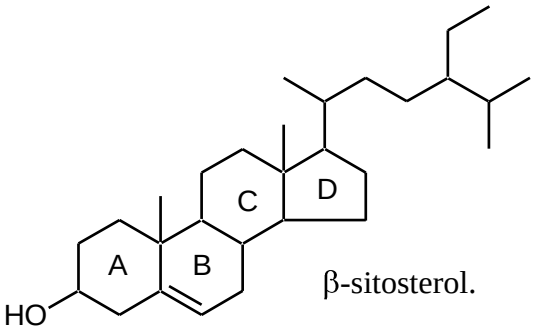
In the ^{1}H NMR spectrum of the compound in acetone-d_6 (Fig. 3.15), the aromatic protons of the A ring appeared as meta related doublets (J=2 Hz), at δ 6.27 (H-6, d, J = 2.0 Hz) and at δ 6.58 (H-8, d, J = 2.0 Hz) while those of the B ring appeared at δ 7.41 (H-2' and H-6', s). The proton on ring C was observed at δ 6.77 (H-3, s). The singlet at δ 3.98 (6H, s) suggested the presence of two methoxy (-OMe) groups. The singal at δ13.04 was typical for a C-5 hydrogen-bonded hydroxyl group. Two very weak signals observed at δ 8.21 and δ 9.70 represented the two hydroxyl groups on carbons 4'and 7.

In the ^{13}C NMR spectrum (Fig. 3.16), the carbonyl carbon, C-4, resonated at δ 182.2 and the methoxyl carbon at δ 56.0 ppm. The signals at δ 148.2 (C-3'= C-5') and at δ140.0 (C–4') corresponded to the aromatic oxygenated carbon atoms with ortho/para (O/P) oxygenation. The four signals at δ values of 164.1, 157.9, 163.9 and 162.4 represented the four aromatic oxygenated carbon atoms without O/P oxygenation (C-2, C-5, C-7 and C-9). The other six signals which appeared at δ values of 103.7, 98.8, 94.0, 104.3, 121.4 and 104.4 corresponded to the aromatic nonoxygenated carbons with O/P oxygenation (C-3, C-6, C-8, C-10, C-1' and C-2'= C-6').

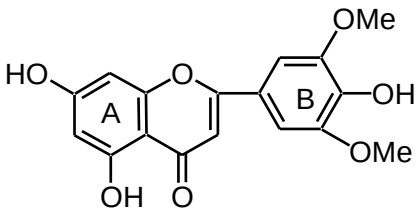
A molecular ion at m/z 330 (M^{+}) was detected by EI-MS analysis (Fig. 3.17). The melting point of the compound was found to be 267-268^{o}C. Elemental analysis gave the values of 61.30% for C and 3.82% for H which agreed closely with the calculated values.

On the basis of the UV spectral data, IR, ^{1}H NMR, ^{13}C NMR and EI-MS, the compound was identified as tricin (Figure 3.12) which is of special interest phytochemically because of its rare occurrence [195]. In the rice plant (*oryza sativa*), it has been previously reported to be present in the leaves [195] and in rice husk [196]. It was also reported in other cereals like oats [197] and Japanese barnyard millet [198]. Based on the spectral data, the compound was identified as tricin (Figure 3.12) which has not been reported in rice bran before.

The crude extract, enriched fractions, and the pure compounds were subsequently used for their antioxidant activity assays using standard *in-vitro* models.



β-sitosterol.



Tricin

Fig.3.12. Chemical structures of compound 1 (β-sitosterol) and compound 2 (tricin).

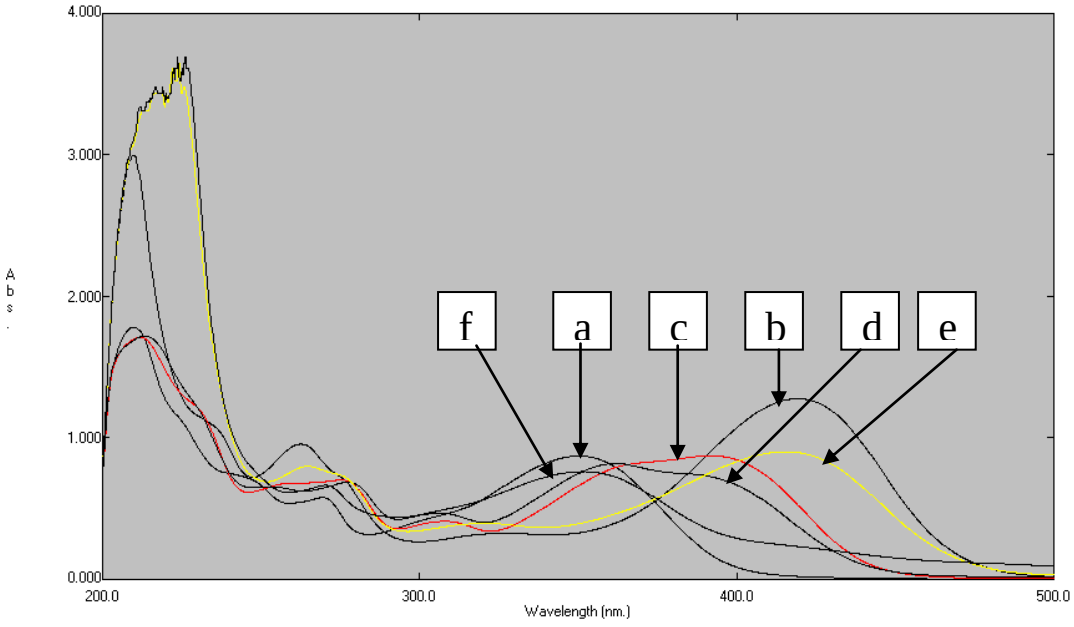


Fig.3.13. UV spectrum of compound 2 with various shift reagents. a: MeOH ; b: +NaOMe; c: +$AlCl_3$; d: +$AlCl_3$/HCl ; e: +NaOAc; f: +NaOAc/H_3BO_3.

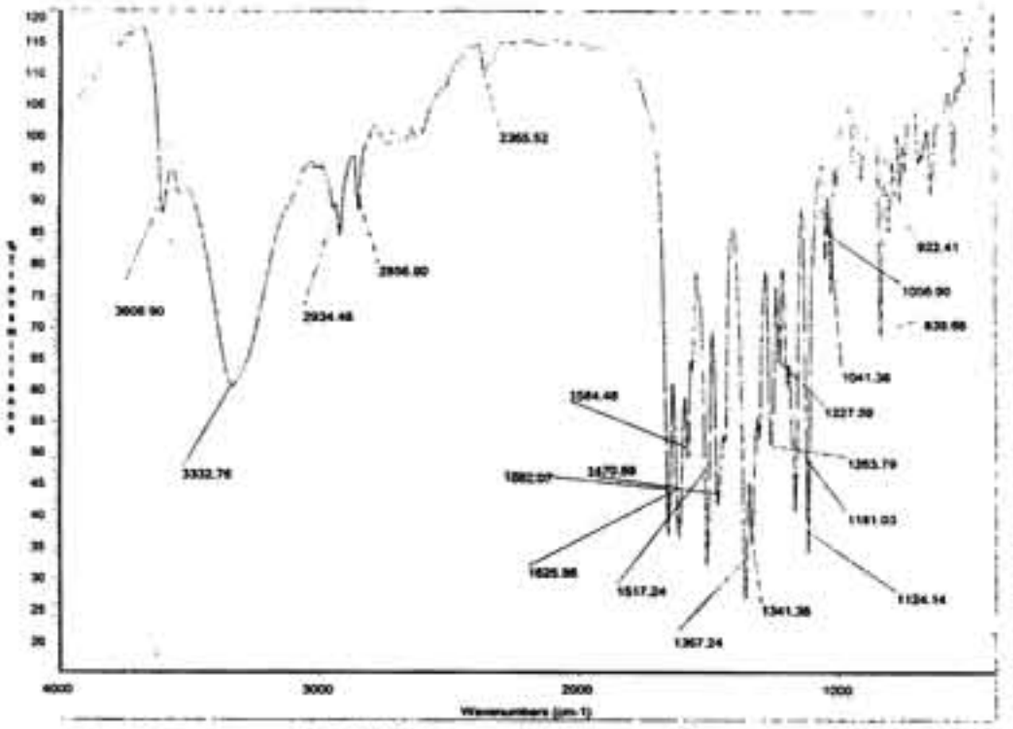


Fig. 3.14. IR spectrum of compound 2

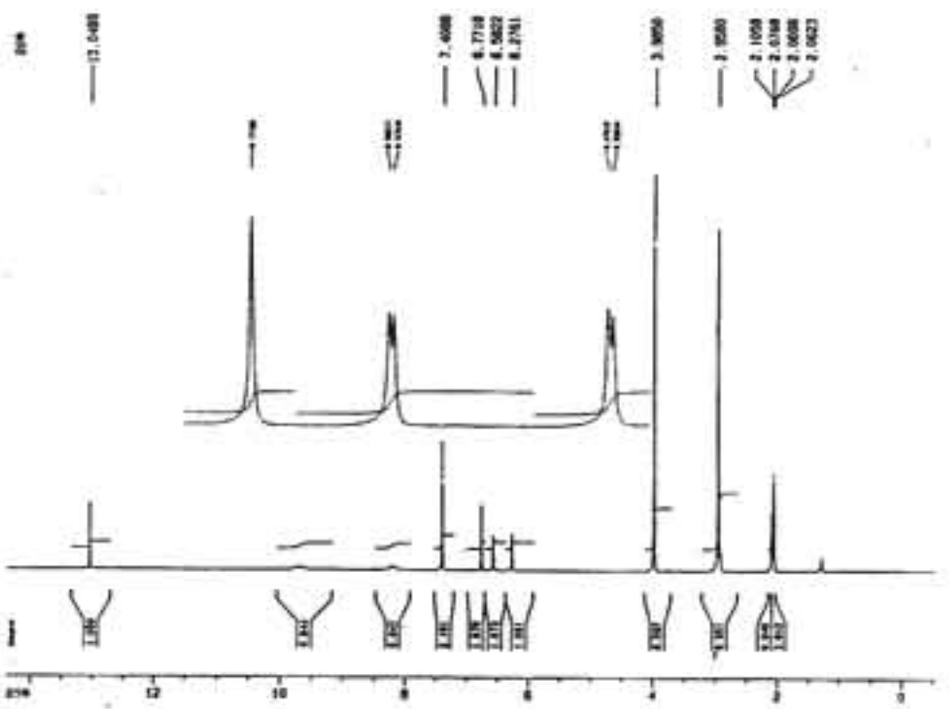


Fig. 3.15. ^{1}H NMR spectrum of compound 2

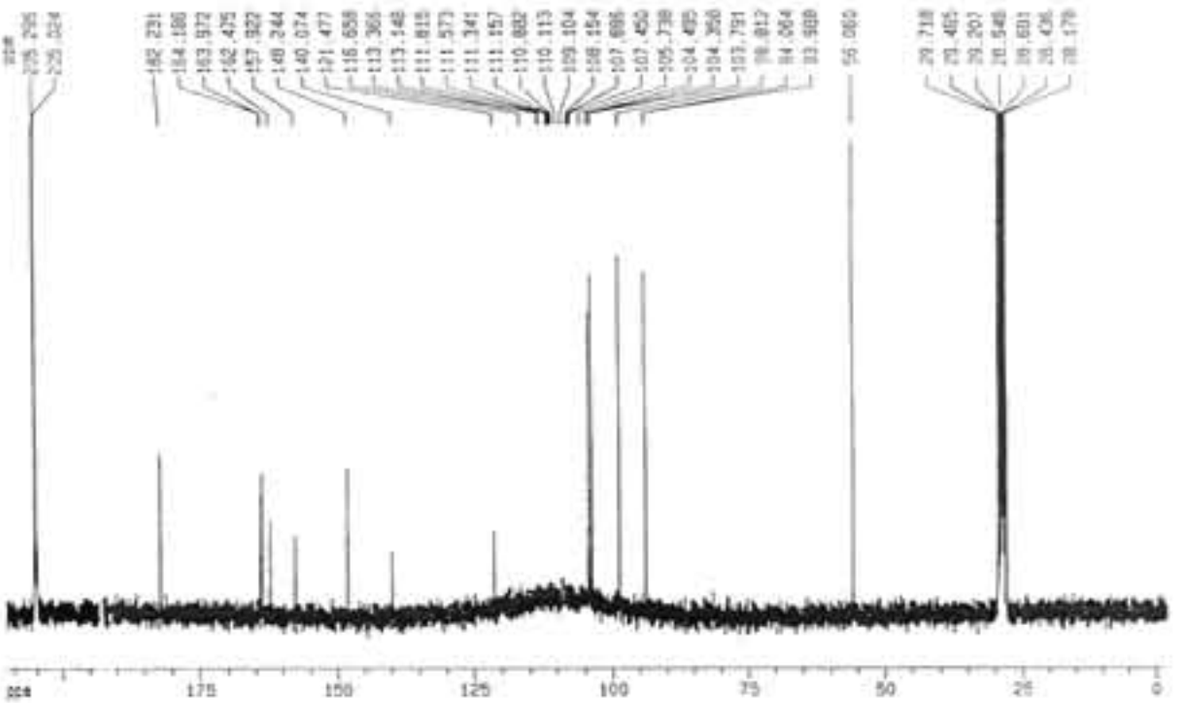


Fig. 3.16. ^{13}C NMR spectrum of compound 2

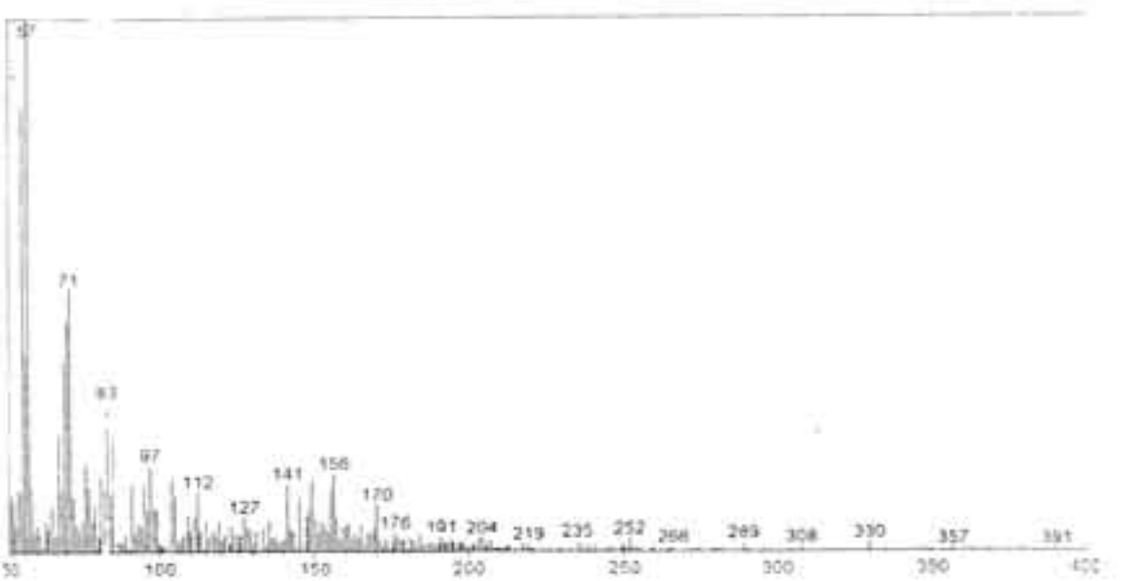


Fig. 3.17. Electron impact-mass spectrum of compound 2

3. 3. Antioxidant efficacy of phytochemical extracts from defatted rice bran in the bulk oil system

There are several standard methods to assess oxidative status of vegetable oils. Since the process of lipid oxidation is known to be very complex, the use of different methods for its assessment can give comprehensive information, especially when the effectiveness of multicomponent natural extracts is investigated [199]. In the present investigation, Schall Oven Test method (60^{o}C) and DSC method (150^{o}C) were used to understand the antioxidant potential of DRB extracts and their phytochemical constituents in comparison with well known synthetic antioxidants like BHT, TBHQ etc. These two models widely differing in temperatures were employed to know the stability and effect of DRB extracts in protecting edible oil (RBD soybean oil) at higher temperature and under accelerated oxidation.

3. 3.1. Schall Oven Test

Schall oven test at 60^{0}C was conducted to evaluate antioxidant extracts of DRB against oxidation during the accelerated storage conditions [136]. The antioxidant extracts and standard compounds were added to fresh, RBD soybean oil (without synthetic antioxidant) at various concentrations. The antioxidant treatments included defatted rice bran extracts viz. CME, AE, AE-LP and AE-PP at 100, 200 and 500 ppm levels and synthetic antioxidants viz. BHT and TBHQ at 100 and 200 ppm levels, and pure phytochemicals identified in defatted rice bran extracts viz. oryzanols (OYL), ferulic acid (FA) and a 1:1 mixture of α-T and γ-T$_3$ (Tmix) at 100 and 200 ppm levels. The oil samples were drawn at three days intervals during 15 days storage period and analysed for peroxide value [130], *p*-anisidine value [137], and diene value [138]. The RBD soyben oil without antioxidant was used as control.

During 15 days of storage at 60^{o}C in the dark, soybean oil treated with various DRB extracts, and their pure phytochemical constituents and the synthetic antioxidants had significantly lower PV than that of the control (Fig.

3.18). The control reached a maximum peroxide value of 78.9 meq/kg after 15 days of storage from an initial value of 1.8. At the end of the storage period, the order of antioxidant activity expressed in terms of percent inhibition values was TBHQ (86.3) > BHT = AE-PP (42.1) > AE = AE-LP (40.0) > CME = T_{mix} (31.8) > FA (22.7) > OYL (16.8), with the inhibition rates given in parenthesis (Table 3.18). The corresponding peroxide values were 10.8, 45.6, 47.3, 53.7, 60.9, and 65.6.

The activity of the extracts followed the order AE-PP>AE-LP = AE > CME and the order for the pure compounds was TBHQ > BHT > T_{mix} > FA > OYL. Thus out of the extracts, the polar phase of acetone extract (AE-PP) was found to be the most efficient, the activity being equivalent to that of BHT (200 ppm) followed by acetone extract, the activity of which was equivalent to that of the lipophilic phase of acetone extract (AE-LP). Crude methanolic extract (CME) offered the least protection, yet the activity was equivalent to that of a mixture containing equal amounts of α-T and γ-T_3, the predominant tocopherol and trienol homologs in DRB extracts. An interesting observation towards the end of the storage period was that all the DRB extracts performed either equally or better than the phytochemical constituents tested which are known to be very good antioxidants. Moreover, the activity of one of the purified fractions viz. AE-PP equalled that of BHT, a widely used synthetic antioxidant in food product. However, TBHQ was the most active as compared to those of extracts or other pure compounds with AE-PP showing about 45% of the activity of TBHQ at 200 ppm level. In general, increasing the concentration from 100 to 200 ppm for pure compounds and from 100-500 ppm for the extracts was effective in offering significantly higher antioxidant protection through the storage period and was more or less linear.

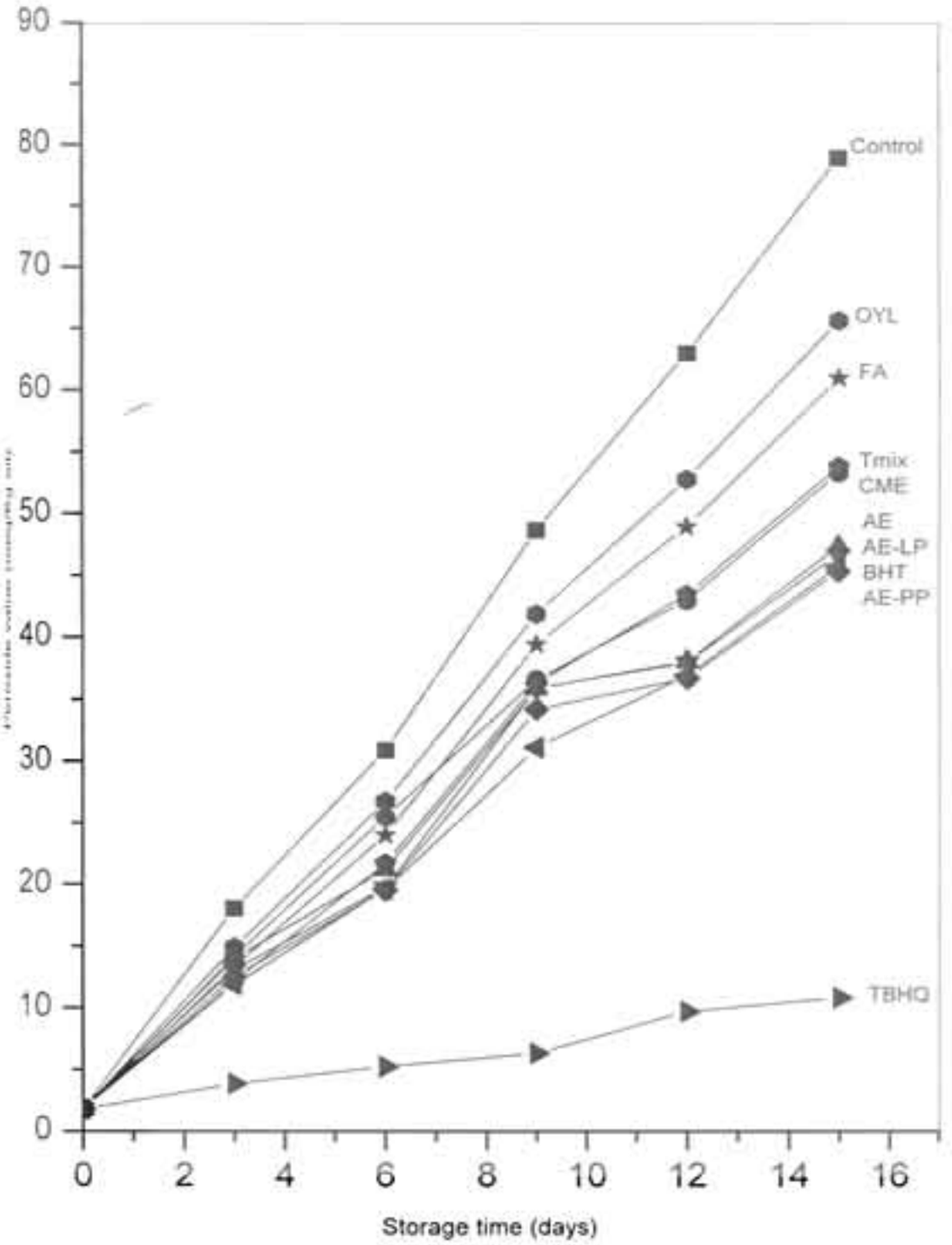


Fig.3.18.Effect of rice bran extracts and synthetic antioxidants at 200 ppm levels on the peroxide value of RBD soybean oil by Schaal oven method at 60°C.

Table.3.18. Inhibition of hydroperoxide (PV) formation by ricebran extracts and phenolic compounds added to RBD soybean oil (% mean inhibition ± S.D, n=4) [a,b]

QTY. (ppm)	Treatments									
	CON	CME	AE	AE-LP	AE-PP	BHT	TBHQ	OYL	FA	TMix
				3 days						
100	0.00 ±0.00 (Aa)	15.19 ±0.53 (Ab)	17.88 ±0.36 (Ac)	20.08 ±0.65 (Ad)	25.29 ±1.08 (Af)	33.65 ±1.16 (Ah)	74.66 ±2.74 (Ai)	16.30 ±0.70 (A b,c)	23.56 ±0.87 (Ae)	30.03 ±1.21 (Ag)
200	0.00 ±0.00 (Aa)	21.62 ±.0.76 (Bc)	22.52 ±0.83 (Bc)	27.60 ±0.98 (Bd)	30.62 ±1.02 (Ae)	34.23 ±1.30 (Af)	78.72 ±2.76 (Ag)	17.69 ±0.61 (Bb)	26.35 ±1.03 (Bd)	32.74 ±1.14 (Bf)
500	0.00 ±0.00 (Aa)	28.05 ±0.98 (Cb)	28.98 ±1.05 (Cb)	38.34 ±1.35 (Cc)	44.31 ±1.30 (Ad)					
				6 days						
100	0.00 ±0.0 (Aa)	15.12 ±1.09 (Ac)	26.28 ±1.96 (Ad)	31.06 ±1.96 (Ae)	31.27 ±1.69 (Ae)	34.71 ±1.60 (Af)	74.21 ±2.15 (Ag)	7.00 ±0.44 (Ab)	16.59 ±0.64 (Ac)	25.02 ±1.46 (Ad)
200	0.00 ±0.00 (Aa)	17.49 ±0.85 (Bc)	31.34 ±1.21 (Be)	36.15 ±2.30 (Bf)	36.81 ±1.82 (Bf)	36.53 ±2.10 (Af)	83.20 ±3.92 (Bg)	13.40 ±0.43 (Bb)	22.21 ±0.48 (Bd)	29.76 ±1.91 (Be)
500	0.00 ±0.00 (Aa)	31.19 ±2.05 (Cb)	37.50 ±0.96 (Cc)	37.59 ±1.81 (Bc)	39.44 ±1.80 (Bc)					
				9 days						
100	0.00 ±0.0 (Aa)	23.41 ±0.54 (Ae)	23.93 ±0.26 (Ae)	25.09 ±0.59 (Af)	26.50 ±0.38 (Ag)	33.33 ±0.25 (Ah)	81.23 ±1.03 (Ai)	12.88 ±0.13 (Ab)	14.20 ±0.38 (Ac)	21.18 ±0.28 (Ad)
200	0.00 ±0.00 (Aa)	24.83 ±0.24 (Bd)	26.34 ±0.22 (Be)	26.29 ±0.69 (Be)	29.80 ±0.82 (Bf)	36.15 ±0.50 (Bg)	87.08 ±1.49 (Bh)	14.05 ±0.16 (Bb)	19.08 ±0.20 (Bc)	25.48 ±0.21 (B d,e)
500	0.00 ±0.00 (Aa)	26.61 ±0.31 (Cb)	29.41 ±0.78 (Cc)	34.31 ±0.66 (Cd)	35.49 ±0.38 (Ce)					

Table. 3.18. continued

QTY. (ppm)	Treatments									
	CON	CME	AE	AE-LP	AE-PP	BHT	TBHQ	OYL	FA	TMix
				12 days						
100	0.00 ±0.00 (Aa)	28.54 ±0.46 (Ae)	36.84 ±0.88 (Af)	38.56 ±0.73 (Ag)	40.52 ±1.28 (Ah)	37.78 ±0.89 (Af,g)	83.54 ±1.96 (Ai)	12.41 ±0.29 (Ab)	18.50 ±0.44 (Ac)	26.61 ±0.70 (Ad)
200	0.00 ±0.00 (Aa)	31.92 ±0.75 (Bd)	39.68 ±1.10 (Be)	39.85 ±0.91 (ABe)	41.82 ±0.90 (Af)	41.43 ±0.96 (Bf)	84.67 ±1.99 (Ag)	16.25 ±0.18 (Bb)	22.35 ±0.55 (Bc)	31.11 ±0.74 (Bd)
500	0.00 ±0.00 (Aa)	34.77 ±0.48 (Cb)	41.24 ±0.96 (Bc)	41.52 ±1.68 (Bc)	44.63 ±1.02 (Bd)					
				15 days						
100	0.00 ±0.00 (Aa)	28.93 ±0.41 (Ae)	37.44 ±0.57 (Af)	39.45 ±0.60 (Ag)	41.24 ±0.78 (Ah)	38.85 ±0.60 (Ag)	85.20 ±1.06 (Ai)	12.54 ±0.40 (Ab)	18.88 ±0.42 (Ac)	27.24 ±0.39 (Ad)
200	0.00 ±0.00 (Aa)	32.45 ±0.44 (Bd)	40.03 ±0.62 (Be)	40.85 ±0.57 (Be)	42.60 ±0.60 (Bf)	42.13 ±0.66 (Bf)	86.32 ±1.20 (Ag)	16.82 ±0.40 (Bb)	22.70 ±0.34 (Bc)	31.83 ±0.61 (Bd)
500	0.00 ±0.00 (Aa)	35.38 ±0.75 (Cb)	41.95 ±0.68 (Cc)	44.08 ±0.80 (Cd)	45.43 ±0.69 (Ce)					

[a] % inhibition=[{(PV control-PV sample)/PV control}x100].

[b] a-j : means within a row with different letters are significantly different (P< 0.05) according to DMRT.

A-E : means within a column with different letters are significantly different (P< 0.05) according to DMRT.

The *p*-AV is indicative of the amount of secondary oxidation products produced during oxidative degradation of oil during storage. The *p*-AV of the storage studies of soybean oil are shown in Fig.3.19. It is clear from the figure that antioxidant treated samples showed significantly lower *p*-AV compared to that of control during storage. The control reached a maximum *p*-AV of 211.1 from an initial value of 18.0 after 15 days of storage. At the end of the storage period, at 200 ppm levels, the order of antioxidant activity expressed in terms of percent inhibition values was TBHQ (73.1)> BHT (43.4)> AE-PP (36.5)> Tmix (33.9) >AE-LP (30.3)> FA (27.4)> AE (25.1)> CME (21.9) > OYL (19.5), with the inhibition rates given in parenthesis (Table 3.19). The corresponding order for *p*-anisidine values were 56.8, 119.3, 133.9, 139.4, 147.0, 153.1, 158.0, 164.8 and 169.9.

None of the extracts equalled to the activity of TBHQ, but AE-PP was close to BHT. However all the extracts and pure compounds offered protection in varying degrees either equal to or better than oryzanols. For the extracts the order of ranking was AE-PP > AE-LP > AE> CME and for the pure compounds the order was TBHQ > BHT > Tmix > FA > OYL. With increase in concentration of extracts or pure comounds the protective effect increased almost in a linear fashion, similar to the results obtained for PV.

Diene value measures conjugation of double bonds before formation of fatty acid hydroperoxides. Diene value therefore is indication of primary stage of oxidation. Changes in diene value of the experimental samples are given in Fig. 3.20. It is evident from the figure that all the antioxidant treated samples showed significantly lower diene values as compared to that of control during storage. The control reached a maximum D.V of 9.2 from an initial value of 2.9 after 15 days of storage. At the end of the storage period, the order of activity obtained was TBHQ (56.0) > BHT=AE-PP (28.4) > AE= AE-LP (25.2) > CME = Tmix (24.3) > FA (22.8) > OYL (19.9), with the inhibition rates given in parenthesis (Table 3.20). The corresponding diene values were 3.9, 6.4, 6.7, 6.8, 6.9, and 7.1. TBHQ was the most potent antioxidant under the

experimental conditions studied and all the extracts and pure compounds inhibited diene formation at varying degrees as in the case of PV and *p*-AV. As was the case with PV and *p*-AV, increasing the concentration was generally effective in increasing the antioxidant protection. However, the difference in the diene values between the samples were in narrow range.

The primary purpose of using antioxidants in lipids is to delay a significant accumulation of primary oxidative products and thus to improve oxidative stability. The primary products of lipid oxidation are hydroperoxides and peroxide value (PV) and conjugated diene measurements are both well-established methods for their determination. Primary oxidation products, i.e. lipid hydroperoxides are consequently broken down by a free radical mechanism in which the O–O bond is cleaved on either side of the carbon atom bearing the oxygen atom to give the hydroxyl free radical and many types of secondary products such as ketones, alcohols, aldehydes and malonaldehydes which give off flavours and *p*-AV is an index of secondary oxidation products. To elucidate the antioxidant effectiveness of the extracts on the secondary stages of oxidation, para anisidine value (*p*-AV) was also measured which determines the amount of aldehydes (principally two alkenals) in oils and fats [200].

The results of PV, DV and *p*-AV estimations clearly showed the antioxidant potential of defatted rice bran extracts against soybean oil oxidation at accelerated storage at 60^{o}C.

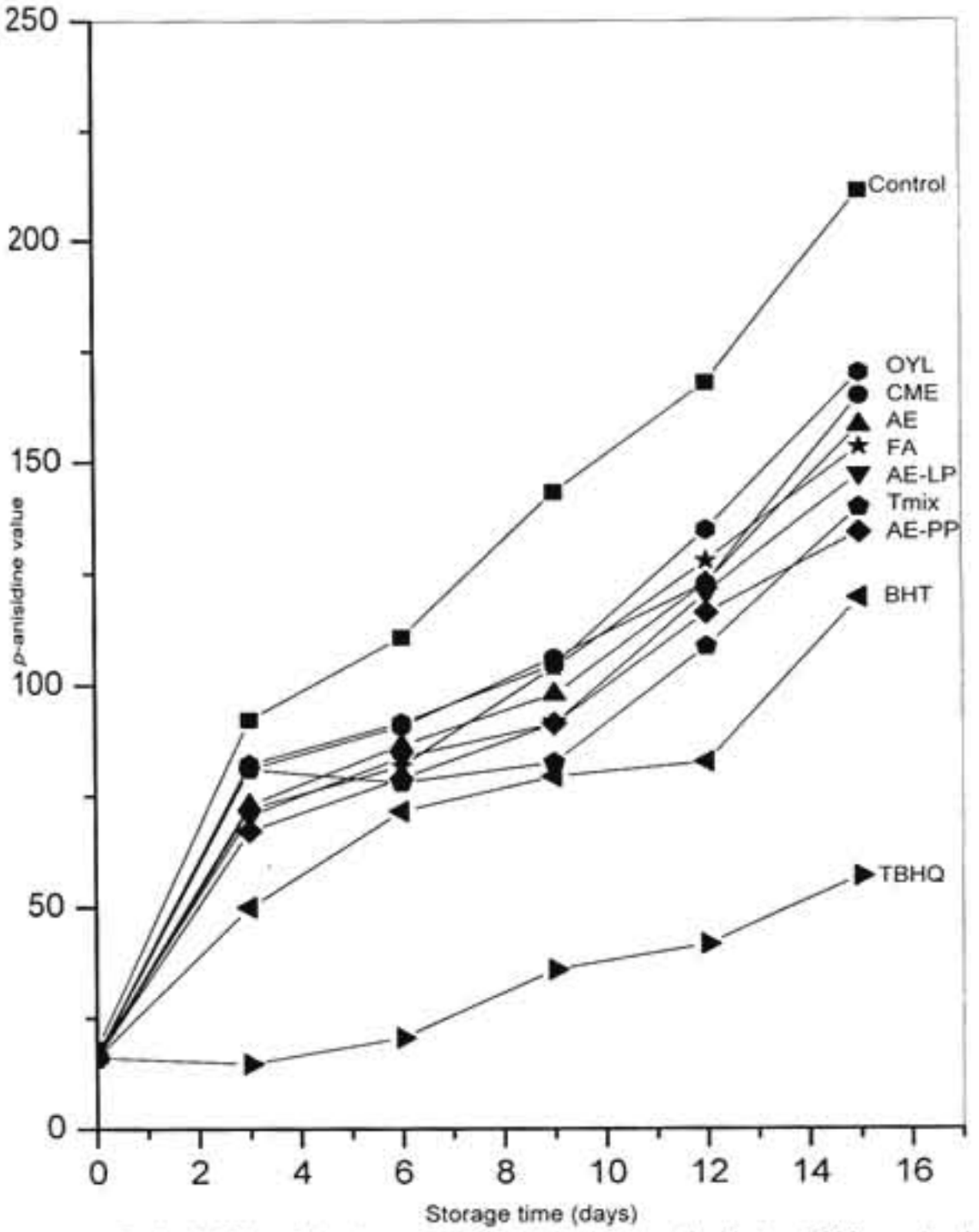


Fig.3.19. Effect of rice bran extracts and synthetic antioxidants at 200 ppm levels on the *p*-anisidine value of RBD soybean oil by Schaal oven method at 60°C.

Table. 3.19. Inhibition of *p*-anisidine value (PAV) formation by ricebran extracts and phenolic compounds added to RBD soybean oil (% mean inhibition ± S.D, n=4) [a,b]

QTY. (ppm)	Treatments									
	CON	CME	AE	AE-LP	AE-PP	BHT	TBHQ	OYL	FA	TMix
				3 days						
100	0.00 ±0.00 (Aa)	7.82 ±0.17 (Ab)	20.94 ±0.40 (Ae)	23.14 ±0.43 (Af)	25.16 ±0.48 (Ag)	32.65 ±0.65 (Ah)	81.97 ±1.74 (Ai)	7.75 ±0.15 (Ab)	16.09 ±0.32 (Ac)	17.54 ±0.34 (Ad)
200	0.00 ±0.00 (Aa)	12.11 ±.0.23 (Bc)	20.86 ±0.39 (Ad)	23.47 ±0.45 (Af)	27.19 ±.0.55 (Bg)	45.80 ±0.87 (Bh)	84.22 ±1.58 (Ai)	10.90 ±0.22 (Bb)	21.89 ±0.44 (Be)	22.24 ±0.23 (Be)
500	0.00 ±0.00 (Aa)	29.10 ±0.55 (Cb)	30.18 ±0.57 (Bc)	32.62 ±0.62 (Bd)	35.81 ±0.68 (Ce)					
				6 days						
100	0.00 ±0.00 (Aa)	10.16 ±0.15 (Ac)	19.70 ±0.30 (Ad)	20.89 ±0.35 (Ae)	24.35 ±0.37 (Af)	30.97 ±0.48 (Ah)	78.06 ±1.21 (Ai)	7.47 ±0.14 (Ab)	19.14 ±0.30 (Ad)	28.29 ±0.43 (Ag)
200	0.00 ±0.00 (Aa)	18.07 ±0.29 (Bb)	21.96 ±0.33 (Bc)	24.46 ±0.37 (Bd)	28.79 ±0.44 (Bf)	35.43 ±0.56 (Bh)	81.57 ±1.25 (Bi)	17.40 ±0.30 (Bb)	26.21 ±0.41 (Be)	29.60 ±0.44 (Bg)
500	0.00 ±0.00 (Aa)	24.39 ±0.38 (Cb)	26.29 ±0.41 (Cc)	25.99 ±0.41 (Cc)	31.66 ±0.50 (Cd)					
				9 days						
100	0.00 ±0.00 (Aa)	21.19 ±0.52 (Ad)	26.55 ±0.66 (Ae)	31.30 ±0.77 (Af)	31.54 ±0.78 (Af)	30.76 ±0.75 (Af)	72.72 ±1.80 (Ah)	6.11 ±0.16 (Ab)	9.57 ±0.24 (Ac)	34.17 ±0.85 (Ag)
200	0.00 ±0.00 (Aa)	25.91 ±0.65 (Bb)	31.60 ±0.78 (Bc)	36.27 ±0.90 (Bd)	36.25 ±0.91 (Bd)	44.62 ±1.09 (Bf)	74.99 ±1.87 (Ag)	27.03 ±0.39 (Bb)	27.36 ±0.68 (Bb)	42.40 ±1.07 (Be)
500	0.00 ±0.00 (Aa)	30.61 ±0.75 (Cb)	37.11 ±0.87 (Cc)	40.51 ±1.03 (Cd)	42.40 ±1.06 (Ce)					

Table. 3.19. continued

QTY. (ppm)	Treatments									
	CON	CME	AE	AE-LP	AE-PP	BHT	TBHQ	OYL	FA	TMix
				12 days						
100	0.00 ±0.00 (Aa)	17.94 ±0.35 (Ad)	19.85 ±0.37 (Ae)	19.65 ±0.38 (Ae)	26.29 ±0.52 (Af)	41.24 ±0.81 (Ah)	72.72 ±1.44 (Ai)	9.61 ±0.20 (Ab)	13.10 ±0.25 (Ac)	28.32 ±0.56 (Ag)
200	0.00 ±0.00 (Aa)	26.95 ±.0.53 (Bd)	26.93 ±0.53 (Bd)	28.19 ±0.55 (Be)	30.85 ±0.61 (Bf)	50.83 ±1.01 (Bh)	75.26 ±1.48 (Bi)	19.83 ±0.38 (Bb)	23.96 ±0.48 (Bc)	35.34 ±0.70 (Bg)
500	0.00 ±0.00 (Aa)	37.72 ±0.75 (Cb)	41.60 ±0.82 (Cc)	43.34 ±0.86 (Cd)	49.40 ±0.97 (Ce)					
				15 days						
100	0.00 ±0.00 (Aa)	16.64 ±0.34 (Ac)	16.73 ±0.34 (Ac)	27.91 ±0.57 (Ae)	28.38 ±0.58 (Ae)	38.99 ±0.81 (Ag)	69.72 ±1.38 (Ah)	14.17 ±0.29 (Ab)	20.92 ±0.43 (Ad)	29.99 ±0.62 (Af)
200	0.00 ±0.00 (Aa)	21.92 ±0.45 (Bc)	25.15 ±0.52 (Bd)	30.35 ±0.63 (Bf)	36.57 ±0.75 (Bh)	43.46 ±0.90 (Bi)	73.12 ±1.51 (Bj)	19.50 ±0.40 (Bb)	27.45 ±0.56 (Be)	33.94 ±0.70 (Bg)
500	0.00 ±0.00 (Aa)	27.88 ±0.58 (Cb)	33.64 ±0.69 (Cc)	39.89 ±0.83 (Cd)	47.83 ±0.99 (Ce)					

[a] % inhibition=[{(PAV control-PAV sample)/PAV control}x100].

[b] a-j : means within a row with different letters are significantly different (P< 0.05) according to DMRT.

A-E : means within a column with different letters are significantly different (P< 0.05) according to DMRT.

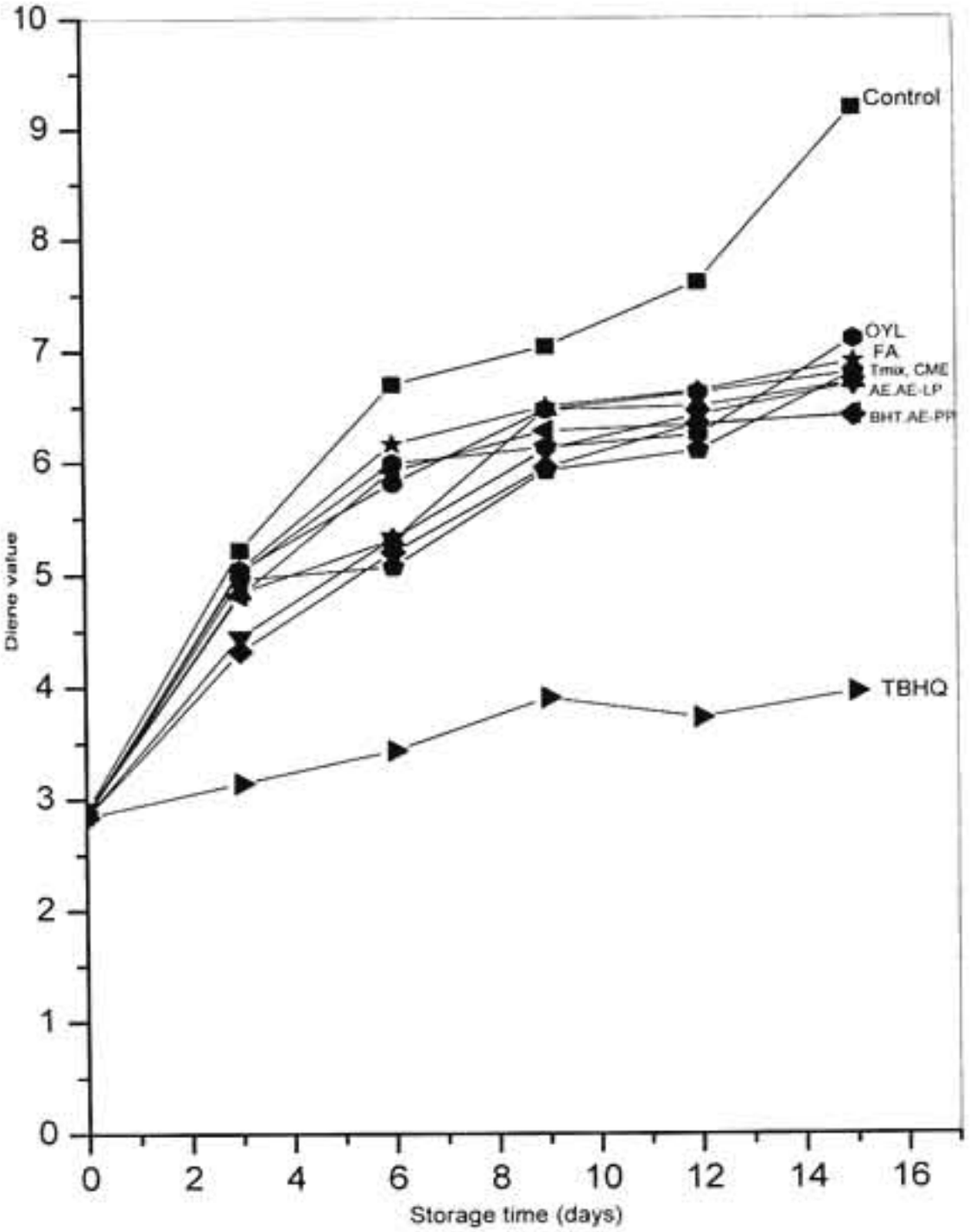


Fig.3.20.Effect of rice bran extracts and synthetic antioxidants at 200 ppm levels on the diene value of RBD soybean oil by Schaal oven method at 60°C.

Table. 3.20. Inhibition of diene value (DV) formation by ricebran extracts and phenolic compounds added to RBD soybean oil (% mean inhibition ± S.D, n=4) [a,b]

QTY. (ppm)	Treatments									
	CON	CME	AE	AE-LP	AE-PP	BHT	TBHQ	OYL	FA	TMix
				3 days						
100	0.00 ±0.00 (Aa)	1.97 ±0.19 (Ac)	4.17 ±0.11 (Ad)	4.03 ±0.02 (Ad)	9.83 ±0.11 (Bf)	5.56 ±0.02 (Ae)	28.43 ±0.16 (Ag)	1.53 ±0.10 (Ab)	1.68 ±0.10 (Ab)	3.98 ±0.10 (Ad)
200	0.00 ±0.00 (Aa)	3.45 ±0.01 (Cc)	7.29 ±0.03 (Bf)	14.77 ±0.06 (Ch)	17.26 ±0.07 (Ci)	7.67 ±0.03 (Bg)	39.76 ±0.20 (Bj)	3.84 ±0.02 (Bd)	3.12 ±0.10 (Bb)	4.84 ±0.10 (Be)
500	0.00 ±0.00 (Aa)	2.21 ±0.12 (Bb)	4.99 ±0.02 (Ac)	7.67 ±0.03 (Bd)	9.40 ±0.39 (Ae)					
				6 days						
100	0.00 ±0.0 (Aa)	2.73 ±0.07 (Ab)	7.13 ±0.08 (Ad)	10.31 ±0.16 (Ag)	11.69 ±0.22 (Ah)	4.29 ±0.26 (Ac)	42.91 ±0.52 (Ai)	9.45 ±0.14 (Af)	2.61 ±0.14 (Ab)	8.07 ±0.20 (Ae)
200	0.00 ±0.00 (Aa)	13.30 ±0.21 (Ce)	20.76 ±0.29 (Cf)	20.50 ±0.35 (Cf)	22.37 ±0.44 (Cg)	11.65 ±0.25 (Bd)	48.81 ±0.60 (Bi)	10.50 ±0.25 (Bc)	8.07 ±0.23 (Bb)	24.31 ±0.48 (Bh)
500	0.00 ±0.00 (Aa)	11.45 ±0.19 (Bb)	14.49 ±0.23 (Bc)	17.89 ±0.33 (Bd)	20.50 ±0.39 (Be)					
				9 days						
100	0.00 ±0.0 (Aa)	6.90 ±0.12 (Ac)	7.82 ±0.17 (Ad)	9.53 ±0.21 (Af)	12.83 ±0.37 (Ah)	5.30 ±0.16 (Ab)	22.57 ±0.44 (Ai)	12.05 ±0.23 (Ag)	6.97 ±0.15 (Ac)	8.53 ±0.26 (Ae)
200	0.00 ±0.00 (Aa)	8.11 ±0.18 (Bb)	8.07 ±0.16 (Ab)	13.22 ±0.29 (Bd)	15.43 ±0.35 (Be)	9.39 ±0.21 (Bc)	44.65 ±0.99 (Bf)	12.87 ±0.40 (Bd)	7.65 ±0.29 (Bb)	15.93 ±0.35 (Be)
500	0.00 ±0.00 (Aa)	9.67 ±0.21 (Cb)	13.22 ±0.29 (Bc)	13.58 ±0.39 (Bc)	18.06 ±0.40 (Cd)					

Table.3.20. continued

QTY. (ppm)	Treatments									
	CON	CME	AE	AE-LP	AE-PP	BHT	TBHQ	OYL	FA	TMix
				12 days						
100	0.00 ±0.0 (Aa)	10.22 ±0.18 (Ac)	11.89 ±0.38 (Ad)	12.97 ±0.17 (Ae)	16.36 ±0.17 (Ag)	8.64 ±0.05 (Ab)	45.73 ±0.81 (Ah)	14.39 ±0.19 (Af)	9.03 ±0.13 (Ab)	13.11 ±0.15 (Ae)
200	0.00 ±0.00 (Aa)	13.01 ±0.27 (Bc)	14.62 ±0.34 (Bd)	15.70 ±0.33 (Be)	16.72 ±0.34 (Af)	11.63 ±0.14 (Bb)	51.21 ±1.03 (Bi)	18.03 ±0.30 (Bg)	12.78 ±0.20 (Bc)	19.87 ±0.28 (Bh)
500	0.00 ±0.00 (Aa)	15.57 ±0.34 (Cb)	18.20 ±0.39 (Cc)	19.35 ±0.39 (Cd)	22.04 ±0.35 (Be)					
				15 days						
100	0.00 ±0.00 (Aa)	21.18 ±0.39 (Ac)	22.32 ±0.49 (Ae)	22.37 ±0.51 (Ae)	24.26 ±0.28 (Af)	24.16 ±0.25 (Af)	52.22 ±0.64 (Ag)	17.95 ±0.25 (Ab)	22.16 ±0.20 (Ad)	20.96 ±0.30 (Ac)
200	0.00 ±0.00 (Aa)	24.58 ±0.44 (Bd)	25.21 ±0.32 (Be)	25.30 ±0.34 (Be)	28.58 ±0.42 (Bf)	28.48 ±0.38 (Bf)	56.09 ±0.68 (Bg)	19.99 ±0.29 (Bb)	22.85 ±0.31 (Bc)	24.30 ±0.33 (Bd)
500	0.00 ±0.00 (Aa)	27.36 ±0.44 (Cb)	30.51 ±0.42 (Cc)	30.59 ±0.48 (Cc)	33.58 ±0.50 (Cd)					

[a] % inhibition=[{(DV control-DV sample)/DV control}x100].

[b] a-j : means within a row with different letters are significantly different (P< 0.05) according to DMRT.

A-E : means within a column with different letters are significantly different (P< 0.05) according to DMRT.

3. 3. 2. Application of Differential Scanning Calorimetry (DSC), to monitor stability of edible oils

Accelerated stability methods are designed to expedite the oxidation process by manipulating pro-oxidant conditions such as temperature, metal catalysts, oxygen pressure, shaking, and light exposure, in order to determine the oxidative stability of fats or fat-containing foods within short time [201]. Schaal oven (forced-air oven) test, oxygen bomb test, Rancimat method, active oxygen method (AOM), and differential scanning calorimetry (DSC) are the major stability studies widely used for the purpose employing accelerated conditions of aeration and heating. All these methods except DSC require large amount of samples (of the order of several grams) and are more time consuming. Oil samples, which require 15 days by Schall oven method could be evaluated in less than one hour by DSC. Isothermal DSC has thus many advantages over the other methods including small sample size (less than 20 mg), minimal sample preparation, simplicity of operation and results in minutes [202].

DSC compares the rate of heat flow of a sample to that of an inert reference material as both are heated or cooled. Lipid oxidation in vegetable oil is primarily an exothermic reaction and from the resulting exotherms, the onset of oxidation time was determined graphically by the instrument by extrapolating the tangent back to the chart baseline of the data plotted as a function of time [139]. Initially the changes are slow and induction time (IT) is taken as the period over which the oil is resistant to oxidation with or without the presence of antioxidants. The results are expressed as % stabilisation effectiveness (% SE)=[{(IT antioxidant-IT control)/IT control}x100] as given by Yen and Lee [203]. For the present study, antioxidant treatments included CME, AE, AE-LP and AE-PP at 100, 200, 500 and 1000 ppm levels and BHT, TBHQ, oryzanols (OYL), ferulic acid (FA) and Tmix , tricin and β-sitosterol (sterol) at 100 and 200 ppm levels.

During oxidation of soybean oil under the standardized conditions of aeration (40 mL/min) and isothermal heating (150^{o}C), all the antioxidant treatments including samples and standards had significantly higher induction periods compared to that of control (Figs.3.21 and 3.22 respectively). For the control, the onset of oxidation was 6.1 minutes. The antioxidant activity expressed as percent stabilization effectiveness values (% SE) followed the order TBHQ > AE-PP > AE-LP > Tmix > AE > BHT > CME > tricin > FA> OYL > sterol at 100 ppm levels. At 200 ppm levels, the activity order was TBHQ (109.5) > AE-PP (72.5) > AE-LP (70.3) > BHT (59.9) > AE = CME (48.3) ≥ Tmix (47.5) > tricin (43.2)> FA (38.1)> OYL (12.1) > sterol (7.5), with the activity indices given in parenthesis (Table 3.21). The corresponding induction times were 12.8, 10.7, 10.6, 9.6, 9.2, 9.0, 8.8, 8.3, 7.0 and 6.5.

It could also be seen that the activity of the extracts followed the order AE-PP > AE-LP > AE=CME and for the pure compounds the order was TBHQ > BHT > Tmix > tricin> FA > OYL> sterol. The fractions AE-PP and AE-LP performed significantly better than BHT and all the extracts were more efficient than tricin, ferulic acid, oryzanol and sterol. The increase in activity with fractionation might be due to the enhanced levels of antioxidants in the resultant fractions compared to CME. In general, increasing the concentrations from 50 to 1000 ppm for the extracts and from 100 to 200 ppm for the pure compounds was effective in extending the induction periods. Results of the DSC experiments thus not only supported the results of the Schall oven test but has also shown that the DRB extracts were stable at high temperature and therefore capable of protecting soybean oil against oxidation even at elevated temperatures.

Antioxidant activity was found to differ between the methods employed here. While the order of activity of various extracts and pure compounds by the SOT (PV and DV data) was TBHQ > BHT= AE-PP > AE= AE-LP > CME=Tmix > FA > OYL, by DSC it was TBHQ > AE-PP > AE-LP > BHT

> AE = CME ≥ Tmix > FA > OYL, at 200 ppm levels. It could be seen that antioxidant efficacy of AE-PP was close to that of TBHQ and far greater than BHT as evident from DSC results. But in the case of SOT, the results for AE-PP was comparable or less than that of BHT. Synergistic action and protective effects of proteins, sugars present and other unidentified phenolic compounds at higher temperatures in the case of DSC could be reasons for higher activity for AE-PP fraction.

The fact that in the bulk oil system (SOT & DSC), AE-PP performed better than AE-LP could be explained on the basis of the "polar paradox" theory. In this dry system, the lipophilic antioxidants would remain in the oil, while more polar hydrophilic antioxidants will be oriented on the air-oil interface where oxidation occurs initially and thus more effective in reducing or preventing oxygen assessibility for oil oxidation [204].

Thus the results of the present study demonstrated that in bulk oils, some of the DRB extracts (AE-PP) were either equally efficient or better than BHT and that at identical concentrations AE-PP, AE-LP and AE, performed better than the phytochemical constituents oryzanols, ferulic acid, tricin, sterol and tocols as pure compounds with respect to PV, DV and DSC results. Similar reports on the superior activities of crude extracts compared to the isolated compounds are not uncommon. For example, Xing and White have observed that oat groat and hull extracts both had significantly more inhibition of oxidation than the pure phenolics at the same amounts and proportions and they attributed it to the possible presence of additional, unidentified antioxidants in the extracts, such as flavonoids and other phenolics [205].

The extracts evaluated here also performed either comparable to or even better than some of the currently available natural extracts of plant origin. For example, mung bean hull extracts were reported to be more effective than α-T in inhibting PV and TBARS development in SBO at 60^{o}C at 100 ppm levels [206]. Xing and White observed that oat extracts had little antioxidative effect on SBO at 60^{o}C at concentrations less than 0.05% (w/w) based on TPC and to

get comparable protections with 0.02% TBHQ, the groat and hull extracts were required at 0.3% levels [205]. According to Duh and Yen, methanolic extracts of peanut hulls at 1200 ppm offered less protection than 200 ppm BHA on accelerated oxidation (60^{o}C) of soybean oil on the 8^{th} day of storage [207]. Lee and co-workers noted that far infra red treated rice hull extracts added to irradiated turkey meat at 0.1% showed a similar level of antioxidant activity to sesamol at 0.01% or commercial rosemary oleoresin at the 0.1% level [91]. The results from the present study showed that the activity of DRB extracts (AE-PP) was comparable to BHT in preventing the primary oxidation of soybean oil, based on PV, DV, and DSC data.

The extracts were also effective at the secondary stage of oxidation. More over, the extracts protected soybean oil against oxidation even at the high temperature (150^{0}C) employed in DSC. Compounds identified and quantitated in the DRB extracts and concentrates are oryzanols, tocols, ferulic acid, tricin, sterol etc. However, the fact that their concentrations are far lower in the extracts used for evaluation here but their antioxidant efficacy was far greater than that of the individual pure compounds need explanation in the light of synergistic activity and other constituents such as proteins, free amino acids, sugars, and several other unidentified phenolics. Since the extract is derived from an oleaginous source, it will be more compatible with vegetable oils. In addition, the extracts have the benefits of antioxidants such as oryzanols, tocols, ferulic acid, tricin, and sterol which are known to have a multitude of biological effects as well.

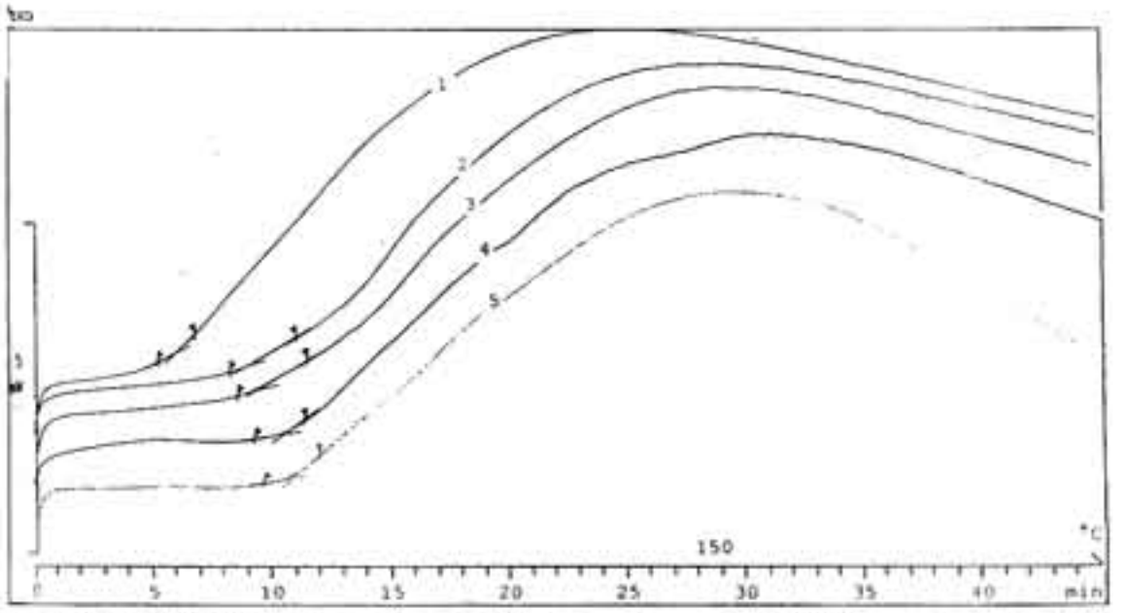


Fig. 3. 21. DSC profiles of oxidative stability of soybean oil with 200 ppm levels of DRB extracts . Sample codes: 1, Contro¹ (6.05); 2, CME (9.05); 3, AE (9.29); 4, AE-LP (10.63); 5, AE-PP (10.79). The onset times in minutes are given in brackets.

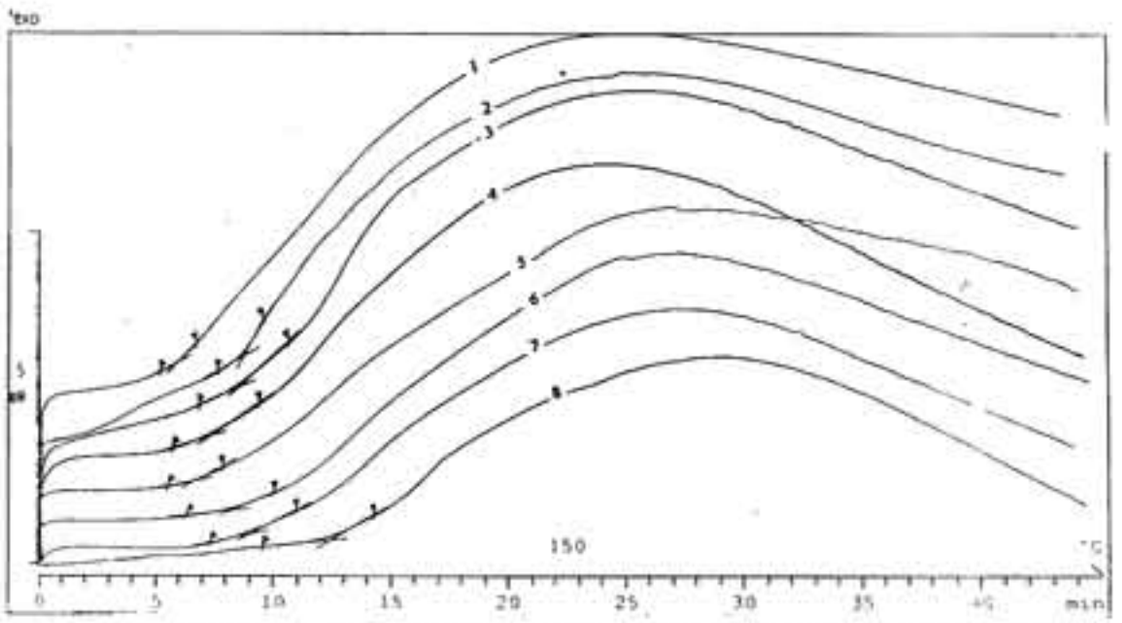


Fig. 3. 22. DSC profiles of oxidative stability of soybean oil with 200 ppm levels of added antioxidants. Sample codes: 1, Control (6.05); 2, BHT (9.68); 3, Tricin (8.81); 4, OYL (7.03); 5, Sterol (6.52); 6, FA (8.39); 7, Tmix (9.05); 8, TBHQ (12.80) . The onset times in minutes are given in brackets.

Table. 3.21. Antioxidant activity of rice bran extracts and phenolic antioxidants as measured by differential scanning calorimetry (% stabilization effectiveness).

Treatments[a]											
CON	CME	AE	AE-LP	AE-PP	BHT	TBHQ	OYL	FA	Tmix	Tricin	Sterol
					50	ppm					
0.00 ±0.00 (Aa)	20.32 ±0.42 (Ab)	20.12 ±0.48 (Ab)	23.39 ±0.48 (Ac)	23.63 ±0.42 (Ac)	-	-	-	-	-		
					100	ppm					
0.00 ±0.00 (Aa)	24.51 ±0.50 (Bf)	37.42 ±0.77 (Bh)	55.32 ±1.14 (Bj)	57.25 ±1.18 (Bk)	32.42 ±0.67 (Ag)	86.44 ±1.78 (Al)	10.81 ±0.22 (Ac)	11.77 ±0.24 (Ad)	38.87 ±0.80 (Ai)	21.91 ±1.69 (Ae)	6.45 ±0.13 (Ab)
					200	ppm					
0.00 ±0.00 (Aa)	48.38 ±0.99 (Cf,g)	50.00 ±1.03 (Cg)	70.32 ±1.45 (Ci)	72.57 ±1.49 (Cj)	59.99 ±1.23 (Bh)	109.1 ±2.25 (Bk)	12.14 ±0.19 (Bc)	38.15 ±0.47 (Bd)	47.58 ±0.98 (Bf)	43.16 ±2.21 (Be)	7.58 ±0.16 (Bb)
					500	ppm					
0.00 ±0.00 (Aa)	65.80 ±1.35 (Db)	64.83 ±1.33 (Db)	85.48 ±1.76 (Dc)	85.80 ±1.76 (Dc)	-	-	-	-	-		
					1000	ppm					
0.00 ±0.00 (Aa)	72.25 ±1.49 (Eb)	74.35 ±1.53 (Eb)	96.93 ±1.99 (Ec)	97.73 ±2.01 (Ec)	-	-	-	-	-		

$$\% \text{ stabilization effectiveness} = \frac{\text{IT antioxidant} - \text{IT control}}{\text{IT control}} \times 100$$

[a]Each value in the table represents the mean ± standard deviation of four analyses from 2 replications.

A-E : means within a column with different letters are significantly different ($P < 0.05$) according to DMRT.

a-j : means within a row with different letters are significantly different ($P < 0.05$) according to DMRT.

3. 4. Antioxidant efficacy of phytochemicals from DRB in *in- vitro* model systems.

Since most natural and synthetic antioxidants are required to function in a wide variety of systems like, lipophilic and hydrophilic environments, food emulsions, physiological micelle etc. protocol employed should be relevant to either foods or biological systems [208]. In the present study, two widely recognized food relevant emulsion models viz. the linoleic acid emulsion method and the β-carotene bleaching test were selected for evaluating the antioxidant potentials of DRB extracts and its various phytochemical constituents.

Besides these methods, the ability of the antioxidants to donate electron were also evaluated. Generally, the electron-donating ability of chemical substances results in their antioxidant activity towards lipid oxidation and radical scavenging studies have therefore been emerged as a convenient methodology for its measurement [209]. $DPPH^{\bullet}$, $ABTS^{\bullet+}$, AAPH etc. are some of the synthetic radicals widely used for the purpose. In the present investigation, the radical scavenging effects of DRB extracts and its various phytochemical constituents were studied using the stable $DPPH^{\bullet}$ radical and the superoxide radicals generated by the xanthine-xanthine oxidase system.

3. 4.1. Linoleic acid emulsion method

Autoxidation of linoleic acid in ethanol-buffer system is one of the simplest conditions of oxidation in heterogenous system for evaluation of antioxidant efficacy [210]. Hydroperoxides resulting from the oxidation of linoleic acid decompose further to give many secondary oxidation products which oxidize Fe^{2+} to Fe^{3+} that in turn forms ferric thiocyanate with blood red colour with ammonium thiocyanate. In presence of added antioxidants in the system, the extent of inhibition of linoleic acid oxidation could be measured [141] and calculated as inhibition %=[{1-(abs sample at 500 nm)/ (abs control at 500 nm)}x100] [140]. The treatments included DRB extracts viz. CME, AE, AE-LP and AE-PP, synthetic antioxidants viz. BHT and TBHQ and also pure

phytochemical constituents identified in DRB extracts viz. oryzanols, ferulic acid, Tmix, tricin and β-sitosterol tested at 100 and 200 ppm levels. A control was performed with linoleic acid but without the extracts.

All the antioxidant treatments performed significantly better than the control (Fig. 3.23). At 100 and 200 ppm levels, the activity indexed as percent inhibition values followed the order TBHQ (83.9)> BHT (60.1) > AE-PP = AE-LP =OYL= tricin (51.7) > Tmix (48.0) > FA (44.1)> AE (36.0) > CME (29.9) >sterol (22.8) with the inhibition percentages at 200 ppm levels given in parenthesis. The activity of the extracts followed the order AE-PP = AE-LP> AE > CME and the effects of AE-PP and AE-LP were similar to that of oryzanols and tricin. Thus in this oil-in-water emulsion system, the phytochemicals oryzanols and tricin were equally effective and more efficient than Tmix which in turn was better than ferulic acid (Table 3.22). Phenolic acids like ferulic and caffeic acids were reported to be more hydrophilic than tocopherols [211]. Thus the observed order OYL> Tmix > FA could be explained on the basis of the polar paradox theory which state that non-polar antioxidants performed better in emulsions than the polar ones which are more efficient in bulk oils [204]. It was also reported by Kikuzaki *et al*. that in the linoleic acid emulsion model, γ-oryzanol was more effective than ferulic acid [87]. Of the various phytochemicals tested here, sterol was the least efficient, its activity being less than that of CME.

From the present investigation, it could be stated that extracts prepared from DRB also possessed activity in the emulsion system. More over, the finding that the various phytochemical constituents of DRB extracts viz. oryzanols, ferulic acid, Tmix, tricin and sterol were shown to be effective in this model directly support results of DRB extracts. The antioxidant potentials of the DRB extracts in bulk oils have already been discussed (section 3.3). Since the extracts were also effective in the emulsion system, as demonstrated here by the linoleate model, the DRB extracts could find applications in heterogeneous food matrix.

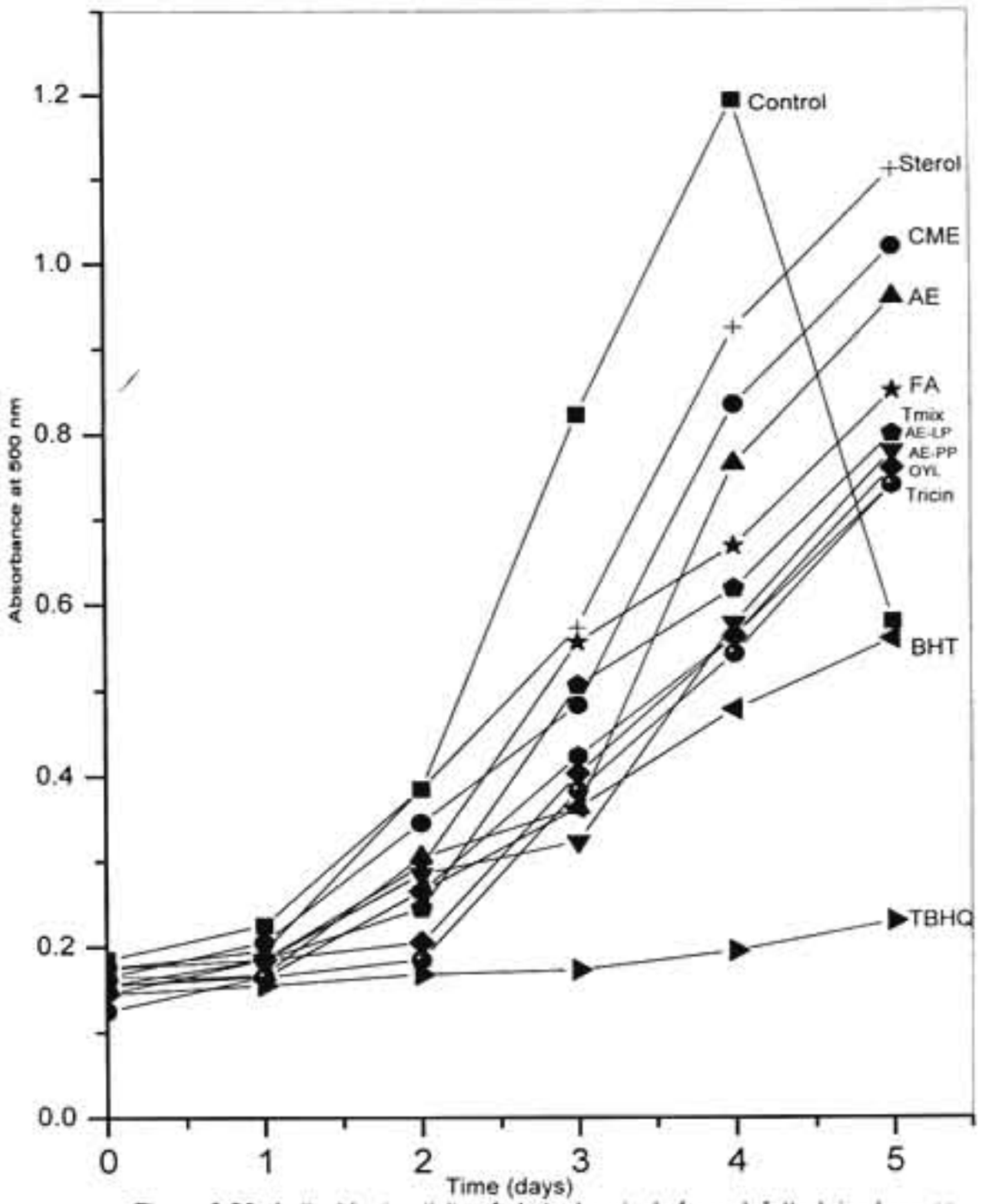


Figure.3.23. Antioxidant activity of phytochemicals from defatted rice bran as measured by the linoleic acid emulsion (thiocyanate) method .

Table. 3.22. Antioxidant activity of phytochemicals from defatted rice bran as measured by the linoleic acid emulsion (thiocyanate) method .

Treatments[a]											
CON	CME	AE	AE-LP	AE-PP	BHT	TBHQ	OYL	FA	Tmix	Tricin	Sterol
					100	ppm					
0.00 ±0.00 (Aa)	22.23 ±1.18 (Ac)	25.86 ±1.32 (Ad)	48.28 ±2.67 (Ag)	49.73 ±2.63 (Ag)	53.18 ±2.63 (Ah)	78.06 ±3.61 (Ai)	49.10 ±1.64 (Ag)	41.75 ±1.36 (Ae)	44.93 ±2.32 (Af)	48.97 ±2.18 (Ag)	15.07 ±1.12 (Ab)
					200	ppm					
0.00 ±0.00 (Aa)	29.96 ±1.80 (Bc)	36.02 ±2.27 (Bd)	51.73 ±2.63 (Ag)	52.93 ±2.58 (Ag)	60.19 ±3.36 (Bh)	83.97 ±4.18 (Ai)	52.06 ±1.08 (Bg)	44.18 ±2.76 (Ae)	48.07 ±2.27 (Af)	54.17 ±2.50 (Bg)	22.87 ±1.30 (Bb)

Inhibition %= [{1-(abs sample at 500 nm)/ (abs control at 500 nm)}x100]

[a]Each value in the table represents the mean ± standard deviation of four analyses from 2 replications.

a-i : means within a row with different letters are significantly different ($P < 0.05$) according to DMRT.

A-B : means within a column with different letters are significantly different ($P < 0.05$) according to DMRT.

3. 4. 2. β-carotene/linoleic acid assay

In this assay, antioxidant capacity is determined by measuring their ability to inhibit the formation of volatile organic compound and conjugated diene hydroperoxide from linoleic acid oxidation [212]. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated β-carotene molecules. As β-carotene molecules lose their double bonds by oxidation, the compound loses its chromophore and characteristic orange colour which can be monitored spectrophotometrically. Antioxidants can retard the β-carotene bleaching by intercepting the linoleate free radical and other free radicals formed in the system thereby preventing oxidation of β-carotene molecules [141]. The percent inhibition of β-carotene bleaching which is the measure of antioxidant activity is thus calculated as $[100\{1-(A_0-A_t)/(A_0'-A_t')\}]$ where A_0 and A_0' are the absorbance values measured at zero time of the incubation for test sample and control respectively. A_t and A_t' are the absorbances measured in the test sample and control, respectively, after incubation for 120' [213].

Figure 3.24 shows the degree of antioxidant potential of the experimental sets at 200 ppm levels. The antioxidant treatments included CME, AE, AE-LP, AE-PP, BHT, TBHQ, oryzanols (OYL), ferulic acid (FA), Tmix, tricin and β-sitosterol at both 100 and 200 ppm levels and a control without any antioxidants added. At 100 and 200 ppm levels, the antioxidant activity followed the order TBHQ (75.2)> BHT (54.1)> OYL=tricin =AE–LP =AE–PP (47.2)> Tmix (43.9) > FA (41.0)> AE (29.9) > CME (25.1) > sterol (20.1), with the antioxidant activity indexes at 200 ppm levels given in parenthesis (Table 3.23). As in the case of linoleic acid emulsion model, here also the activity of the phytochemical constituents followed the order OYL=tricin > Tmix > FA>sterol and the order for the extracts remained as AE-PP = AE-LP > AE > CME.

Though with unidentical levels of TPC, oryzanols, and ferulic acid , the activities of AE-LP and AE-PP were similar here also and was equal to that of oryzanols and tricin. Contrary to the bulk oil system where the DRB extracts (AE-PP) were either equally efficient or better than BHT, the latter was more effective in emulsions. It was shown earlier that in the bulk oil system (SOT & DSC), AE-PP performed better than AE-LP in accordance with the "polar paradox" theory. However, in the emulsion system, AE-LP did not perform better than AE-PP, but was equally effective. This departure from the polar paradox concept could be attributed to the well known fact that the antioxidant activity in an emulsion is hard to evaluate because of the partition phenomenon between hydrophilic and hydrophobic phases and the complex interfacial affinities between air-oil and oil-water interfaces involved [214]. More over, on fractionation AE-PP was enriched in ferulic acid and AE-LP in oryzanols compared to AE and CME. But both of them were equally effective in the emulsion systems. This may also be due to the presence of additional antioxidants, synergistic or additive effects as has been suggested by Koleva *et al.* [215].

Recently, Shih *et al.* have reported that the antioxidant activity of methanolic extracts of rice seeds and milled-rice co-products ranged from 45 to 86% as assayed by the β-carotene bleaching model [92]. A number of other natural extracts were also reported to be active by this method. For instance, Moure *et al.* have reported that to get comparable protections with BHT, they used 10 times more of the ethanol extrats of Gevuina avellana (a native Chilean oilseed) hulls equivalent to 2200 mg/L [216]. Koleva *et al.* have reported that apolar extracts of Sideritis (Labiatae) plant species applied at 0.1% levels possessed activity in the β-carotene bleaching method close to that of 0.01% BHT [217]. On the contrary, BHT was better only by about a factor of 8% compared to either AE-PP or AE-LP at the identical concentrations tested in the present study. More over, the finding that the various phytochemical

constituents of DRB extracts viz. oryzanols, ferulic acid, Tmix, tricin and sterol all proved to be effective in this emulsion model also, confirms the observed antioxidant potentials of the DRB extracts. Thus the results of this assay indicated that the DRB extracts could be used for protecting both linoleic acid and β-carotene against oxidation. Carotenoids including β-carotene are widely used as food colour and their discolouration due to oxidation could affect the quality of food adversely. Carotenoids can also perform the functions of free radical scavengers on account of the diene system of the molecule thus protecting food [218]. Stability of carotenoids as food colourant and as an antioxidant could thus be enhanced by addition of natural extracts like DRB as demonstrated here [219].

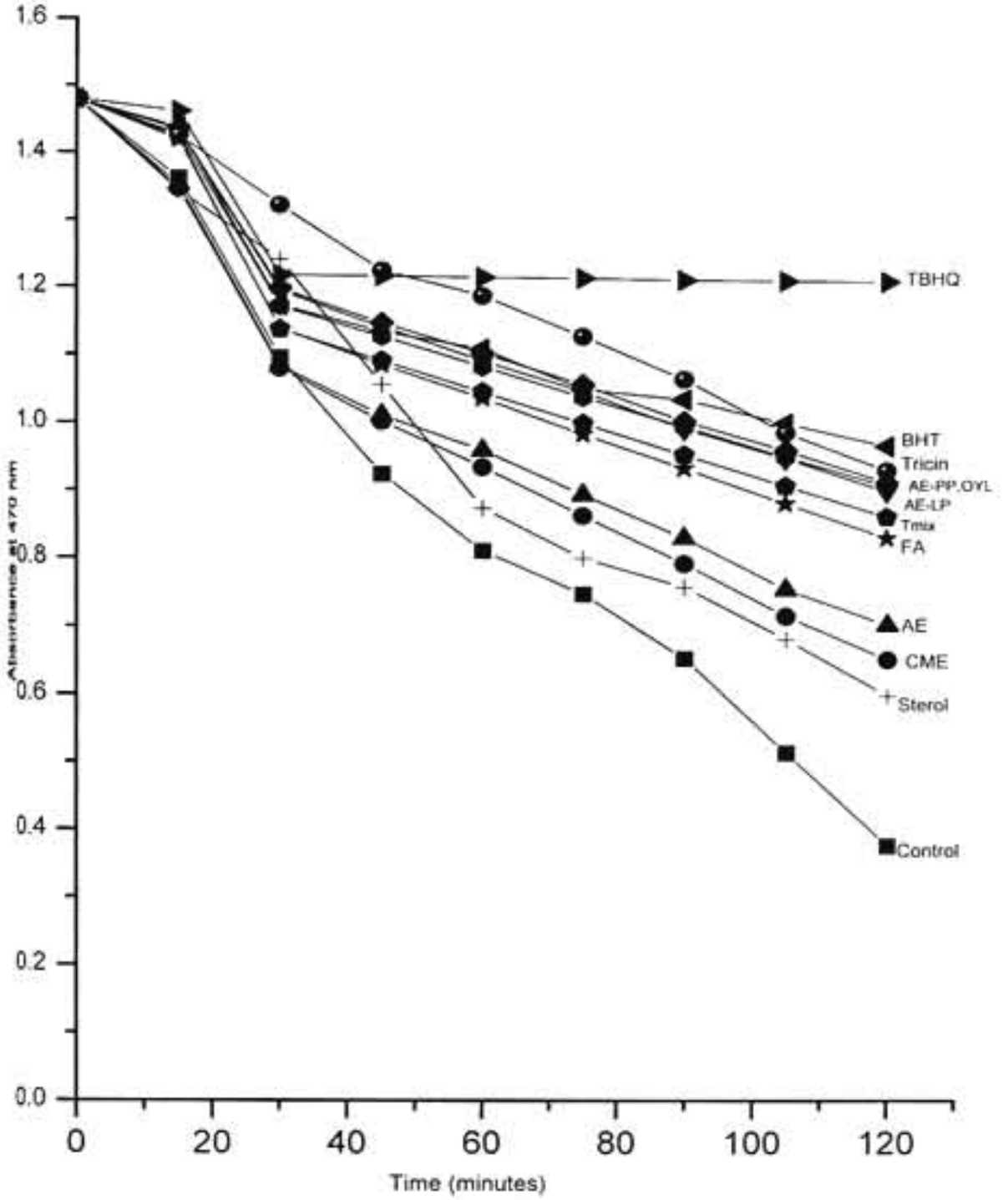


Figure.3.24. Antioxidant activity of phytochemicals from defatted rice bran in the β-carotene-linoleate model.

Table. 3.23. Antioxidant activity of phytochemicals from defatted rice bran as measured by the β-carotene-linoleate model system.

Treatments[a]											
CON	CME	AE	AE-LP	AE-PP	BHT	TBHQ	OYL	FA	Tmix	Tricin	Sterol
					100	ppm					
0.00 ±0.00 (Aa)	22.96 ±1.15 (Ac)	28.03 ±1.16 (Ad)	45.02 ±1.72 (Ag)	46.52 ±1.80 (Ag)	51.81 ±2.22 (Ah)	71.96 ±2.80 (Ai)	46.97 ±2.18 (Ag)	38.32 ±1.76 (Ae)	41.88 ±1.91 (Af)	44.85 ±2.01 (Ag)	13.57 ±1.02 (Ab)
					200	ppm					
0.00 ±0.00 (Aa)	25.10 ±1.11 (Bc)	29.90 ±1.31 (Ad)	47.21 ±1.74 (Ag)	48.47 ±1.72 (Ag)	54.17 ±2.50 (Ah)	75.26 ±2.56 (Ai)	48.04 ±1.98 (Ag)	41.06 ±1.90 (Ae)	43.92 ±1.50 (Af)	49.71 ±2.24 (Bg)	20.10 ±1.00 (Bb)

Antioxidant activity = $[1- (A_0 - A_t) / (A_o' - A_t')]100$. A_0 and $A_o'$are the absorbance values at 0' for test sample and control respectively and A_t and A_t' are the absorbance values after incubation for 120' for test sample and control respectively.

[a]Each value in the table represents the mean ± standard deviation of four analyses from 2 replications.

a-i : means within a row with different letters are significantly different ($P< 0.05$) according to DMRT.

A-B : means within a column with different letters are significantly different ($P< 0.05$) according to DMRT.

3. 4. 3. DPPH radical scavenging activity

Measurement of radical scavenging activity using discoloration of 1,1-diphenyl-2-picrylhydrazyl radicals ($DPPH^{\bullet}$) has been widely used due to its stability, simplicity and reproducibility [220]. In its radical form, $DPPH^{\bullet}$ has an absorbance at 515 nm which disappears on reduction by an antioxidant compound (AH) or a radical species $R^{\bullet}$ to become a stable diamagnetic molecule with the result the colour changes from purple to yellow [142].

$$DPPH^{\bullet} + AH \rightarrow DPPH{-}H + A^{\bullet}$$

$$DPPH^{\bullet} + R^{\bullet} \rightarrow DPPH{-}R$$

The new radical $A^{\bullet}$ can undergo radical-radical coupling to yield stable molecules [221].

$$DPPH^{\bullet} + A^{\bullet} \rightarrow DPPH{-}A$$

$$A^{\bullet} + A^{\bullet} \rightarrow A{-}A$$

In the present study, various concentrations of extracts or pure compounds were allowed to react with DPPH. The antioxidant treatments included DRB extracts viz. CME, AE, AE-LP, and AE-PP, its phytochemical constituents oryzanols, ferulic acid, Tmix, tricin, and β-sitosterol and synthetic antioxidants viz. BHT and TBHQ. At each concentration, a graph was plotted with time versus $\%DPPH_{REM}$ (DPPH remaining) (Figs.3.25 to 3.35 respectively) for 30 minutes. This graphic presentation showed the behaviour of each antioxidant compound. The $\%\ DPPH_{REM}$ was then plotted against the standard concentration to obtain the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% and is denoted as EC_{50}. The EC_{50} values (g antioxidant/kg DPPH) were taken as a measure of antiradical activity. The lower the EC_{50}, higher the antioxidant potential [142].

The EC_{50} (g antioxidant/kg DPPH) values of CME, AE, AE-LP, AE-PP, oryzanols (OYL), ferulic acid (FA), Tmix , tricin, β-sitosterol, BHT, and TBHQ

were 1977, 1945, 7985, 1072, 972, 174, 164, 3947, 21416, 1120, and 61 respectively. The order of antiradical activity was TBHQ > FA = T_{mix} > OYL > AE-PP = BHT > CME = AE > tricin > AE–LP> β-sitosterol (Fig. 3.36). The activity of the phytochemical constituents followed the order FA=Tmix > OYL > tricin > sterol whereas for the extracts the order was AE-PP > CME = AE > AE-LP. The activity of AE-PP equalled that of BHT and for ferulic acid and Tmix it was several fold higher than that of BHT.

The number of hydroxyl groups on an aromatic ring is the major factor contributing to the efficacy of phenolic antioxidants [222]. This may explain the higher activity of TBHQ (a diphenolic antioxidant) compared to that of BHT, a monophenolic antioxidant. The antiradical activity of BHT, ferulic acid and tocols is due to their phenolic hydrogen atoms. The presence of electron releasing substituents in positions ortho and/or para to the hydroxy function increases the electron density of the active centers by an inductive effect. This facilitates the homolytic fission of the O-H bond with consequent increase in the stability of the resulting phenoxyl radicals [13]. In BHT, both the ortho positions to the hydroxy function are substituted by tertiary butyl groups with strong inductive effects which can enhance the stability of the phenoxyl radical as described above. More over, the steric hindrance exerted by the bulky t-butyl groups reduces the rate of possible propagation reactions involving antioxidant radicals thus increasing its stability [13]. The hydroxyl group in ferulic acid is para-substituted on an aromatic ring which is connected to a highly conjugated side chain which allows the phenoxyl radical to be delocalised across the entire molecule and therefore stabilized [223]. The ortho substitution with the electron donor methoxy group is another factor that enhances the stability and hence the antioxidant and antiradical activities of ferulic acid [224]. The chromanoxyl radicals ($TO^{\bullet}$) formed in the case of tocopherols and tocotrienols (chromanols) also are stabilized by methyl groups in ortho positions. In addition to forming harmless adducts with other radicals,

$TO^{\bullet}$ can also undergo self coupling to form dimers which too possess antioxidant acivity [225]. Apart from these structural aspects, kinetic factors also contribute to the observed activity order of FA=Tmix> BHT. Tocopherols are reported to have intermediate kinetic behaviour reaching the steady state within 5-30 minutes [143], whereas BHT and ferulic acid showed slow kinetic behaviour taking more than 30 minutes to reach the steady state [143, 226]. The reported TEC_{50} values (time to reach the steady state at the concentration corresponding to EC_{50}) for α-tocopherol, ferulic acid and BHT were 10, 50, and 300 minutes respectively [143, 226]. In the present study, the EC_{50} values were determined after 30 minutes of the reaction time and not at the steady state in order to provide identical experimental conditions for all the test compounds. The higher activity of ferulic acid and Tmix compared to that of BHT could readily be followed when the above mentioned structural and kinetic factors were taken into consideration. It is also reported by Chen & Ho [227] and by Nenadis & Tsimidou [228] that the DPPH activities of ferulic acid and α-tocopherol were greater than that of BHT.

The observed order FA> OYL is supported by other lierature reports too. For example, Kikuzaki *et al.* have found that at 20 μM levels, the DPPH radical scavenging effect of ferulic acid was more than that of oryzanols [87] and Xu and Godber have attributed the antioxidant activity of oryzanols to the phenolic hydroxyl group in the ferulate portion of their structure [82]. Also, the EC_{50} value of 163 for ferulic acid as reported by Sanchez-Moreno *et al.* [143] was in close agreement with the values obtained in the present study. The lower activity of tricin could be attributed to the methylation of the hydroxyl groups (3 and 5) in ring B as the 3', 4'-dihydroxy structure (catechol structure) in flavonoids is important for the expression of radial scavenging activity [198].

For the fractions AE, AE-LP and AE-PP, it could be seen that both the order of DPPH radical scavenging activity and their respective phenolic

contents (TPC) could be correlated and TPC content followed the order AE-PP > AE > AE-LP (Table 3.17). Further, the ferulic acid content of these fractions (Table 3.17) could be responsible for the lower EC_{50} values for these fractions. The DPPH scavenging activities therefore could be largely attributed to the levels of TPC and FA in the fractions. As stated before, concentrations of the antioxidants identified in the extracts are far lower as compared to other constituents such as proteins, amino acids, sugars, and unidentified phenolics. These compounds could also contribute to the higher antioxidant efficacy observed here.

Free radical scavenging is considered a good in-vitro model widely used to assess antioxidant efficacy in relatively short time [142]. Larrauri *et al.* have reported that the EC_{50} values of Spanish red wines ranged from 1030 to 2450 g/kg DPPH [229]. Davalos *et al.* have reported EC_{50} values of 147626 for straw berry extracts [230]. The EC_{50} values of moringa leaf extracts have been shown to be in the range of 1050 to 3070 g/kg DPPH [213].

DPPH activity is also reported for coloured rices [85] and rice hulls [90] and procyanidins have been attributed to the $DPPH^{\bullet}$ activity of red hulled rice [85] while dihydro ferulic acid and dihydro sinapic acid have been suggested as the major $DPPH^{\bullet}$ active compounds of Kurosu (Japanese unpolished rice vinegar) [86]. The results of the present study indicated that the various phytochemical constituents of DRB extracts viz. oryzanols, ferulic acid, tricin and Tmix exhibited considerable $DPPH^{\bullet}$ activity and their synergistic effects could largely be responsible for the observed antiradical efficacies of DRB extracts. The $DPPH^{\bullet}$ activities of some of the DRB extracts (AE-PP) was equal to that of BHT and the activities of the phytochemical constituents viz. ferulic acid and Tmix was several fold higher that of BHT. More over, Tmix and ferulic acid possessed about one third the activity of TBHQ, a synthetic compound with very high antioxidant potentials. The fact that the DRB extracts

were found to be very efficient in the DPPH$^{\bullet}$ assay as demonstrated here is relevant in biological system. Because H-atom donating capacity is an important biologically significant property of antioxidants to convert potentially damaging ROS (oxyl and peroxyl radicals) into non-toxic species [231] and in this context DRB could be a good source of such antioxidants.

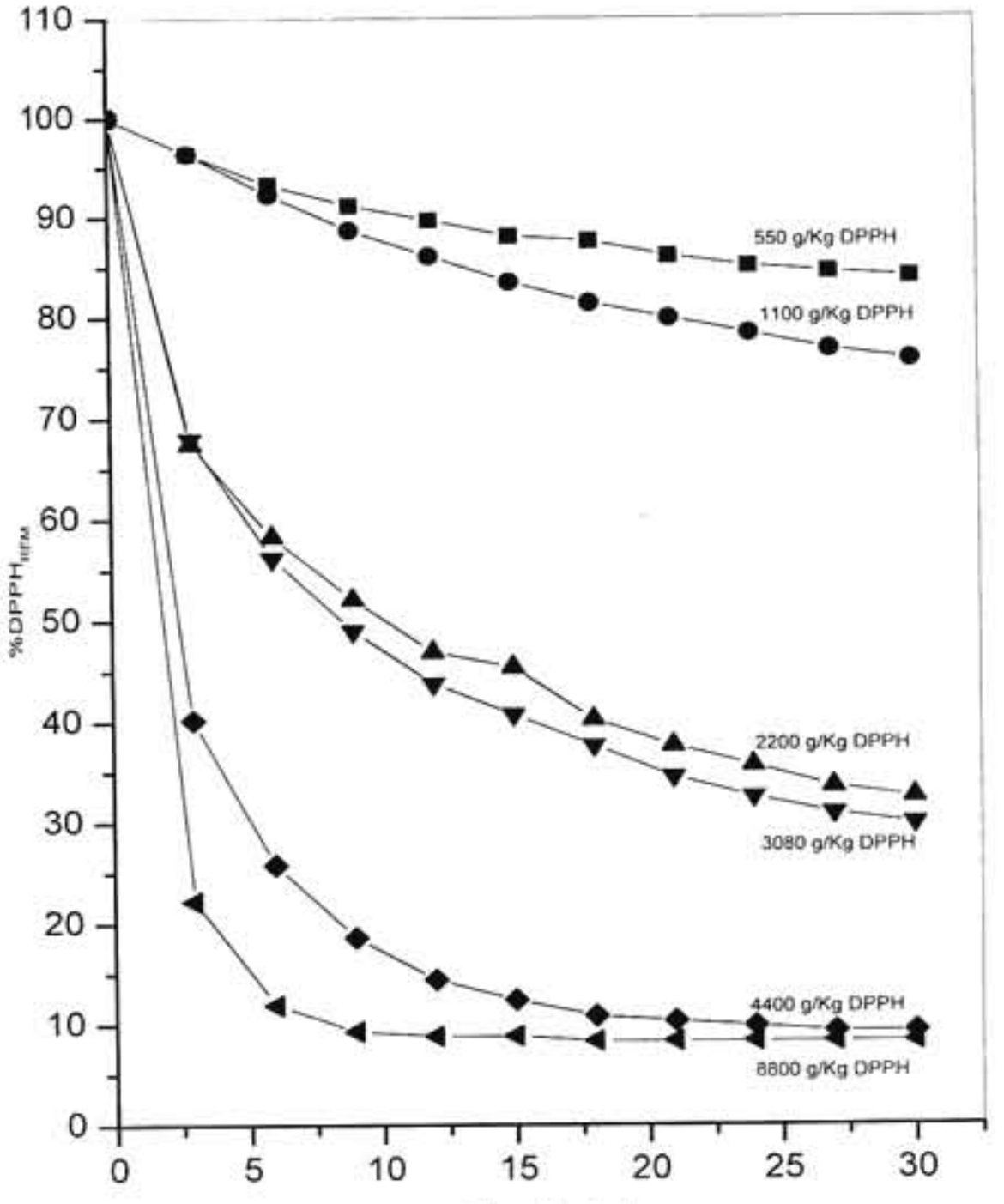


Fig. 3.25. DPPH scavenging effect of crude methanolic extract (CME).

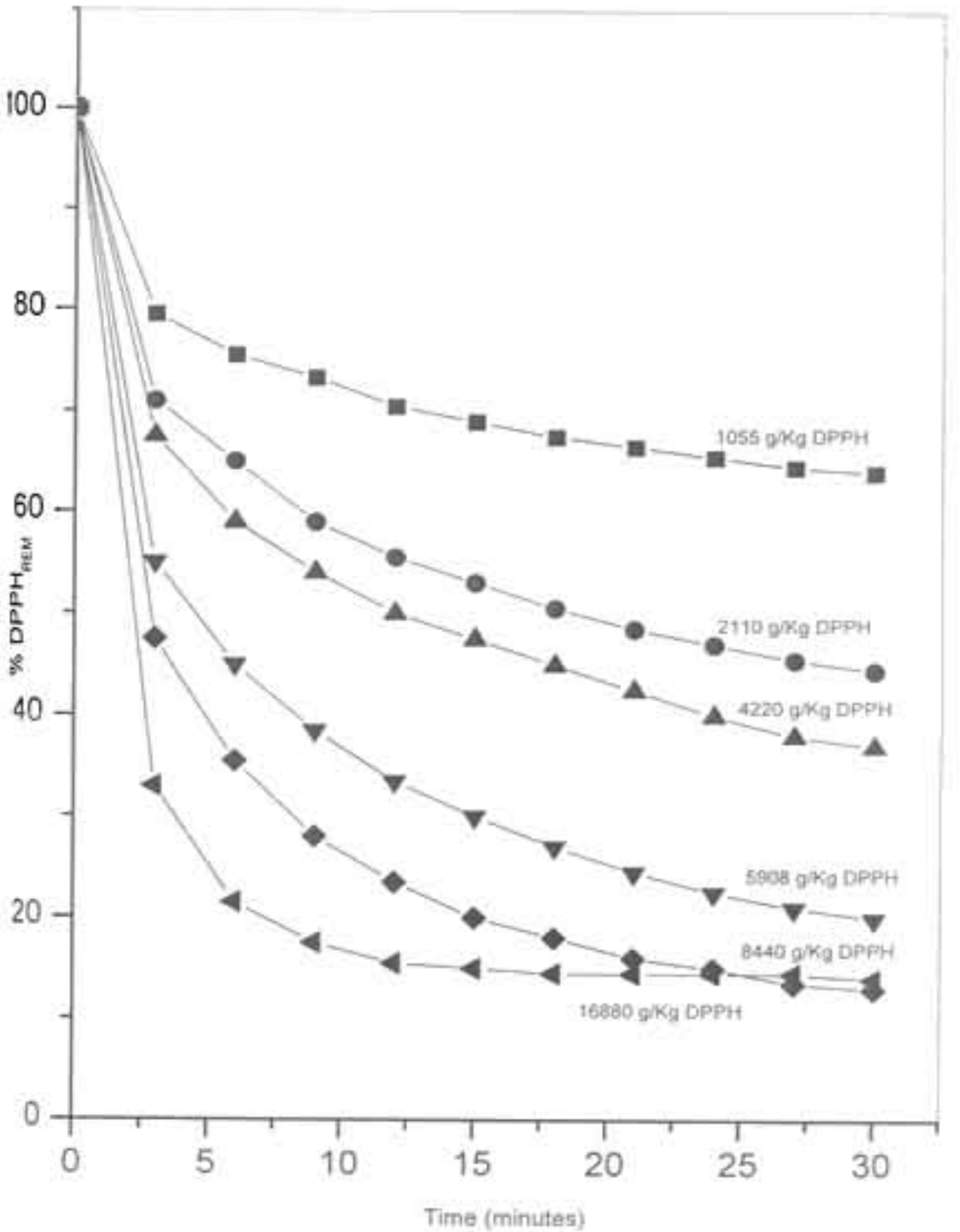


Fig.3.26.DPPH scavenging effect of acetone extract (AE).

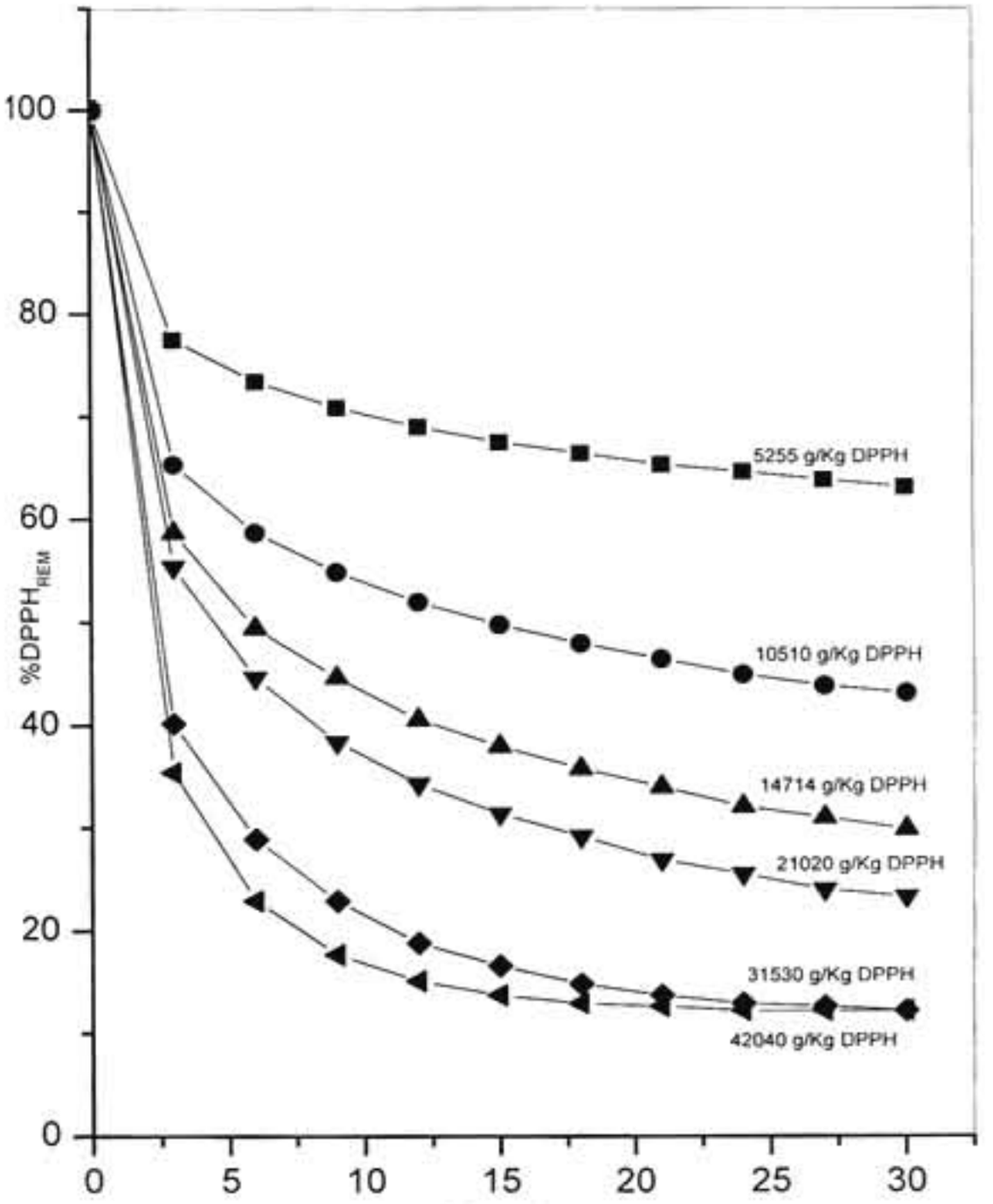


Fig.3.27.DPPH scavenging effect of lipophilic phase of acetone extract (AE-LP).

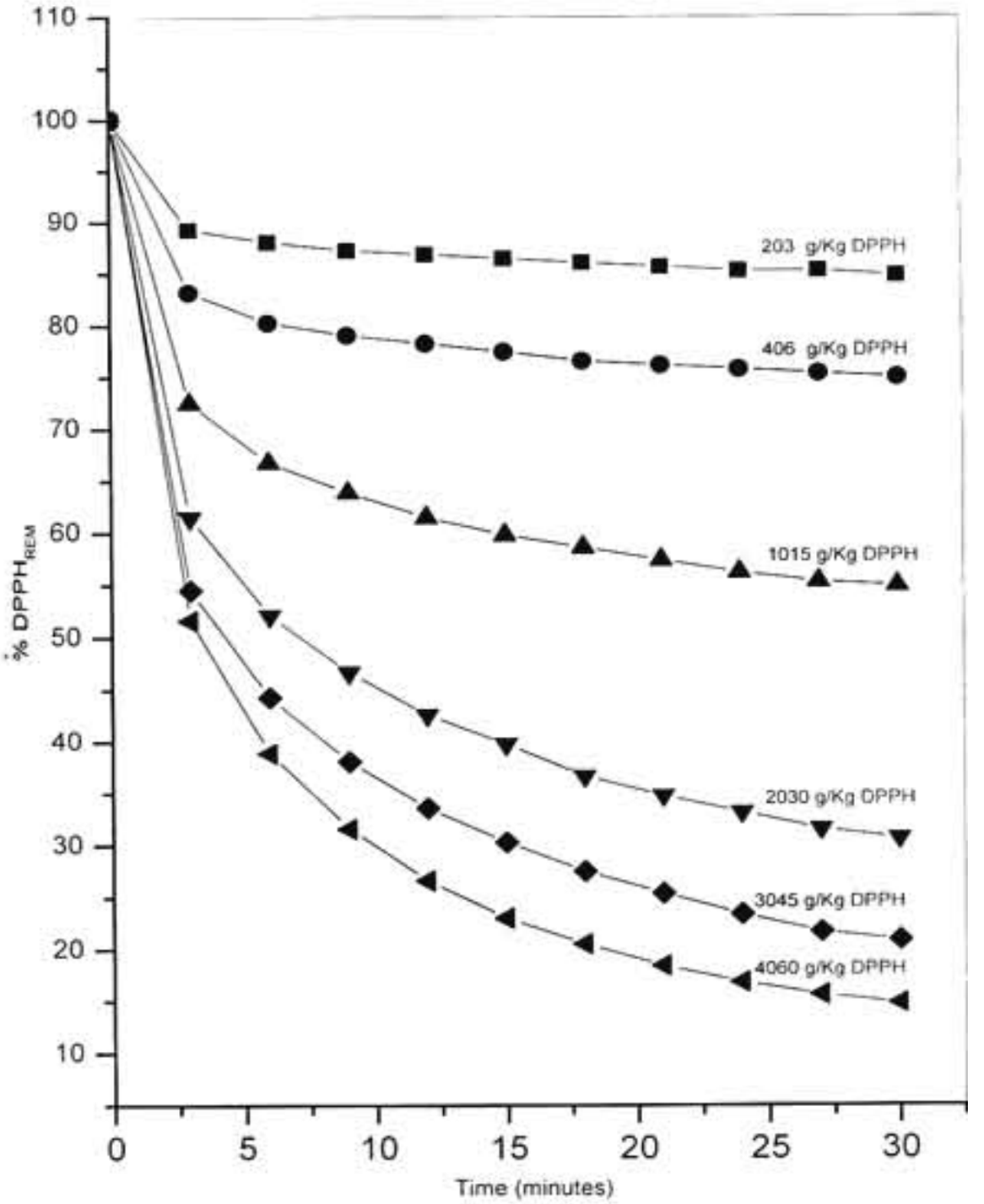


Fig.3.28.DPPH scavenging effect of polar phase of acetone extract (AE-PP)

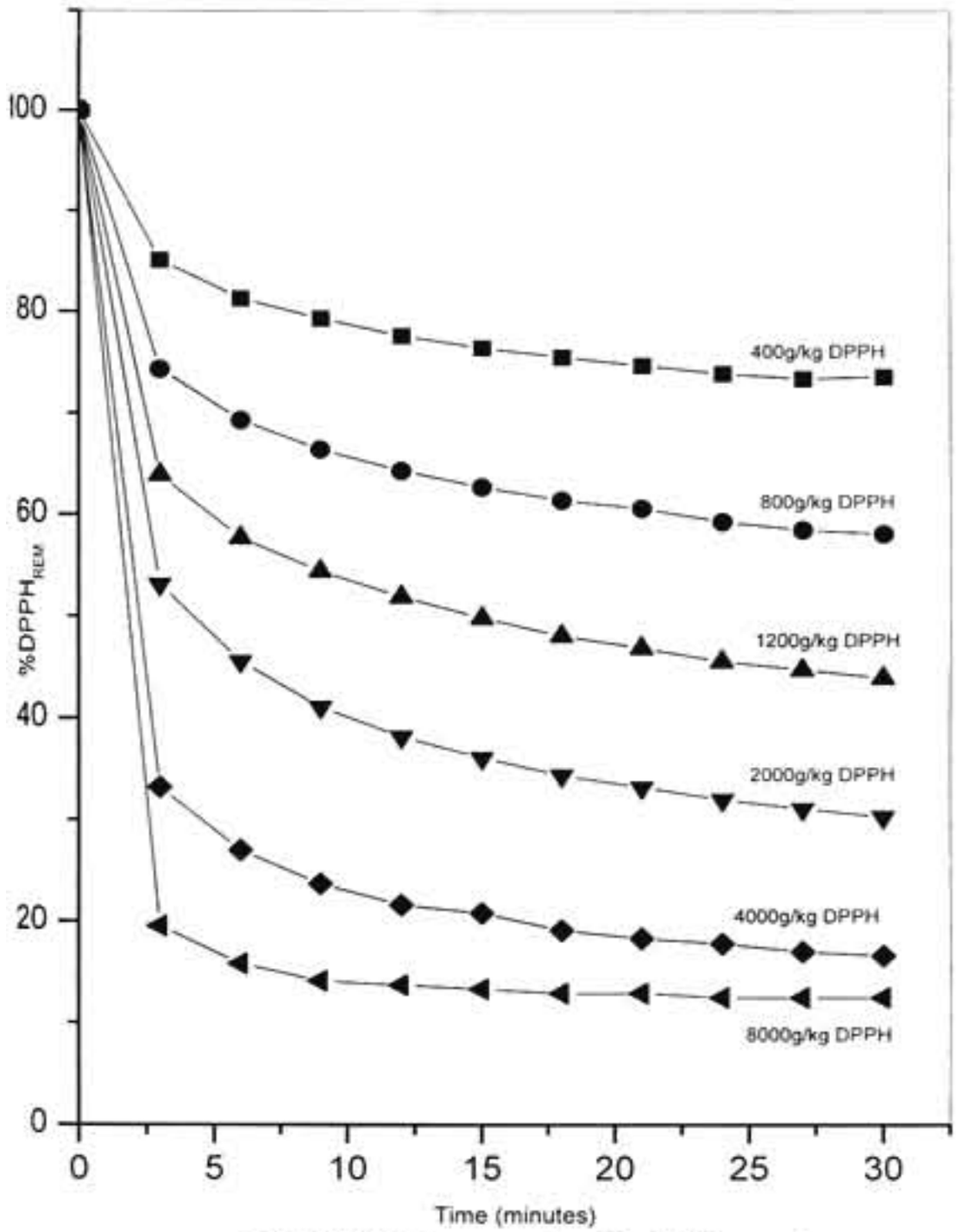


Fig.3.29.DPPH scavenging effect of Oryzanol

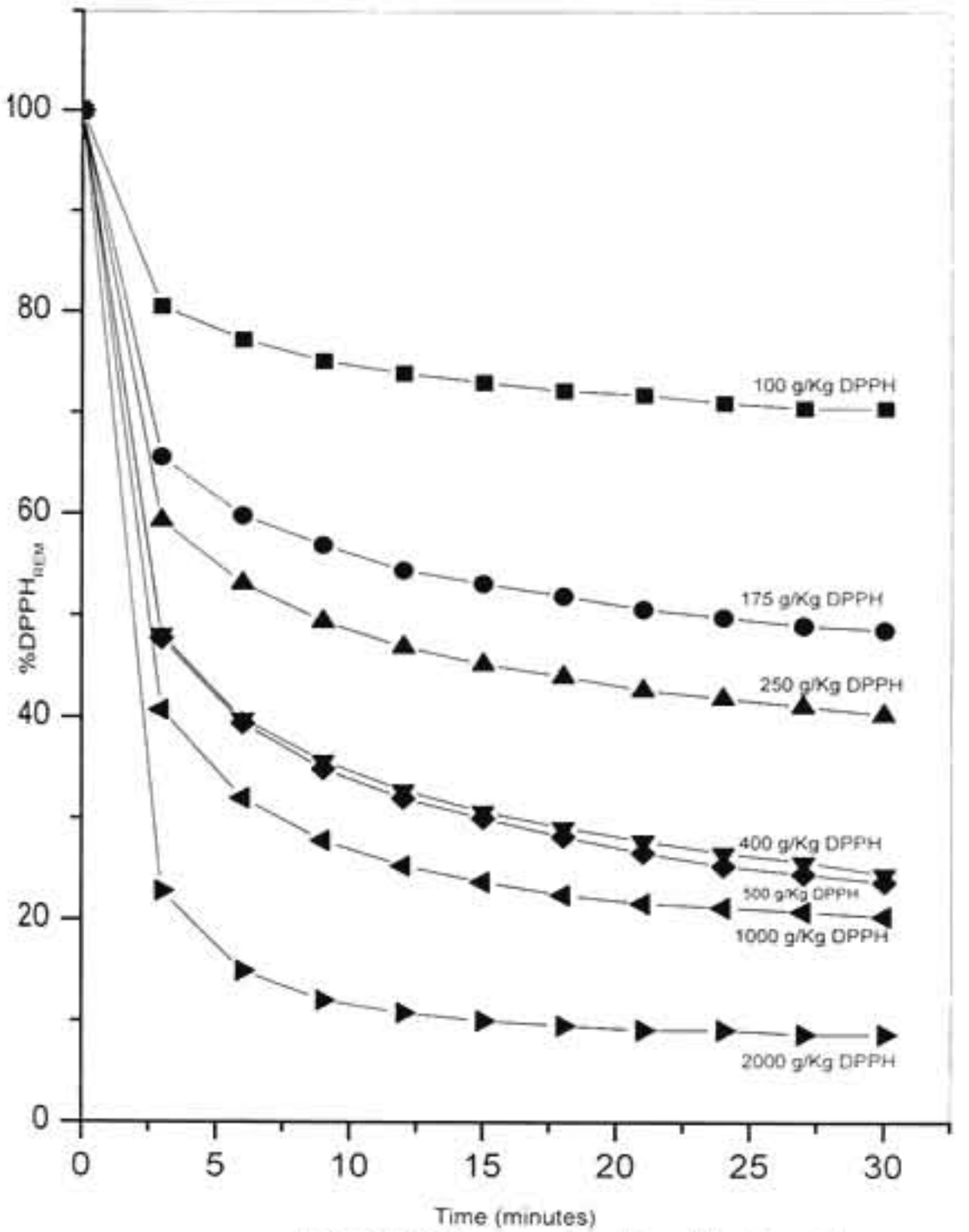


Fig.3.30.DPPH scavenging effect of Ferulic acid

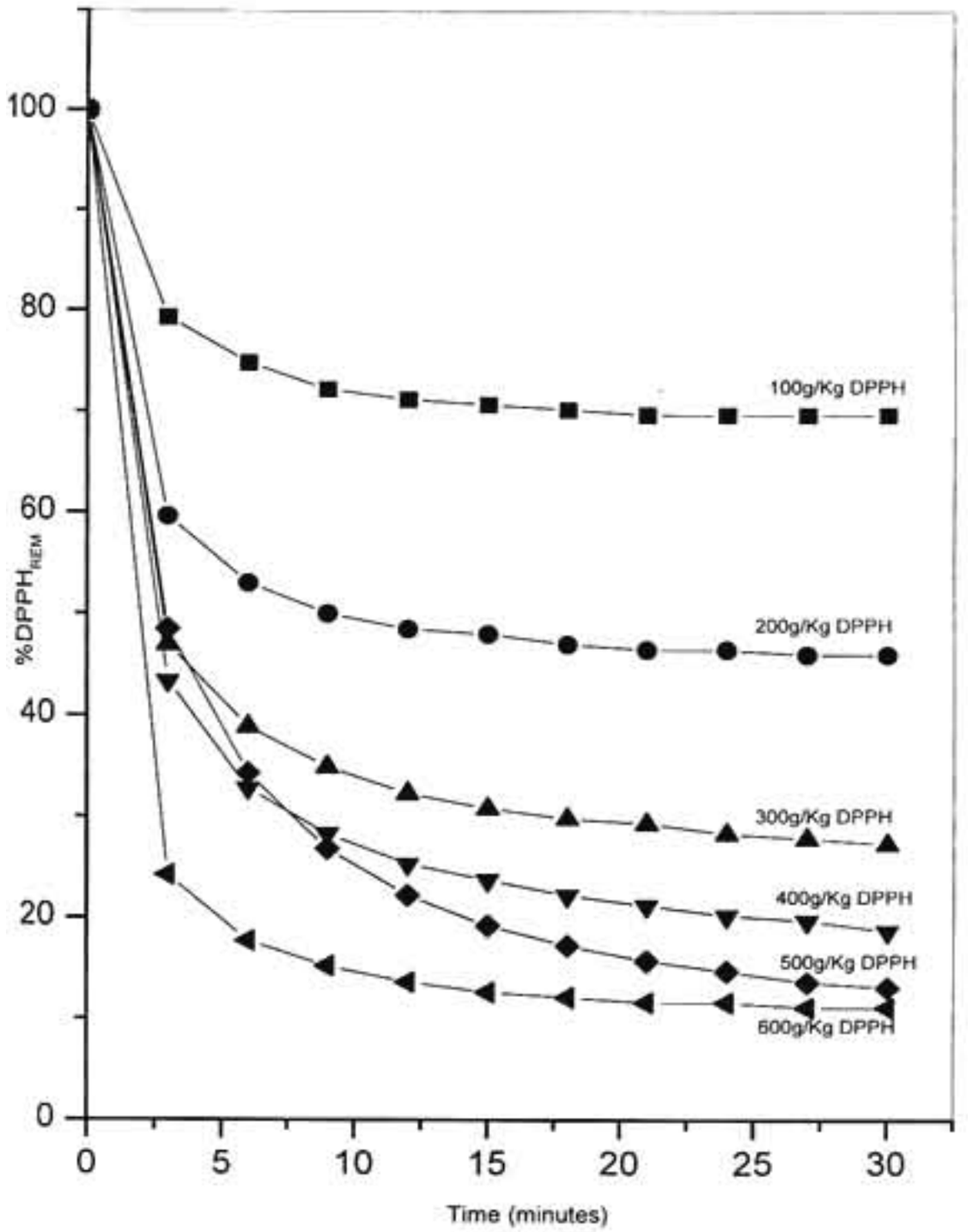


Fig.3.31. DPPH scavenging effect of Tmix

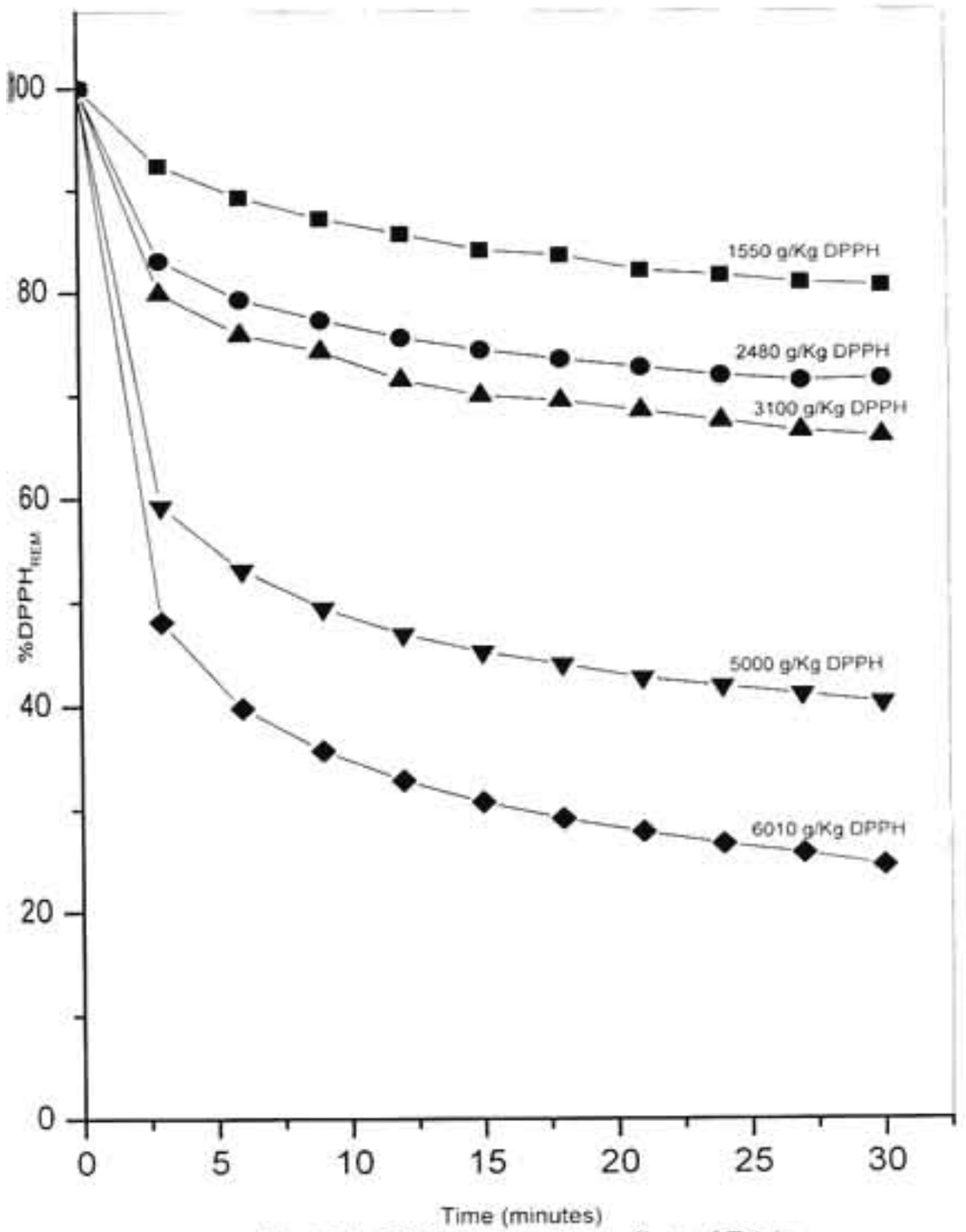


Fig.3.32. DPPH scavenging effect of Tricin

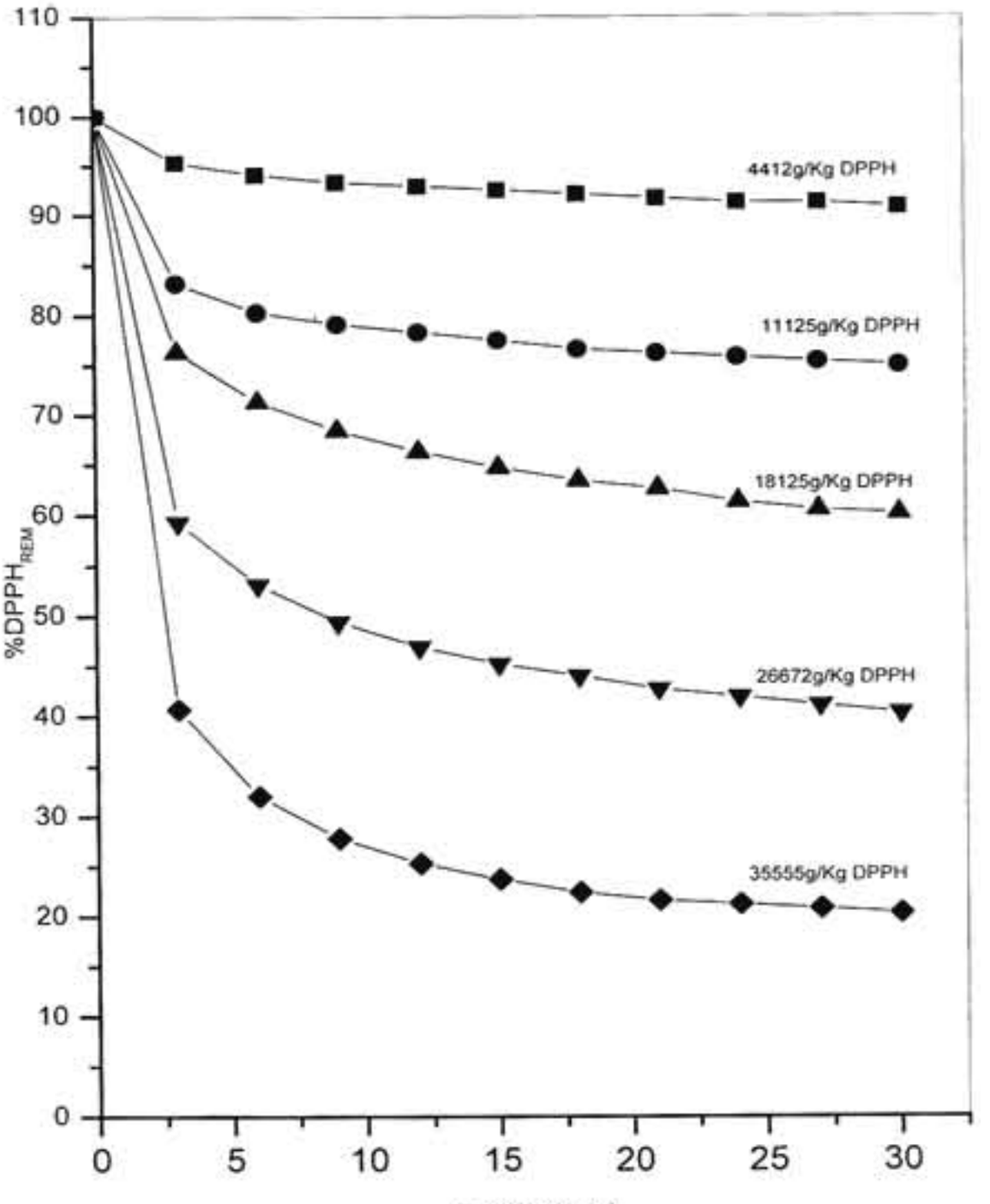


Fig.3.33.DPPH scavenging effect of β-sitosterol.

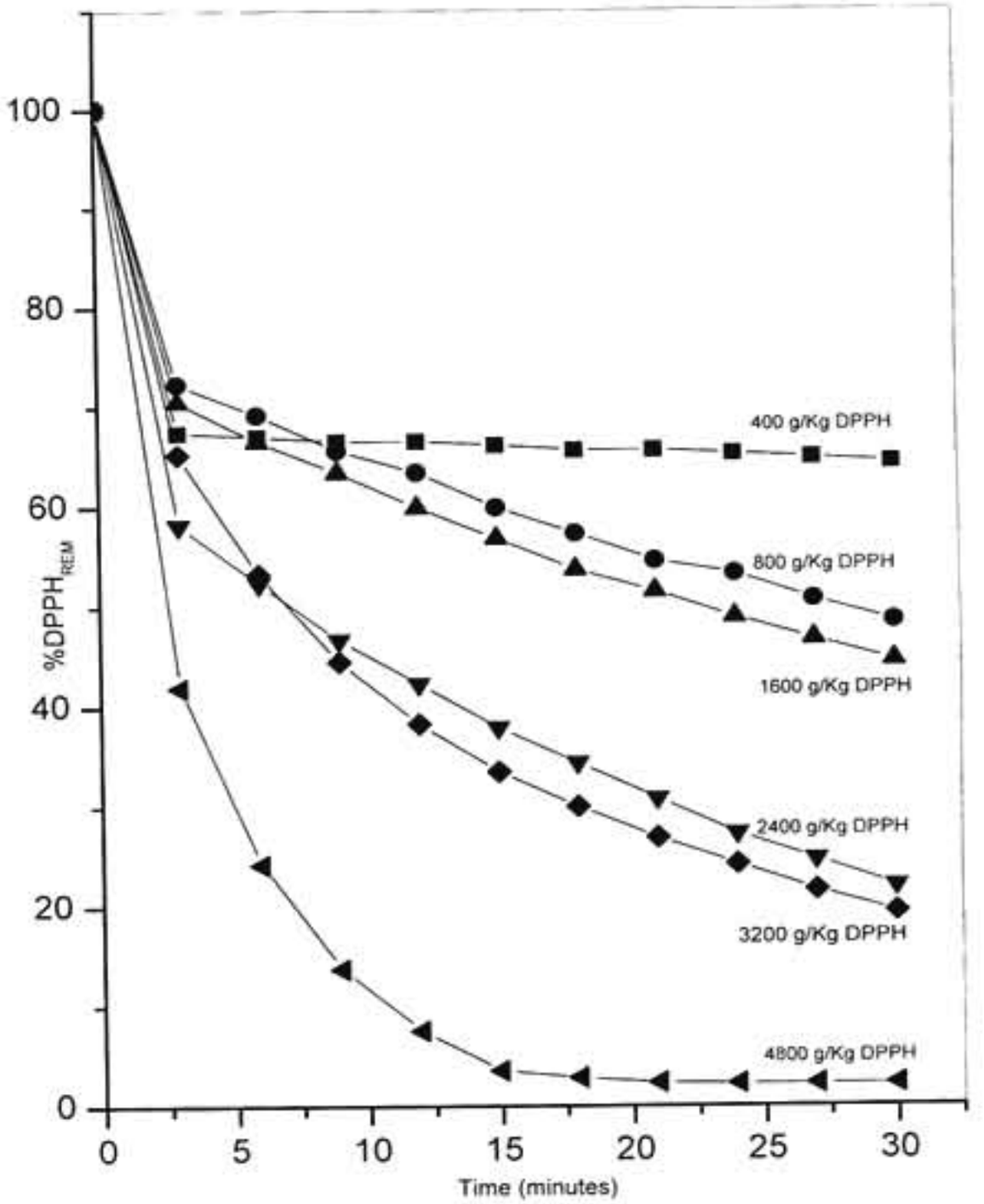


Fig.3.34.DPPH scavenging effect of BHT

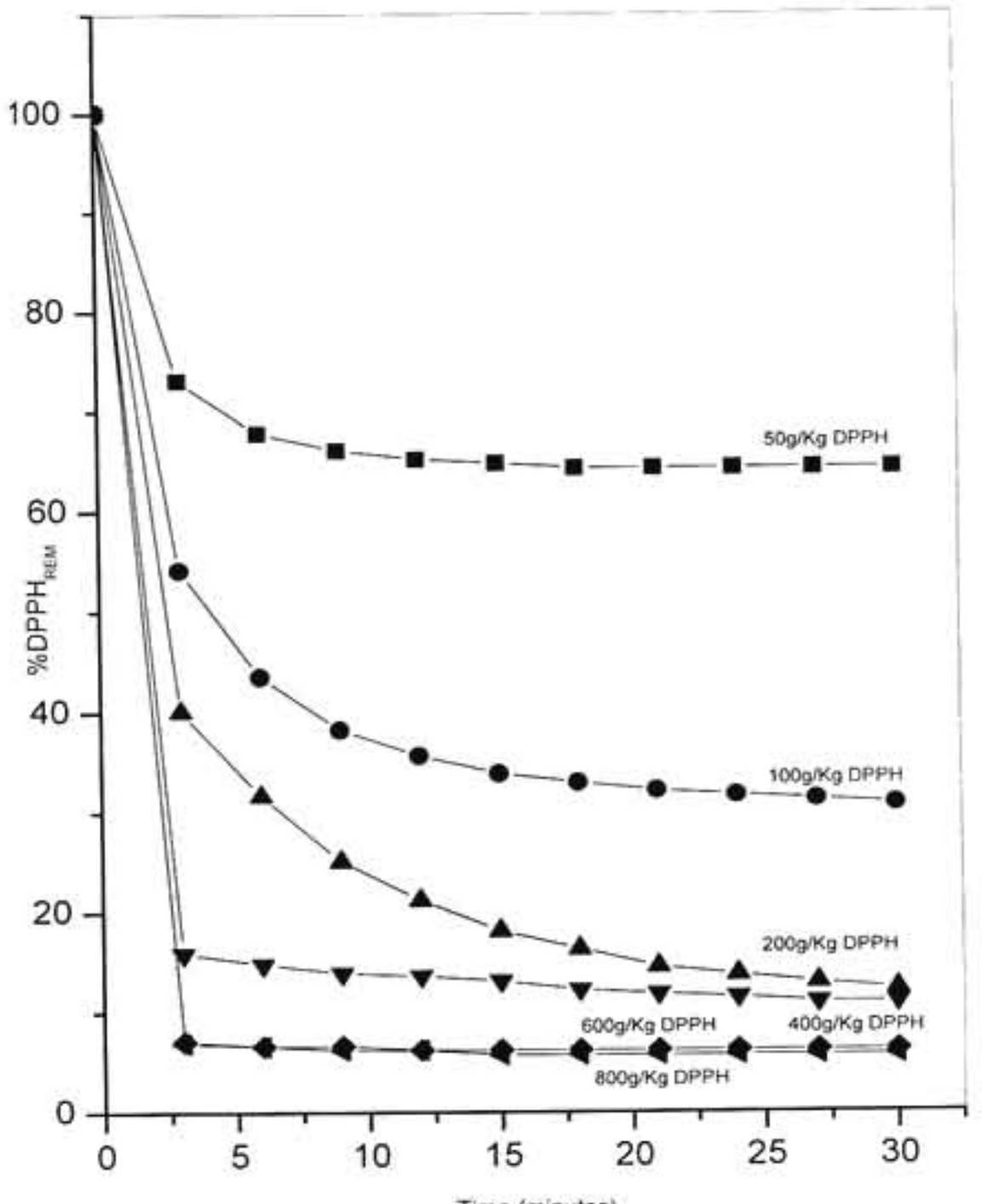


Fig.3.35.DPPH scavenging effect of TBHQ

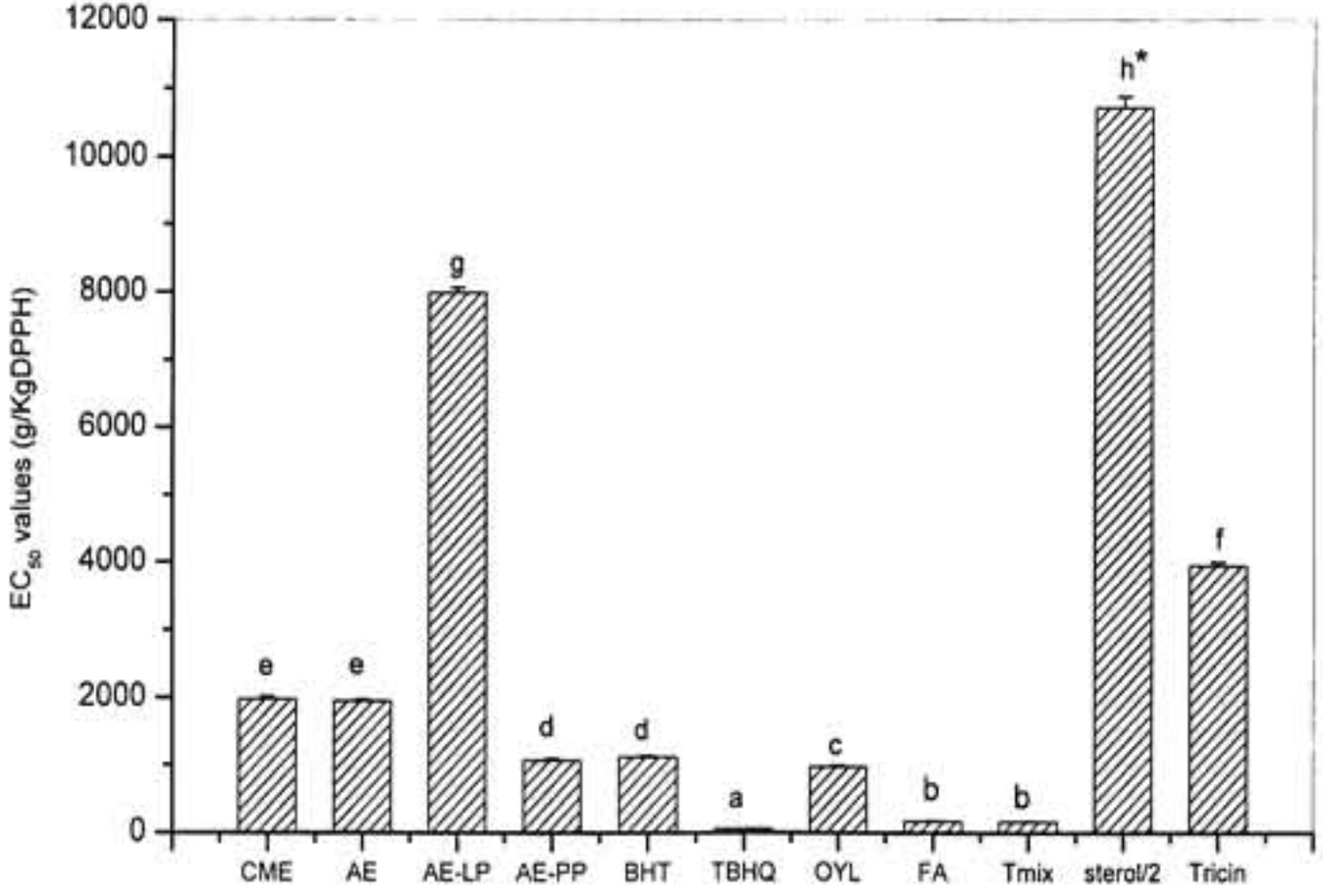


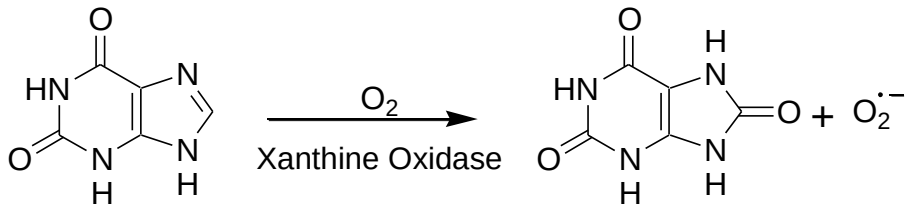
Fig.3.36. Effect of DRB extracts and its phytochemical constituents on DPPH radical scavenging. Values show mean± SD from two experiments performed in duplicate. Bars with different lower case letters are significantly different ($P<0.05$) according to DMRT.* For sterol, half the EC_{50} value is given inorder to accomodate it in the histogram.

3. 4. 4. Superoxide radical scavenging activity

Reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), H_2O_2, and hydroxyl radicals are byproducts of normal metabolism and their excess production and consequent oxidation of membrane lipids, proteins and DNA leads to tissue damage and degenerative diseases [232]. Xanthine oxidase (XOD) is one of the main enzymatic sources of reactive oxygen species (ROS) *in vivo*. Xanthine oxidase oxidizes xanthine or hypoxanthine to uric acid, superoxide anions and H_2O_2 [233]. Under normal physiological conditions, the endogenous superoxide scavengers in the system protect tissues by neutralizing these radicals. This *in vivo* reaction is simulated in the *in-vitro* model so as to use it as an analytical tool to evaluate the ROS scavenging abilities of natural products.

3.4.4.1. Superoxide radical scavenging activity by Xanthine-Xanthine oxidase/Cytochrome C method

For evaluating superoxide scavenging activity, the cytochrome C method was followed in which superoxide is generated with a xanthine - xanthine oxidase system which reduces the ferricytochrome C with consequent increase in absorbance at 550 nm. After the addition of superoxide scavengers, the reduction rate of cytochrome C decreases. Antiradical activity is expressed as IC_{50} which is the amount of antioxidant required to decrease initial superoxide concentration by 50% and was calculated as scavenging capacity (%)= [100-{(abs.of medium containing the additive)/ (abs.of control)}x100].



Xanthine

Uric acid

$Fe^{3+} + O_2^{\bullet -} \longrightarrow Fe^{2+} + O_2$

Superoxide scavenging effects of DRB extracts and its phytochemical constituents tested at 20μg levels is shown in Fig. 3.37. The IC_{50} values interpolated from dose response curves for CME, AE, AE-LP, AE-PP, FA, Tmix, tricin, BHT, and TBHQ were 47, 38, 57, 28, 34, 38, 35, 17, and 5 μg respectively. Thus the activity of the experimental sets followed the order TBHQ > BHT > AE-PP > FA=tricin > AE = T_{mix} > CME > AE–LP (Fig. 3.38). The IC_{50}values of oryzanols and β-sitosterol could not be determined by this method owing to their low solubility and consequent turbidity that affected spectrophotometric measurement. The activity of ferulic acid and tricin were similar and was more than that of Tmix and the activity order for the extracts was AE-PP >AE >CME >AE-LP.

3.4.4.2. Superoxide radical scavenging using Xanthine–Xanthine oxidase/ NBT method

Xanthine/Xanthine oxidase generated superoxide radicals reduce tetrazolium blue into formazan blue (λmax 560 nm), but in presence of radical scavengers, the formation of formazan blue is inhibited and therefore absorption at 560 nm decreases. Antiradical activity was defined as the amount of antioxidant necessary to decrease initial $O_2^{\bullet -}$ concentration by 50% [234]. The superoxide scavenging effects of DRB extracts and pure compounds at 40 μg levels is shown in Fig.3.39. The IC_{50} values interpolated from dose response curves for CME, AE, AE–LP, AE–PP, FA, and tricin were 125, 97, 171, 77, 39, and 41 μg respectively. The activity of the experimental sets followed the order FA=tricin >AE-PP > AE >CME> AE–LP (Fig.3.40). The IC_{50} values of BHT, TBHQ, and Tmix could not be determined by this method as these compounds interfered with measurement by reducing NBT directly. Oryzanols and sterols were poorly soluble in this assay system too and hence their IC_{50} values could not be determined by this method also. Activities of ferulic acid and tricin were similar and the activity of the extracts followed the order AE-PP >AE >CME >AE-LP.

Though $O_2^{\bullet -}$ cannot directly initiate lipid oxidation, in the presence of metal ions, the highly reactive hydroxyl radical ($OH^{\bullet}$) can be generated by the Fenton reaction [235]. Xanthine oxidase-derived superoxide radical has been linked to the postischemic tissue injury and generation of neutrophil chemotoxins [236]. The DRB extracts when assayed by cytochrome C method and NBT method showed positive radical scavenging effects. But the activities were found to be higher for the former method. The order of antioxidant efficacies by the two methods were found to be similar for the extracts and it was in the order AE–PP > AE > CME > AE–LP, which also followed the order of their TPC and ferulic acid contents.

According to Khanom *et al.*, the superoxide scavenging activity of Bangladeshi medicinal plants determined by the cytochrome method were mostly higher than that of the NBT method and they suggested that the former method measured not only the superoxide scavenging activity but also other biochemical properties [144]. In the present study, the IC_{50} values for the DRB extracts by the cytochrome C method were in the range of 28-57 μg and those by the NBT method were in the range of 77-171 μg. These assays make use of the competition kinetics of reduction of cytochromeC/NBT (probe) and $O_2^{\bullet -}$ scavenger (antioxidant compound) by superoxide. But many antioxidants are capable of reducing the probes directly [237]. In the present study, such a problem was not encountered with the cytochrome C method whereas many tested antioxidants viz. BHT, TBHQ and Tmix was found to reduce NBT directly. This can in turn result in a lower estimate of the true superoxide scavenging activity of the tested compounds as only lesser amounts of the added compounds would now be available for scavenging $O_2^{\bullet -}$. Complications can also arise in determining the extent to which the probe is reduced by the antioxidant and by $O_2^{\bullet -}$. The situation will be more complex in the case of multicomponent natural extracts like DRB especially when it is not fully characterized.

These methods were used by various authors for natural extracts. Hot water extracts of budrock were required at 1 mg levels to obtain 65% inhibition [238] and the IC_{50} value of tomato pulp was reported to be 1640 μg on dry weight basis [239]. Superoxide scavenging effects of fermented rice bran extracts were attributed to proteins by Miyamoto *et al.* [77]. Itani *et al.* have attributed the superior scavenging effects of red and purple black hulled rices compared to that of white hulled rices to the tannins and anthocyanins of the former [240]. Kim *et.al*. have studied the SOD-like activity of 24 lipophilic antioxidants including γ-oryzanol by measuring the inhibition of pyrogallol autoxidation catalysed by superoxide radicals [241]. The superoxide scavenging activity of ferulic acid was reported by Graf [223]. The reduction rate constant ($K \sim 10^2$-10^6 $M^{-1}S^{-1}$) of superoxide radicals by flavonoids is reported to be the highest among biological compounds [242]. The results of the present study demonstrated that the various phytochemical constituents of DRB extracts viz. ferulic acid, tricin and Tmix exhibited excellent superoxide radical scavenging activity thus directly supporting the superior antiradical efficacies of DRB extracts. However, as mentioned before, the presence of amino acids, sugars and other unidentified phenolics that constituted the bulk of the extracts would also have affected their antioxidant efficacy either synergistically or antagonistically.

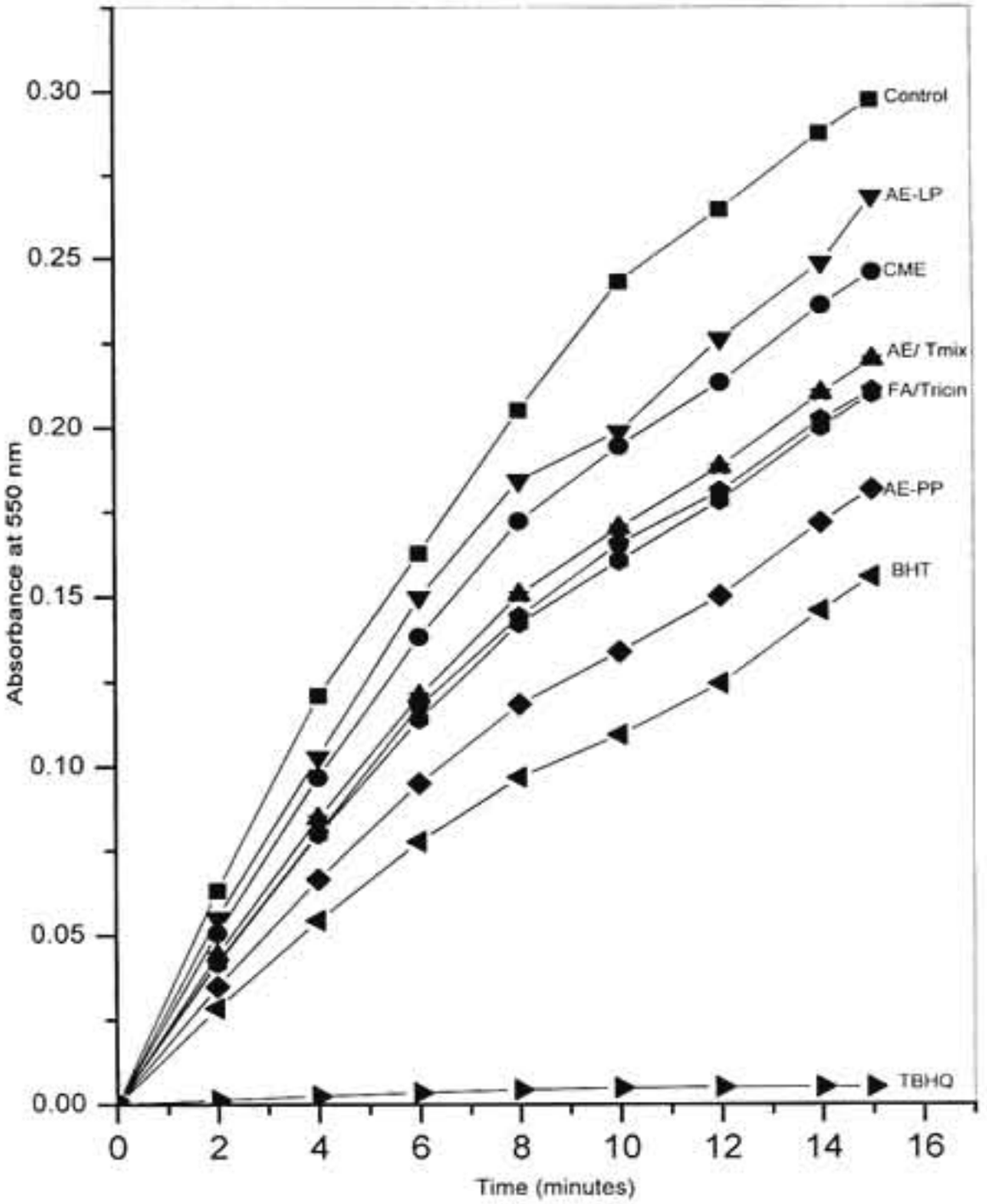


Fig.3.37.Superoxide radical scavenging effects of DRB extracts and its phytochemical constituents at 20 ug levels by Xanthine-Xanthine oxidase/Cytochrome method.

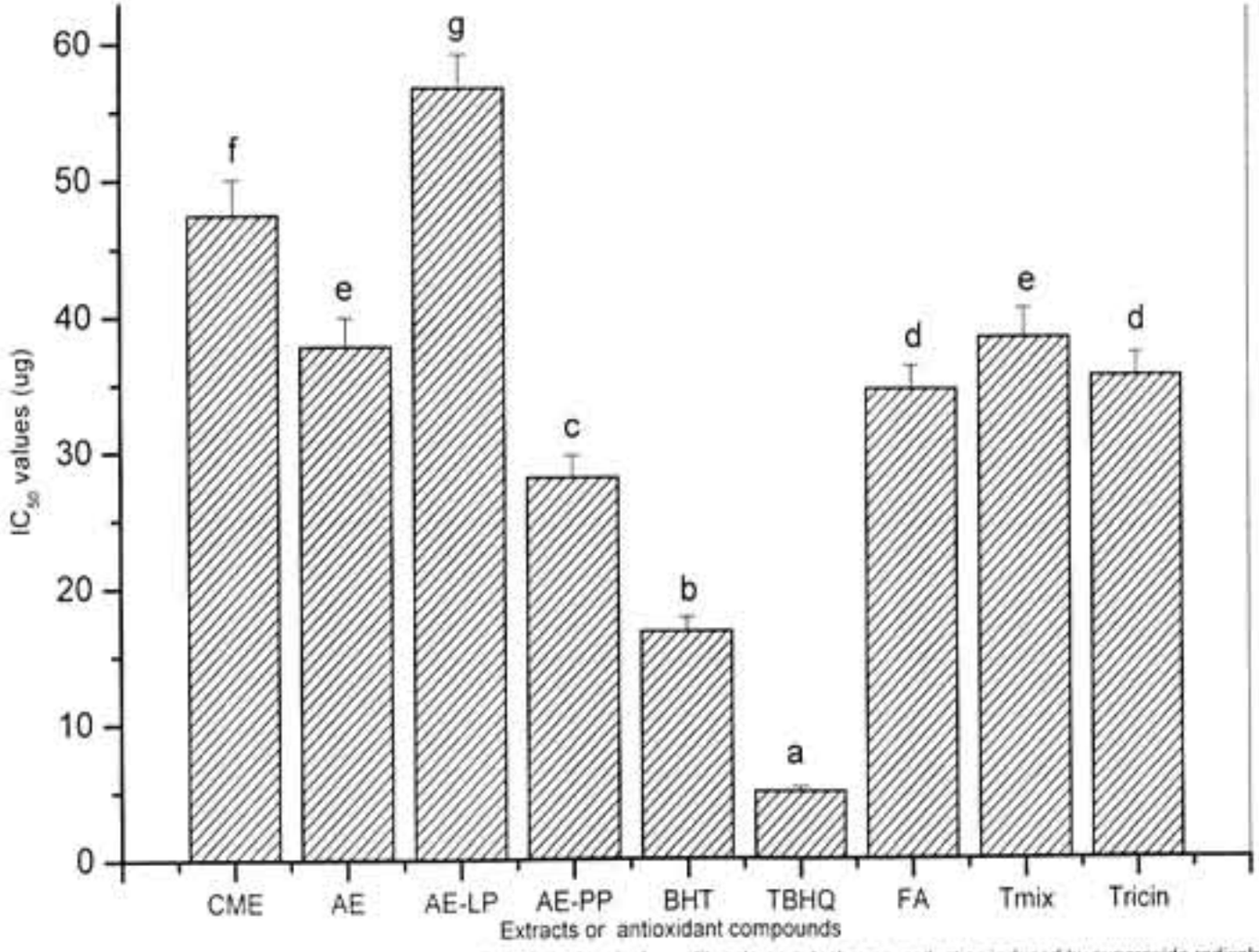


Fig.3.38 Effect of DRB extracts and its phytochemical constituents on cytochrome reduction induced by superoxide radicals generated in an X/XO system. Values show mean ± SD from two experiments performed in duplicate. Bars with different lower case letters are significantly different ($P<0.05$) according to DMRT

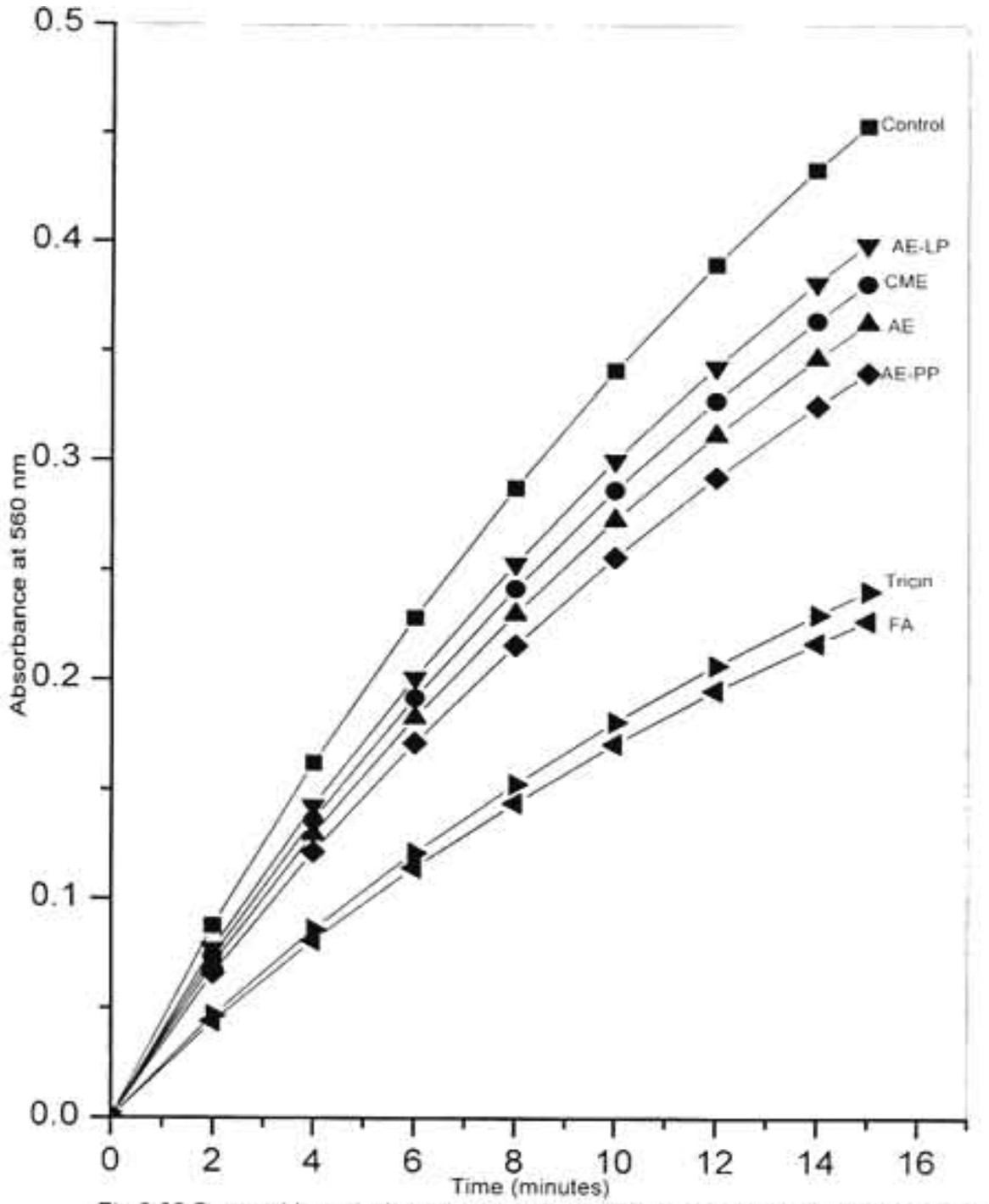


Fig.3.39.Superoxide radical scavenging effects of defatted rice bran extracts and its phytochemical constituents at 40 ug levels by Xanthine-XOD/NBT method.

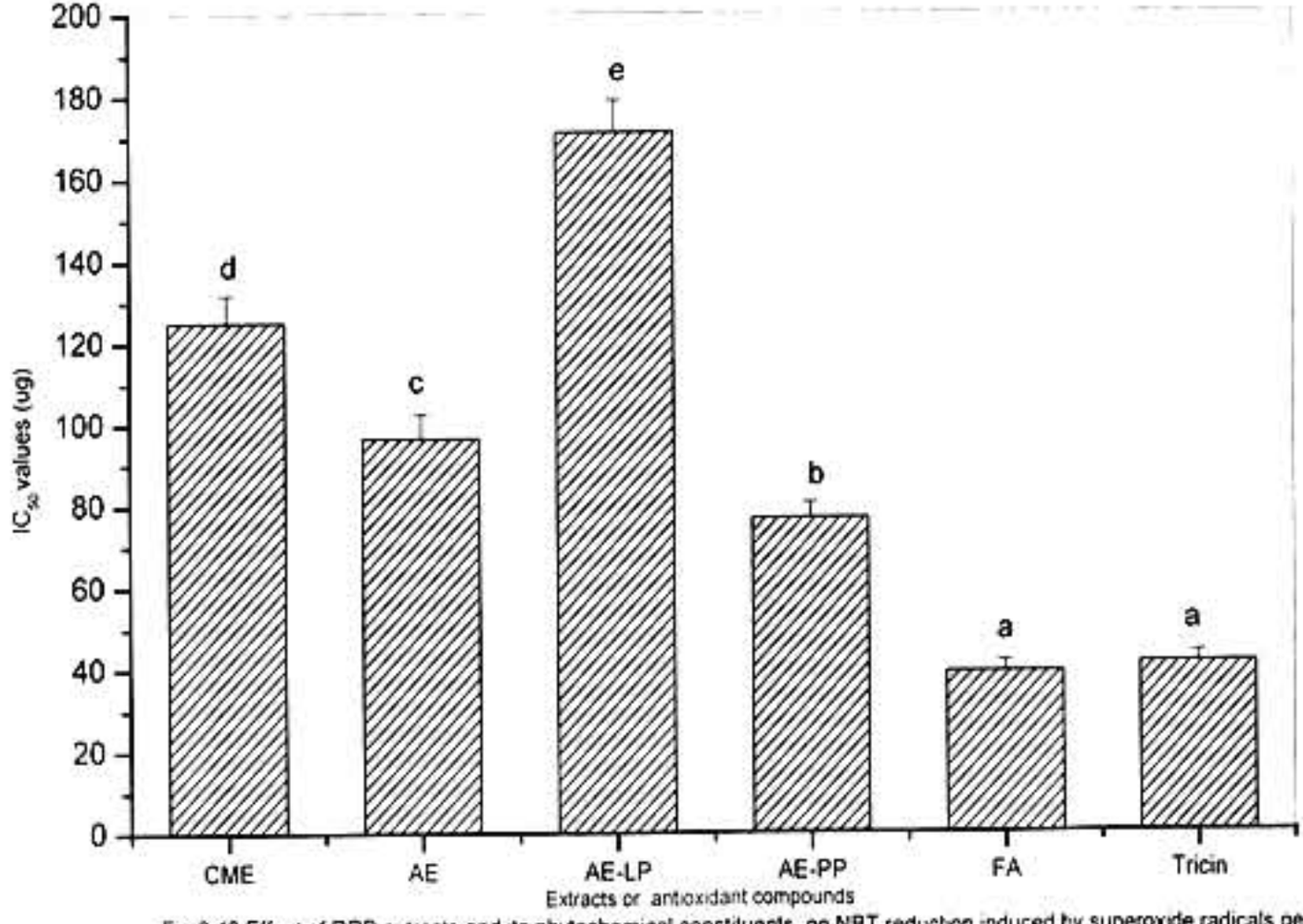


Fig 3.40 Effect of DRB extracts and its phytochemical constituents on NBT reduction induced by superoxide radicals generated in an X/XO system. Values show mean ± SD from two experiments performed in duplicate. Bars with different lower case letters are significantly different ($p<0.05$) according to DMRT

CHAPTER 4

SUMMARY AND CONCLUSIONS

4.0. Phytochemicals as antioxidants are gaining importance due to their dual role in the food industry as lipid stabilizers and as chemo preventive agents that could modulate cancer, CVD, cataract, ageing etc. Although synthetic antioxidants like BHA, BHT, TBHQ etc. are widely used as antioxidants in foods, their adverse effects on long term coupled with consumer preference for natural products have resulted in the resurgence in the research on natural antioxidants and consequently food industry is in search of potential natural antioxidants from edible sources. The major dietary sources of antioxidant phytochemicals are cereals, legumes, fruits, vegetables, oilseeds, beverages, spices and herbs. The present study focused on rice bran and its byproducts with special emphasis on defatted rice bran (DRB), an abundantly available byproduct of the rice bran oil industry (RBO). The main objective of the present study was to investigate whether DRB could be a source material for natural antioxidants/ nutraceuticals which is otherwise used as cattle feed. Thus the approach has been to characterise rice bran from prominent rice varieties grown in the region for their antioxidant phytochemicals; development of protocols for extraction and enrichment of the phytochemicals; and evaluation of their antioxidant efficacy using standard *in-vitro* models.

4.1. Rice bran, from major cultivars of the region were analysed for their chemical profile. The mean values for major constituents were dry matter (89.1%), fat (16.8%), protein (10.1%), crude fiber (11.3%), ash (11.4%), and available carbohydrates (50.5%). The mean energy content was 393.5 Kcal/100g. The mean values (ppm) of various minerals followed the order P (13608) > K (9520) > Mg (3844) > Ca (362) > Fe (216) ~ Na (190) > Mn (99) > Zn (39) > Cu (4), with P and Cu being the most and least abundant minerals respectively.

4.2. Rice bran oil obtained from the major cultivars were analysed for their chemical characteristics as well as fatty acid composition. The values obtained for FFA, saponification value, iodine value, and unsaponifiable matter were 12.3%, 182.9, 97.3 and 4.8% respectively. RBO had exceptionally high

unsaponifiables as compared to that of other edible oils. Major fatty acids of RBO were 16:0, 18:0, 18:1, and 18:2 with mean values of 21.6%, 2.0%, 41.8%, and 32.5% respectively, with saturated to unsaturated ratio of approximately 1:3. The fatty acid profile of RBO is thus close to the ideal ratio of saturated: mono unsaturated: poly unsaturated of 1:1.5:1.

4.3. To understand the distribution of the major antioxidant phytochemicals (oryzanols, tocols, and ferulic acid) among full fat rice bran (FFB), defatted rice bran (DRB), and rice bran oil (RBO); these process streams from the selected cultivars were characterized for the above phytochemicals using standard extraction protocols and HPLC. This included a systematic approach to separation, identification, and quantitation of various oryzanol and tocol components by HPLC. The various oryzanol components identified in RBO include stigmasteryl ferulate, cycloartanyl ferulate, 24-methylene cycloartanyl ferulate, cycloartenyl ferulate, campesteryl ferulate and β-sitosteryl ferulate. Seven tocols except β-T_3 were present in all the rice varieties studied. The total oryzanols varied between 2169-3473 ppm in FFB, 12648-19348 ppm in RBO and 94-217 ppm in DRB. Corresponding values for tocols were 183-316 ppm, 1042-1648 ppm, and 3-9 ppm. The ferulic acid content of FFB and DRB from the varieties ranged from 66-166 ppm and from 81-201 ppm respectively. However, ferulic acid was not present in the hexane extracted RBO. Thus DRB, as the byproduct of RBO extraction could contain substantial amounts of antioxidant phytochemicals that could be harnessed as a source for natural antioxidants.

4.4. Kinetic studies were designed to select appropriate solvent and to optimise other process parameters like material-solvent ratio, time, temperature etc. for extraction of antioxidants from defatted rice bran. Methanol was found to be the most efficient solvent, with respect to the yield of TPC, oryzanols and ferulic acid from DRB. Other optimized conditions included a material-solvent ratio of 1:15 and a time of extraction of 10 hours using a Soxhlet extractor. The yield of methanol extracts of DRB from the major cultivars ranged from 3.2 to

5.0%. The sugar content of the extracts ranged from 18.8 to 33.8%, protein from 17.9 to 25.0%, TPC from 5.3 to 8.4% and ash from 3.9 to 5.1 %. The oryzanol content of the extracts ranged from 2358 to 6602 ppm, ferulic acid from 2541 to 4376 ppm and tocols from 110 to 284 ppm. 24-methylene cycloartanyl ferulate (~45%), and cycloartenyl ferulate (~25%) represented the major oryzanols of the DRB extracts. γ-tocotrienol (~70%), and α-tocopherol (~10%) were the major chromanols.

4.5. Enrichment of antioxidants in crude methanol extract was achieved by sequential extraction and fractionation. For this, the CME was re-extracted with less polar organic solvents like ethyl acetate, acetone, ether etc. From this, acetone was found to be the best solvent for ferulic acid and tocols. For further purification of the acetone extract (AE), sequential extraction technique was employed. For this, the dry AE was re-extracted with hexane to give a soluble fraction enriched in lipophilic compounds (AE-LP) and a residue enriched in polar compounds (AE-PP). Considering the bioactive phytochemicals of interest, AE-LP was enriched in oryzanols, and tocols and AE-PP in ferulic acid. Column chromatography was employed to isolate components present in the crude extract. The two pure compounds obtained were identified to be β-sitosterol and tricin based on UV, IR, NMR and MS data. Of these, the flavone tricin is of special phytochemical interest because of its rare occurrence.

4.6. The crude extract (CME), the enriched fractions (AE, AE-LP, and AE-PP) and the pure phytochemicals (oryzanols, tocols, ferulic acid, tricin and sterol) were then subjected to a number of antioxidant and antiradical activity assays using standard *in-vitro* models. To evaluate the antioxidant potential of the extracts and its phytochemical constituents in bulk oils, Schall Oven Test method and differential scanning calorimetry (DSC) were used. The results demonstrated that in bulk oils, some of the DRB extracts (AE-PP) were either equally efficient or better than BHT and that at identical concentrations AE-PP,

AE-LP, and AE performed better than the phytochemical constituents ozyzanols, ferulic acid and tocols with respect to PV, DV and DSC data. The increase in activity with fractionation might be due to the enhanced levels of antioxidants in the resultant fractions compared to CME. To evaluate the antioxidant potential in food relevant systems, linoleic acid emulsion method and the β-carotene bleaching test were used. The DRB extracts and its phytochemical constituents proved to have significant activity in these emulsion models as well. Contrary to the bulk oil system (SOT & DSC), where the DRB extracts (AE-PP) were either equally efficient or better than BHT, the latter was more effective in emulsions. None of the pure phytochemicals tested performed better than BHT or TBHQ, both in bulk oils and emulsions indicating that the synergistic effects of phytochemicals in the extracts including that of proteins, sugars and unidentified polyphenols could be contributing to the observed efficacy of DRB extracts. The results further suggest that the DRB extracts could be used both in bulk oils and in food emulsions as natural antioxidants.

4.7. The antiradical efficacies of DRB extracts and their phytochemical constituents were studied using the stable DPPH radical and the superoxide radicals generated *in-situ* by the xanthine-xanthine oxidase system. The DPPH radical scavenging activity of ferulic acid, and Tmix was greater than that of BHT and the activity of AE-PP was equal to that of BHT. It was also found that the DPPH scavenging activity of the fractions AE, AE-LP and AE-PP could be largely attributed to the levels of TPC and ferulic acid in the fractions. H-donating capacity (as evaluated by the DPPH method here) is an important biologically significant property of antioxidants to convert potentially damaging ROS (oxyl and peroxyl radicals) into non-toxic species, and in this context DRB could be a good source of such antioxidants. For evaluating the superoxide scavenging activity of the extracts, the cytochrome C and NBT methods were used. The superoxide scavenging activities of the

extracts also followed the order of their TPC and ferulic acid contents. Moreover, the various phytochemical constituents of DRB extracts viz. ferulic acid, tricin and T_{mix} also exhibited excellent superoxide radical scavenging activity thus directly supporting the superior antiradical efficacies of DRB extracts.

Quest for health friendly phytochemicals as food additives, chemo preventive and therapeutic agents is far more stronger than ever before. The resurgence in natural products in recent past and consequent scientific studies have brought out a variety of potential natural sources of such phytochemicals. However from the commercial point of view, not many sources have been identified to meet the requirements for food products, nutraceuticals and therapeutic agents. Because of the abundant availability and renewability, rice bran could be an economically viable source for antioxidant phytochemicals for the future as demonstrated through the results of this study. However more work is suggested for further studies on complete chemical characterisation and development of a process to obtain a fraction with higher purity active compounds.

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LIST OF PUBLICATIONS AND PRESENTATIONS

Publications

1. Tocopherol and tocotrienol profiles of some vegetable oils by HPLC

   R.Renuka Devi, K.P.Suja, A.Jayalekshmy, and C.Arumughan, Journal of the Oil Technologists' association of India, vol.32, No.4, 2000, 176-182.

2. Changes in carotenoids of raw palm oil during heating, storage and light exposure.

   K.P.Suja, **R.Renuka Devi**, A.Jayalekshmy, and C.Arumughan, Journal of the Oil Technologists' association of India, Vol.34, No.2, 2002, 43-48.

Presentations

1. Determination of Isomers of Tocopherols, Tocotrienols and Carotenoids of Red Palm Oil by modified HPLC method.

   K.P.Suja, **R.Renuka Devi**, A.Jayalekshmy, and C.Arumughan presented at the fourth International Food Convention, CFTRI, Mysore, Nov. 23-27, 1998.

2. Effect of parboiling on the fatty acid composition and antioxidant constituents of Rice Bran Oil from some popular Indian rice varieties.

   R.Renuka Devi, A.Jayalekshmy and C.Arumughan, presented at the 55th Annual Convention and Seminar on Indian Oilseeds and Edible Oil Sector, Challenges Ahead, IICT, Hyderabad, Nov. 18-19, 2000.